

**Cross-resistance to Phage Infection in *Listeria monocytogenes*
Serotype 1/2a Mutants and Preliminary Analysis of their Wall
Teichoic Acids**

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Abstract

Listeriosis, a foodborne illness that may lead to serious infections and/or death in immunocompromised individuals, is caused by the Gram-positive bacterial pathogen *Listeria monocytogenes*. Gram-positive bacteria contain in their cell walls a thick layer of peptidoglycan, which attaches surface glycopolymers known as wall teichoic acids (WTA). WTA are vital for many functions in the cell, but the primary interest within these studies concerns their role as bacteriophage receptors. Bacteriophages, viruses that exclusively infect bacteria, have been used for over a decade as antimicrobial agents to control *L. monocytogenes* in ready-to-eat foods and food processing facilities. However, an ever present concern is the possibility of resistance to phage developing after use of such products. Phage-resistance arises from bacterial mutations affecting biosynthesis or glycosylation of WTA. The objective of the first study was to assess the cross-resistance of mutant strains of *L. monocytogenes* to a diverse collection of bacteriophages. The objectives of the second study were to develop a method for preliminary analysis of purified *L. monocytogenes* WTA using silylation and gas chromatography with flame ionization detection (GC-FID); to apply the same methods for silylation and GC-FID for analysis of sugar standards; and to determine if this methodology could be streamlined to assess cruder samples. The first study found that the mutant strain lacking rhamnose on its WTA (FSL D4-0119) was the most resistant to phages, with only one phage able to infect it. These results can be applied in the formulation of *Listeria* phage biocontrol products better able to prevent phage-resistance. The second study obtained chromatograms for standards which included N-acetylglucosamine, glucosamine, galactose, glucose, rhamnose, and ribitol. All *L. monocytogenes* strains analyzed contained ribitol, and only FSL D4-0119 did not contain a peak for rhamnose. It was found that the methods used can be streamlined to analyze cells that have not been processed further than

autoclaving. However, to confirm the presence of the predicted WTA monomer units, standards should be synthesized and run using the same silylation and GC-FID methods. Results obtained using these methods with synthesized standards and the phage-resistance study will contribute to better understanding the mechanisms behind phage-resistance.

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Chapter I: Literature Review

Pathogenesis & Virulence of *Listeria monocytogenes*

Listeria monocytogenes is a Gram-positive, non-spore forming, facultative anaerobic bacteria that is most well-known for its role as an opportunistic foodborne pathogen. As such, illness caused by *L. monocytogenes*, called listeriosis, is most dangerous for elderly, pregnant, and immunocompromised individuals. In healthy individuals it will result in non-invasive listeriosis, which includes typical symptoms of a foodborne infection, such as gastroenteritis and fever. However, in susceptible individuals, much more severe symptoms may present as invasive listeriosis. These symptoms include septicemia, bacteremia, meningitis, meningoencephalitis, or even death in people ≥ 65 years old, children, the immunocompromised, or perinates. In pregnant women, listeriosis is not typically dangerous for the mother as symptoms will present as a mild flu; however, this generally precedes an abortion of the fetus (152). Additionally, pregnant women have a 12-fold increased risk of contracting listeriosis after eating contaminated foods as compared to healthy, non-pregnant individuals (71).

The amount of time between the ingestion of contaminated food and the emergence of symptoms (incubation period) varies depending on the severity of the infection. In non-susceptible individuals, symptoms of non-invasive listeriosis usually occur within 24 hours (110). Conversely, symptoms of invasive listeriosis typically commence after three weeks, although this period can differ significantly (5). Infection from listeriosis begins approximately 20 h after ingestion of food contaminated with *L. monocytogenes*. However, the dose required for infection is variable due to differences in host immunity and the bacterial strain (152). In susceptible groups, doses of around 10^2 - 10^4 bacterial cells per gram of contaminated food can lead to serious illness. For healthy, non-pregnant individuals, less serious illness is caused from food

contaminated with closer to 10^6 - 10^9 bacterial cells per gram of food (117, 152). The high doses necessary for illness in such individuals is at least partially due to the acid environment of the stomach. Studies have found that individuals taking antacids or H₂-blocking agents have an increased susceptibility to listeriosis (70, 134). Additionally, studies have found differences in the LD₅₀ for mice with *L. monocytogenes* administered either parenterally (10^5 - 10^6 cells) or orally (10^9 cells). The *Listeria* cells that avoid succumbing to the low pH of gastric acids move on to initiate pathogenesis in the host (152), which is orchestrated by many different virulence factors.

Regulation of virulence factors mainly occurs at transcriptional or post-translational levels. Transcriptional regulation is controlled by either positive regulatory factor A (PrfA), PrfA with Sigma B, or VirR regulons. PrfA regulates virulence factors including the actin assembly inducing protein (ActA), phospholipase A (PlcA), and *Listeria* adhesion protein B (LapB); PrfA with Sigma B together regulate internalin A (InlA), internalin B (InlB), listeriolysin O (LLO), phospholipase B (PlcB), and p60; and VirR regulates the multiple peptide resistance factor (MprF) as well as the Dlt operon. Post-translational regulation is controlled by the enzyme sortase A (SrtA) or the accessory secretion system SecA2. SrtA regulates internalin J (InlJ), and has influence over the regulation of InlA and LapB, while SecA2 regulates *Listeria* adhesion protein (LAP) and fibronectin-binding protein A (FbpA), with influence over the regulation of p60 (20). Regulation of flagellar expression is also important for virulence. *L. monocytogenes* expresses flagella at temperatures below 30°C; this is due to the inhibition of a transcriptional repressor, MogR, by the anti-repressor GmaR. GmaR is activated by a response regulator known as DegU. However, at temperatures $\geq 37^\circ\text{C}$, MogR is not inhibited and the gene coding for

flagellin (*flaA*) is down-regulated in order to avoid detection by the host innate immune system (65, 83).

Adhesion of the bacteria to host cells is the crucial first step required for pathogenesis to occur (143). Adhesion is made possible by many different factors present on the cell. One of the most important is LAP, which binds preferentially to intestinal cells, both in pathogenic and non-pathogenic *Listeria* species (79). LapB is another *Listeria* adhesion protein, but is specific to pathogenic species (120). FbpA is novel in that it acts to both adhere to cells via binding to human fibronectin, and acts as a chaperone for other virulence factors LLO and InlB (42). InlJ binds specifically to MUC2 in intestinal mucus (95). The gene coding for InlJ is only found in *L. monocytogenes*, making it a unique marker for identification (124). Ami is an autolytic enzyme anchored to the cell wall that also aids in adherence to host cells (103).

Invasion is the next stage of pathogenesis. *L. monocytogenes* is either engulfed by phagocytes or enters into non-phagocytic cells (117). Non-phagocytic entry into M cells of the intestine can occur without any known virulence factors (34). However, it is more common that *Listeria* initiates a zipper mechanism of endocytosis through receptor-mediated binding (35). Receptor-mediated entry of intestinal cells occurs through interaction between bacterial InlA and E-cadherin of epithelial goblet cells (109, 117). To access endothelium cells and hepatocytes, *L. monocytogenes* utilizes InlB to bind their c-Met receptors (36). The pathogen also employs different virulence factors to resist host defenses during invasion. MprF is a protein that adds lysine to the membrane phospholipid diphosphatidylglycerol, giving it a positive charge. This helps the membrane to resist cationic antimicrobial peptides (CAMPs) produced by the host

(146). Similarly, the prolipoprotein Lgt is a diacylglyceryl transferase which lipidates prelipoproteins, also making *L. monocytogenes* more resistant to CAMPs (100).

Once *L. monocytogenes* has successfully invaded a cell, it must escape the phagosome created by its endocytic entry. It does so by using LLO (encoded by the gene *hly*) to degrade the phagosome (132). Two other important elements in vacuole escape are the phospholipases PI-PLC and PC-PLC. PI-PLC is also known as PlcA, a phosphatidylinositol-specific phospholipase C; PC-PLC is also known as PlcB, a broad-range phospholipase C (20).

ActA is a protein that enables *L. monocytogenes* to spread from one host cell to another by actin-based propulsion (7, 152). It is necessary for the pathogen to polymerize actin from within the cytoplasm of the host cell for motility (43), as flagella are not expressed at physiological temperatures (138). The protein p60 is also necessary for *L. monocytogenes* to utilize the polymerized actin (115). The propelled bacterium eventually contacts the host cell membrane, forming a protrusion that is taken up by an adjacent host cell (71). The protein internalin C (InlC) was found to play a role in the formation of this protrusion (119). This process enables *L. monocytogenes* to spread throughout the body. The majority of the bacterial load ends up in the liver and multiplies there (7, 152). The next stages of infection depend upon the responsiveness of the host immune system. In healthy individuals, the immune system contains the pathogen within the liver and proceeds to eliminate it from the body. Conversely, in susceptible individuals, the diminished or altered immune response leads to bacteremia, enabling the pathogen to infect the central nervous system, multiple organs (septicemia) or, in pregnant women, the placenta and/or fetus (152).

Treatment of *Listeria monocytogenes*

Treatment of listeriosis infections is accomplished by administration of antibiotics, which may include amikacin, amoxycillin, ampicillin, azlocillin, ciprofloxacin, chloramphenicol, clindamycin, coumermycin, doxycycline, enoxacin, erythromycin, gentamicin, imipen, netilmicin, penicillin, rifampin, trimethoprim or vancomycin (162), though the most commonly used are amoxycillin, penicillin, or ampicillin, or one of these in combination with gentamicin (3, 72). In individuals sensitive to these antibiotics, trimethoprim-sulfamethoxazole can be administered to kill the pathogen (123). Cephalosporins, fosfomycin, and first-generation quinolones are not effective on *L. monocytogenes* (3, 144). *L. monocytogenes* is considered Beta-lactam tolerant, hence the use of gentamicin in combination with such antibiotics for treatment in humans (73). Antibiotic resistance though is primarily an issue in animals, such as those used for human food sources (144). Patients with invasive listeriosis are much more likely to survive when treated promptly with suitable antibiotics; however, it is not a guarantee that a susceptible individual will recover from the illness without complications, or at all. Moreover, patients may not be diagnosed in time or correctly to receive proper treatment, in which case they are at an even greater risk of suffering irreversible neurological conditions or death (147). The possibility of such severe health consequences is the reason that foodborne outbreaks of listeriosis are such a serious threat.

***Listeria monocytogenes* as a Foodborne Pathogen**

One of the major factors leading to listeriosis outbreaks is the organism's ability to survive in the same conditions that many food processing facilities maintain. *L. monocytogenes* is a psychrotroph, meaning it can survive in cold temperatures such as those used for refrigeration of

food products (126). In fact, a procedure known as cold-selection in which bacteria are grown in non-selective media at refrigeration temperature (4°C) can be used to select for *Listeria* (162). The temperature range for growth of *L. monocytogenes* is from -0.1 to 45°C, with optimal growth from 30 to 37°C (154, 162). Aside from its ability to thrive in cold temperatures, *L. monocytogenes* can survive in a wide variety of environmental conditions, such as alkaline, acid, and high salt (98, 162). The optimal pH range for *L. monocytogenes*' survival and growth is between 6 and 9, but it has been shown experimentally to survive at a pH as low as 4.19 (32, 162). *L. monocytogenes* typically has a minimum water activity (a_w) of 0.92, meaning foods that have a a_w of ≥ 0.92 will promote its growth (48). However, a study demonstrated that *L. monocytogenes* was able to grow in an environment with an a_w of 0.91, created using a solution of 13-14% NaCl (48). It has also been shown to survive in concentrations of NaCl as high as 18% (w/v) (32). However, growth and survival in these extremes is dependent on the other conditions of the environment and the strain of *L. monocytogenes* (12). Additionally, it was demonstrated in another study that at the lowest a_w tested (0.80), *L. monocytogenes* still survived approximately 8 days (102). This is relevant to recent recalls involving *L. monocytogenes* in nut butters (55, 56). Although an a_w of 0.70 for peanut butter (54) is lower than what is required for pathogen growth, there is potential for it to survive long enough to cause illness. Additionally, the bacterium can develop resistance to heat under food processing conditions that first expose it to low levels of heat, or to solutes like salt or sugar (41). *L. monocytogenes* is also capable of persisting in food processing environments for long periods of time (i.e. years) within biofilms (105, 111).

In 2011, data regarding cases of foodborne illness from 2000 to 2008 was compiled to develop a picture of the prevalence and severity of 31 major foodborne pathogens, including *L. monocytogenes*. It was found to be the third leading cause of foodborne illness, contributing to 19% of deaths. All cases of listeriosis reported (average of 1,455 annually) resulted in hospitalization; of which an average of 255 resulted in death. Listeriosis is also almost entirely (99%) contracted through eating food contaminated with *L. monocytogenes* (128). More recent data regarding the occurrence of cases of listeriosis in the U.S. was collected from 2009 to 2011. Within this time frame, 1,651 invasive listeriosis cases were reported, which included 292 deaths/fetal losses (21% mortality rate) and 93% of patients having been hospitalized. The majority of the cases (58%) involved adults aged 65 or older, while 14% of cases occurred in pregnant women. Among those affected that were not in the ≥ 65 years or pregnant groups, a high prevalence (74%) of patients with an underlying medical condition was reported (140). The most current data regarding the 41 most prevalent foodborne pathogens, chemicals and toxins involved in U.S. outbreaks consists of information gathered from 2009 to 2015. *L. monocytogenes* contributed to over half of the deaths (52%) within these parameters, demonstrating the continuing severity of such outbreaks (40). In addition to the physical and emotional trauma caused by the pathogen, there is a significant financial burden that must be considered as well. Even though the number of listeriosis cases is relatively low compared to the other 14 major foodborne pathogens considered, it ranks as the second most costly per case. This is attributed to its high rates of hospitalization (~94%) and death (~16%) (74). For these reasons, many efforts have been made in an attempt to reduce the occurrence of listeriosis outbreaks, or at least lessen their impact as much as possible.

Government organizations such as state health departments, the CDC (Centers for Disease Control and Prevention), the FDA (Food and Drug Administration) and the USDA-FSIS (United States Department of Agriculture – Food Safety and Inspection Service) all work together under the *Listeria* Initiative, a program started in 2004 to process outbreak data more efficiently and determine its source as quickly as possible (27). These organizations have also effected legislation in an effort to decrease or eliminate the occurrence of *L. monocytogenes* in food. The CDC declared listeriosis a nationally notifiable disease in 2000, meaning that health officials are required to report any patients diagnosed with the illness to their local public health department (28). In 2003, the USDA-FSIS with support from the FDA issued the *Listeria* Rule, which maintains a zero-tolerance policy for the presence of *Listeria* on ready-to-eat (RTE) products (57). This policy remains in effect, although temporarily it was not applied to foods that should not support the growth of the bacteria. However, the decision was made to reverse these changes, probably due to the increasing presence of *L. monocytogenes* on novel foods items (6).

The history of *L. monocytogenes* as a pathogenic organism dates back to 1924, when E.G.D. Murray first isolated it from rabbits. The first recorded outbreak of listeriosis in humans occurred in 1949 in Germany. It affected 85 infants, who were either stillborn or died shortly after birth (71). However, the earliest known foodborne outbreak of listeriosis was not for another 30 years. In 1979, raw vegetables were presumed to be the source of a listeriosis outbreak in Boston, MA, although this was not able to be confirmed (70). In 1981, an outbreak of listeriosis in Nova Scotia, Canada identified cabbage as the confirmed source of *L. monocytogenes*, and solidified the link between consumption of foods contaminated by *L. monocytogenes* and contraction of listeriosis (130). It has been well-established that *L. monocytogenes* is ubiquitous in nature, in

which it functions as a saprophyte living off decaying plant matter. Soil is a common source from which *L. monocytogenes* can be isolated, indicating the possibility of it being present on any type of produce grown in the soil (158, 160). Therefore, it is not surprising that it was the causative agent in these outbreaks.

Subsequent outbreaks of *L. monocytogenes* have linked the pathogen to other foods such as RTE meats and dairy products (22). The infamous 1985 outbreak in Los Angeles, California, was due to contamination of Mexican-style cheese. This outbreak caused 142 people to become ill; of these, 93 cases were pregnant women or perinates. There was a total of 48 deaths, of which nearly all cases were in perinates or immunocompromised individuals (96). Of the 17 most noteworthy listeriosis outbreaks in the U.S. from 1979 through 2008, seven were caused by RTE meat products, and seven were caused by dairy products (155). However, a closer look at the latter end of this timeline reveals a shift from RTE meats to other RTE foods. Additionally, a summary of the 24 confirmed outbreaks of listeriosis in the U.S. between 1998 and 2008 shows that nine out of the ten outbreaks from RTE meats occurred in the early part of the timeline, while different types of RTE items (nacho/taco salad, sprouts) appeared later (22).

A recent review of U.S. outbreaks has revealed produce specifically as a reoccurring commodity linked to outbreaks of listeriosis (19). These outbreaks were linked to produce items including celery in 2010 (62), romaine lettuce in 2011 (165), sprouts in 2014 (30), stone fruits in 2014 (76), caramel apples in 2014-2015 (4), and bagged lettuce in 2015-2016 (137). An outbreak involving frozen vegetables in four states in the U.S. also occurred recently, with cases identified from 2013 to 2016 (25). The most notorious U.S. listeriosis outbreak involving produce (to date)

was in 2011, when cantaloupe grown at a farm in Colorado was identified as the commodity responsible for 147 cases of listeriosis in 28 states. Of these cases, at least 143 were hospitalized, and 33 died (101).

Although foods such as RTE red meats and produce have had a shifting prevalence as sources of outbreaks, dairy associated outbreaks have remained fairly constant (19, 140). Between the years of 1998 and 2011, 90 outbreaks of *L. monocytogenes* occurred in the U.S. which were linked to dairy products. Approximately half of these outbreaks were from products made with pasteurized milk (64). From 2010 to 2015, an outbreak associated with ice cream products from a single company caused illness in 10 individuals in four states (116). Listeriosis outbreaks associated specifically with soft cheeses have even increased since the mid-2000's. Most of these were from cheese products that had undergone pasteurization, indicating that the products were contaminated at some point after this process (78). However, one of the most recent U.S. listeriosis outbreaks was confirmed to be from an unpasteurized soft raw milk cheese product, causing 2 deaths out of the 8 people affected. This outbreak spanned from September 2016 to March 2017 and caused illnesses in four states (26).

A review of outbreaks occurring in Europe, the U.S. and Canada from 2005 to 2008 has demonstrated that although the incidences of outbreaks in food items like deli meats have declined, they have not disappeared (148). In the U.S., an outbreak occurring in late 2018 due to contaminated RTE pork led to the hospitalization of four individuals in four states (29). Outside of the U.S., the largest outbreak to date occurred in South Africa by contamination of RTE meat. Over 1,000 cases and over 200 deaths were reported from 2017 to 2018 due to the presence of

Listeria within a facility producing a RTE meat known as polony (2, 125). Although many efforts have been made to reduce the occurrence of outbreaks from *L. monocytogenes*, it remains to be a significant public health threat and can have devastating effects if not properly controlled for.

Detection of *Listeria monocytogenes*

To verify that *L. monocytogenes* is the cause of illness in suspected cases of invasive listeriosis, patient samples are collected from blood or cerebrospinal fluid. In patients with gastroenteritis, stool samples can be used, however these must be first selectively enriched for *Listeria* spp. due to competing bacteria (3). An epidemiologic investigation of a listeriosis outbreak makes use of whole genome sequencing (WGS) to identify clusters of listeriosis cases with information from patient samples. This method of identification was employed in 2013 as a major upgrade to the previous standard known as pulse-field gel electrophoresis or PFGE, which differentiates fragments of DNA based on molecular weight (27, 61, 77). WGS is able to analyze DNA sequences in more detail to account for differences and similarities not evident with PFGE (27, 77). Once clusters have been established, information collected from patients (i.e. food consumption history) is applied to determine a common food source that could be causing the outbreak (27). However, the prolonged incubation period of *L. monocytogenes* often creates difficulties for food recall in affected individuals (59). Suspected food products and environmental samples from their production facilities are tested for the pathogen using the same methods as clinical isolates (77).

The zero-tolerance policy for *L. monocytogenes* has motivated many RTE food production facilities to routinely test their products and production areas for the presence of *Listeria*. Often, these samples are sent to third-party laboratories for analysis. To test food samples for *L. monocytogenes* (or *Listeria* spp.), they are first enriched in selective media (69). Most selective medias for *L. monocytogenes* contain (in addition to required nutrients) high salt concentrations, nalidixic acid, acriflavine, esculin and ferric ions (68, 107). Salt, nalidixic acid, and acriflavine inhibit growth of most other bacteria besides *Listeria* spp. (68). Esculin is used to differentiate colonies as *Listeria* spp. is able to hydrolyze it, producing 6,7-dihydroxycoumarin which reacts with ferric ions to produce a dark color. These colonies can then be used for further testing. For routine analyses, *L. monocytogenes* is detected using automated immunoassays including enzyme-linked immunosorbent assays (ELISA) or enzyme-linked fluorescent assays (ELFA). Both of these methods make use of antibodies that bind specifically to virulence proteins. If binding occurs, a fluorescent marker will be activated that can then be measured by the instrument. A more precise measurement used in the case of a presumptive positive sample is the polymerase chain reaction (PCR). PCR employs primers that target a specific genetic sequence, which if present will be amplified to high enough levels that it can be detected (80).

Taxonomy and Classification of *Listeria monocytogenes*

The genus *Listeria* is currently comprised of at least 17 recognized species, including *Listeria monocytogenes*, *Listeria grayi*, *Listeria innocua*, *Listeria welshimeri*, *Listeria seeligeri*, *Listeria ivanovii*, *Listeria marthii*, *Listeria rocourtiae*, *Listeria fleischmannii*, *Listeria weihenstephanensis*, *Listeria floridensis*, *Listeria aquatica*, *Listeria cornellensis*, *Listeria riparia*, *Listeria grandensis*, *Listeria booriae*, and *Listeria newyorkensis* (117, 159). Of these,

only *L. monocytogenes* and *L. ivanovii* are pathogenic. However, illness caused by *L. ivanovii* is rare, mainly occurring in ruminant animals. These species have been defined and refined using progressively advancing approaches including numerical taxonomy, biochemical analyses, DNA composition, and 16S rRNA gene sequencing. The same techniques have been used to determine similarities to other organisms within the *Listeriaceae* family. The most closely related genus to *Listeria* is *Brochothrix* (162). *Staphylococcus* and *Bacillus* have also demonstrated high similarity to *Listeria* based on the comparison of their 23S rRNA sequences (127). One major commonality between these closely related genera is the low G+C (guanine and cytosine) content of their DNA (127, 162). A paramount study in 2001 reported the full genome sequences of *L. monocytogenes* and *L. innocua*. Both have circular genomes consisting of 2,944,528 base pairs (bp) and 3,011,209 bp, respectively, with G+C contents of 39% and 37%, respectively. These two species share very similar genomes; most of the differences that do exist between them are known or suspected to be related to virulence (63).

L. monocytogenes has been mainly organized by two types of classification; lineages and serogroups/serotypes (ST). Currently, four lineages are used to group isolates of the species by phenotypic and genotypic similarities. Lineage I consists of different strains belonging to ST 1/2b, 3b, 3c, and 4b; Lineage II consists of strains belonging to 1/2a, 1/2c, and 3a ST; and Lineages III and IV consist of strains belonging to ST 4a, 4b, and 4c. Isolates from lineage I and II are responsible for nearly all outbreaks and sporadic cases of listeriosis in humans (112). Prior to the introduction of lineages, serotyping was used as the primary method for classification (113). *L. monocytogenes* currently consists of at least thirteen ST to include 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7. Previously, *L. monocytogenes* was also included in

serogroups 5 and 6, but the strains associated with them have subsequently been reassigned as *L. ivanovii* and *L. welshimeri*, *L. seeligeri* or *L. innocua*, respectively (49, 112, 136, 162). *L. monocytogenes* serogroups 1/2, 3, and 4 are further organized according to somatic or flagellar antigens. Serogroups 1/2 and 3 are subdivided into ST based on flagellar agglutination, and serogroup 4 is subdivided into ST based on somatic agglutination (133, 162). ST 1/2a, 1/2b, and 1/2c are the most commonly isolated from food products, and ST 1/2a and 1/2b are most often implicated in outbreaks of gastrointestinal listeriosis. Conversely, ST 4b is extremely frequent in outbreaks of invasive listeriosis (144). Additionally, outbreaks occurring in Northern Europe are often attributed to ST 1/2a, while in the U.S. the 4b ST (within lineage I) is most common (112). However, some recent outbreaks suggest that these geographical associations are becoming less distinct (99).

***Listeria monocytogenes*' Cell Structure**

L. monocytogenes' cells normally consist of parallel, short rods with blunt ends that are approximately 1-2 μm long and 0.4-0.5 μm wide. When grown at temperatures between 20 and 30°C, they develop 2-6 peritrichous flagella which aid in motility. Grown below 20°C, flagella still develop but are involved with adhesion rather than motility (162). *L. monocytogenes* is a Gram-positive bacterium, therefore its cell wall consists of a thick layer of peptidoglycan (PG). The PG comprises about 30-40% of *Listeria* cell walls, and helps to protect the cell from changes in osmotic pressure. It is made up of glycan chains of N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) disaccharide units, which are cross-linked by pentapeptide (L-alanine-D-glutamic acid-meso-diaminopimelic acid-D-alanine-D-alanine) stems. *Listeria* PG is the A1- γ type, which is characterized by cross-linking at the meso-diaminopimelic acid; in this

case, to the penultimate alanine of another stem (39, 50, 51). Modifications to the PG are important for bacterial survival in the host. It was found that O-acetylation (catalyzed by OatA) of PG MurNAc and N-deacetylation of PG GlcNAc is essential for *L. monocytogenes*' resistance to lysozyme, a cell wall hydrolyzing enzyme produced by the host (66, 82, 118). N-deacetylation of the PG MurNAc also aids in *Listeria*'s escape from the hosts' innate immune system (14).

Structures and Functions of Teichoic Acids

All Gram-positive bacteria possess within their PG glycopolymer structures that extend to the cell surface. The most abundant and well-studied of these structures are the teichoic acids. Teichoic acids consist of both lipoteichoic acids (LTA) and wall teichoic acids (WTA) (157). Both are important for cell survival; it was shown that *B. subtilis* mutants without either LTA or WTA are not viable (129). LTAs are not attached to the PG but anchored to the membrane of the cell via a glycolipid. LTAs are amphiphilic molecules; *L. monocytogenes* possesses type I LTAs which consist of an unbranched polyglycerol-phosphate chain that may have subunits substituted with D-alanine and galactose residues. Mutations which affect LTA synthesis can lead to cells with increased sensitivity to lysozyme and antibiotics. Additionally, LTA deficient cells are impaired in cell division and form smaller colonies, and have decreased abilities for biofilm formation, cation homeostasis, and virulence (1, 121, 156). Similar effects have been observed in cells lacking WTA (18, 121, 145).

Although WTA and LTA share many functions, they also possess some important distinctions. WTA polymers of *Listeria* do not contain a hydrophobic portion; they are made of 20-30 repeating units of a ribitol molecule that usually includes glycosyl substituents such as GlcNAc,

glucose, galactose, rhamnose, or hexose (81, 139). Additionally, LTAs have not been shown to demonstrate much variation (23, 50, 157), but WTA are highly diverse, as *L. monocytogenes* utilizes different combinations and positions of the aforementioned glycosyl substituents to modify their structure. WTA are in fact the O-antigens of the *Listeria* cell and are essential for differentiating between different ST. There are two types of WTA structures in *L.*

monocytogenes. Type I WTA consist of a ribitol with glycosyl substitutions bound to C2 and/or C4, and are connected to each other through phosphodiester bonds between C5 and C1. Type II WTA also have a ribitol backbone, but C2 or C4 is bound to a GlcNAc. This GlcNAc is linked through a phosphodiester bond to C1 of the next repeat unit. However, it is possible that this substitution is not acetylated, i.e., replaced by glucosamine (45, 108, 139).

The repeating unit of serogroup 1/2 strains is a type I WTA, which consists of a ribitol molecule attached to GlcNAc at C2 and rhamnose at C4. This structure has been elucidated using a number of different methods. After extraction of the WTA from the cell wall followed by purification and hydrolysis, samples have been analyzed for molecular weight by gel filtration chromatography (58, 84); for chemical composition by gas-liquid chromatography (GLC) (52, 84) coupled with flame ionization detection (FID) (161) or mass spectrometry (MS) (81), ultra-performance liquid chromatography (UPLC) with electrospray ionization (ESI) and tandem MS (MS/MS) (139) or ESI-MS/MS (46); for molecular connectivity within structures by Smith degradation, acid hydrolysis, and oxidation/reduction reactions (58, 84, 150); and anomeric configurations by nuclear magnetic resonance (NMR) spectrums (81, 139). Similar methods were used to elucidate the WTA structures of *L. monocytogenes* within serogroups 3 (46, 52, 58, 139, 150), 4 (46, 52, 58, 139, 150, 161) and 7 (52, 139, 150). Serogroups 3 and 7 also elicit type

I WTAs. The repeating WTA unit of serogroup 3 strains is the same as that of serogroup 1/2 strains, but without the rhamnose substitution. Serogroup 7 strains have a WTA monomer structure consisting of a ribitol that may be unsubstituted, or bound to a hexose at positions C2 or C4 (45, 139). Serogroups 3 and 7 were in fact discovered to be mutants of the 1/2 serogroup. Serogroup 3 strains have a single nucleotide polymorphism (SNP) mutation in at least one of the genes necessary for WTA rhamnosylation (*lmo 1080*, *lmo1081*, *lmo1082*, *lmo1083*, or *lmo1084*). Serogroup 7 strains have a SNP mutation in at least one of the genes required for WTA rhamnosylation, and at least one of the genes required for WTA N-acetylglucosaminylation (*lmo1079*, *lmo2549*, or *lmo2550*) (47).

Serogroup 4 differs from serogroups 1/2, 3 and 7 as it contains type II WTAs, and its ST are divided by somatic antigens rather than flagellar antigens. As such, its different ST represent much more variable WTA structures than serogroups 1/2, 3 and 7. ST 4a WTAs usually consist of a ribitol with a GlcNAc substitution at C2. ST 4b WTAs typically have a glucose and a galactose bound to a GlcNAc at C4. The repeating unit of ST 4c has a galactose substituent connected to GlcNAc on C2, and ST 4d WTAs have a glucose substituent connected to GlcNAc on C4 (45, 58, 139). The WTA structures of ST 4e and 4ab are less well-studied. However, it has been demonstrated that similar to ST 4d, 4ab contains predominantly glucose and GlcNAc, while studies on 4e have found it to contain GlcNAc with either galactose or glucose as the primary glycosyl units (50, 58).

Many of the studies that analyzed *L. monocytogenes* ST 1/2, 3, or 7 WTAs also found glucose as a minor component of the WTA composition. This glucose is not part of the WTA repeating

unit; rather it is part of the linkage unit which connects the WTA polymer to the PG. In these ST, the unit consists of glucose-glucose-glycerol-phosphate-ManNAc-GlcNAc, in which the glucose end is bound to the WTA polymer by a phosphodiester bond, and the GlcNAc end is bound to the MurNAc of the PG by a phosphodiester bond. The whole unit is fairly conserved amongst the species; however ST 4b and 4d have a glucose-GlcNAc unit instead of glucose-glucose, and ST 4a and 4c have only GlcNAc in this position (84, 88, 139). The ManNAc-GlcNAc-glycerol-phosphate segment seems to be highly conserved amongst organisms in the *Listeriaceae* family, as it is also found in *B. subtilis* and *S. aureus* (33, 84).

WTA comprise between 30 and 75% of the dry weight of the cell wall (10, 17, 51, 58, 145, 151). It is estimated that there is one WTA polymer unit attached to approximately every tenth MurNAc of the PG (52). In *S. aureus*, the placement of WTA regulates the cross-linking of the PG (8). Additionally, placement of WTA throughout the cell wall ensures the proper spacing of autolysins and cell-division machinery; WTA deficient cells do not divide properly and have an altered shape (157). The presence of many phosphate groups within the structure of the WTA makes them negatively charged. This phosphate store can subsequently provide a reservoir for magnesium (Mg^{+2}) as well through the formation of ionic bonds (93, 108). WTA are also indispensable for survival in the harsh environments of the host (17). They have been found to affect the expression of virulence factors involved in adherence (LAP) to and invasion (InlB) of host cells. *L. monocytogenes* cells pre-treated with tunicamycin, an antibiotic that disrupts WTA biosynthesis, had reduced levels of both these virulence factors (166). In addition to attachment to host cells, WTAs also play a role in adherence to abiotic surfaces (i.e. biofilm formation) (17, 145). In a recent study, biofilm resistance to rinsing and cleaning was assessed in the EGE-e (a

ST 1/2a strain) wild-type strain vs. mutants lacking genes *lmo2549* or *lmo2550*, essential for GlcNAcylation of WTA. Biofilms established by mutant strains were found to detach more easily with washing than wild-type biofilms (15). *L. monocytogenes* ST 4nonb mutants lacking galactose decorations in their WTA were also found to have reduced actin tail lengths, diminishing their capability for cell-to-cell spread in the host (142). Additionally, ST 1/2 mutants deficient in WTA rhamnose have demonstrated a decrease in the functionality of the virulence factors *ami* and *InlB* (24).

Much of the resistance to antibiotics and antimicrobials displayed by Gram-positive pathogens can be attributed to modifications to their teichoic acids. CAMPs are molecules produced by many organisms to defend against bacteria. By lessening the negative charge of their cell wall pathogens are better able to resist such antimicrobials (108, 146). This is achieved through D-alanylation of WTA and/or LTA, in which the structures are modified using D-alanine. This process is controlled by the *Dlt* operon present in many Gram-positive bacteria. In *L. monocytogenes*, LTA are D-alanylated which contributes to their virulence, but this modification has not been found in their WTA (1, 23, 46, 81, 139). Resistance to lysozyme has also been demonstrated in *B. subtilis* through D-alanylation of teichoic acids (66). WTA are necessary for beta-lactam antibiotic resistance in *B. subtilis* and methicillin resistant *S. aureus*, possibly due to their N-acetylglucosaminylation (18). In *L. monocytogenes*, mutants lacking *Fri*, a ferritin-like protein, had decreased resistance to beta-lactam antibiotics due to an inability to upregulate WTA and therefore control autolysin activity (90). Additionally, *L. monocytogenes*' WTA rhamnosylation has been shown to promote resistance to antimicrobial peptides. The rhamnose helps to physically block antimicrobials from contacting the cell membrane (23).

WTA have also been found to play roles in transferring genetic information between cells. A recent study explored the potential of WTA in *B. subtilis* to aid in transformation of genetic material into cells. It was found that competent cells (i.e. cells able to receive genetic material) treated with tunicamycin, an antibiotic inhibiting WTA synthesis, bound significantly less exogenous DNA. It was also found that competent cell WTA were localized around the protein ComGA, found only in competent cells. This suggests that WTA act as scaffolding for exogenous DNA in preparation for transformation into the recipient cell (104). Additionally, it was found that WTA of *S. aureus* and *L. monocytogenes* contribute to horizontal gene transfer by enabling transduction of genetic material (163).

However, one of the most pivotal roles of WTA is ultimately to the detriment of the cell. WTA are the receptors for bacteriophage adsorption to Gram-positive bacteria. Bacteriophages are viruses that specifically infect bacteria in order to replicate. They are used to combat pathogenic bacteria in both clinical and food industry settings (60). It has been demonstrated that genes contributing to WTA glycosylation in *L. monocytogenes* are also associated with phages' ability to adsorb to the host in STs 1/2a (37) and 4b (31). WTA glycosylation is not required for all instances of phage binding, as demonstrated in *S. aureus* (164). However, in *L. monocytogenes*, strains lacking glycosylated WTA have been found to be resistant to phage binding (9, 37, 47, 142). This resistance comes at a cost to the cell, as resistant mutants were found to have attenuated virulence *in vivo* (9, 142).

Wall Teichoic Acid Biosynthesis

The biosynthesis of WTA occurs within the cytoplasm of the cell. As *L. monocytogenes* contains ribitol teichoic acids, the proteins involved in these processes are referred to as the Tar (teichoic acid ribitol) group of proteins as opposed to the Tag (teichoic acid glycerol) group of proteins. The first steps of WTA biosynthesis involve the formation of the linkage unit, initiated by the specific protein TarO (17). TagO and TarO are targets for the antibiotic tunicamycin which is used to inhibit WTA synthesis; when used at higher concentrations, it also inhibits PG synthesis (104, 166). Previously, it was thought that glycosylation of all WTA polymers took place in the cytoplasm, before the molecule was transferred outside of the cell (17). However, a recent study proposed that N-acetylglucosaminylation of *L. monocytogenes* ST 1/2a strains 10403S and EGDe takes place after the molecule has been transferred out of the cytoplasm. This was hypothesized after the discovery that *lmo1079* in *L. monocytogenes*, responsible for moving GlcNAc residues from a C55-P—GlcNAc-lipid intermediate onto the WTA polymer, is an ortholog of YfhO in *B. subtilis*, which glycosylates its LTA in this fashion. Another gene involved in *L. monocytogenes* WTA glycosylation is *lmo2550*, which produces the C55-P—GlcNAc-lipid intermediate. The protein GtcA, encoded by *lmo2549* is thought to act as a flippase, or an enzyme that flips the C55-P-GlcNAc-lipid intermediate across the cell wall. (121). Rhamnosylation also occurs in ST 1/2 strains. The genes *lmo1081*, *lmo1082*, *lmo1083* and *lmo1084* are involved with the biosynthesis of rhamnose in *L. monocytogenes*. Collectively they comprise the *rmlACBD* locus, which is present in STs 1/2a, 1/2b, 1/2c, 3c, and 7. However, ST 3c has a mutation in *rmlA*, and ST 7 has a mutations in *rmlB*, and therefore cannot produce rhamnose. To successfully add rhamnose to WTAs, the gene *lmo1080* (also known as *rmlT*) is required, as it produces a putative rhamnosyltransferase (23).

Bacteriophages

As mentioned, bacteriophage are viruses that exclusively infect and replicate in bacterial cells. They were first characterized and named in 1917 by Felix D’Herelle, and were gaining popularity in treating bacterial infections until the advent of commercial antibiotics in 1940. Phage research continued with vigor in countries such as Poland and Georgia, but in most other parts of the world was largely overlooked in favor of antibiotics. However, research and use of phage has seen a resurgence in western countries over the past 3 decades, primarily in response to antibiotic resistant bacteria (85, 92).

The ability of phages to successfully infect and replicate in their host is dependent on the very specific structure of their organelles. The basic structure of most bacteriophages consists of a capsid head containing genetic material, a tail, a baseplate, and long and short tail fibers. Variation in these features is used to differentiate taxonomical groups. Generally, the long tail fibers are used to probe the surface of a bacterial cell after the phage has contacted it. Once these fibers bind enough receptors, the baseplate moves close to the cell surface, allowing the short tail fibers to interact with receptors. In bacteriophages targeting Gram-positive bacteria, this proximity also allows baseplate enzymes known as virion-associated peptidoglycan hydrolases (VAPGH) to degrade the cell wall enough for the tail to transverse it and insert the phages’ genetic material (60, 67, 122).

Bacteriophages are categorized as either temperate or virulent depending on their replication cycle. Temperate phages initiate a lysogenic cycle, meaning that their injected genetic material is integrated into the bacterial genome as a prophage (85). This is accomplished by a protein known

as an integrase (38). When the bacterial cell is introduced to environmental stress (e.g. UV light), this signals the prophage to transition to a lytic cycle. The lytic cycle, used by both virulent phages and temperate phages, involves the exploitation of the bacterial cell machinery to produce progeny bacteriophage. PG degrading enzymes known as endolysins are produced simultaneously in order to lyse the cell and release the fully constructed virions (44, 85).

***Listeria* Phages**

Phages that specifically infect bacteria in the *Listeria* genus belong to the order *Caudovirales*, which includes the families *Myoviridae*, *Podoviridae*, and *Siphoviridae*. *Listeria* phages are found only within the *Myoviridae* and *Siphoviridae* families, which consist of phages with long, contractile tails or long, non-contractile tails, respectively. The genomes of all *Listeria* phages contain dsDNA (87, 91). *Listeria* phages have been organized by orthoclusters, or groups that share orthologous genes. Five different orthoclusters exist, grouping *Listeria* phages by genome size, G+C content, and morphology. Orthocluster I contains *Myoviridae* *Listeria* phages with a large genome size (~131-138 kb) and approximately 36% G+C content. Orthoclusters II, III and IV contain *Siphoviridae* (B1) *Listeria* phages with a small genome size (~36-43 kb) and approximately 35-40% G+C content. Orthocluster V contains *Siphoviridae* (B3) *Listeria* phages with a medium genome size (~65-67 kb) and approximately 33% G+C content. Phages from the genus *P100virus* (91) in orthocluster I have a capsid head diameter of approximately 86 nm and tail dimensions (length by diameter) of 206 x 18 nm. Phages from orthoclusters II, III and IV have similar head diameters ranging from 53 – 57 nm, but a much larger size range of tail dimensions. Phages in the genus 2671 (orthocluster IV) have the largest tail dimensions at 297 x 8 nm; phages in the genus 2389 (orthocluster III) have tail

dimensions of 160 x 7-10 nm, and phages in the genus P35 (orthocluster II) have tail dimensions of 100 x 8 nm. Phages in orthocluster V have head dimensions of 123 x 44 nm and tail dimensions of 162 x 7-8 nm (38).

Use of *Listeria* Phages Against *Listeria monocytogenes*

Bacteriophage research is primarily focused on exploiting their ability to combat pathogenic bacteria. Phage applications include use within clinical, veterinary, agricultural, and food microbiology sectors (92). One useful role of phage is in the detection of pathogenic bacteria. In 1989, Seeliger & Langer noted the importance of using phage typing to accurately determine *L. monocytogenes* isolates by epidemiologists (135). Although more precise technologies are currently used to identify *L. monocytogenes* isolates during an outbreak (27, 77), phages are still employed to detect for the presence of the pathogen in food products. One method of using *Listeria* phage for pathogen detection involves the use of their endolysins. Endolysins are composed of a cell wall binding domain (CBD) at the C-terminal of the protein, and an enzymatically active domain (EAD) at the N-terminal of the protein. Fusion of the highly specific CBD to a fluorescent marker enables both detection and differentiation of *Listeria* cells to the strain level. This method provides results faster and with higher specificity than those currently employed (i.e. PCR, ELISA) (131). However, CBD that has not bound to cells must be washed away to achieve accurate results, which can be difficult to accomplish in food matrixes. Additionally, this method cannot determine live cells from dead ones. Fortunately, another method of detection known as reporter phage based rapid detection can overcome these shortcomings. In this method, recombinant reporter phage are engineered to emit color/fluorescence upon integration of their DNA into the host cell. Naturally, only live host cells

will be affected by these phage, and the signal indicating a positive for the bacteria is emitted as soon as infection occurs, with no need for washing. A reporter *Listeria* phage engineered to encode luciferase could detect six different strains of *L. monocytogenes* (11).

The main use of *Listeria* phages in the food industry is to eliminate the presence of *L. monocytogenes*. As such, two products have been developed using *Listeria* phages for application in food processing facilities for use on RTE foods (106). They include ListShield™ by Intralytix, Inc. (Baltimore, MD, USA) and PhageGuard Listex™ by Micros Food Safety (Wageningen, Netherlands). ListShield™ is a product consisting of a cocktail of six phages, and was approved by the FDA in 2006 as a food additive in RTE foods for antimicrobial purposes (16, 75, 97, 114). PhageGuard Listex™ (formerly Listex P100) is a phage product that targets *Listeria* strains using just one bacteriophage with a broad host range, P100. It was found to be effective against over 95% of the 250 isolates of *Listeria* tested, including strains from ST 1/2 and 4 (21, 106, 114). Additionally, P100 has been shown to be effective against biofilms of strains representing all ST of *L. monocytogenes* (141). Both ListShield™ and PhageGuard Listex™ are currently approved by the USDA-FSIS as processing aids when applied to RTE meat and poultry products (FSIS Directive 7120.1) (75, 114). In 2006, PhageGuard Listex™ was approved as “generally recognized as safe” (GRAS) by the FDA. In 2014, ListShield™ was re-approved as GRAS (11).

However, considerations regarding the conditions to which phage are applied must be taken into account for them to work properly. Temperature is an important variable which affects the ability of *Listeria* phages to successfully adsorb and/or replicate. It has been demonstrated that different

Listeria phages within the *P100virus* and *P70virus* genera were unable to form plaques at 37°C. Adsorption and plaquing at other temperatures was also varied, and additionally affected by the *L. monocytogenes* strain used (91, 149). Other environmental conditions also have an effect on phage activity. A study by Fister et al. found that P100 is stable under a pH range of 4 – 10, but was inactivated at pH values ≤ 2 and ≥ 12 . It was also found to be stable after storage in 2 M NaCl for 24 h. Lutensol detergent (5%) did not affect phage infectivity after 24, but storage in 5% SDS (sodium dodecyl sulfate) solution after 24 h caused a significant reduction in phage infectivity (53). Conversely, another study which used two different mixtures of 6 and 14 distinct lytic *Listeria* phages found that neither was able to reduce bacterial populations on apple slices at a pH of 4.4 (94). These results highlight that environmental tolerances differ among *Listeria* phages, as well as the variables which much be considered when selecting them for food safety applications.

Resistance to *Listeria* Phages

Although *Listeria* phages have been demonstrated as a viable alternative antimicrobial agent in the food industry, *L. monocytogenes* can still become resistant to them. Resistance develops through genetic alterations, which may be dependent on environmental conditions such as temperature to be expressed phenotypically (86, 149). Phage-resistance may also develop as a direct response to predation by bacteriophage. Genetic mutations which alter WTA biosynthesis and/or glycosylation in *L. monocytogenes* confer resistance to phage. The phage are unable to bind to, and therefore infect such cells. In an environment where infectious phage are present, such mutations are necessary for survival (47). These mutations occur randomly (*de novo* mutations), enabling those bacteria to survive and pass on the same genes to the next generation.

As such, the bacterial population will shift towards one that is resistant to the phages present. However, bacteriophage also mutate and those that exhibit phenotypes capable of overcoming such resistance will be able to propagate, again shifting the balance of the population to bacteria susceptible to phage. This process is called co-evolution, and it is occurring constantly in environments that contain bacteria and bacteriophage able to infect them (89).

In *L. monocytogenes* ST 1/2a strains, bacterial resistance has been observed due to specific genetic alterations which affect WTA glycosylation with rhamnose and/or GlcNAc. In a study by Biemann et al., it was demonstrated that the *Siphoviridae* *Listeria* phage P35 requires both GlcNAc and rhamnose in WTA for phage binding to occur. A temperate *Listeria* phage in the same family, A118, required only rhamnose as a phage receptor in ST 1/2 *L. monocytogenes* strains. Mutant strains of EGDe which lacked either rhamnose or GlcNAc were used to assess which glycosylation units were required for phage binding (13). Denes et al. showed that the phage LP-048 was unable to bind to a *L. monocytogenes* 10403S mutant strain that was lacking rhamnose in its WTA, and the phage LP-125 was unable to bind to mutant strains lacking rhamnose or GlcNAc in their WTA. This study demonstrated that phage-resistance occurs when phage are unable to adsorb to their host, and not through other mechanisms (37). Similarly, it was shown by Eugster et al. that mutations affecting WTA glycosylation in EGDe, another ST 1/2a *L. monocytogenes* strain, resulted in phage-resistance. Specifically, the phages A118 and P40 could not bind to mutants deficient in rhamnose, and the phages P35 and A511 could not bind to mutants deficient in either GlcNAc or rhamnose (47).

It has also been shown that ST 4a, 4b and 4c strains are more sensitive to phages than ST 1/2 or 3, however this is also highly dependent on the specific *L. monocytogenes* strain being tested and not just the ST (153). Phages must be considered on an individual basis to determine their host range and environmental limitations in the development of phage based products for use as antimicrobial agents in food processing facilities.

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Chapter II: Cross-resistance to Phage Infection in *Listeria monocytogenes* Serotype 1/2a

Mutants

Publication Information

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Abstract

Bacteriophage-based biocontrols are one of several tools available to control *Listeria monocytogenes* in food and food processing environments. The objective of this study was to determine if phage-resistance that has been characterized with a select few *Listeria* phages would also confer resistance to a diverse collection of over 100 other *Listeria* phages. We show that some mutations that are likely to emerge in bacteriophage-treated populations of serotype 1/2a *L. monocytogenes* can lead to cross-resistance against almost all types of characterized *Listeria* phages. Out of the 120 phages that showed activity against the parental strain, only one could form visible plaques on the mutant strain of *L. monocytogenes* lacking rhamnose in its wall teichoic acids. An additional two phages showed signs of lytic activity against this mutant strain; although no visible plaques were observed. The findings presented here are consistent with other studies showing mutations conferring phage-resistance through loss of rhamnose likely pose the greatest challenge for phage-based biocontrol in serotype 1/2a strains.

Introduction

The Gram-positive foodborne pathogen *Listeria monocytogenes* is the causative agent of listeriosis. Listeriosis can cause serious health problems for susceptible populations, including septicemia, bacteremia, meningitis, and meningoencephalitis (37). Illness due to listeriosis leads to a high rate of hospitalizations (approx. 91%) and deaths (approx. 16%). Listeriosis in humans is contracted almost exclusively through consumption of contaminated foods (32). The largest *L. monocytogenes* outbreak to-date occurred in South Africa and was caused by contamination of polony, a ready-to-eat meat, leading to over 1,000 illnesses and 200 deaths from 2017 to 2018 (2, 30). *L. monocytogenes* can be particularly problematic due to its abilities to replicate at refrigeration temperatures (31) and to persist for years in food processing facilities (26, 28). *L. monocytogenes* is categorized into at least thirteen different serotypes (ST). ST 1/2a, 1/2b, and 4b are the most commonly associated with human illness (29). From 1998 to 2008, serotype 1/2a caused 40% of the confirmed *L. monocytogenes* outbreaks in the U.S. (8). The largest outbreak of *L. monocytogenes* in the U.S. occurred in 2011 and was caused by cantaloupe contaminated with serotype 1/2a and 1/2b *L. monocytogenes*. Illnesses associated with the outbreak occurred in 28 states and were responsible for 143 hospitalizations and 33 deaths (25).

Bacteriophages (or “phages”), viruses that specifically target bacteria, have been approved for use in the U.S. since 2006 for control of *L. monocytogenes* in foods and on food processing surfaces (7). There are currently two commercial *Listeria* phage products approved for food safety applications, and, more recently, products have been approved to target *Salmonella enterica*, *Escherichia coli*, and *Shigella* spp. (27). Previous work has shown that phage-resistance is consistently selected for in *L. monocytogenes* populations infected with *Listeria*

phages (15). A screen of *L. monocytogenes* strains that were isolated from European processing facilities from 1987 to 2012 found that phage resistant *L. monocytogenes* only occurred after those processing facilities had used or experimentally tested a *Listeria* phage product; strains that were isolated prior to the intentional introduction of phage were all found to be sensitive (18). In *L. monocytogenes* serotype 1/2a strains 10403S and EGDe, mutations conferring phage-resistance were consistently found in genes that affect known *Listeria* phage binding receptors: terminal N-acetylglucosamine (GlcNAc) and rhamnose (Rha) decorations of the wall teichoic acids (WTA) (15, 17). Surprisingly, no other binding receptors have been conclusively shown to be used by *Listeria* phages that are able to infect *L. monocytogenes* serotype 1/2a strains.

Here, we measure activity of a diverse collection of 120 *Listeria* phages, representing four of the five known orthoclusters, against three mutant strains of *L. monocytogenes* 10403S (serotype 1/2a). *Listeria* phages have been shown to form five distinct genomic clusters, referred to as orthoclusters. Each orthocluster has unique morphological characteristics; four contain siphoviruses and one contains myoviruses (11, 16). None of the phages from orthocluster IV that were available to us showed activity against our serotype 1/2a strains, so they were not included in this study. The lysis profiles of a majority of the phages we tested were previously described (38). The three mutant strains represent three distinct phenotypes of phage-resistance. Two of these mutants were previously sequenced and characterized (15) and the third is characterized here. We aimed to determine if these characterized phage-resistant mutants were broadly resistant to *Listeria* phages and if any phages could overcome these common resistance types.

Materials & Methods

Growth of Bacterial Strains

Propagation host strains 10403S and 10403S-derived mutants (Table 2.1 in the appendix) were prepared from stocks stored at -80°C in Brain Heart Infusion (BHI; Becton Dickinson, Sparks, MD) broth with 15% (wt/vol) glycerol and incubated at 30°C on 1.5% (wt/vol) BHI agar plates. Overnight cultures were prepared by inoculating BHI broth with a single colony from a streak plate, that was prepared less than two weeks prior, and then incubated for 16±2 h at 30°C shaking at 160 RPM. Bacterial lawns were prepared using an agar overlay method as described previously (35) with modifications. The agar underlay used here was prepared in square 6 x 6 grid plates (Simport Scientific, Beloeil, QC, Canada). Forty µL of overnight culture was aliquoted into 4-4.5 mL of 0.7% (wt/vol) modified lysogeny broth (LB) morpholino-propane sulfonic acid (MOPS) overlay agar supplemented with 0.1% (wt/vol) glucose and 1 mM each MgCl₂ and CaCl₂, after equilibrating to 56°C. The mixture was vortexed briefly, poured onto the agar underlay and allowed to solidify for 20-30 min. UTK P1-0001 was isolated from a 10403S culture infected by LP-048 under previously described conditions (15).

Phage Amplification

Phage stocks used for the experiment (Table 2.2 in the appendix) were first amplified to a titer of at least 1×10^7 pfu/mL using the plate lysate method. Plates were prepared in duplicate using an agar overlay method as described previously (38) with modifications. Thirty µL of overnight culture and 100 µL of the phage dilution in saline magnesium (SM) buffer with gelatin (Fisher, Fair Lawn, NJ) was aliquoted into 3-3.5 mL of modified LB MOPS overlay agar after equilibrating to 56°C. The mixture was vortexed briefly, poured onto the agar underlay and

allowed to solidify for 20-30 min. Plates were then incubated for 18-24 h at 25°C. Five mL of sterile SM buffer with gelatin was aliquoted onto each plate with confluent lysis and allowed to sit for 1-2 h. The overlay agar was then carefully broken into pieces and scraped to the side of the plate using a sterile cell scraper (VWR, Radnor, PA). The phage-containing buffer was then siphoned off the plate using a serological pipette, centrifuged at 3000 RPM for 15 min at 4°C to remove debris, and then sterile filtered into an amber vial using a 0.20 µm-pore size surfactant-free cellulose acetate (SFCA) syringe filter (Corning, Incorporated, Corning, NY). Phage stocks were stored at 4°C.

Screening for Phage Activity Against *Listeria monocytogenes* 10403S

From the lab collection, phage stocks with a titer of at least 1×10^7 pfu/mL whose propagation host was not *L. monocytogenes* Mack (ST 1/2a) were tested for activity against *L. monocytogenes* 10403S (ST 1/2a) as described previously (15). Five µL of undiluted phage stocks were spotted onto bacterial lawns of 10403S in duplicate. All phages with a titer $\geq 1 \times 10^7$ pfu/mL, with a propagation host of Mack or with observed activity (visible inhibition of bacterial growth) against 10403S, were included in the study (120 total).

Preparation of Standardized Stocks

Working stocks of each phage were prepared at a theoretical concentration of 1×10^7 pfu/mL based on recent (< 1 month old) titers.

Phage Dilution Spot Assays

Phages were tested for activity and/or plaque formation against 10403S and the three mutant strains relative to their propagation host as described above. Ten μL of phage, either directly from a standardized stock or serially diluted in phosphate buffered saline (PBS) were spotted onto bacterial lawns. All phages were spotted within 4 h of bacterial lawns being poured. Plates were incubated at 30°C for 18-24 h. Phage titers were calculated after observation of visible plaques. Phage activity against each strain was measured as the greatest phage dilution where either visible plaques were observed or visible inhibition of bacterial growth was observed (if no countable plaques were seen) as compared to the propagation host. Inhibition of bacterial growth was confirmed by visual comparison to controls of 10 μL spots of phage-free SM buffer with gelatin or PBS. Three replicates were conducted.

Adsorption Assay

The adsorption of *L. monocytogenes* strains 10403S, FSL D4-0119 and UTK P1-0001 to bacteriophages LP-048 and LP-125 was determined as described previously (15). Working phage stocks of LP-125 and LP-048 at a titer of 1×10^9 pfu/mL were prepared using SM buffer with gelatin as the diluent. Sterile microcentrifuge tubes (2 mL) were prepared with 912 μL BHI, 20 μL of phage (at 1×10^9 pfu/ml) and 9 μL each 1 M MgCl_2 and 1 M CaCl_2 before the addition of either 50 μL bacterial overnight culture (as described above) or 50 μL sterile BHI (negative control). After addition of the overnight culture or sterile BHI, tubes were incubated for 15 min at 30°C with shaking at 160 RPM. Samples were then centrifuged for 1 min at $17,000 \times g$ and 4°C . The supernatant was filter sterilized (0.20 μm -pore size SFCA syringe filter; Corning, Incorporated, Corning, NY), serially diluted in PBS, and enumerated on duplicate plates using

the agar overlay method (described above under ‘Phage Amplification’) to determine the number of unbound phage present. Three replicates were conducted.

Cluster Analysis (Phage Spot Assays)

Heatmaps were generated using the heatmap hierarchical clustering tool from the HIV databases website which can be accessed at

<https://www.hiv.lanl.gov/content/sequence/HEATMAP/heatmap.html>. Clustering parameters included a complete method using Euclidean distances, which were calculated by taking the square root of the usual sum of squared differences distances between elements of vectors X [columns (*L. monocytogenes* strains)] and Y [rows (*Listeria* phages)]. Cluster stability was calculated using standard bootstraps with 100 iterations.

Statistical Analysis (Adsorption Assays)

Log₁₀ reduction was analyzed using JMP (version 13; SAS Institute Inc., Cary, NC). Linear models were constructed with the means of three replicates using strain as a factor. Log₁₀ reduction was used as the model response. A Fit Y by X platform was used to compare means by one way ANOVA using Tukey’s HSD test. Values in Figure 2.5 in the appendix represent the log₁₀ reduction of phage in the supernatant. This was calculated by subtracting the log-transformed concentration of phage in the supernatant by the log-transformed concentration of phage in the negative control. An alpha of 0.05 was used to determine significant differences.

Sequencing and Variant Analysis of 10403S and UTK P1-0001

DNA was extracted using a QIAamp extraction kit (Qiagen, Hilden, Germany) as previously described (15). NexteraXT Library preparation (Illumina, San Diego, CA) and sequencing was conducted at the University of Tennessee Genomics Core. Sequencing was performed on an Illumina MiSeq instrument using 300 bp paired-end read chemistry. Raw reads were uploaded to the NCBI sequencing read archive (SRA IDs SRR9115406 and SRR9115405) and then trimmed with Trimmomatic version 0.35 (6). Trimmed reads were then quality-checked with FastQC version 0.11.7 (3). McCortex (36) was used for variant calling in the *L. monocytogenes* mutant (UTK P1-0001) and control (10403S) isolates. The *L. monocytogenes* 10403S RefSeq assembly (RefSeq ID 376088) was downloaded and used as the reference. The McCortex pipeline was run with joint calling, the “vcfs” target, and a kmer size of 57 (optimum kmer size determined with KmerGenie version 1.7048 (12)). SnpEff version 4.3t (13) was used to annotate the vcf output files.

Results & Discussion

***Listeria* Phages Isolated from NY Dairy Farms Almost All Require Rhamnose in Their Serotype 1/2a Host’s Wall Teichoic Acids**

Out of 120 phages tested, only LP-018 formed visible plaques on FSL D4-0119, the mutant strain of 10403S lacking rhamnose in its WTA (Figure 2.1 in the appendix), and only two other phages from the collection showed any phage activity against the strain (Figure 2.2 in the appendix). This supports previous observations that serotype 3 strains were largely resistant to a collection of 16 *Listeria* phages (24). Although a mutant of a serotype 1/2a strain, FSL D4-0119 would be expected to be classified as a serotype 3 strain. Serotype 3 strains are known to possess

only N-acetylglucosamine decorations in their WTA (11, 16). It has also been shown that loss of rhamnose due to phage selection in a serotype 1/2a strain converts that strain to serotype 3, and that serotype 3 strains typically resemble serotype 1/2 strains that have accumulated a mutation or mutations affecting rhamnosylation genes (17). As spontaneous mutations affecting rhamnosylation of WTAs are consistently selected for in *L. monocytogenes* 1/2a populations infected with bacteriophages (15), it may be common for phage-resistance to emerge under the selective pressure of a single phage that confers cross-resistance to a majority of phages. This suggests that it will be a challenge for phage-based biocontrol to prevent the emergence of phage-resistant mutants in treated environments; however, the risk of *L. monocytogenes* contamination causing human illness may still be considerably reduced as rhamnose has been shown to promote virulence of serotype 1/2 strains by increasing resistance to host antimicrobial peptides (9) and by promoting association virulence factors to the cell wall (10). This suggests that rhamnosylation-affecting mutations may reduce the virulence of *L. monocytogenes*. This is consistent with the rarity of serotype 3 strains association with outbreak or illness (8, 29, 34). If *Listeria* phage applications are expected remain effective against all *L. monocytogenes* strains in the same environment for an extended period of time, it may be critical to include a phage like LP-018 that can infect mutants lacking rhamnose in their WTA. Future studies should be conducted to characterize LP-018 and to determine its binding receptors.

Mutations conferring phage-resistance through loss of GlcNAc in WTA (15) are less problematic for *Listeria* phage applications than those that confer resistance through the loss of rhamnose, as there are many available phages that can infect the GlcNAc deficient 1/2a strain. Thirteen out of the 120 *Listeria* phages could form visible plaques on FSL D4-0014, the mutant strain of 10403S

lacking GlcNAc in its WTA (Figure 2.1 in the appendix), and one additional phage showed phage activity against the strain (Figure 2.2 in the appendix). Phages that can infect FSL D4-0014 include the well-characterized LP-048, which is a myovirus from the *P100virus* genus (21) that may serve as an important model phage for studies on *Listeria* phage applications that aim to reduce the emergence of phage-resistance. Specifically, previous studies of LP-048 infection kinetics showed consistent bursts of approximately 13 phage particles under laboratory conditions (15), and plaquing efficiency was shown to increase by up to 50% at cooler temperatures that are more relevant to food and food processing conditions (35).

L. monocytogenes strain UTK P1-0001 was included in this study as a phage-resistant mutant of 10403S because it showed a phenotype different from the mutants lacking rhamnose and GlcNAc in their WTA, i.e., UTK P1-0001 showed resistance to phage LP-048 and susceptibility to LP-125 (Figures 2.1 and 2.2 in the appendix). Adsorption assays show UTK P1-0001 does not support binding of LP-048 (Figure 2.5a in the appendix), which likely uses rhamnose as its primary receptor. However, UTK P1-0001 does support a reduced level of binding of LP-125 (Figure 2.5b in the appendix), which is unable to bind to the rhamnose deficient mutant. Sequencing revealed that the only mutation present in UTK P1-0001 was a deletion in *LMRG_00544* (GAATA to G at nucleotide position 1,098,886; this variant was supported by 146/146 sequencing reads that covered the position) that would cause a frameshift and early termination, leading to a truncated protein product. *LMRG_00544* encodes RmlC, which is part of the dTDP-L-Rhamnose pathway (19). It is possible that loss of function of RmlC would result in incorporation of dTDP-6-deoxy-D-xylo-4 hexulose (the substrate of RmlC) being incorporated into the WTA instead of L-rhamnose; this would be consistent with our observation that this

mutant has a distinct phage susceptibility phenotype from FSL D4-0119, which lacks L-rhamnose in the WTA. Forty-two of the *Listeria* phages showed visible plaques on UTK P1-0001, and an additional 61 phages showed activity against the mutant strain. Out of the 120 *Listeria* phages tested, only 17 showed no phage activity against UTK P1-0001. This specific type of mutation is likely not a great challenge to *Listeria* phage applications as only a few *Listeria* phages are fully affected by it; however, the mutant strain may be useful for differentiating and better understanding receptor requirements of *Listeria* phages.

Phage Activity was Frequently Observed Without the Formation of Visible Plaques

Efficiency of plaquing (or “plating”) experiments are often conducted to evaluate the host range of bacteriophages (22). Each phage-resistant mutant we used in this study showed several phages that failed to produce visible plaques, yet showed phage activity (Figures 2.1 and 2.2 in the appendix). These phages were capable of lysing or significantly inhibiting the growth of the target strains; however, if we only performed efficiency of plaquing experiments, this activity would not be observed. The formation of a visible plaque is a complex biological process and absence of plaque formation does not necessarily indicate virion inviability (1). If evaluating phages for potential in biocontrol applications, visible plaque formation on all target strains may not be necessary. For example, if constructing a cocktail that is designed to limit the emergence of phage-resistance, it may be effective to include a phage that only shows activity against a phage-resistance type (such as rhamnose deficient WTA); those specific mutations are likely to be rare in the target *L. monocytogenes* population, so as long as the cocktail exerts selective pressure on those mutants, they are unlikely to grow to concentrations capable of causing illness.

Conclusions

We have identified only one bacteriophage, LP-018, capable of infecting all three of the phage-resistant mutants of serotype 1/2a *L. monocytogenes* that were used in the study. We conclude that mutations conferring phage-resistance through loss of rhamnose likely pose the greatest challenge for phage-based biocontrol in serotype 1/2a strains, as we found that they confer resistance to almost all of the *Listeria* phages (119/120) in the diverse collection tested. These results have the potential to aid in the rational design of *Listeria* phage cocktails that aim to reduce the emergence of phage-resistance to ensure long-term efficacy.

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Appendix

Table 2.1: *Listeria monocytogenes* Strains Used in this Study.

Strain	Features	Reference or Origin
Propagation Strains:		
Mack	Lineage II; 1/2a Serotype	(20)
F2365	Lineage I; 4b Serotype	(23, 39)
FSL J1-175	Lineage I; 1/2b Serotype	(4, 33)
FSL J1-208	Lineage IV; 4a Serotype	(14)
Model Strains:		
10403S	Lineage II; 1/2a Serotype	(5)
FSL D4-0014	10403S mutant; nonsense mutation in <i>LMRG_00541</i> ; deficiency of N-acetyl glucosamine in WTA	(15)
FSL D4-0119	10403S mutant; nonsense mutation in <i>LMRG_00542</i> ; deficiency of Rhamnose in WTA	(15)
UTK P1-0001	10403S mutant; frameshift mutation caused by a deletion in <i>LMRG_00544</i> ; truncated RmlC protein	This study

Table 2.2: *Listeria* Phages Used in this Study and Their Propagation Strains.

Phage	Propagation Host
A511	MACK
LP-018	MACK
LP-019	MACK
LP-025	MACK
LP-031	MACK
LP-038	MACK
LP-039	MACK
LP-048	MACK
LP-049	MACK
LP-064	MACK
LP-066	MACK
LP-069	MACK
LP-077	MACK
LP-083-1	MACK
LP-097	MACK
LP-098	MACK
LP-099	MACK
LP-100	MACK
LP-101	MACK
LP-102	MACK
LP-103	MACK
LP-104	MACK
LP-120	MACK
LP-123	MACK
LP-125	MACK
LP-179	MACK
LP-180	MACK
LP-095	F2365
LP-096	F2365
LP-105	F2365
LP-106	F2365
LP-116	F2365
LP-124	F2365
LP-127	F2365
LP-177	F2365
LP-178	F2365
LP-017	J1-175
LP-040	J1-175

Table 2.2 Continued.

Phage	Propagation Host
LP-056	J1-175
LP-009	J1-208
LP-010	J1-208
LP-011	J1-208
LP-012	J1-208
LP-013	J1-208
LP-014	J1-208
LP-015	J1-208
LP-016	J1-208
LP-022	J1-208
LP-023	J1-208
LP-026	J1-208
LP-029	J1-208
LP-032	J1-208
LP-034	J1-208
LP-035	J1-208
LP-036	J1-208
LP-037	J1-208
LP-041	J1-208
LP-042	J1-208
LP-043	J1-208
LP-044	J1-208
LP-045	J1-208
LP-046	J1-208
LP-050	J1-208
LP-051	J1-208
LP-052	J1-208
LP-058	J1-208
LP-059	J1-208
LP-060	J1-208
LP-061	J1-208
LP-062	J1-208
LP-065	J1-208
LP-067	J1-208
LP-070	J1-208
LP-071	J1-208
LP-072	J1-208
LP-073	J1-208

Table 2.2 Continued.

Phage	Propagation Host
LP-078	J1-208
LP-079	J1-208
LP-080	J1-208
LP-081	J1-208
LP-082	J1-208
LP-084	J1-208
LP-086	J1-208
LP-087	J1-208
LP-089	J1-208
LP-090	J1-208
LP-091	J1-208
LP-092	J1-208
LP-108	J1-208
LP-109	J1-208
LP-110	J1-208
LP-111	J1-208
LP-112	J1-208
LP-113	J1-208
LP-114	J1-208
LP-115	J1-208
LP-118	J1-208
LP-121	J1-208
LP-122	J1-208
LP-126	J1-208
LP-128	J1-208
LP-129	J1-208
LP-130	J1-208
LP-131	J1-208
LP-132	J1-208
LP-133	J1-208
LP-134	J1-208
LP-135	J1-208
LP-136	J1-208
LP-137	J1-208
LP-138	J1-208
LP-139	J1-208
LP-141	J1-208
LP-142	J1-208

Table 2.2 Continued.

Phage	Propagation Host
LP-170	J1-208
LP-171	J1-208
LP-172	J1-208
LP-173	J1-208
LP-174	J1-208
LP-175	J1-208

Figure 2.1: Average Efficiencies of Plaquing Heatmap. Values represent the titer of each *Listeria* phage on each bacterial strain compared to the titer on the phage's propagation host. "No visible plaques" indicates that no titer was obtained, but does not necessarily indicate no activity (Fig. 2.2). Colored dendrogram lines indicate stability of clustering. Bootstrap probabilities of $\geq 70\%$ are highlighted in red; bootstrap probabilities of $\geq 90\%$ are highlighted in blue. *Listeria* phage names are colored to indicate genome size categories. Purple indicates a large genome size (approx. 97 to 140 kb), predicted to be within Orthocluster I; green indicates a medium genome size (approx. 57 to 70 kb), predicted to be within Orthocluster V; orange indicates a small genome size (approx. 31 to 43 kb), predicted to be within Orthoclusters II-IV. **Bold** font indicates genome size obtained by sequencing; *italic* font indicates genome size obtained by PFGE. 10403S possesses WTA with terminal GlcNAc and Rha; FSL D4-0014 possesses WTA with terminal Rha; FSL D4-0119 possesses WTA with terminal GlcNAc. The means of three replicates are shown (See Fig. 2.3 for values from each replicate).

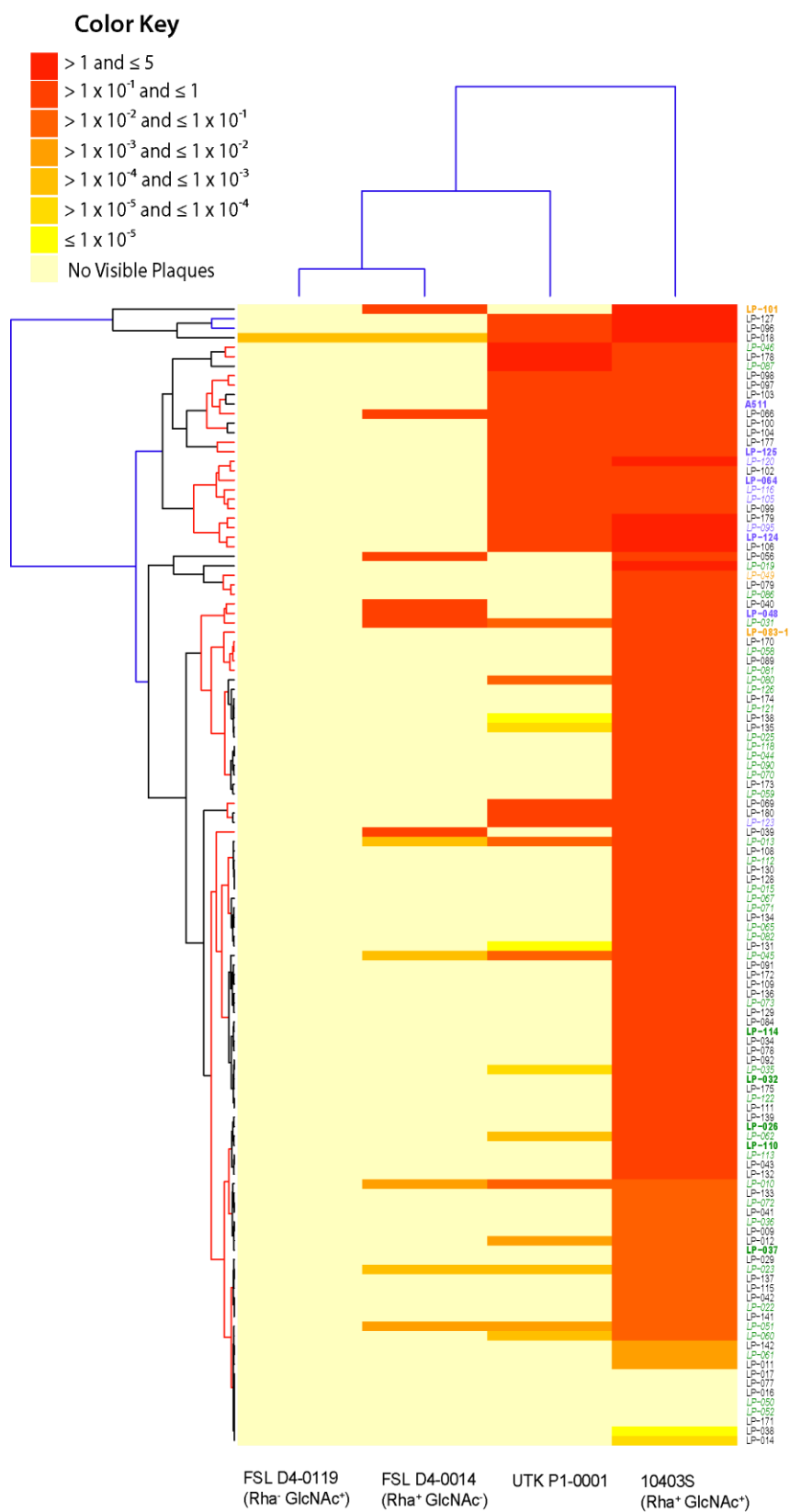


Figure 2.2: Average Relative Phage Activities Heatmap. Values represent the highest dilution of *Listeria* phage with visible activity against the specified bacterial strain compared to the highest dilution of that phage showing activity against its propagation strain. Colored dendrogram lines indicate stability of clustering. Bootstrap probabilities of $\geq 70\%$ are highlighted in red; bootstrap probabilities of $\geq 90\%$ are highlighted in blue. *Listeria* phage names are colored to indicate genome size categories. Purple indicates a large genome size (approx. 97 to 140 kb), predicted to be within Orthocluster I; green indicates a medium genome size (approx. 57 to 70 kb), predicted to be within Orthocluster V; orange indicates a small genome size (approx. 31 to 43 kb), predicted to be within Orthoclusters II-IV. **Bold** font indicates genome size obtained by sequencing; *italic* font indicates genome size obtained by PFGE. 10403S possesses WTA with terminal GlcNAc and Rha; FSL D4-0014 possesses WTA with terminal Rha; FSL D4-0119 possesses WTA with terminal GlcNAc. The means of three replicates are shown (see Fig. 2.4 for values from each replicate).

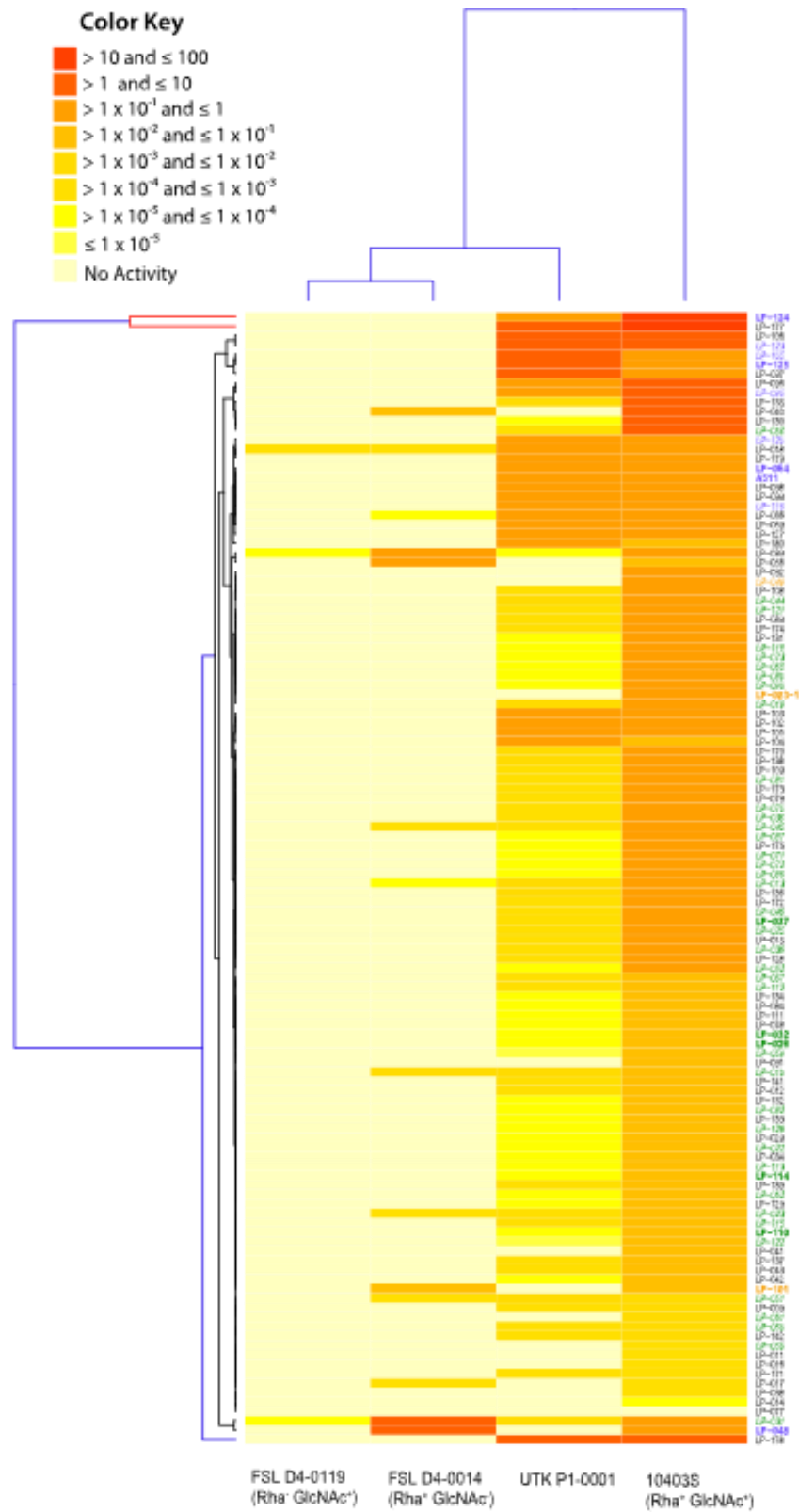
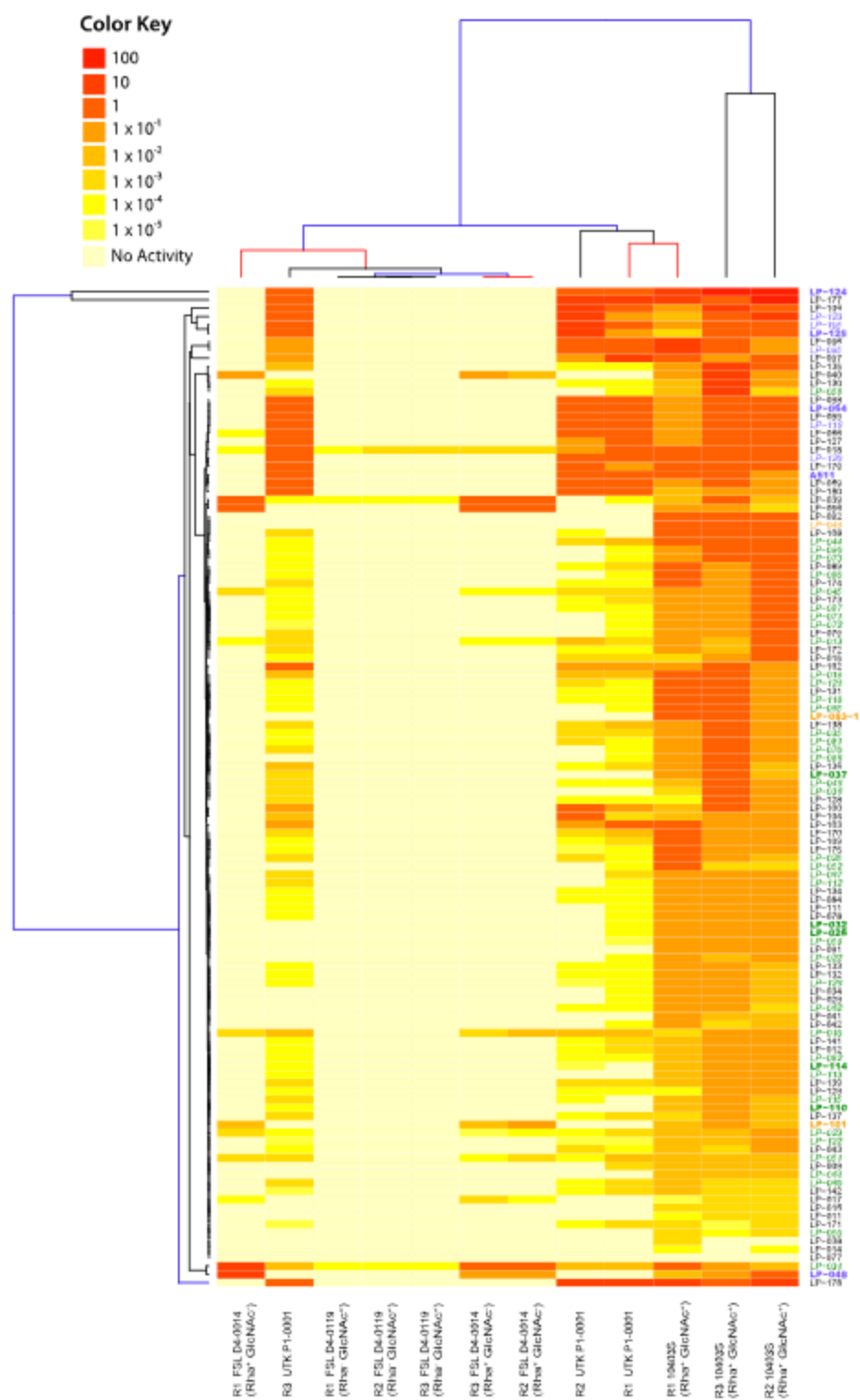


Figure 2.3: Efficiencies of Plaquing Heatmap. Values represent the titer of each *Listeria* phage on each bacterial strain compared to the titer on the phage's propagation host. "No visible plaques" indicates that no titer was obtained, but does not necessarily indicate no activity (Fig. 2.2). Colored dendrogram lines indicate stability of clustering. Bootstrap probabilities of $\geq 70\%$ are highlighted in red; bootstrap probabilities of $\geq 90\%$ are highlighted in blue. *Listeria* phage names are colored to indicate genome size categories. Purple indicates a large genome size (approx. 97 to 140 kb), predicted to be within Orthocluster I; green indicates a medium genome size (approx. 57 to 70 kb), predicted to be within Orthocluster V; orange indicates a small genome size (approx. 31 to 43 kb), predicted to be within Orthoclusters II-IV. **Bold** font indicates genome size obtained by sequencing; *italic* font indicates genome size obtained by PFGE. 10403S possesses WTA with terminal GlcNAc and Rha; FSL D4-0014 possesses WTA with terminal Rha; FSL D4-0119 possesses WTA with terminal GlcNAc. The results of three individual replicates are shown (see Fig. 2.1 for mean values).

Figure 2.4: Relative Phage Activities Heatmap. Values represent the highest dilution of *Listeria* phage with visible activity against the specified bacterial strain compared to the highest dilution of that phage showing activity against its propagation host. Colored dendrogram lines indicate stability of clustering. Bootstrap probabilities of $\geq 70\%$ are highlighted in red; bootstrap probabilities of $\geq 90\%$ are highlighted in blue. *Listeria* phage names are colored to indicate genome size categories. Purple indicates a large genome size (approx. 97 to 140 kb), predicted to be within Orthocluster I; green indicates a medium genome size (approx. 57 to 70 kb), predicted to be within Orthocluster V; orange indicates a small genome size (approx. 31 to 43 kb), predicted to be within Orthoclusters II-IV. **Bold** font indicates genome size obtained by sequencing; *italic* font indicates genome size obtained by PFGE. 10403S possesses WTA with terminal GlcNAc and Rha; FSL D4-0014 possesses WTA with terminal Rha; FSL D4-0119 possesses WTA with terminal GlcNAc. The results of three individual replicates are shown (see Fig. 2.2 for mean values).



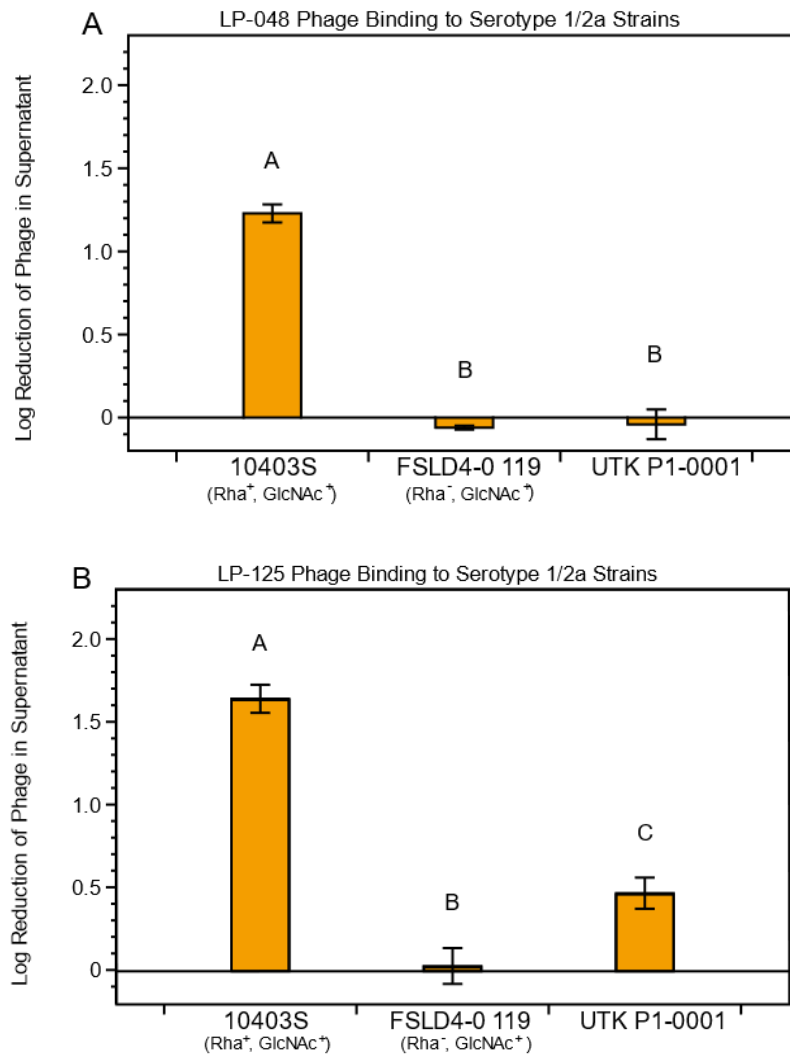


Figure 2.5: Phage Binding of LP-048 (a) and LP-125 (b) to 10403S and Mutant Strains. Panel A shows the binding of LP-048 (requiring rhamnose for binding ST 1/2a strains) to wild-type 10403S as compared to 10403S mutant strain FSL D4-0119, deficient in WTA rhamnose, and 10403S mutant strain UTK P1-0001, a unique and previously uncharacterized mutant. Panel B shows the binding of LP-125 (requiring rhamnose and GlcNAc for binding ST 1/2a strains) to the same strains. Values represent the log₁₀ reduction of phage in the supernatant, which indicates phage binding. In each panel, bars that do not share letters are significantly different from each other. Error bars represent the standard error of the mean.

Chapter III: Preliminary Analysis of the Wall Teichoic Acids of *Listeria monocytogenes*

Serotype 1/2a Mutants

Abstract

Listeria monocytogenes is a Gram-positive foodborne pathogen capable of causing listeriosis, an illness that may result in serious health consequences or death. Wall teichoic acids (WTA) are external cell wall glycopolymers present in Gram-positive bacteria and are important for many cell functions. Additionally, they act as receptors for bacteriophages, viruses that solely infect bacteria. Commercial products have been developed using phages to combat *L. monocytogenes* in ready-to-eat products and in food processing facilities. However, an important issue that must be considered when applying phage products is the bacteria's ability to develop resistance against them. Spontaneous mutations which affect WTA biosynthesis or glycosylation often result in resistance to bacteriophages, as they can no longer successfully adsorb onto their host. The main objective of this study was to develop alternative methods for the purification and hydrolysis of *L. monocytogenes* WTA components, as well as the methods for their derivatization and analysis using gas chromatography with flame ionization detection (GC-FID). The methods for derivatization and GC-FID were also applied to sugar and amino sugar standards. The chromatograms obtained with the methods used here provide peak retention times of the compounds present in the WTA of four *L. monocytogenes* strains. However, this data must be compared to synthesized standards of the presumed WTA monomer units to confirm these findings. A secondary objective was to determine how much the methodology could be streamlined while still providing similar results. It was demonstrated that the WTA monomer can still be detected using GC-FID in cells that have been autoclaved without further refinement. Once further developed, these methods can potentially be applied to rapidly assess the presence of previously determined WTA structures, saving ample time and materials.

Introduction

Listeria monocytogenes is a foodborne pathogen that is known for its potential to cause listeriosis, a disease that may lead to serious illness or death in the young, old, immunocompromised, and pregnant (29). Major outbreaks caused by listeriosis have been associated with various ready-to-eat (RTE) foods, including dairy products, produce, and deli meats (18, 19, 23). Listeriosis currently ranks as one of the most deadly and costly foodborne illnesses in the U.S. (15, 24, 26).

L. monocytogenes is a Gram-positive bacterium, indicated by the thick peptidoglycan (PG) layer comprising approximately 30-40% of its cell wall (10). This substantial layer of PG allows for the placement of cell wall glycopolymers (CWG), attached either to the PG itself or to the cell membrane. In *Listeria*, CWG attached to the cell membrane are the lipoteichoic acids (LTA), and CWG attached to the PG are the wall teichoic acids (WTA) (30). LTA and WTA share some important functions in the cell, including proper cell division and morphology, biofilm formation, ion regulation, and virulence (3, 22). Although *Listeria* can survive without teichoic acids or even a cell wall as L-form bacteria, such cells require very specific growth conditions to do so, and lose functions associated with the cell wall and CWG (5).

Unlike LTA, *Listeria* WTA display considerable variation within their glycosylation units, acting as the O-antigens for the cell and are major determinants of the different serotypes (ST) (4, 7, 25). *L. monocytogenes* consists of at least 13 different ST, including 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4ab, 4c, 4d, 4e and 7 (21). The ST 1/2, 3 and 7 display a type I WTA structure, consisting of a ribitol backbone with either N-acetylglucosamine (GlcNAc), rhamnose, or a hydrogen group

bound to carbons 2 and/or 4. Specifically, the 1/2 ST has a GlcNAc substituent at C2 and a rhamnose substituent at C4 (25). ST 3 WTA units are affected by a mutation in at least one of the genes necessary for WTA rhamnosylation, and therefore are lacking rhamnose in this structure. ST 7 strains lack GlcNAc and rhamnose due to a mutation in at least one of the genes required for WTA rhamnosylation, as well as in at least one of the genes required for WTA N-acetylglucosaminylation (9). Individual type I WTA units are bound at C1 and C5 of the ribitol by a phosphodiester linkage to form polymer chains approximately 21 units long (16, 25).

A pivotal role of WTA is that they serve as the receptors for bacteriophage, viruses that exclusively infect bacteria (13, 30). In order to replicate, bacteriophage inject genetic material into the bacterial host, where the cellular machinery of the host is utilized to produce progeny phage. At the end of this process, the host cell bursts and dies and then releases the progeny phage (13). Research has demonstrated that bacterial resistance to *Listeria* phage occurs when the phage are unable to adsorb to their host. This process is hypothesized to occur through mutations in WTA glycosylation; in *L. monocytogenes* ST 1/2a strains, mutations that affect WTA rhamnosylation or N-acetylglucosaminylation significantly reduce the ability of bacteriophages to adsorb and infect (1, 6, 9).

The structures of *Listeria* WTA have been analyzed using many different analytical methodologies. Usually the methods first require extraction, purification, and hydrolysis of the WTA polymer before the sample can be analyzed. The published methods are time-consuming and laborious. For the analysis of WTA structural components, methods including gel filtration chromatography (12, 17), gas chromatography (11, 17) with flame ionization detection (31) or

mass spectrometry (MS) (16), ultra-performance liquid chromatography with electrospray ionization (ESI) and tandem MS (MS/MS) (25), and ESI-MS/MS alone (8) have been employed. For analysis of molecular connectivity within the WTA structure, Smith degradation as well as general oxidation and reduction reactions have been used (12, 17, 28), and nuclear magnetic resonance (NMR) spectrums have been produced to determine anomeric configurations within the structure of the WTA (77, 133). Although the methods used to determine WTA structures have progressively advanced over the past few decades, there has not been a significant reduction in the time and materials needed to obtain and analyze pure, isolated WTA monomer units.

The primary goal of this research was to develop methods for the preliminary analysis of WTA monomer units of *L. monocytogenes*; these included methods for WTA purification and hydrolysis, derivatization by silylation, and analysis of the products using gas chromatography with flame ionization detection (GC-FID). Sugar and amino sugar standards normally present in *L. monocytogenes* WTA were analyzed using the same silylation and GC-FID methods. Additionally, all three methods were performed on four different strains of *L. monocytogenes*, including the wild-type 10403S, and phage-resistant mutant strains demonstrated to lack rhamnose (FSL D4-0119) or GlcNAc (FSL D4-0014) in their WTA (6), as well as a unique phage-resistant mutant strain isolated in this laboratory (UTK P1-0001) which possesses a truncated RmlC protein (27). Another goal of this research was to determine if the chromatogram peaks seen in the purified sample could still be detected with progressively cruder samples. This streamlining procedure can be applied to rapidly determine the presence or absence of a WTA

monomer standard without the need for a time-consuming, arduous and costly purification process.

Materials & Methods

The methods used for bacterial growth, cell lysis, cell wall treatments, extraction and purification of wall teichoic acids, establishment of the phosphate standard curve, determination of wall teichoic acid containing fractions, and hydrofluoric acid hydrolysis were similar to those used by Eugster et al., with modifications (8).

Bacterial Growth Conditions

Working stocks of *Listeria monocytogenes* strains (Table 3.1 in the appendix) used were stored at -80°C in Brain Heart Infusion (BHI; Becton Dickinson, Sparks, MD) broth with 15% (wt/vol) glycerol. Plates containing 1.5% (wt/vol) BHI agar were used for streaking out working stocks, then incubated at 30°C for approximately 24 h. Erlenmeyer flasks (125 mL) containing 30 mL BHI broth were used for overnight (ON) cultures and inoculated with three similar sized colonies from a streak plate prepared less than two weeks prior. They were then incubated for 16 h at 30°C shaking at 160 rpm. ON culture was added to Erlenmeyer flasks ¼ full with sterile BHI broth in a 1:100 ratio (ON culture to BHI) and incubated at 30°C shaking at 160 rpm. Cells were grown to an OD₆₀₀ between approximately 0.8 and 1.0 (GENESYS 30 Visible Light Spectrophotometer; Thermo Scientific, Waltham, MA), then autoclaved for 30 min at 121.1°C. Flasks were cooled to approximately 4°C in an ice water bath, then the culture was aliquoted into centrifuge bottles and centrifuged (Beckman J2-HS; Beckman Coulter Life Sciences,

Indianapolis, IN) at 7000 x g for 10 min at 4°C to pellet cells. The autoclaved cell pellets were collected and frozen for storage at -20°C.

Cell Lysis

Frozen cell pellets were thawed to room temperature (RT) and resuspended in Saline Magnesium (SM) buffer to a density of approximately 0.75 g cells/mL. Cells were lysed by at least two passages through a French Press Pressure Cell (French Press Cell Disrupter; Thermo Electron Corporation, Milford, MA) at 270 MPa. Lysed cells were centrifuged (Eppendorf 5804 R; Eppendorf, Hamburg, Germany) at 1400 x g for 5 min to remove unbroken cells. The supernatant was collected and centrifuged (Beckman J2-HS; Beckman Coulter Life Sciences, Indianapolis, IN) at 20,000 x g for 30 min at 4°C to recover cell walls. Pellets were collected from the supernatant until no more visible solid material remained (9 collections total per strain) which were then washed twice with sterile ultrapure water (20,000 x g for 30 min at 4°C). Cell wall material was pooled into Nalgene Oak Ridge tubes (Thermo Scientific, Waltham, MA), combined and frozen for storage at -20°C.

Cell Wall Treatments

Frozen cell wall materials were thawed to RT, weighed and resuspended in 10 mM Tris-Cl (pH 7.6) for a combined volume of 36,867 µL. DNase (Alfa Aesar, Tewksbury, MA) working solution was prepared by mixing DNase powder (lyophilized by manufacturer in 2.5 mM calcium acetate and 2.5 mM magnesium sulfate) with sterile ultrapure water to a concentration of 10 mg/mL. RNase (Alfa Aesar, Tewksbury, MA) working solution was prepared by mixing lyophilized RNase powder with 100 mM Tris-Cl (pH 7.6) to a concentration of 10 mg/mL.

Proteinase K (Fisher Scientific, Fair Lawn, NJ) working solution was prepared by mixing lyophilized Proteinase K powder with 10 mM Tris-Cl (pH 7.6) to a concentration of 10 mg/mL. Cell wall materials were mixed with DNase and RNase working solutions (376 μ L each) together with each enzyme at a final concentration of 100 μ g /mL, then inverted 20 times and incubated at 25°C for 3.5 h with two inversions. Following this, 380 μ L of proteinase K working solution was added for a final concentration of 100 μ g /mL, inverted 20 times and incubated at 25°C for 2 h with inversions every 30 min. After enzyme treatments, cell walls were pelleted by centrifugation at 20,000 x g for 30 min at 4°C (Beckman J2-HS; Beckman Coulter Life Sciences, Indianapolis, IN). Supernatant was discarded and pellets were stored overnight at 4°C. Following this, pellets were resuspended with 30 mL 4% (w/v) Sodium Dodecyl Sulfate (SDS) solution and aliquoted into glass tubes (~10-12 mL per tube). Tubes were incubated in water for 30 min at 100°C. After cooling to RT, sample aliquots were re-combined into Nalgene Oak Ridge tubes and SDS-insoluble material (cell pellet) was collected by centrifugation at 20,000 x g for 30 min at 20°C (Beckman J2-HS; Beckman Coulter Life Sciences, Indianapolis, IN). Detergent was removed after washing the pellet five times with sterile ultrapure water at 20,000 x g for 30 min at 20°C. The resulting carbohydrate fraction was then resuspended in 5 mL sterile ultrapure water and transferred into 50 mL centrifuge tubes, frozen on an angle at -20°C, then lyophilized (VirTis Advantage Plus EL-85; SP Scientific, Gardiner, NY) and stored at -20°C with desiccant.

Extraction of Wall Teichoic Acids

The lyophilized carbohydrate fraction of treated cells was mixed with 25 mM glycine/HCl buffer (pH 2.5) in Reacti-Vials (Thermo Fisher Scientific Inc., Waltham, MA) and hydrolyzed for 10 min at 100°C. After cooling to RT, samples were centrifuged (Avanti J-26 XP; Beckman Coulter

Life Sciences, Indianapolis, IN) at 30,000 x g for 30 min at 4°C to pellet insoluble materials. The supernatant was collected and the pellet was resuspended in the same buffer. Hydrolysis and centrifugation was repeated twice; all collected supernatant was pooled and dialyzed (20 mL D-Tube Dialyzer Mega, MWCO 3.5 kDa; MilliporeSigma, Burlington, MA) at 4°C against 2 L ultrapure water for approximately 24 h (with one change of water at 12 h) to remove buffer. The WTA solution was then frozen on a slant at -20°C, lyophilized, and stored at -20°C with desiccant.

Purification of Wall Teichoic Acids

Crude WTA (10 mg) was dissolved in starting buffer (750 µL of 10 mM Tris-HCl, pH 7.5) and manually loaded onto the ÄKTA pure (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) Liquid Chromatography (LC) system. WTA purification was performed with anion-exchange chromatography using a HiTrap DEAE FF Column (5 mL; GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The column was first equilibrated with two column volumes of the starting buffer at a flow rate of 5 mL/min, then 20 column volumes of fractions were collected at a flow rate of 1 mL/min into tubes by elution using a linear gradient of 0-1 M NaCl solution. Starting and elution buffers were filtered through a 0.45 µm Nylon membrane (Whatman – GE Healthcare, Buckinghamshire, United Kingdom) before use in the ÄKTA system. All glassware used for buffer preparation and storage was acid washed with a 10% HCl solution and rinsed with de-ionized (DI) and ultrapure water before use to prevent phosphate contamination.

Establishment of the Phosphate Standard Curve

A working stock solution of phosphate standard with a concentration of 10 mg/L PO₄ was prepared from a phosphate standard solution (Merck, Darmstadt, Germany) with a concentration of 1,000 mg/L PO₄. From the working stock solution, 5 mL phosphate standards were prepared in concentrations ranging from 0 to 5 mg/L PO₄ (Table 3.2 in the appendix). A phosphate test kit (Spectroquant; Merck, Darmstadt, Germany) was applied to each standard as well as an ultrapure water blank as per manufacturers' instructions. Absorbencies of each standard were read in 10 mm cuvettes with a spectrophotometer (GENESYS 30 Visible Spectrophotometer; Thermo Fisher Scientific, Madison, WI) at a wavelength of 690 nm. A standard curve (Figure 3.1 in the appendix) was developed using Microsoft Excel (Version 1811) to establish a linear regression formula ($y = 0.1613x + 0.0094$) for determining unknown phosphate values based on sample absorbencies at 690 nm.

Determination of Wall Teichoic Acid Containing Fractions

Fractions obtained after WTA purification were tested for UV activity using 1 mL samples aliquoted into acid-washed quartz cuvettes. Absorbance was read at a wavelength of 205 nm using the NanoDrop One Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA) against a blank containing a 1:1 mixture of 10 mM Tris-HCl, pH 7.5 and 1 M NaCl. A 30 µL subsample was taken from fractions showing absorbency at 205 nm, diluted to a total volume of 5 mL with ultrapure water, and treated with decomposition reagent (NANOCOLOR NanOx Metal; Macherey-Nagel, Düren, Germany) as per manufacturer's instructions. A blank of ultrapure water was treated the same way. A phosphate test kit (Spectroquant; Merck, Darmstadt, Germany) was then applied to the treated subsamples as well as the blank as per manufacturers'

instructions. Absorbencies were read by spectrophotometry (GENESYS 30 Visible Light Spectrophotometer; Thermo Scientific, Waltham, MA) at a wavelength of 690 nm to calculate phosphate concentration using the formula obtained from the phosphate standard curve ($y = 0.1613x + 0.0094$). Fractions calculated as having over 25 mg/L PO_4 were dialyzed (20 mL D-Tube Dialyzer Mega, MWCO 3.5 kDa; MilliporeSigma, Burlington, MA) at 4°C against 2 L ultrapure water for 24 h (with one change of water at 12 h) to remove buffer. The WTA solution was then frozen on a slant at -20°C, lyophilized and stored at -20°C with desiccant.

Hydrofluoric Acid Hydrolysis

Samples (2-4 mg purified WTA polymer from each strain and 10 mg each streamlining sample) were subjected to hydrolysis using 200 μL Hydrofluoric Acid (HF) (48-51%) at 0°C for 20 h prior to evaporation over NaOH pellets in a chamber under vacuum. Samples were subjected to vacuum until HF evaporation was complete; the sample was then mixed with 500 μL of ultrapure water, frozen at -20°C, lyophilized and stored at -20°C with desiccant.

Wall Teichoic Acid Analysis by Chromatography

Liquid Chromatography with Mass Spectrometry (LC-MS)

LC-MS analysis of the purified WTA monomer of 10403S was performed with an Agilent 1260 series HPLC system (Agilent Technologies Inc., Santa Clara, CA). The system was equipped with an autosampler, a BIN Pump SL binary pump, a TCC SL thermostated column compartment, and a DADSL diode array detector, interfaced to a 6410 triple-quadrupole LC-MS mass selective detector equipped with an API-ESI ionization source. Prior to injection, the sample was dissolved in methanol to a concentration of 1 mg/mL. Chromatographic separations

for 10 μ L injection volumes were performed using a Gemini column (250×4.6 mm i.d.; 5.0 μ m particle size) (Phenomenex, Torrance, CA). The column temperature was set at 25°C and operated at a 1.0 mL/minute flow rate. DI water with 0.1% formic acid (A) and methanol with 0.1% formic acid (B) were employed in the binary mobile phase with a linear gradient of 5-55% B over 50 min; 55-90% over 5 min; elution at 90% for 5 min, followed by re-equilibration over 10 min. Data acquisition and analysis were performed using Mass Hunter Workstation Data Acquisition, Qualitative Analysis, and Quantitative Analysis software. LC-MS analysis was performed in both negative and positive ion mode with ionization parameters set at capillary voltage, 3.5 kV; nebulizer pressure, 35 psi; drying gas flow, 13.0 mL/min; drying gas temperature, 350°C; and mass scan range, m/z 300-2000. Quantitative analysis of the sample was performed in negative ion mode with the same ionization parameters as described above.

Sample Derivatization for Gas Chromatography (GC)

Prior to analysis using gas chromatography (GC), standards and samples were derivatized similarly to the methods used by Munafo et al. (20) with modifications. Approximately 0.5 to 1 mg of the purified WTA monomer sample for each strain and approx. 1 mg each of the streamlining samples were derivatized in Reacti-Vials (Thermo Fisher Scientific Inc., Waltham, MA) at approximately 70°C for 1 h using a mixture of 7 parts anhydrous pyridine and 3 parts BSTFA (N,O-Bis(trimethylsilyl)trifluoroacetamide) with 1% TMCS (Trimethylchlorosilane) for a total volume of 100 μ L (Thermo Scientific, Bellefonte, PA). Standards of GlcNAc, rhamnose, ribitol, galactose, glucose, and glucosamine were derivatized similarly, using 1 mg of each with a total volume of 1 mL derivatizing reagents. Samples were then analyzed using GC coupled with flame ionization detection (FID).

Gas Chromatography – Flame Ionization Detection (GC-FID)

GC-FID (6890 Series; Agilent Technologies, Santa Clara, CA) analysis was conducted similarly to the methods used by Munafo et al. (20) with modifications. GC-FID was performed by manual injection (Hamilton Company, Bonaduz, Switzerland) with 1 μ L of the derivatized sample and a split ratio of 3:30 (1:10). Helium was used as the carrier gas at a flow rate of 1-1.5 mL/min. The oven was set to an initial temperature of 80°C (held for one min) with ramp of 6°C/min to a maximum temperature of 240°C, which was then held 27 min for purified WTA samples (total run time of 55 min), and 15 min for standards and streamlined samples (total run time of 42.67 min). The column used was an HP-5 with capillary size 30.0 m x 320 μ m x 0.25 μ m nominal (Agilent). Data was analyzed using GC ChemStation Rev. A 10.02 [1757] software (Agilent).

Gas Chromatography – Mass Spectrometry (GC-MS)

GC-MS was performed on an Agilent 6890 series gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) coupled to an Agilent 5973 mass spectrometer detector. The capillary column used for chromatographic separation was a fused silica GC column HP-FFAP (30 m $\times$ 0.25 mm $\times$ 0.25 μ m; Agilent). A 1 μ L split/spitless injection (1:1 split) of the derivatized purified WTA monomer of 10403S was made by an autosampler using a 10 μ L syringe. Helium was used as a carrier gas with a constant flow of 1 mL/min. The oven temperature was initially held at 35°C for 1 min followed by an increase in temperature at a rate of 60°C/min until the oven temperature reached 60°C. The oven was then heated at a rate of 6°C/min to reach 250°C and held at this temperature for 5 min. The mass spectrometer detector was coupled to the GC via a

transfer line heated at 250°C and operated in electron ionization (EI) mode at 70 eV. The detector scan range was m/z 50–350.

Results & Discussion

Analysis of Standards and Preliminary Analysis of the Wall Teichoic Acid Monomers of *L. monocytogenes* Serotype 1/2a Mutants using GC-FID

Sugar and amino sugar standards including ribitol, rhamnose, GlcNAc, glucosamine, glucose, and galactose were analyzed using GC-FID (Figures 3.1 - 3.6). The WTA of *L. monocytogenes* wild-type strain 10403S (Rha⁺, GlcNAc⁺) (25) and mutant strains FSL D4-0014 (Rha⁺, GlcNAc⁻) (6), FSL D4-0119 (Rha⁻, GlcNAc⁺) (6), and UTK P1-0001 (truncated RmlC protein) (27) and were also analyzed using the same method (Figures 3.7 - 3.10). All samples contain a peak at ~18.2 min, corresponding to ribitol as demonstrated by the chromatogram of the ribitol standard (Figure 3.1). The wild-type strain 10403S also possesses notable peaks at ~16.2 min, ~30.1 min, and ~41.3 min (Figure 3.7). The mutant strain UTK P1-0001 possesses the same pattern of peaks, however those at ~16.2 min and ~30.1 min are attenuated compared to 10403S (Figure 3.10). The peak at ~16.2 min corresponds to rhamnose, as confirmed by analysis of the rhamnose standard (Figure 3.2). UTK P1-0001 has a mutation affecting genes coding for RmlC (27), an epimerase which is involved in the biosynthesis of rhamnose (14). SnpEff version 4.3t was used to determine that the mutation found in this strain is affecting the end of the protein (nucleotide position at 1,098,886; end of gene at nucleotide position 1,098,895), therefore its functioning may not be completely inhibited (27). Additionally, this strain has demonstrated binding to the *Listeria* phage LP-125, which requires both GlcNAc and rhamnose for adsorption (6, 27). The

possibility of RmlC functioning on some level could also be indicated by the small amount of rhamnose present in UTK P1-0001.

The peak at ~30.1 min is also present in FSL D4-0014, but not in FSL D4-0119 (Figures 3.8 – 3.9). Sequencing data and phage spot tests/adsorption assays demonstrated that FSL D4-0119 is deficient in rhamnose (6). Therefore the peak at ~30.1 min might correspond with a WTA monomer consisting of rhamnose and ribitol. Conversely, the peak at ~41.2 min is present in FSL D4-0119, but not in FSL D4-0014 (Figures 3.8 – 3.9), which is deficient in GlcNAc (6). This could indicate that this peak corresponds to a WTA monomer consisting of ribitol and GlcNAc. To further assess these hypothesized compounds, MS analysis was performed on the purified WTA monomer of wild-type strain 10403S.

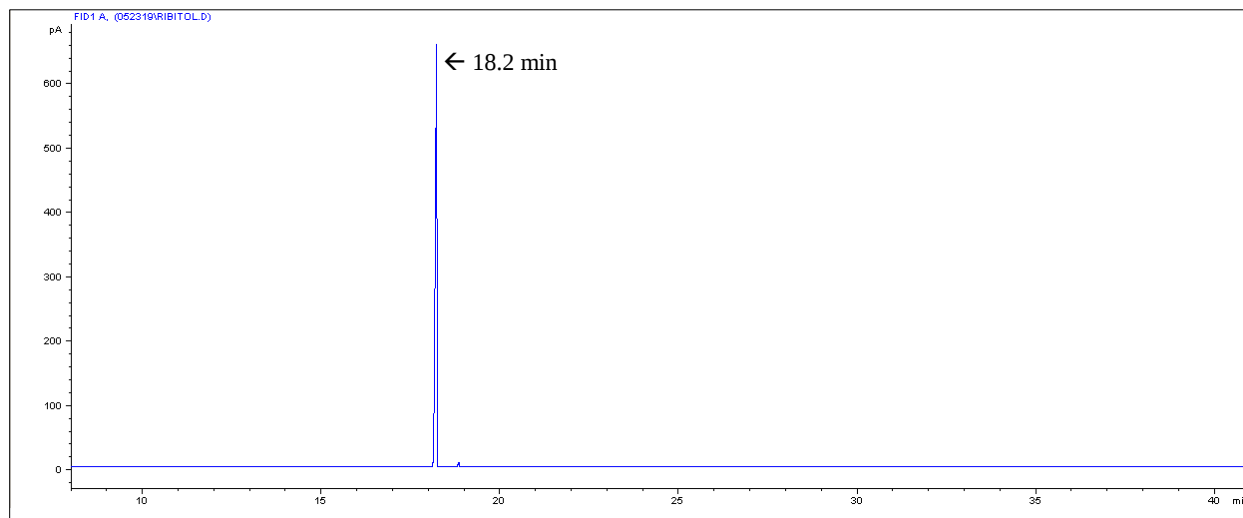


Figure 3.1: GC-FID Chromatogram of Ribitol Standard. Peak retention time of approximately 18.2 min.

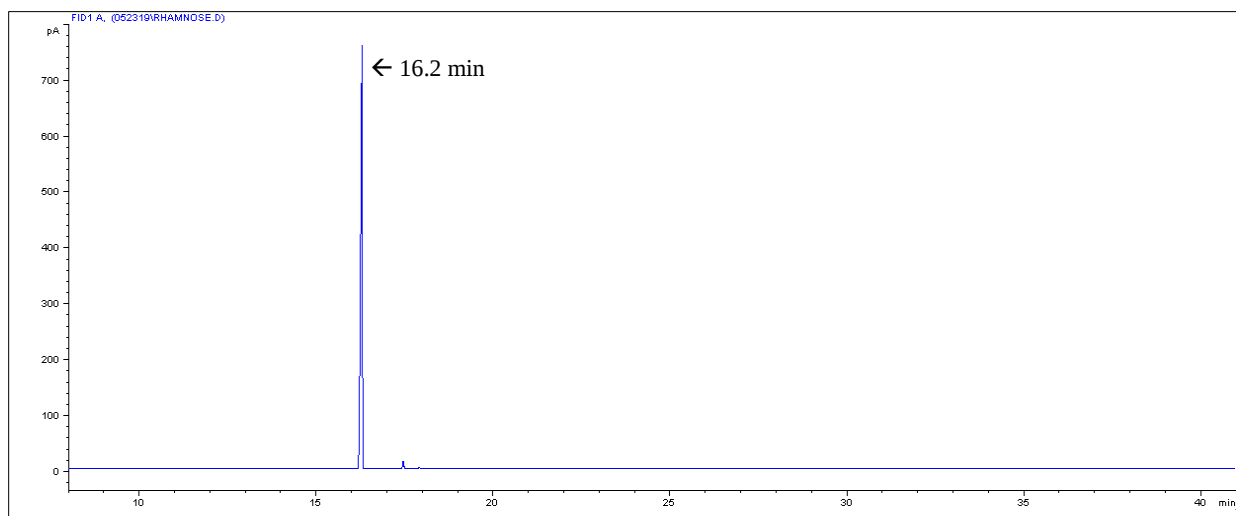


Figure 3.2: GC-FID Chromatogram of Rhamnose Standard. Peak retention time of approximately 16.2 min.

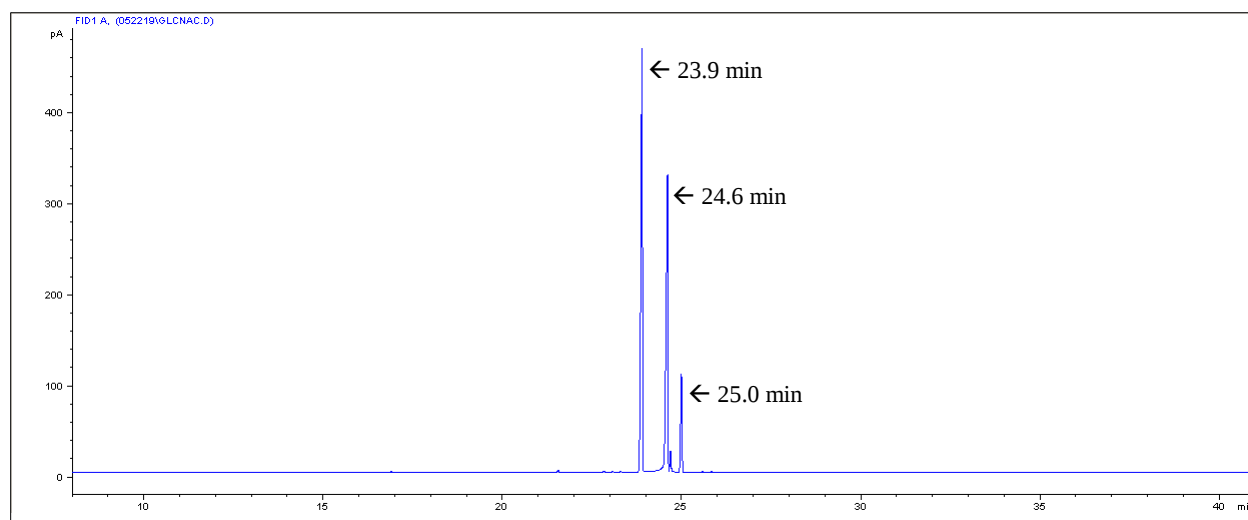


Figure 3.3: GC-FID Chromatogram of N-acetylglucosamine Standard. Peak retention times of approximately 23.9, 24.6 and 25.0 min.

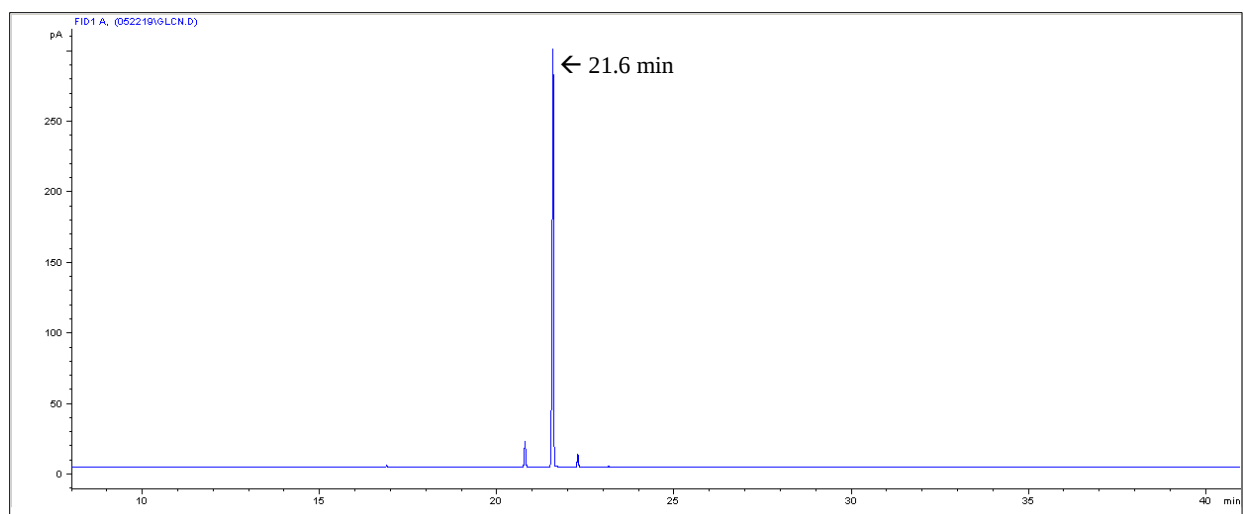


Figure 3.4: GC-FID Chromatogram of Glucosamine Standard. Peak retention time of approximately 21.6 min.

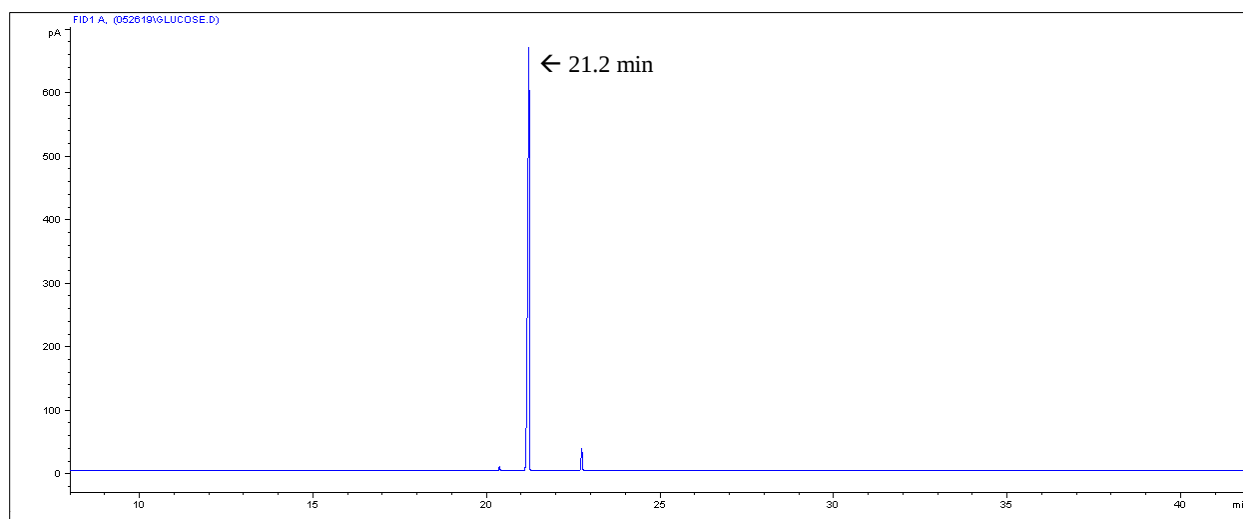


Figure 3.5: GC-FID Chromatogram of Glucose Standard. Peak retention time of approximately 21.2 min.

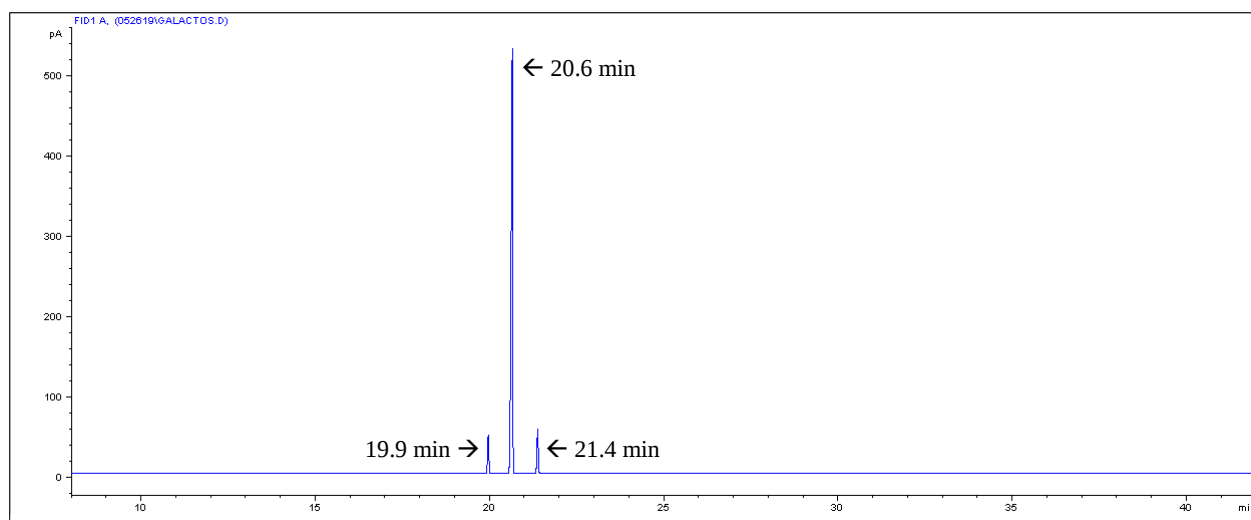


Figure 3.6: GC-FID Chromatogram of Galactose Standard. Peak retention times of approximately 19.9, 20.6, and 21.4 min.

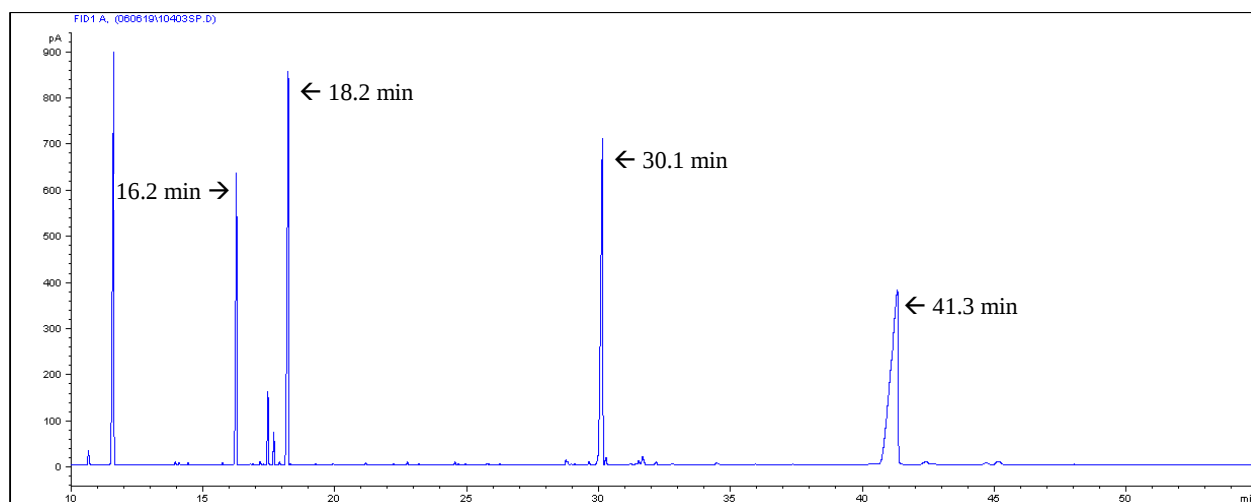


Figure 3.7: GC-FID Chromatogram of Purified WTA Monomer of *Listeria monocytogenes* 10403S. Notable peaks at retention times of approximately 16.2, 18.2, 30.1, and 41.3 min.

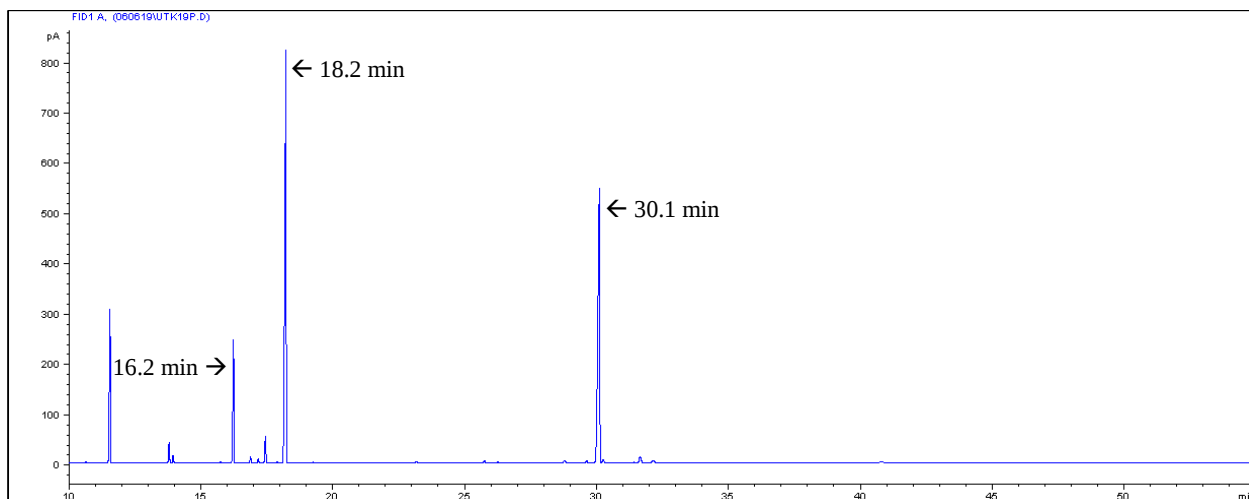


Figure 3.8: GC-FID Chromatogram of Purified WTA Monomer of *Listeria monocytogenes* FSL D4-0014. Notable peaks at retention times of approximately 16.2, 18.2, and 30.1 min.

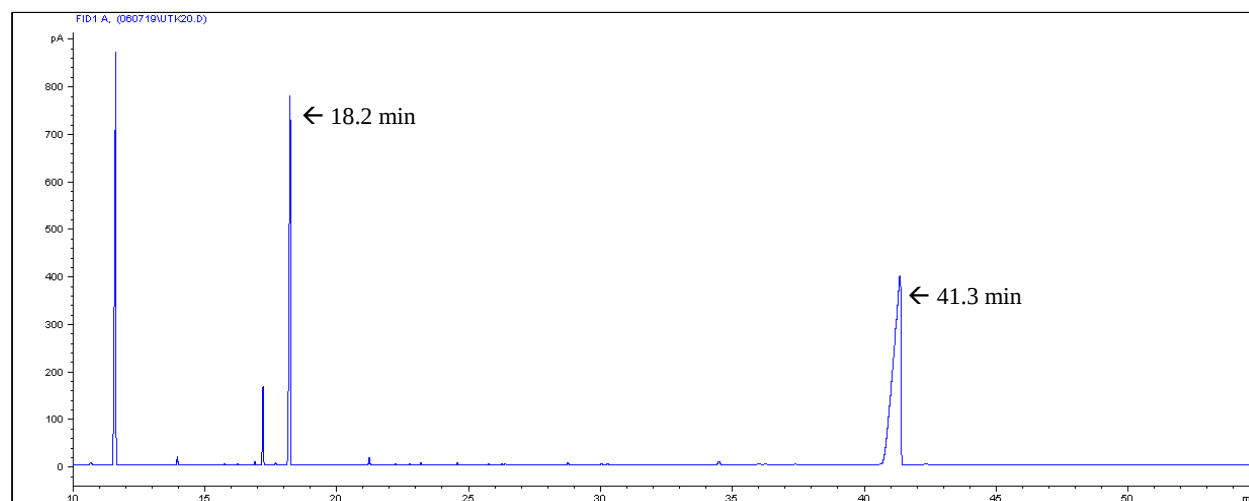


Figure 3.9: GC-FID Chromatogram of Purified WTA Monomer of *Listeria monocytogenes* FSL D4-0119. Notable peaks at retention times of approximately 18.2 and 41.3 min.

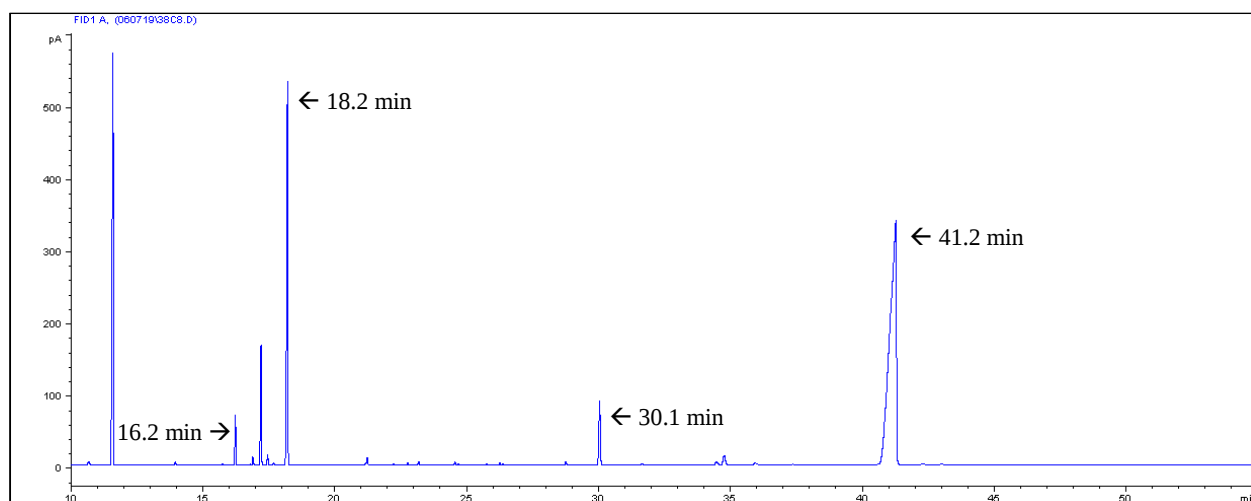


Figure 3.10: GC-FID Chromatogram of Purified WTA Monomer of *Listeria monocytogenes* UTK P1-0001. Notable peaks at retention times of approximately 16.2, 18.2, 30.1, and 41.2 min.

Analysis of Wall Teichoic Acid Monomers of *L. monocytogenes* 10403S using MS

The presence of the full WTA monomer unit of *L. monocytogenes* 10403S (containing ribitol, rhamnose, and GlcNAc) was confirmed using LC-MS (Figure 3.11). The resulting spectra indicated that the major fraction showed a base ion peak m/z 500.2 (100, $[M - H]^-$), consistent with a molecular weight (MW) of 501.2. Also a peak was observed in the MS spectrum at m/z 1001.5 (18, $[2M - H]^-$), consistent with a MW of 501.2. This was the same m/z value previously reported to correspond with the full WTA monomer unit of a ST 1/2a strain of *L. monocytogenes* (8). This supports that the methods used to isolate and hydrolyze the purified WTA monomer unit were successful. To further assess the peaks displayed using GC-FID, GC-MS was performed on the wild-type strain 10403S. Four major peaks were obtained at retention times of 14.4 min, 14.8 min, 25.5 min, and 30.7 min. The spectra obtained from these peaks (Figures 3.12 – 3.15) seem to correspond to the molecular weights of silylated and fractionated molecules of rhamnose, ribitol, a ribitol-rhamnose compound, and a putative ribitol-GlcNAc compound; however, these results must be confirmed by comparison to synthesized standards analyzed using the same methods.

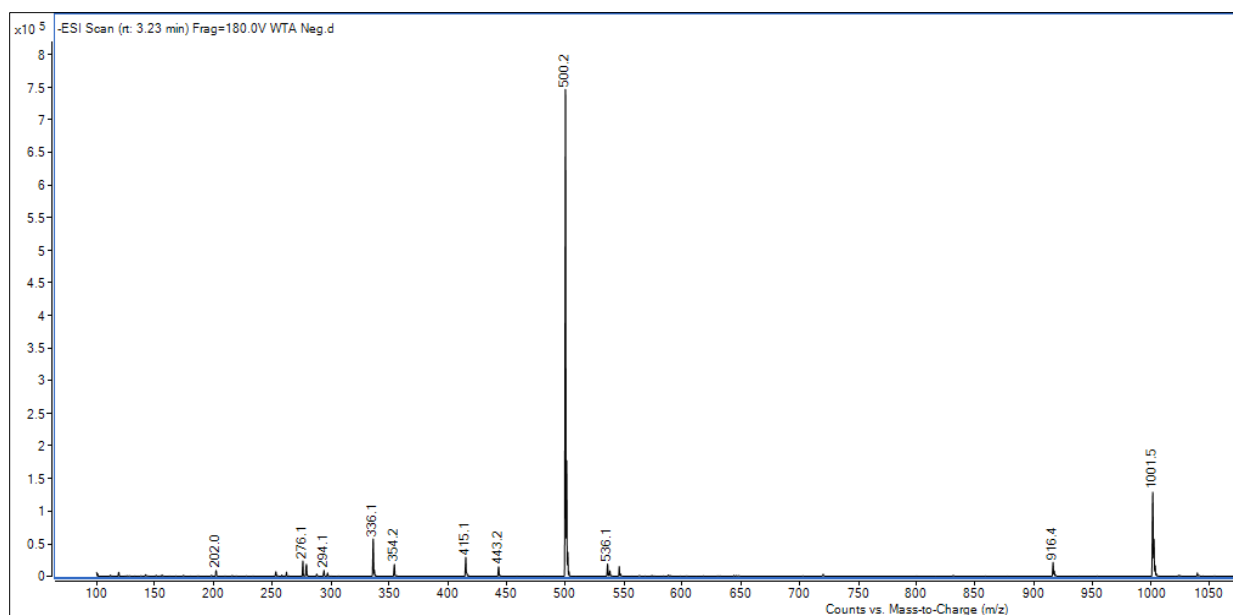


Figure 3.11: LC Mass Spectrum of Purified WTA Monomer of *Listeria monocytogenes* 10403S. Peaks observed at m/z 500.2 (100, $[M - H]^-$) and 1001.5 (18, $[2M - H]^-$).

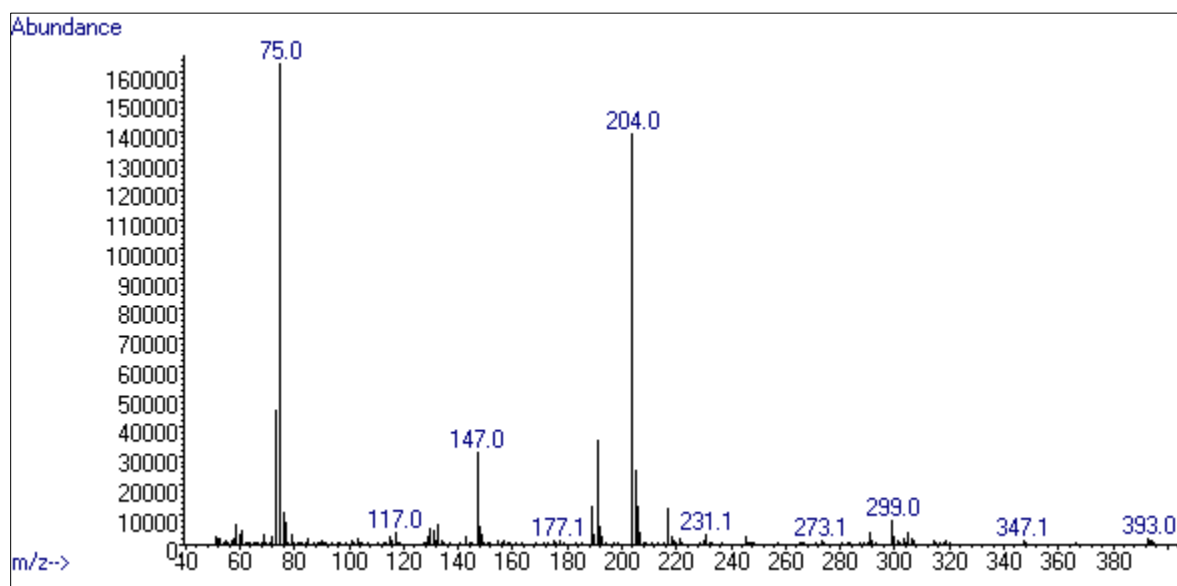


Figure 3.12: GC Mass Spectrum of Rhamnose Peak. Peak retention time of 14.4 min.

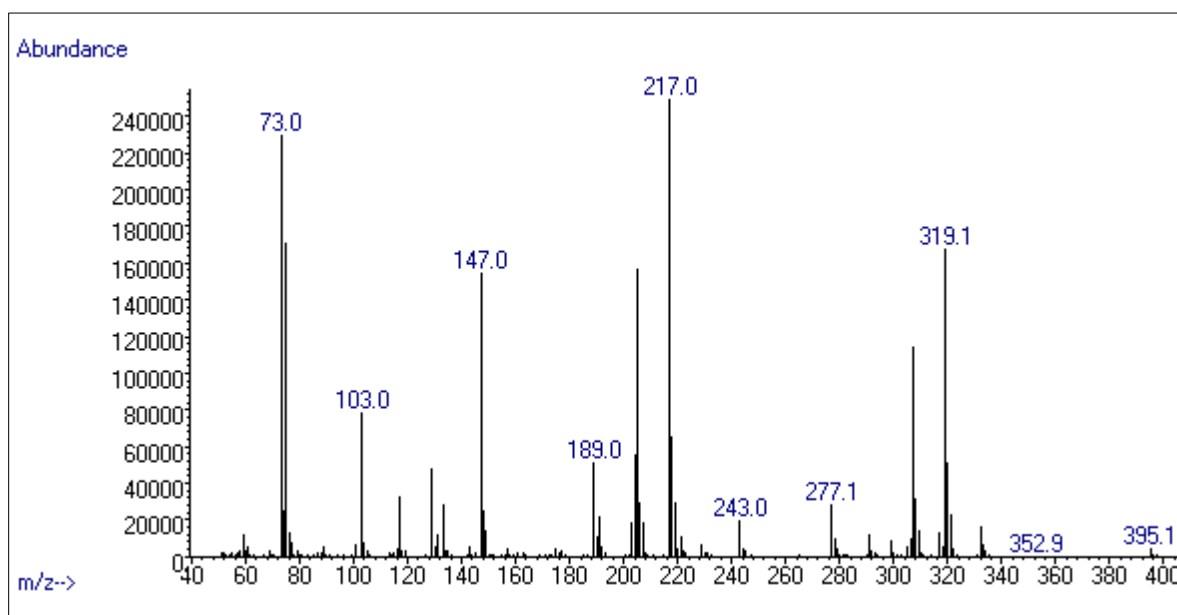


Figure 3.13: GC Mass Spectrum of Ribitol Peak. Peak retention time of 14.8 min.

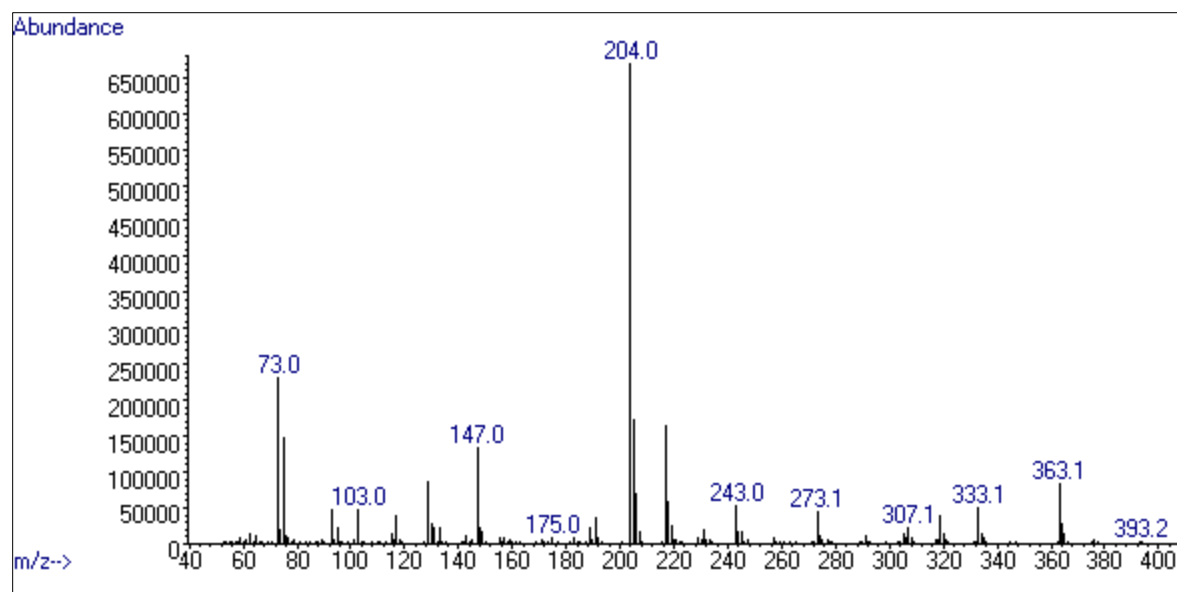


Figure 3.14: GC Mass Spectrum of Ribitol-Rhamnose Peak. Peak retention time of 25.5 min.

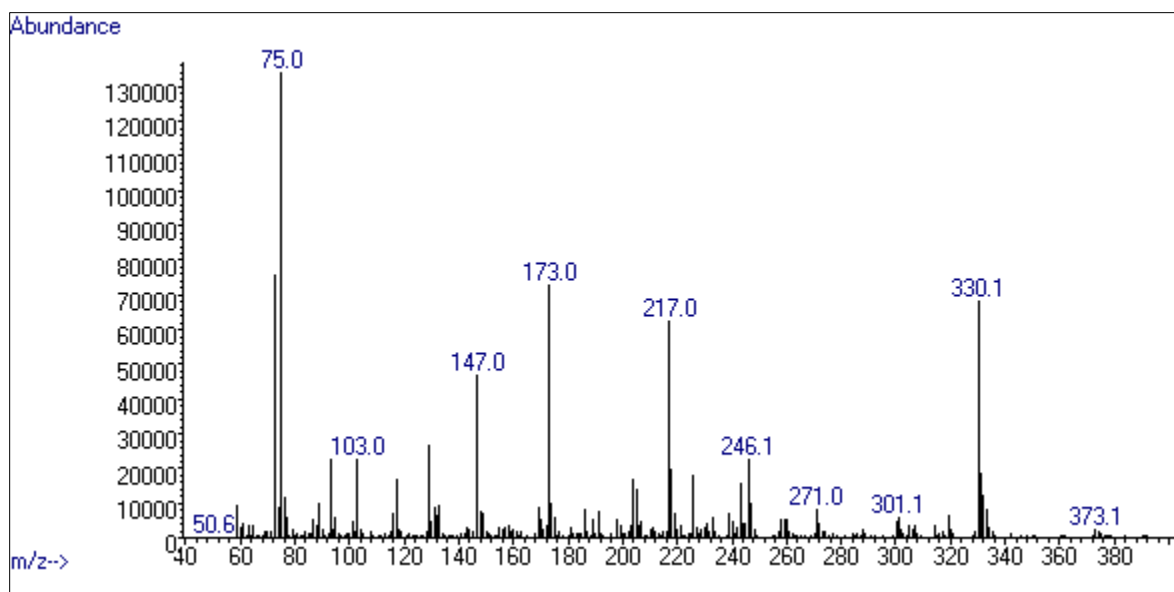


Figure 3.15: GC Mass Spectrum of Putative Ribitol-GlcNAc Peak. Peak retention time of 30.7 min.

Streamlining the Analysis of Wall Teichoic Acid Monomers

Results of the streamlining process indicate that the peaks for the compounds present in the purified WTA sample are indeed still detectable in all samples analyzed, including cells that were only steam-killed (Figures 3.16 – 3.18 below). However, the same amounts of progressively cruder samples produced progressively weaker intensities of the chromatogram peaks. Using higher concentrations of cruder cell materials could aid in obtaining stronger chromatogram peaks for analysis. Additionally, the crudest sample (autoclaved cell pellet) displays many more peaks earlier in the chromatogram (i.e. before ~40 min), as elements of cell proteins, nucleic acid material, lipids, and BHI media were not removed from the sample prior to analysis. There is also a slight shift in retention time for some of the peaks observed in the chromatograms of the less refined samples, the most significant being the peak at ~41.3 min (as observed in the purified WTA sample). This is a factor that should be taken into consideration when analyzing crude cell samples, especially when assessing for the presence of compounds which elute from the column earlier.

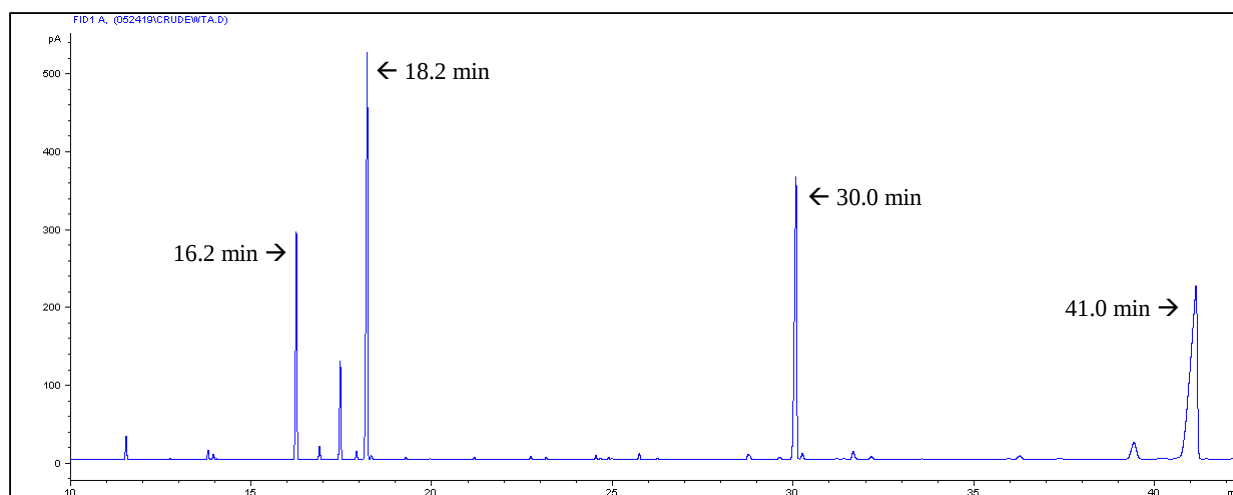


Figure 3.16: GC-FID Chromatogram of Crude WTA Monomer of *Listeria monocytogenes* 10403S. Notable peaks at retention times of approximately 16.2, 18.2, 30.0, and 41.0 min at a slightly decreased intensity compared to the purified WTA monomer sample.

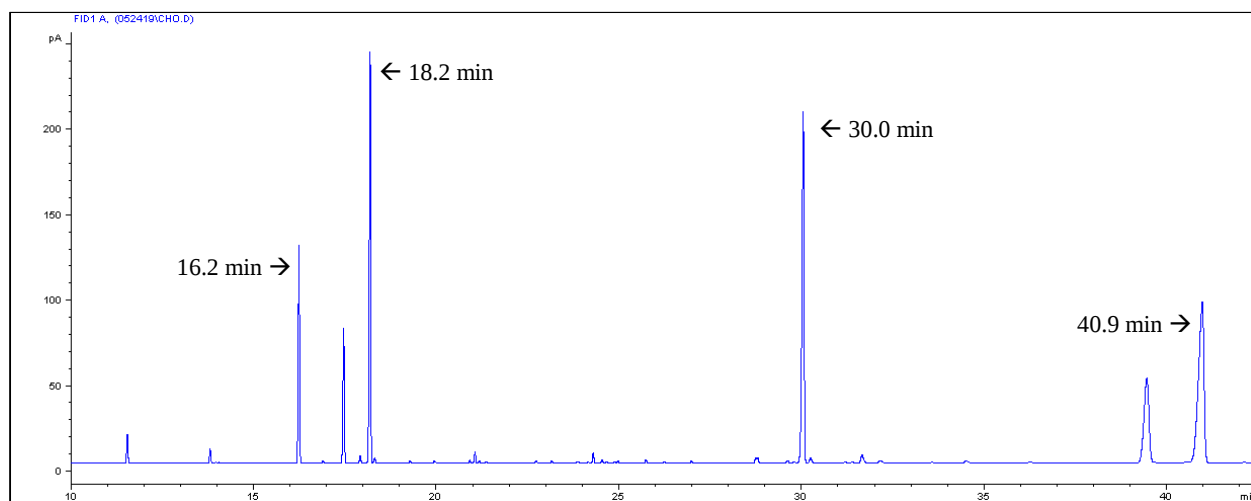


Figure 3.17: GC-FID Chromatogram of Carbohydrate Fraction of Treated Cells of *Listeria monocytogenes* 10403S. Notable peaks at retention times of approximately 16.2, 18.2, 30.0 and 40.9 min at a decreased intensity compared to the purified WTA monomer sample.

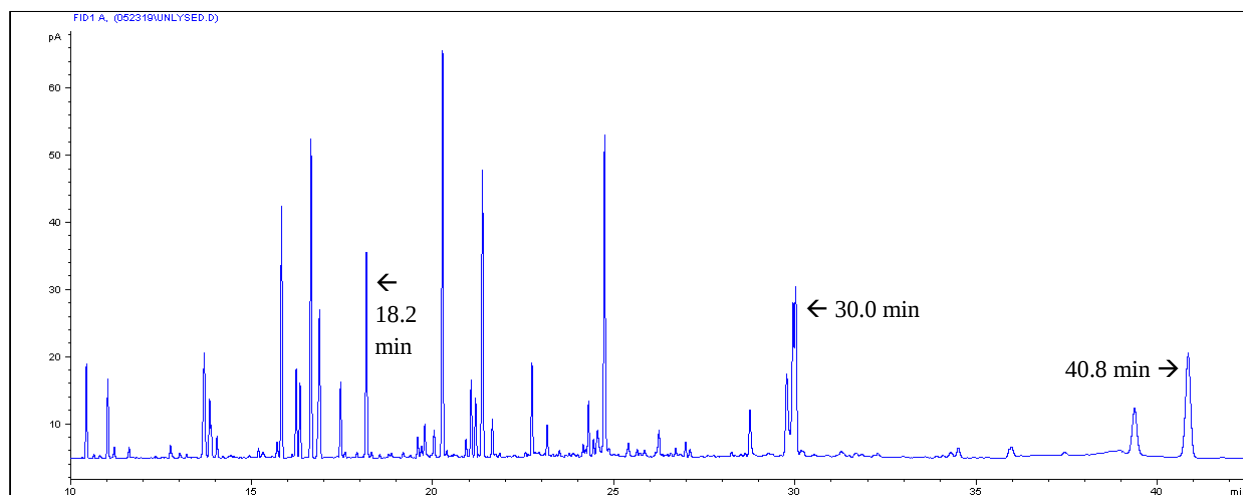


Figure 3.18: GC-FID Chromatogram of Autoclaved Cell Pellet of *Listeria monocytogenes* 10403S. Notable peaks at retention times of approximately 18.2, 30.0, and 40.8 min at a markedly decreased intensity compared to the purified WTA monomer sample, with many additional peaks before ~40 min.

Conclusion

Although it is necessary to purify samples of WTA polymers to some extent for their initial analysis, samples can be successfully “streamlined” in order to more rapidly determine the presence or absence of specific WTA compounds. Although peaks of interest are still detectable using an autoclaved cell pellet that has not been further processed, the carbohydrate fraction of treated cells produced a “cleaner” chromatogram without extensive purification methods. The ability to analyze a sample using far less time, materials, and equipment will allow for the analysis of more samples and enable laboratories with less resources to contribute to this work. Although the results obtained here provide a preliminary analysis of molecules that may be present in the WTA analyzed, further analysis using synthesized standards of WTA monomers must be applied to confirm the presence of these predicted structures. This work has further developed methods for purification and HF hydrolysis of WTA monomers of *L. monocytogenes* that provides standards that can be used for preliminary analysis, as well as a methods for the successful silylation and GC-FID analysis of sugar and amino sugar standards. Further analysis using synthesized standards can provide information that further supports results obtained through whole genome sequencing and phage adsorption assays of *L. monocytogenes* mutant strains.

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Appendix

Table 3.1: *Listeria monocytogenes* Strains Used in this Study.

<i>Listeria monocytogenes</i> Strain	Features	Reference or Origin
Wild-type Laboratory Strain: 10403S	Lineage II; 1/2a serotype (GlcNAc and rhamnose in WTA)	(2)
Mutant Strains: FSL D4-0014	10403S mutant; nonsense mutation in <i>LMRG_00541</i> ; deficiency of GlcNAc in WTA	(6)
FSL D4-0119	10403S mutant; nonsense mutation in <i>LMRG_00542</i> ; deficiency of rhamnose in WTA	(6)
UTK P1-0001	10403S mutant; frameshift mutation caused by a deletion in <i>LMRG_00544</i> ; truncated RmlC protein	(27)

Table 3.2: Preparation of the Phosphate Standard Curve.

Concentration PO₄	Volume of Working Stock (10 mg/L PO₄)	Volume of Ultrapure Water
0	0	5000 µL
0.5 mg/L	250 µL	4750 µL
1.0 mg/L	500 µL	4500 µL
1.5 mg/L	750 µL	4250 µL
2.0 mg/L	1000 µL	4000 µL
2.5 mg/L	1250 µL	3750 µL
3.0 mg/L	1500 µL	3500 µL
3.5 mg/L	1750 µL	3250 µL
4.0 mg/L	2000 µL	3000 µL
4.5 mg/L	2250 µL	2750 µL
5.0 mg/L	2500 µL	2500 µL

A working stock solution of phosphate standard with a concentration of 10 mg/L PO₄ was prepared from a phosphate standard solution with a concentration of 1,000 mg/L PO₄. From the working stock solution, 5 mL phosphate standards were prepared in concentrations ranging from 0 to 5 mg/L PO₄. A phosphate test was applied to each standard as well as an ultrapure water blank as per manufacturers' instructions. Absorbencies of each standard were read in 10 mm cuvettes with a spectrophotometer at a wavelength of 690 nm to develop a standard curve.

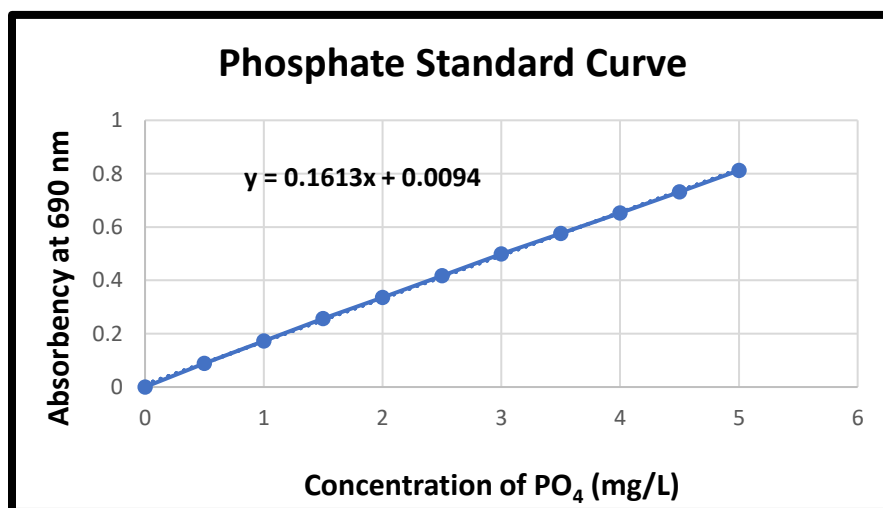
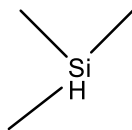
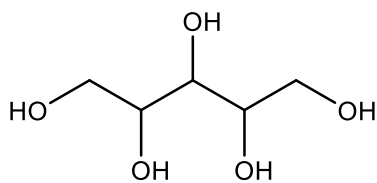


Figure 3.19: Phosphate Standard Curve. A standard curve was developed using Microsoft Excel (Version 1811) to establish a linear regression formula ($y = 0.1613x + 0.0094$) for determining unknown phosphate values based on sample absorbencies at 690 nm.

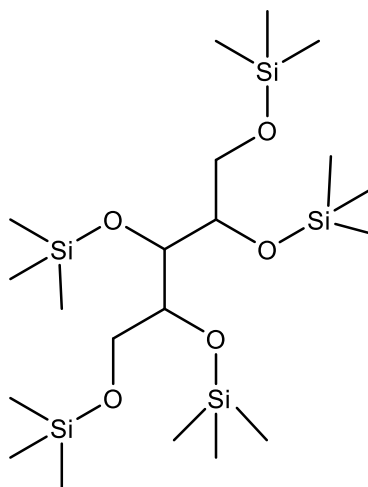


Molecular Weight: 74.20

Figure 3.20: Illustration of Trimethyl Silane Molecule.

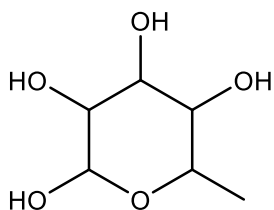


Molecular Weight: 152.15

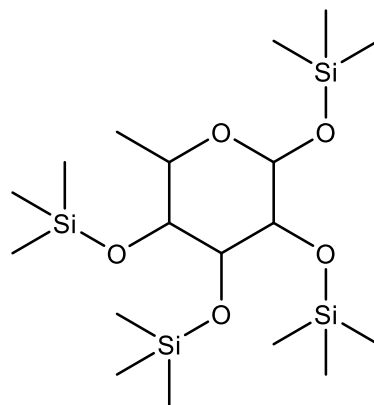


Molecular Weight: 513.06

Figure 3.21: Illustration of Ribitol Molecule (left) and Silylated Ribitol Molecule (right).

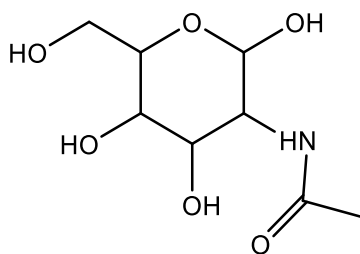


Molecular Weight: 164.16

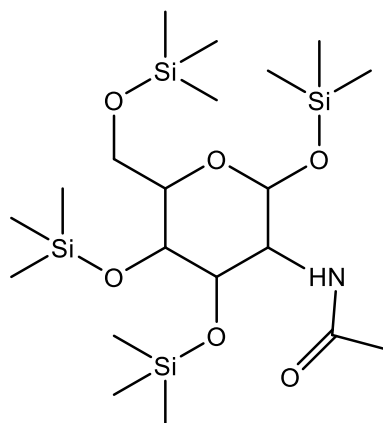


Molecular Weight: 452.89

Figure 3.22: Illustration of Rhamnose Molecule (left) and Silylated Rhamnose Molecule (right).

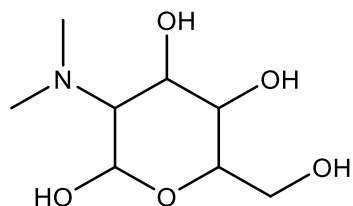


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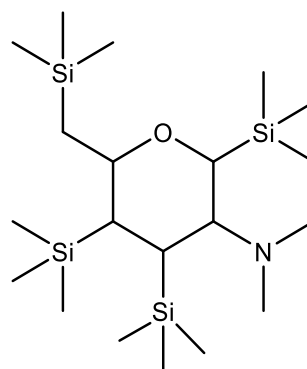


Molecular Weight: 509.94

Figure 3.23: Illustration of N-acetylglucosamine Molecule (left) and Silylated N-acetylglucosamine Molecule (right).

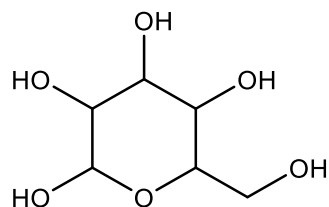


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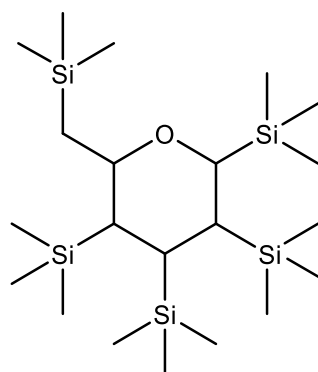


Molecular Weight: 431.96

Figure 3.24: Illustration of Glucosamine Molecule (left) and Silylated Glucosamine Molecule (right).

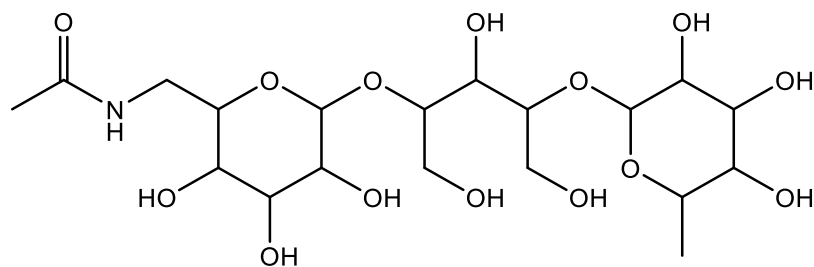


Molecular Weight: 180.16

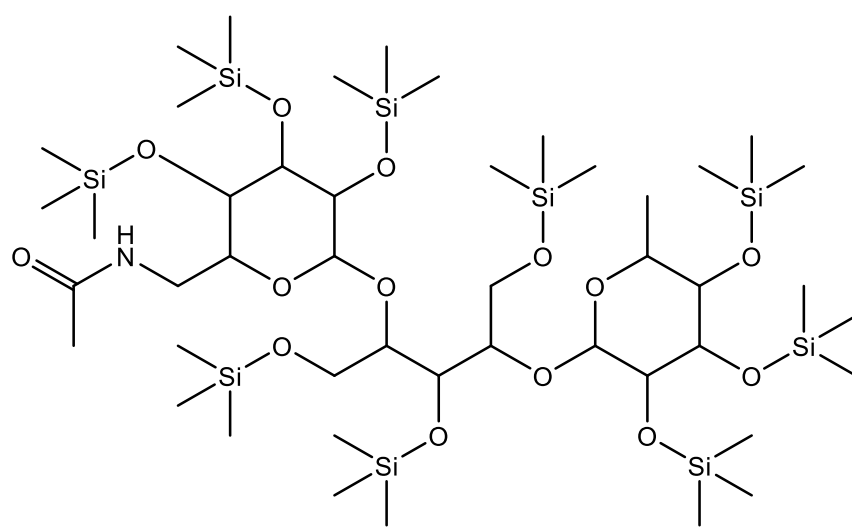


Molecular Weight: 461.07

Figure 3.25: Illustration of Glucose or Galactose Molecule (left) and Silylated Glucose or Galactose Molecule (right).

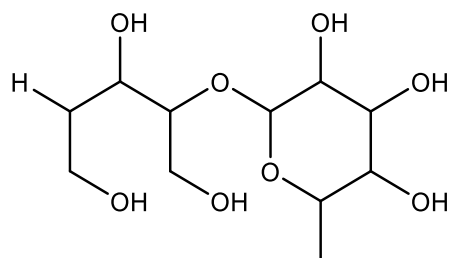


Molecular Weight: 501.48

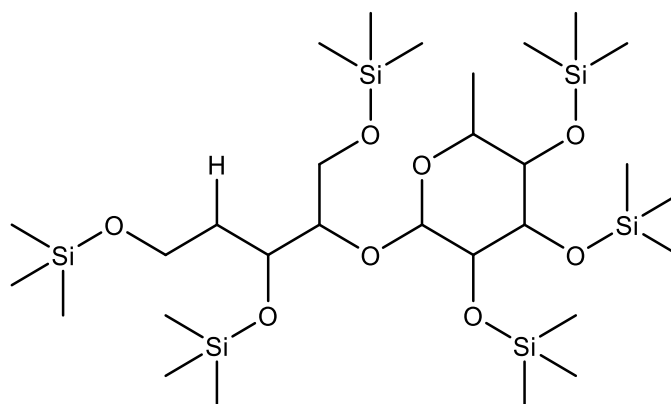


Molecular Weight: 1151.12

Figure 3.26: Illustration of *Listeria monocytogenes* Serotype 1/2a WTA Monomer (top) and Silylated *Listeria monocytogenes* Serotype 1/2a WTA Monomer (bottom).

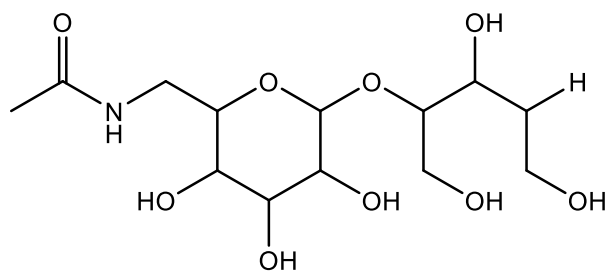


Molecular Weight: 282.29

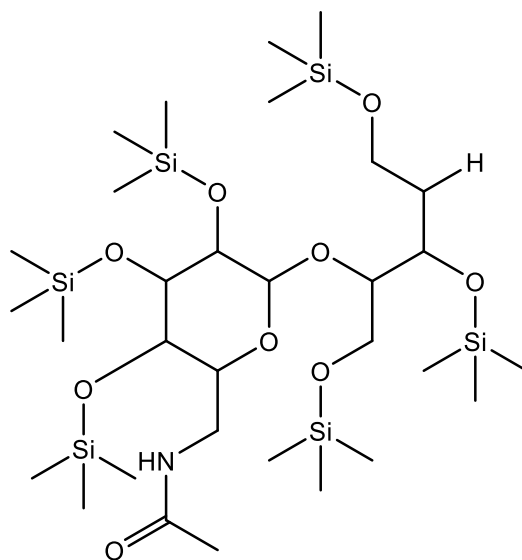


Molecular Weight: 715.38

Figure 3.27: Illustration of *Listeria monocytogenes* FSL D4-0014 WTA Monomer (top) and Silylated *Listeria monocytogenes* FSL D4-0014 WTA Monomer (bottom).



Molecular Weight: 339.34



Molecular Weight: 772.43

Figure 3.28: Illustration of *Listeria monocytogenes* FSL D4-0119 WTA Monomer (top) and Silylated *Listeria monocytogenes* FSL D4-0119 WTA Monomer (bottom).

Chapter IV: Conclusion

The objectives of the studies presented here included determining the efficiency of plaquing and/or activity of a collection of phages against *L. monocytogenes* 10403S and three mutants derived from this strain; FSL D4-0014, FSL D4-0119, and UTK P1-0001, as well as a preliminary analysis of the WTA structures of all these strains. One bacteriophage was identified (LP-018) that was capable of infecting all three of the phage resistant mutants of serotype 1/2a *L. monocytogenes*. It was concluded that mutations conferring phage-resistance through loss of rhamnose likely pose the greatest challenge for phage-based biocontrol in serotype 1/2a strains, as we found that they confer resistance to almost all of the *Listeria* phages (119/120) in the diverse collection tested. Additionally, we have provided a visual comparison of the WTA components of these *L. monocytogenes* strains via chromatograms obtained using GC-FID analysis, as well as chromatograms for sugar and amino-sugar standards present in *L. monocytogenes* WTA. Although the results obtained here give us a better picture of what types of molecules may be present in these WTA, a comparison to synthesized standards must be performed to confirm these predicted compounds. It is necessary to purify samples of WTA polymers to some extent for their initial analysis, but subsequent unknown samples can be successfully “streamlined” in order to more rapidly determine the presence or absence of such previously determined WTA monomer peaks. The chromatograms of the confirmed WTA monomers can be organized into a reference library, as the retention times of peaks should remain nearly the same if using the same GC-FID program and column. The ability to analyze a sample using far less time, materials, and equipment will allow for the analysis of more samples and enable laboratories with less resources to contribute to this work. These results together contribute to our understanding of phage-resistance in *L. monocytogenes* as well as *Listeria*

phage binding receptors. Additionally, they have the potential to aid in the rational design of *Listeria* phage cocktails used as antimicrobial agents in food industries that aim to reduce the emergence of phage-resistance and ensure long-term efficacy.

Vita

Danielle Trudelle was born on August 27th, 1986 in Glen Cove, NY. She graduated from Locust Valley High School in Locust Valley, NY in 2004 to attend SUNY Stony Brook University in Stony Brook, NY from August 2004 until May 2006. She then transferred to SUNY Plattsburgh in Plattsburgh, NY and graduated with a Bachelor of Science in Food and Nutrition in May 2009. After graduation, Danielle worked in the dietary departments of three different long term care facilities, a laboratory rodent breeding facility, and finally a laboratory that performed analyses on different food commodities. She then began the M.S. program in the Food Science department at the University of Tennessee, Knoxville in August of 2017, and is expected to graduate in August of 2019.