ABSTRACT

CHENG, YING. Genetic Characterization of Genes Specific to *Listeria monocytogenes* Epidemic-Associated Serotype 4b Strains (Under the direction of Dr. Kathariou Sophia.)

Listeria monocytogenes, a food-borne pathogen of humans and animals, can cause severe listeriosis with relatively high mortality. A cluster of closely related strains of *L. monocytogenes* (designated Epidemic Clone I) have been implicated in numerous outbreaks in Europe and North America, including the California outbreak of 1985. *L. monocytogenes* strains implicated in the 1998-1999 and the 2002 multistate outbreaks in the USA represent a unique epidemic-associated clonal group, designated Epidemic Clone II (ECII). Comparative genomic analyses across five genomes from different *L. monocytogenes* isolates and *Listeria* species identified a genomic region (region-18) in serotype 4b strains that may have been acquired by horizontal gene transfer. Region-18 is either absent or markedly divergent in ECII strains but conserved among other serotype 4b strains. Region-18 is flanked by a large gene encoding a putative cell-wall associated protein (*wap*) on one side and a well-known virulence gene internalin A (*inl*A) on the other side in serotype 4b strains. PCR primers and DNA probes derived from this ECII-specific region-18 can readily differentiate ECII strains from other serotype 4b strains. This facilitates the detection and monitoring of these strains belonging to ECII clonal group in foods, clinical samples, and the environment. Genetic characterization of *wap* by the construction of deletion mutants suggested that the ECII *wap* mutant but not the ECI *wap* mutant may be involved in specific environmental adaptations such as surface adherence and possibly biofilm formation in ECII strains. Mutational and functional analyses showed that the deletion mutant of region-18 in ECII had an enhanced death rate during post-stationary incubation at 42℃, suggesting that the ECII-specific region-

18 may be implicated in post-stationary stress responses. Two c72.44-negative variants of epidemic-associated *L. monocytogenes* serotype 4b strains were isolated from laboratory cultures. Naturally occurring c74.22-negative variants that exist under laboratory conditions without any noticeable phenotypic differences from their original forms may complicate the analysis of phage sensitivity and pathogenic characteristics.

Genetic Characterization of Genes Specific to *Listeria monocytogenes* Epidemic-Associated Serotype 4b Strains

by
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Biography

Ying Cheng is from China. She received the M.S. degree in microbial genetics from the Fudan University in China in 1999. She joined the food pathogen laboratory at Food Science Department in NC State University in 2003, where her research is to address the evolution of *Listeria monocytogenes* virulent strains, including identification and characterization of potential virulence genes by using molecular biological methods along with genetic, physiologic approaches.

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CHAPTER I Literature Review

1.1 *Listeria monocytogenes* and listeriosis

Listeria monocytogenes is the only species of genus *Listeria* that is capable of causing serious invasive disease (listeriosis) in both humans and animals. *L. monocytogenes* is a small (1-1.5 μm × 0.5 μm), Gram-positive, facultative anaerobic, non-spore-forming rod that is ubiquitous in environment and can grow over a wide range of pH values (4.3-9.6), temperatures (1-45 ℃) and salt concentrations (up to 10%) (40, 49, 71).

Epidemiologic and laboratory studies associated with the Maritime Provinces outbreak in 1981, traced to contaminated coleslaw, first demonstrated the transmission of *L. monocytogenes* to humans by contaminated food (69). Afterwards, numerous epidemiological, population genetics and evolutionary studies of *L. monocytogenes* using various subtyping tools have greatly improved our understanding of how *L. monocytogenes* is transmitted from animals or the environment through foods to humans (43, 66). *L. monocytogenes* can cause both sporadic cases and outbreaks of listeriosis. Although a variety of food products have been found contaminated with *L. monocytogenes*, including coleslaw, unpasteurized cheese, pasteurized milk, seafood, and processed meat products, most human listeriosis infections appear to be caused by processes and refrigerated ready-to-eat (RTE) foods (70). The capability of *L. monocytogenes* to commonly colonize the food processing environment and contaminate food makes *Listeria* an issue of particular concern to the food industry.

Human listeriosis is a rare but severe disease, which causes approximately 2,500 human cases and 500 deaths each year in the United States (51). Although relatively rare compared to other food-borne diseases, listeriosis frequently has serious complications, including meningitis, septicemia, central nervous system (CNS) infections, or abortion. Elderly,

neonates, and pregnant women, immuno- compromised patients are particularly at risk (68). In addition, outbreaks of febrile gastroenteritis due to consumption of foods highly contaminated with the pathogen have occurred. The widespread occurrence of *L. monocytogenes* in nature and its ability to grow at refrigeration temperature, coupled with a high mortality rate of 20-30% in those developing listeriosis, make this organism and listeriosis a serious public health risk.

1.2 Pathogenesis and virulence determinants

1.2.1 Overview of pathogenesis of *L. monocytogenes*

As a facultative intracellular pathogen, *L. monocytogenes* can invade and replicate in epithelial cells and macrophages. In mouse and guinea pig models, *L. monocytogenes* has been shown to be taken up by enterocytes or M cells near Peyer's patches in the small lining of the small intestine and then multiplies in underlying phagocytic cells (50). Bacteria are disseminated from the intestine through circulating macrophages cells and reach the primary target organs (liver and spleen), where most of them are killed by resident macrophages (47, 62). If the host's T cell-mediated immune response is compromised, the bacteria multiply in hepatocytes and macrophages and are carried in the blood to various organs, particularly the brain and/or uterus, due to their potential to breach the blood-brain and placental barriers.

Cell culture models have shown that, following invasion of the host cell, the bacteria are internalized in a vacuole and escape into the host cell cytoplasm with the aid of listeriolysin O (LLO). In the cytosol, *L. monocytogenes* can replicate and propel itself intracellularly through an actin-based motility process. It invades neighboring cells through the formation of a double-membrane protruding pseudopods, which are engulfed by adjacent host cells. The double-membrane vacuole is then lysed, allowing a new infection cycle in adjacent cells (22).

1.2.2 Entry to epithelial cells mediated by internalins

To date, several listerial determinants have been found to mediate bacterial adherence into target cells and invasion. Most intensively studied are two major internalins, internalin A (InlA) and internalin B (InlB). *L. monocytogenes* can cause infection by invading intestinal epithelial cells following ingestion of contaminated food. Since epithelial cells are not actively phagocytic, *L. monocytogenes* must enter these cells by an invasive process that requires the association of a bacterial surface protein (internalin) with a receptor on the surface of the host (23, 24, 28, 29). InlA (800 amino acids) is essential for internalization by intestinal epithelial cells. It is characterized by leucine-rich repeats (LRRs) and is covalently linked to peptidoglycan through LPXTG motif at the carboxyl terminus. Interaction between InlA and its receptor (E-cadherin) on the host cells mediates the internalization process. InlB (600 amino acids) is another member of the internalin family, which is involved in entry of *L. monocytogenes* to a broad range of cell lines including hepatocytes and nonepithelial cells. The LRR region of InlB is sufficient for bacterial adherence. The GW module at the carboxyl terminal domain mediates attachment of InlB to the bacterial cell wall through noncovalent interaction with lipoteichoic acid. The hepatocyte growth factor/scatter factor receptor, or Met, has been identified as the main receptor of InlB on target cells. The interaction between internalins and their receptors stimulates the phosphorylation of various host cell proteins and initiates a complex signal transduction cascade that results in internalization of the bacteria (25).

1.2.3 Intracellular life cycle

After entering the host cells, *L. monocytogenes* is enclosed within a vacuole that is lysed by listeriolysin O (LLO) in synergy with the phospholipases (PLCs). Listeriolysin O

(LLO), a bacterial pore-forming toxin, is essential for bacterial escape from primary and secondary intracellular vacuoles. The optimal activity of LLO is obtained at pH5.5 and 6.0 (typical pH of the early phagosome) in the vacuole. A PEST-like motif might indicate destiny of LLO for degradation in cytosol. This precise control of LLO restricts its function to the vacuole environment and inhibits the destruction of the host cell membrane (19, 25).

Two PLCs implicated in the lysis of intracellular vacuoles are phosphatidylinositol (PI)-PLC and phosphatidylcholine (PC)-PLC. Both enzymes act synergistically with LLO in lysing primary and secondary vacuoles (11, 30).

After lysis of the vacuole, a surface protein of *L. monocytogenes*, ActA, induces polymerization of actin filaments in the host cell cytoplasm, promoting bacterial intracellular movement and cell-to-cell spread. Bacteria move along these filaments to the cell membrane and form protrusions that are engulfed by neighboring cells (25).

1.2.4 Regulation of virulence factors

The genes encoding many of the factors involved in listerial cellular pathogenesis are clustered together on an 8.2-kb pathogenicity island and regulated by the transcriptional activator *prfA* gene, which is also located in this island. This virulence gene island includes *prfA, plcA, hly, mpl, actA,* and *plcB* (encoding transcriptional activator PrfA, (PI)-PLC, LLO, metalloprotease, ActA, and PC-PLC, respectively) (FIG.1). Two other well-characterized virulence genes, *inlA* and *inlB* are located elsewhere in the chromosome under the partial control of *prfA* gene. A 14-bp palindromic sequence ("PrfA box") in genes regulated by PrfA is found to bind to the putative helix-turn-helix motif of PrfA *in vitro* (72).

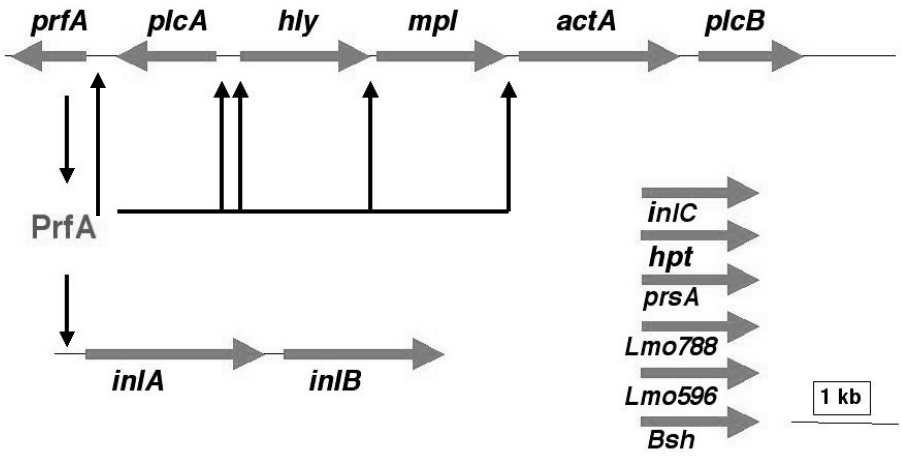

FIG.1: The organization and coordinate regulation of virulence gene expression by PrfA in *L. monocytogenes.* Diagram modified from Sheehan et al.(73).

Several other virulence-related determinants have been identified outside the *prfA* regulon. Ami, a GW autolysin, has been shown to be implicated in bacterial adhesion to target cells in a Δ*inlAB* background. *ami* mutants are attenuated, indicating its role in virulence of *L. monocytogenes* (54). p60 is a 60-kDa protein (originally designated *iap* for invasion associated protein, and currently changed to *cwhA*), which is implicated in septum formation. A Δp60 bacterial strain was highly attenuated in virulence in mice (60) . Recent studies indicated that a 570-amino acid protein, FbpA, contributes to cell adherence to HEp-2 cells and could function as a molecular chaperone, preventing degradation of some virulence proteins as well (21). Several Clp proteases and ATPase have been identified as chaperones and proteolytic enzymes and have been shown to play important roles in pathogenesis of *L. monocytogenes* (55).

1.3 Molecular epidemiology

1.3.1 Molecular subtyping tools of special value for surveillance of human listeriosis

Although *L. monocytogenes* has been isolated from a variety of foods, most human listeriosis infections have been traced to contaminated cold-stored, ready-to-eat (RTE) foods, including processed meats, dairy products, seafood, and vegetables. Marketing and distribution practices in current food system have changed. Foods produced by a single manufacturer are distributed over multiple states and nations. These changes have a great impact on the epidemiology of foodborne disease outbreaks. In addition, clinical characteristics of human listeriosis, such as a long incubation period (1 to 90 days) in comparison to that of many other foodborne diseases, complicate the detection and tracking of outbreaks. Surveillance of human listeriosis and detection of listeriosis clusters represent a particular challenge. Classical epidemiologic surveillance systems appear to be limited for detecting many common source outbreaks, which may also occur over a prolonged time periods and a wide geographic area. Thus, rapid and standardized subtyping methods for *L. monocytogenes* are particularly important for effective detection of human listeriosis outbreaks and sporadic cases.

1.3.2 Subtyping tools

Subtyping has been crucial for the detection of human listeriosis outbreaks, foodborne disease surveillance, and source tracking throughout the food chain. Phenotypic and DNA-based subtyping methods not only allow for differentiation of *L. monocytogenes* beyond the species and subspecies level but also promote better understanding of ecology and characteristics of bacterial strains, subtypes, and differences in their abilities to cause human foodborne diseases. Commonly used criteria for evaluating a subtyping method include

typability, reproducibility, discriminatory power, ease of interpretation, and ease of use. In the last five years, tremendous advancements have been made in the development of sensitive, rapid, and automated molecular subtyping methods for *L. monocytogenes*. Bacterial subtyping methods can be divided into conventional and phenotypic, and genetic or DNA-based methods. Although conventional and phenotypic methods (e.g. serotyping, phage typing) have been used for many years to subtype *L. monocytogenes* and other foodborne pathogens, DNA-based subtyping methods have been increasingly used. Traditional subtyping methods, including serotyping and phage typing, show restricted value in epidemiological studies because of their low typability, reproducibility, and discriminatory power (78). Compared to phenotypic subtyping methods, molecular subtyping methods allow more sensitive strain discrimination and a higher level of standardization and reproducibility. Molecular subtyping methods have mostly replaced conventional subtyping methods as laboratory tools for epidemiological studies of *L. monocytogenes*.

Multilocus enzyme electrophoresis (MLEE). MLEE is a protein-based, isoenzyme typing method that differentiates bacterial strains by variations in the electrophoretic mobility of different house-keeping (constitutive expressed) enzymes. Bacterial lysates containing soluble enzymes are separated by size in non-denaturing starch gels, and enzyme activities are determined in the gel using color-generating substrates. Variations in the electrophoretic mobility of different enzymes (or electrophoretic types, ETs) enable differentiation of *L. monocytogenes* strains. Electromorphs of each enzyme are equated with alleles at the corresponding structural gene locus, and electrophoretic types (ETs), which are interpreted as multilocus genotypes representative of the chromosomal genome, provide a basis for estimating levels of genetic diversity and relatedness among isolates from natural populations

(59). This method provides 100% typability, but is difficult to standardize between laboratories (48). Although MLEE is a powerful tool for population genetic, taxonomic and evolutionary studies, its ability to discriminate subtypes for epidemiological investigations is limited (34).

Pulsed-Field Gel Electrophoresis (PFGE). PFGE is based on restriction fragment length polymorphisms (RFLPs) of bacterial DNA. Specifically, complete bacterial DNA is released from the cells (which are embedded in agarose blocks) and subsequently cut into large fragments (40-600 kb) using rarely cutting restriction enzyme (i.e. *Asc*I and *Apa*I). The large fragments are then size-separated by pulsating electrical currents in specialized equipment. In the United States, PulseNet, a national molecular subtyping network for foodborne disease surveillance established by the Centers for Disease Control and Prevention (CDC) is based on PFGE as the preferred method to characterize clinical isolates of *L. monocytogenes.* The method is one of the most discriminatory typing methods available for *L. monocytogenes* when compared with other molecular subtyping methods, and is considered the current "gold standard" molecular subtyping method. It is important to point out, however, that PFGE may also detect small genetic differences (i.e. 1-3 different bands) that may not be epidemiologically significant and it does not provide genetic characterization of isolates.

Ribotyping. Ribotyping is a DNA-based subtyping method which combines restriction enzyme digestion with Southern blot hybridization using probes to the genes encoding the 16S rRNA (34). Ribotyping uses a frequent cutting restriction enzyme to digest bacterial DNA into many (>300-500) of small-sized fragments (1-30 kb). These DNA fragments are separated by agarose gel electrophoresis, and a subsequent Southern blot step uses DNA probes to specifically detect those DNA fragments that contain the bacterial genes encoding

the ribosomal RNA (rRNA). A completely automated ribotyping system has become available commercially (Riboprinter Microbial Characterization System, Qualicon, Wilmington, DE), thus promoting an increasing use of ribotyping for large-scale epidemiologic investigations of human listeriosis.

DNA sequence-based subtyping. Multilocus sequence typing (MLST) uses automated DNA sequencing to characterize the alleles present at different housekeeping genes or virulence-associated genes and to determine the subtypes and genetic relatedness of bacterial isolates. Because it is based on the nucleotide sequence, it provides unambiguous results that are directly comparable ("portable") between laboratories. The development of MLST is favored by the fact that DNA sequences of the *L. monocytogenes* housekeeping and virulence genes are available for one or more strains. However, this method requires the identification of genes in *L. monocytogenes* with high levels of polymorphism that can serve as the basis for a discriminatory sequence-based typing method.

DNA Microarrays. DNA typing with DNA microarrays represents the latest technological advancement for detecting genetic differences that exist among isolates. In essence, a microarray is a dot-blot that uses the same principles of hybridization and detection that have been used by established blotting methods such as Southern and Northern blots. Typically, microarrays are composed of a lattice of spots of densely packed probes robotically imprinted onto the substrate. Probes are usually oligonucleotides or PCR products derived from cloned genes or gene fragments. The test DNA (sequences to be analyzed) is labeled by fluorescence dyes and applied to the microarray slides, where it hybridizes with its complementary probe. The hybridization is then detected by a fluorescence scanner. This technique has the added advantage that, unlike PFGE, ribotyping, and MLST, it can not only

subtype different bacterial isolates but it can also identify specific or unique genes associated with the strains. Furthermore, microarrays permit the rapid characterization of genetic differences at hundreds of loci in a high throughput format.

Currently, PFGE, ribotyping, MLST, and DNA microarrays are commonly used for subtyping. In addition, PCR-based subtyping methods such as random amplification of polymorphic DNA (RAPD), arbitrarily primed PCR (AP-PCR), amplified fragment length polymorphic DNA (AFLP), PCR-restriction fragment length polymorphism (PCR-RFLP), and repetitive element PCR (REP-PCR) have been used as well in epidemiologic investigations (48). It must be pointed out that each typing method presents advantages and shortcomings. The choice of an appropriate subtyping method relies on the intended application and the goal of subtyping.

1.3.3 Applications of molecular subtyping in epidemiological investigations

Listeriosis surveillance and tracking of outbreaks.

Over the last few years, numerous molecular typing methods have been applied to subtyping and detection of listeriosis outbreaks in listeriosis surveillance programs. Analysis of molecular subtyping data in conjunction with epidemiological data has significantly contributed to the detection of outbreaks. Currently, PFGE and automated ribotyping are being used extensively in outbreak investigations and for routine surveillance of strains from clinical, food, or environmental sources. After the introduction of *L. monocytogenes* in the PulseNet system in 1998, this effort has contributed significantly to the detection of at least three multistate human listeriosis outbreaks in the United States. In each case, identification of clinical isolates from different states with identical PFGE patterns and ribotypes provided the first indication of the occurrence of a common-sources multistate outbreak. The outbreak

became subsequently confirmed by epidemiologic and bacteriologic investigations (13, 14, 17). It is noteworthy that more outbreaks caused by *L. monocytogenes* were detected after inclusion of *L. monocytogenes* in the PulseNet program. The increasing use of high-resolution molecular subtyping methods and real-time portability of molecular subtyping data in the surveillance system may contribute to an improved detection of human listeriosis outbreaks.

1.4 Phylogenic divisions, epidemic clones and population structure of *L. monocytogenes*

1.4.1 Introduction

The vast majority of human listeriosis infections appear to be foodborne (51). Several surveys of prevalence of *L. monocytogenes* in food and food-processing plants indicated that *L. monocytogenes* is predominant in certain RTE foods and persistent in a variety of food-processing environments. However, reservoirs of *L. monocytogenes* and sources for contamination of this organism in the food chain are still not completely understood. Previous studies revealed that among 13 serotypes of *L. monocytogenes*, only three serotypes (1/2a, 1/2b, and 4b) account for >95% of the clinical cases (66, 70, 75). Interestingly, serotype 1/2a is most frequently isolated from food, but serotype 4b causes the majority of human epidemics. This suggests that there may be a link between serotype and virulence potential. Further studies on epidemiology, population genetics, and evolution of *L. monocytogenes* using various subtyping methods have been critical to clarify the transmission pathways of this versatile pathogen and to better understand the association of certain subtypes with phenotypic characteristics and pathogenic potential.

1.4.2 Genomic divisions

Various molecular typing methods (e.g. MLEE, ribotyping, PFGE, RFLPs in specific genes, DNA sequencing, PCR-RFLP, DNA microarrays) have been used to explore the population genetics and evolution of *L. monocytogenes*. Data generated with such subtyping methods have shown that genetic structure of *L. monocytogenes* is highly clonal. By these molecular subtyping methods *L. monocytogenes* is consistently divided into two genetic divisions, lineage I (also designated Genomic Division II) and lineage II (also designated Genomic Division I). Serotypes 1/2b, 3b, 4b, 4d and 4e are generally grouped into lineage I, while the lineage II appears to include 1/2a, 3a, 1/2c, and 3c (10, 59, 67). Ribotyping and virulence gene polymorphism data showed the existence of a third lineage, including strains of serotypes 4a and 4c (20, 64, 77, 79), with some investigators suggesting that this lineage may represent a distinct taxonomic unit and may represent a different subspecies of *L. monocytogenes* (20, 64, 77, 79). This clonal partitioning of the species along serotypic groups suggests that strains of certain serotype-associated lineages may pose higher risks to human health than others. From the regulatory perspective, a zero tolerance for the control of *L. monocytogenes* in RTE foods is based on the assumption that each serotype has equal ability to cause human disease. Identification of clonal groups that differ in their abilities to adapt to specific food niches or to cause human foodborne disease may limit the need for zero tolerance to a relatively small number of more pathogenic strains in the future.

1.4.2.1 Lineage I, II, and III

Lineage I and II. Phylogenetic analysis has categorized *L. monocytogenes* strains into two primary phylogenetic divisions. The different molecular subtyping methods have resulted in notably similar strain groupings based on serotype distribution to distinct genomic divisions,

or lineages (36). Two evolutionary lineages were identified initially by early MLEE investigations, with serotype 1/2b, 4a, and 4b in genomic division II (lineage I) and serotype 1/2a and 1/2c in genomic division I (lineage II) (59). Subsequently, the existence of two major lineages was confirmed by identification of DNA sequence variation in the listeriolysin gene (63), ribotyping, PFGE (5) and the combination of ribotyping and allelic analysis of virulence genes (*hly*, *actA*, and *inlA*) (79). More recently, the phylogenetic data generated by MLST, mixed-genome microarray and DNA sequencing of the *prfA* virulence gene cluster (*p*VGC) further verified the presence of two evolutionary lineages within *L. monocytogenes* (10, 67, 77, 81). Additionally, sequence analysis and RFLP of various virulence genes as well as a genomic region responsible for low-temperature growth could differentiate lineage I strains from lineage II strains (82). Genomic fingerprinting via PFGE divided *L. monocytogenes* into two distinct genomic lineages that also correlated with flagella (H) antigen types: Division I (lineage II) contained serovar 1/2a, 1/2c, 3a and 3c strains, and division II (lineage I) contained serovar 1/2b, 3b, 4b, 4d, and 4e strains (5) . At higher discriminatory level, lineage II isolates were further grouped into cluster IA (serovar 1/2c and 3c) and cluster IB (serovar 1/2a and 3a) strains. Likewise, lineage I isolates were further grouped into cluster IIA (serovar 1/2b and 3b) and cluster IIB (serovar 4b, 4d, 4e) strains (5, 74). In agreement with this subgrouping data from PFGE, cluster analysis of microarray data split lineage I into two subgroups that are congruent with serotype distribution; II.2 comprised serotype 1/2b and 3b strains while II.1 comprised serotype 4b, 4d, 4e strains. Lineage II was subgrouped into I.1 (1/2a and 3a) and I.2 (1/2c and 3c) (20).

Lineage III. Rasmussen *et al*. (64) identified for the first time a third *L. monocytogenes* phylogenetic lineage, lineage III, based on allelic analysis of *flaA*, *iap*, and *hly*. The existence

of lineage III was further confirmed by ribotyping and PCR-RFLP analysis of virulence genes, as well as comparative genomics and DNA sequencing of *p*VGC (77). When lineage III was initially identified, it was reported to contain serotype 4a and 4c. Recent data based on the characterization of a larger number of isolates in this lineage also identified serotype 4b strains to be prevalent (77). Phylogenetic analysis of *sigB* and *actA* sequences indicated that lineage III represents three distinct subgroups, termed IIIA, IIIB, and IIIC. Lineage III appears to be rare and has several atypical and unique phenotypic characteristics. Unlike typical *L. monocytogenes*, all subgroup IIIB and IIIC isolates are unable to ferment rhamnose. The majority of subgroup IIIA isolates lack the putative virulence gene *lmaA*, which encodes a listerial antigen capable of eliciting a delayed-type hypersensitivity reaction in *Listeria*-immune mice. In contrast, most IIIB and all IIIC strains carry this gene (65).

1.4.3 Epidemic-associated strains

Previous epidemiological studies strongly suggested that not all strains of *L. monocytogenes* were equally likely to be isolated from human cases of listeriosis. Among 13 described serotypes of *L. monocytogenes*, serotypes 1/2a (classified into lineage II), 1/2b and 4b (classified into lineage I) account for >95% of human infections. Early MLEE-based subtyping data indicated that strains implicated in several major outbreaks were genetically closely related (2, 59). These observations were further confirmed by ribotyping, allelic analysis of virulence genes and by PFGE (4, 7, 38). To date, most human outbreaks of foodborne listeriosis have involved a small number of closely related strains, primarily of serotype 4b (43).

The repeated involvement of some clonal groups (lineages) in major outbreaks has prompted the following hypotheses: (1) these clones may be particularly well adapted for

growth and/or survival in foods and food processing plants; (2) these clones may have high level of pathogenicity to humans. Current data on the prevalence of serotype 4b or epidemic-associated strains in foods and food processing plants are not commonly available. A recent survey of 3,063 ready-to-eat food samples revealed that 2.97% samples were positive for *L. monocytogenes*, and lineage I strains (serotypes 1/2b, 3b, and 4b) were more prevalent than lineage II (57% and 34%, respectively) (74). In addition, surveys of environmental samples from two turkey processing plants in the United States and analysis of isolates from various foods indicated the potential presence of these epidemic clonal groups in food and food processing plants (26, 80).

A number of early studies using cell culture and animal models have failed to identify differences between serotype 4b epidemic-associated strains and sporadic clinical isolates of the same serotype (42). More recently, a population genetic study based on ribotyping and in vitro cytopathogenicity showed that three epidemic lineage I ribotypes (DUP-1042B, DUP-1038B, and DUP-1044A) had increased cytopathogenicity in a tissue culture plaque assay, compared to other ribotypes (35). However, it can not be concluded that these epidemic clones represent enhanced human virulence since the correlation between cell culture-based cytopathogenicity and human virulence characteristics remains speculative. All three epidemic ribotypes have been shown to be overrepresented among human isolates as compared with food isolates (35). On the contrary, another epidemic-associated lineage II ribotype, DUP-1053A, seems less prevalent among human cases and does not seem to have the same increased cytopathogenicity as the other epidemic-associated lineage I ribotypes (35).

The "epidemic clone" concept is introduced in this context to define different clonal groups that consist of genetically related strains implicated in one or more epidemics, and presumably of a common ancestor. Three epidemic-associated clonal groups, designated Epidemic Clone I (ECI), Epidemic Clone II (ECII), Epidemic Clone III (ECIII), have been described based on their historical association with human listeriosis (42) (Table 1). Of these clonal groups, ECI has been involved in numerous outbreaks and has been most extensively studied (37). In contrast, ECII implicated in the 1998-1999 and 2002 multistate outbreaks (13, 14, 16), as well as ECIII implicated in a 2000 multistate outbreak (15), were identified recently and are less extensively characterized. Representatives of these three Epidemic Clones of *L. monocytogenes* have been chosen for whole genome sequencing in 2004. A better understanding of the physiology, ecology, and genetics of these currently identified clones will greatly facilitate the detection of outbreaks and will contribute to the elimination of outbreak sources.

Table 1: Major epidemic clones implicated in food-borne outbreaks of listeriosis modified from reference (42)

Epidemic Clone	Location, year, implicated food	Ribotype (35)	Electrophoretic Type (59)
Epidemic Clone I (ECI)	Nova Scotia, 1981, coleslaw, California, 1985, Mexican-style cheese Switzerland, 1983-1987, Cheese Denmark, 1985-1987, vehicle unknown France, 1992, jellied pork tongue	DUP-1038B	ET1
Epidemic Clone Ia (ECIa)	Massachusetts, 1983, pasteurized milk Boston, 1985	DUP-1042B	ET7
Epidemic Clone II (ECII)	Multistate, USA, 1998-99, hot dogs Multistate, USA, 2002, turkey deli meats	DUP-1044A	
Epidemic Clone III (ECIII)	Multistate, USA, 2000, turkey deli meats	DUP-1053A	

1.4.3.1 Epidemic Clones

Epidemic Clone I (ECI). A cluster of closely related strains (designated Epidemic Clone I) were implicated in numerous outbreaks in Europe and North America, including those in Nova Scotia, Canada (coleslaw, 1981), California (Mexican-style cheese, 1985), France (pork tongue in aspic, 1992), and others (Table 1). Several genetic and phenotypic characteristics specific to Epidemic Clone I strains were identified. Strains from major epidemics of food-borne listeriosis, including Nova Scotia, California, and Switzerland, share

a unique restriction fragment length polymorphism (RFLP) in a genomic region essential for low temperature (4ºC) growth of *L. monocytogenes* (82). These strains also harbor an additional unique RFLP, in a genomic region containing a putative mannitol permease locus (76). Furthermore, genomic DNA from these same strains is resistant to digestion by the enzyme *Sau3*AI, suggesting methylation of cytosine at GATC sites (83). In 2001, Herd and Kocks (37) identified several genomic fragments or gene clusters specific to Epidemic Clone I. DNA fragment 85 was specific to ECI and was internal to a gene with putative involvement in cytosine methylation at GATC sites. Genome sequencing data revealed that this fragment was part of a gene cassette composed of three genes, 85R (5-methyl cytosine restriction), 85M (methylase) and 85S (endonuclease *Sau3*AI) (80). Thus, this ECI-specific cassette is likely to have GATC-specific restriction-modification functions that may be responsible for the observed resistance of the DNA of these epidemic-associated strains to *Sau3*AI digestion. In addition, several genomic fragments and gene clusters unique to ECI strains were identified, although the functional roles of the genes were not determined. Genetic studies of these genes will be necessary to obtain further information on possible contributions of these genes to possible differences in pathogenicity, and the ability of the specific epidemic-associated strains to survive and grow in their respective environmental niches.

MLEE and ribotyping data also identified another serotype 4b clonal group closely related to ECI, but representing distinct epidemic clone (designated ECIa) (35, 59). Strains from the Massachusetts outbreak (1983) are representative of this clone. The genomic fragments unique to ECI are absent in this epidemic clone (37) .These strains appear to lack the RFLPs and cytosine methylation at GATC sites that are unique characteristics of ECI (76,

82, 83). This epidemic clone has remained poorly characterized. Genetic and phenotypic features that may be unique to this clone have not yet been identified.

On the basis of sequence analysis, the G+C content of most genomic fragments that are unique to the epidemic strains was in the range of 24 % to 33 %. This range is noticeably lower than the average for *L. monocytogenes* (38%), suggesting the possibility that these genes were acquired by horizontal transfer from currently unidentified bacteria.

Currently, the whole genome sequence of *L. monocytogenes* F2365 (a serotype 4b ECI strain implicated in the California Jalisco cheese outbreak) is available (56). Comparative genomic analysis between the genome of this strain and the previously published genome of *L. monocytogenes* strain EGD-e (serotype 1/2a) and *L. innocua* strain CLIP 11262 already greatly facilitated identification of strain- and serotype-specific genes (56). These genome sequence-based analyses will provide novel information that improves our understanding of evolution and unique attributes of ECI and other epidemic-associated strains.

Epidemic Clone II. In 1998-1999, a new genotype of *L. monocytogenes* serotype 4b was implicated in a multistate outbreak of listeriosis in the United States that involved contaminated hot dogs and was responsible for 101 human cases, including 21 deaths (13, 14). Strains from this outbreak exhibit unique ribotype and PFGE patterns that had been only rarely encountered before in the national PulseNet database. Upon further characterization, the strains from this outbreak were designated Epidemic Clone II (ECII). In 2002, another multistate outbreak of listeriosis also involved bacteria of serotype 4b and was attributed to contaminated turkey deli meats. PFGE and ribotyping data revealed that the isolates from the 1998-99 and 2002 outbreaks were closely related (17).

Previous studies have found that ECII isolates have diversification in a genomic region ("region 18") that was otherwise conserved among other *L. monocytogenes* of serotype 4b. PCR with primers derived from these DNA fragments of other strains of serotype 4b as well as Southern blots with the amplicons as probes readily differentiated ECII from other serotype 4b strains, with absence of the PCR product or signal in Southern blots from ECII strains (27).

Recently, the genome of strain H7858 representing ECII (a serotype 4b frankfurter isolate from the multistate outbreak of 1998-1999 in the USA) has been sequenced (56). Sequence analysis revealed a number of genes and gene clusters that were unique to ECII strains. Using these sequence data, along with prior findings resulting from the characterization of ECII isolates (37), several ECII-specific genetic makers have been identified which can facilitate the detection and further characterization of strains belonging to this clonal group. For instance, a recent study indicates that the 2002 outbreak isolates harbored the ECII-specific genetic markers, including diversification in genomic region 18. These findings, in conjunction with data from DNA array-based subtyping, confirmed that the closely genetic relatedness between the isolates from the two outbreaks (44). These multiple lines of evidence suggest that both the 1998-1999 and 2002 multistate outbreaks of listeriosis in the United States involved closely related members of a single clonal group (ECII) that had not been identified in outbreaks prior to 1998.

Epidemic Clone III. In 2000, a multistate outbreak of listeriosis in the United States involved contaminated turkey deli meat products and resulted in 29 cases across 10 states (15). These outbreak strains have indistinguishable PFGE pattern and the same ribotype. Unlike most other outbreaks, the implicated strains were serotype 1/2a, belonging to lineage

II. An interesting finding was that the outbreak strains had the same genotype as a strain that was identified in a early human listeriosis case associated with consumption of contaminated turkey franks in 1988 (12). The products implicated in the 2000 multistate outbreak were from same processing plant as the earlier isolate, suggesting that this strain had persisted there over several years, without detectable genotypic changes (42, 58). Identification of ECIII-specific markers probably represents a particular challenge since 1/2a strains belong to lineage II, which harbors more genetic diversity than lineage I (1, 5, 7, 39). Clearly, the previously published genome sequence of *L. monocytogenes* EGD-e (serotype 1/2a) and recently published genome sequence of *L. monocytogenes* F6854 (from the 1988 hot dog isolate) will greatly facilitate the use of genomotyping schemes to identify ECIII-specific genetic markers (56).

1.4.3.2 Reservoirs and ecology

The repeated involvement of the same epidemic clone in different outbreaks suggests that the implicated strains have a reservoir during the interval between outbreaks (41). The widespread incidence of certain epidemic clones suggests that these clonal groups could be ubiquitous in the environment. Animal and human carriers, or asymptomatic individuals, may also serve as reservoir for these clones, although the transmission pathways of *L. monocytogenes* remain unclear. Epidemiological data suggested that ECI strains appear to be present prior to their involvement in outbreaks, along with other sporadic strains that constituted the background levels of human listeriosis (43). Earlier studies indicated that ECI strains, which are clearly virulent to humans and farm animals, appear to be not predominant in foods for unknown reasons. In a recent survey of a group of serotype 4b food isolates, results revealed an unexpectedly high prevalence of strains harboring ECI-specific genetic

markers (80). This may reflect a relatively high fitness of strains with these ECI genetic markers in foods, compared to other serotype 4b strains, and may partially account for the repeated involvement of this epidemic clone in different outbreaks. In another study, survey of environmental samples from two turkey processing plants in the United States showed that environmental and raw product samples from one plant repeatedly yielded isolates with genetic markers typical of the two major serotype 4b epidemic clonal groups, ECI and ECII. The finding suggested that *L. monocytogenes* 4b isolates that harbor ECI- or ECII-specific genetic markers were established and persisted in the processing plants (26). As mentioned previously, ECIII strains appeared to have persisted in the processing plant for more than a decade. These findings suggest that the food manufacturing facility may be one of the key reservoirs for these epidemic clone strains.

Currently, substantial gaps remain in our knowledge of the transmission of foodborne listeriosis. More survey work is necessary to clarify the ecology and reservoirs of *L. monocytogenes*.

1.4.3.3 Genetic diversity of outbreak strains

Although the strains involved in outbreaks are highly clonal, the potential for genome diversification may be significant in *L. monocytogenes*. Depending on the phenotypes that are affected and the strain characterization methods that are employed, such variants may or may not be easily detected genotypically and phenotypically. Twenty-seven percent of the clinical strains from the Nova Scotia outbreak lacked galactose substituents in the teichoic acid of the cell wall, which serves as a major 4b-specific antigen determinant and receptor for 4b-specific phage. Such variants were negative with serotype 4b-specific monoclonal antibodies and resistant to serotype 4b-specific phages. Similar variants with same

phenotypic characteristics were identified in both the California and the Massachusetts outbreaks (18). The mechanisms responsible for the establishment of c74.22-negative phenotype in epidemic strain populations remain to be elucidated. One possibility is that these phenotypes became established during isolation, passage, or storage of the bacteria in the laboratory. An alternative hypothesis is that the observed variants were selected during infection of their human host, or in response to other selective pressures. A detailed PFGE analysis of isolates from the 1998-99 outbreak revealed minor variations in PFGE profiles among isolates from this outbreak (33). In addition, genome diversification was found in 11 strains isolated from independent patients from this outbreak (81). Distinguishable patterns of polymorphism was detected for each strain, although they shared identical ribotype and had a common PFGE pattern with only two strains differing by a single band. Though the data from different outbreaks seem to support the potential for rapid diversification of the genome, there is little evidence for a common underlying mechanism.

1.4.4 Population structure

A sister-group relationship between lineage I and III. As we discussed earlier, all of previous phylogenetic analyses generated concordant results that the species *L. monocytogenes* is indeed composed of two phylogenetic lineages. More recently, some groups have suggested the existence of a third evolutionary lineage containing strains of serotype 4a, 4c, and 4b (64, 77). However, disagreement persists about the evolutionary relationships between the three *L. monocytogenes* lineages. The early MLEE data can only differentiated *L. monocytogenes* into two lineages. Similarly, in two studies based on mixed-genome microarray data (3) and data from ribotyping and RAPD (53), only two lineages were recognized, with lineage III interpreted as a branch of lineage I. In both studies, the

identification of a third lineage is limited most likely due to inadequate sampling of variation within lineage III since both included only two lineage III isolates. A phylogenetic analysis on basis of *prfA* virulence gene cluster sequences (*p*VGC) revealed that average genetic distance between *p*VGC haplotypes was significantly (P<0.001) greater for lineage III than for lineage I (77). This result verified that there are at least three primary evolutionary lineages within *L. monocytogenes*. The combined *p*VGC data derived from both neighbor-joining and maximum-parsimony analyses support a sister-group relationship between lineage I and III, and indicate that lineage I and III share a common ancestor. This conclusion is in agreement with the results derived from MLEE analysis, combined analysis of ribotyping and RAPD typing, and phylogenetic analyses based on genomic microarrays. In addition, this observation is also consistent with phylogenetic data based on two housekeeping genes (*gap*, *prs*) and one stress gene (*sigB*) (57), as well as MLST data of nine genes (*hisJ, cbiE, truB, ribC, comEA, purA, aroE, hisC,* and *addB*) (52).

Distinct genetic structures of two lineages (lineage I and lineage II). In early MLEE studies, the clonal characteristic of population structure is supported by three lines of evidence, including the lack of sharing of serotypes between two major primary lineages, the occurrence of linkage disequilibrium (nonrandom association of alleles) among ETs for many pairs of the enzyme loci assayed, and closely genetic relatedness of geographically unlinked isolates (4, 60) . Genetic linkage can be a useful index to the degree of horizontal transfer (Linkage disequilibrium) occurring within the species *L. monocytogenes*. Commonly, the index of association (I_A) was used to test for linkage disequilibrium. Significant linkage disequilibrium was detected in two sets of data based on the mixed-genome microarray (10) and MLST of nine housekeeping genes, respectively (67). However, no evidence for linkage

disequilibrium was detected when the analysis was performed separately in lineage I and lineage II. These data suggested that the recombination should be rare between strains belonging to different lineages, but the evidence for clonal structure disappears when the lineage I and lineage II data were analyzed separately. Furthermore, more recent studies revealed that lineage I isolates appear to highly clonal with limited horizontal gene transfer and lineage II isolates are more active in recombination (52). It can be concluded consistently from data generated by two different phylogenetic analyses (MLST or combined ribotyping and RAPD) that the vast majority of lineage I isolates cluster tightly and lineage II isolates showed greater genetic diversity (52, 53, 57). In two DNA sequence–based phylogenetic analyses, Sawyer's test for recombination was performed with each of the sequence alignments and data indicated that more recombination events occur in lineage II than lineage I (52, 57). These findings are in agreement with previous studies, which have shown that different bacterial species can demonstrate varied population structures, ranging from *Neisseria*, a species with high genetic diversity to *Salmonella*, a species with highly clonal population structure (45).

1.5 Comparative genomics

L. monocytogenes is widely distributed in nature and it has been isolated from numerous foods and food processing environments. Furthermore, *L. monocytogenes* has the capacity to adapt and survive in extreme environments such as high salt concentration (10% NaCl), a broad pH range (from 4.5 to 9.0) and a wide temperature range (-1 ºC-45 ºC). *L. innocua* is the species genetically closest to *L. monocytogenes*. Even though these two species differ markedly in pathogenicity, they share the same ecological niches in the environment

(including food, vegetation, and soil). In enrichment broths, *L. innocua* can overgrow *L. monocytogenes*, which may lead to *L. monocytogenes* escaping detection (6, 32).

In order to address questions regarding the molecular basis of the pathogenesis, phenotypic differences and the evolution of listeriae, *L. monocytogenes* strain EGD-e (1/2a) and *Listeria innocua* CLIP 11262 (6a) have been fully sequenced. A comparison of these sequences revealed 10.5 % and 14% specific genomic sequences for each strain, respectively (31). Although this initial comparison between two strains provides considerable insight on the virulence attributes of *L. monocytogenes*, the sequencing and comparative genomic analysis of additional *L. monocytogenes* strains was necessary if a core set of *L. monocytogenes*-specific genes was to be defined. To date, *L. innocua* (serotype 6a), *L. monocytogenes* strains EGD-e (serotype 1/2a, lineage II), and F2365 (serotype 4b, lineage I) have been sequenced to closure, whereas the genomes of strains F6854 (serotype 1/2a, lineage II) and H7858 (serotype 4b, lineage I) were sequenced to 8-fold coverage of an estimated 3.5 Mbp genome without gap closure (56). Sequence analysis and in particular comparative genomics will help to better understand the molecular mechanisms of *L. monocytogenes* virulence in humans and survival of this bacterium in food and in the environment. Most recently, 16 of *L. monocytogenes* strains have been sequenced by Broad Institute of MIT and Harvard University.

1.5.1 Pathogenic *L. monocytogenes* vs. nonpathogenic *L. innocua*

The *L. monocytogenes* EGD-e genome is 2.9 Mb long and has an average G+C content of 39%. The *L. innocua* chromosome has a similar size (3.0 Mb) and a similar G+C content (37%). *Listeria*-specific features deduced from sequenced genomes indicated that both genomes contain a large number of surface proteins, an abundance of transport proteins, in

particular proteins dedicated to carbohydrate transport, and an extensive regulatory repertoire (8). Surface proteins have important roles in the interactions of the microorganism with its environment, in particular during host infection. Major virulence factors of *L. monocytogenes* include surface proteins, such as InlA and InlB, necessary to enter eukaryotic cells, or ActA, playing a key role in actin-based motility. A specific feature of the *Listeria* genomes, probably also related to its property to colonize a broad range of ecosystems, is the presence of a large number of genes encoding different transport proteins. The 209 regulatory proteins identified in the *L. monocytogenes* genome should be connected to the capacity of *Listeria* to adapt and respond to a wide variety of different environments, and some global regulatory systems might also be associated with virulence.

Horizontal gene transfer is a key evolutionary force that leads to a mosaic genome structure in bacteria. It includes the transmission of phages, plasmids and the uptake of DNA by naturally competent bacteria. Differences between the two listerial genomes provide evidence for all three mechanisms. The sequenced *L. monocytogenes* EGD-e and *L. innocua* genomes contain one and five prophages, respectively. Furthermore, *L. innocua* carried an 81.9-kbp plasmid encoding heavy metal resistance. Most interesting finding was that both *Listeria* genomes contain putative DNA uptake genes, homologous to *Bacillus subtilis* competence genes. As *Listeria* are not known to be naturally competent, the *Listeria* DNA uptake apparatus may have lost its original function. Alternatively, signals that induce competence in *Listeria* may differ from those in *B. subtilis*, since the counterparts of several *B. subtilis* regulatory genes required for competence gene expression are missing in *Listeria*. In *L. monocytogenes*, 54 of the 100 specific regions had a noticeably lower G+C content than

the flanking regions and 6 had a noticeably higher G+C content, suggesting recent acquisition by horizontal gene transfer (31).

If prophage genes are excluded, 270 (9.5%) *L. monocytogenes* EGD-specific genes and 149 (5%) *L. innocua*-specific genes were identified. These species-specific genes were distributed within different functional categories, including proteins homologous to known virulence-associated proteins from other bacteria or proteins implicated in adaptation to different environments. The *L. monocytogenes* genome sequence revealed the presence of 41 proteins containing an LPXTG motif, 19 of which belong to the LRR/internalin family. Eleven of those are absent from *L. innocua*. InlB and an additional member of the GW protein family were absent from *L. innocua*. Of 86 secreted proteins, 23, including three soluble internalins, were absent from *L. innocua*. In *L. monocytogenes*, 26% of the transporter genes were devoted to carbohydrate transport mediated by phosphoenolpyruvate-dependent phosphotransferase systems (PTS) and corresponding to 39 putative complete or incomplete enzyme II permeases. Eight enzyme II permeases were absent from *L. innocua.* PrfA, a member of the Crp/Fnr family, which activates most of the known virulence genes, was missing from *L. innocua.* Genes encoding glutamic acid decarboxylases (*gad*) involved in acid resistance were identified. One of the three *gad* paralogs of *L. monocytogenes* was absent from *L. innocua*. In addition, three genes possibly involved in the degradation of bile salts were present in *L. monocytogenes* but not in *L. innocua*, probably reflecting the capacity of *L. monocytogenes* to survive in the mammalian gut (31).

1.5.2 Comparative genomic analyses of *Listeria monocytogenes* serotype 4b and 1/2a strains

In 2001, in order to assess whether genomic loci exist that could underlie the increased epidemic potential of epidemic-associated strains, Herd and Kocks subtracted the genome of the virulent prototype *L. monocytogenes* EGD-e from a prototype epidemic strain F4565 (serotype 4b, isolated from the same outbreak as F2365). Five to six percent of the genome of strain F4565 was shown not to hybridize to strain EGD-e DNA. A large number of these DNA regions code for bacterial surface determinants and hence are likely to play roles in the interaction with the environment or with the host organism (37).

Representatives of the two major genomic lineages of *L. monocytogenes* as represented by strains of serotype 1/2a and 4b were chosen for whole genome sequencing in 2004 (56). More specifically, *L. monocytogenes* strain F2365 is a serotype 4b (genomic lineage I) cheese isolate from the Jalisco cheese outbreak of 1985 in California, and represents Epidemic Clone I; *L. monocytogenes* strain H7858 is a serotype 4b frankfurter isolate from the multistate outbreak of 1998-1999 in the USA and represents Epidemic Clone II; *L. monocytogenes* strain F6854 is a serotype 1/2a (genomic lineage II) turkey frankfurter isolate from a sporadic case in 1988 in Oklahoma, and represents Epidemic Clone III. These strains were used in a comparative genome analysis that includes a comparison with the two previously published strains: *L. monocytogenes* EGD-e (serotype 1/2a) and *L. innocua* strain CLIP 11262. A total of 57, 97, 69 and 61 strain-specific genes were identified from strains F2365, F6854, H7858 and EGD-e, respectively. Although strain-specific and serotype-specific genes were identified, the genomes of all four *L. monocytogenes* were remarkably similar in gene content and organization. Comparative genomic analysis has revealed that the

L. monocytogenes genomes are syntenic, with the majority of genomic differences consisting of phage insertions, transposable elements, scattered unique genes, and islands encoding proteins of known function, as well as single nucleotide polymorphisms (SNPs) in many genes, including those associated with virulence functions. With the exception of prophage sequences, genes present in *L. innocua* CLIP 11262 that were absent from *L. monocytogenes* EGD-e were typically also absent from the genomes of the other three *L. monocytogenes* strains, suggesting gene loss from a lineage ancestral to *L. monocytogenes* prior to the genomic diversification into genomic lineage I and lineage II.

The overall similarity in metabolism and transport between the different *L. monocytogenes* serotypes suggests that substrate utilization patterns are similar in serotype 1/2a and 4b. Thus not differences in the utilization of substrate but other physiological differences may underlie the observed differences in ability of the different serotypes to colonize processing plants. Major virulence determinants (i.e. LLO, PlcA, PlcB, ActA, PrfA, InlA, and InlB) are conserved in all four *L. monocytogenes* strains. Strain-specific virulence genes, which may explain possible differences in virulence among strains, remain unidentified through whole genome comparisons, although a number of previously unknown protein sequences containing characteristic motifs of putative and known virulence factors have been identified. Overall, all four *L. monocytogenes* strains shared a high degree of similarity in their genomes. The comparison of the four sequenced *L. monocytogenes* genomes suggest that *L. monocytogenes* strains prevalent in human and animal illness rely on a relatively small number of unique regions for antigenic diversity and epidemiologically relevant attributes.

1.5.2.1 Lineage- and strain-specific genes

Comparative genomic analysis and genome microarrays have revealed that most lineage-specific and strain-specific genes fall into three major categories: genes coding for surface proteins, genes coding for transcriptional regulators, and genes coding for transporter proteins (81). An important gene family in *L. monocytogenes* encodes surface proteins. The macroarray hybridization and the sequence analyses of *L. monocytogenes* serotype 4b strain indicated that a group of genes encoding surface proteins which includes all known internalin genes (*inlA*, *inlB*, *inlG*, *inlH*, *inlE*, *inlC*, and *inlF*) is highly specific for the species *L. monocytogenes*. Each subgroup within each lineage of *L. monocytogenes* appeared to be characterized by a specific set of surface proteins. Interestingly, in the rarely isolated *L. monocytogenes* serovar 4a strains, which are mostly animal pathogens, 13 of 25 *L. monocytogenes*-specific surface proteins, including all internalins except *inlAB*, were missing (20). The lack of these surface proteins may account for lower disease potential of these strains for humans, resulting in lower prevalence in human illness. The fact that different subgroups of each *L. monocytogenes* lineage contain different sets of surface proteins may also reflect possible differences in potential to cause illness or to adapt to different environmental niches. The functional analysis of different surface proteins and the putative strain-specific characteristics that they confer may provide additional insights into our understanding of the tropism of *L. monocytogenes* toward different cell types and the ubiquity of *L. monocytogenes* in environment. In addition, genomic subtraction study also revealed that many epidemic-associated serotype 4b strain F4565-specific genes had homologies to surface proteins of *L. monocytogenes* or to other Gram-positive bacteria (37). Thus, several independent comparative genome studies of *L. monocytogenes* consistently

show a collective bias toward cell surface-related differences in genome content among the *L. monocytogenes* serotypes and *Listeria* species. These findings suggest selection pressures for different combinations of cell surface characteristics, which perhaps distinguish the way the lineages and species interact at the cell surface with host cells or with the external environment.

1.5.2.2 Surface proteins

In Gram-positive bacteria, some components of cell wall are crucial for bacterial viability. The cell wall also serves as a docking site for proteins that interact with environmental factors, bacterial phages and infected hosts and that are involved in bacterial adherence, invasion and interaction with the host immune system (9). A global analysis on the basis of the sequenced genomes of *L. monocytogenes*, which only included proteins displaying a signal peptide, has detected four major types of surface proteins in *Listeria monocytogenes*: proteins that posses an LPXTG carboxyl-terminal sorting signal; proteins anchored by a hydrophobic tail motif; proteins containing GW module; and lipoproteins (9).

A total of 133 surface proteins were identified in *L. monocytogenes* EGD-e, including 41 LPXTG proteins, nine GW proteins with a signal peptide, 11 hydrophobic tail proteins, four P60-like proteins and 68 lipoproteins. Thus, at least 4.7% of the coding capacity of the genome is dedicated to surface proteins. Moreover, 22.6% of genes encoding putative cell-surface proteins are absent from *L. innocua*, indicating that the main differences between these two species can be attribute to their surface protein composition.

1.5.2.3 Cell wall-associated proteins and surface antigens

A comparison across the genomes of four *L. monocytogenes* strains (F2365, F6854, H7858, and EGD-e) has revealed that the serotype 1/2a-specific genes also include an operon that encodes the biosynthetic pathway for the antigenic rhamnose substituents that decorate the cell wall-associated teichoic acid polymer in serotype 1/2a strains, five glycosyltransferases and an adenine-specific DNA methyltransferase (56). A previous study using shotgun DNA microarrays constructed from the serotype 1/2a strain 10403s show two serotype 1/2a-specific regions of genome difference (RD) are related to rhamnose decoration of cell wall teichoic acid (81). Similarly, in *L. monocytogenes* serotype 4b strains, two 4b-specific genes (*gtcA* and *gltAB*) have been identified, which are responsible for galactose decoration of cell wall teichoic acid (46, 61). Strain-specific genes associated with cell wall and teichoic acid biosynthesis, as well as glycosyltransferases, are probably related to differences in somatic antigens and may be involved in virulence and immunogenicity of strains of different serotypes. Comparative genome analyses using subtractive libraries identified a 4b F4565-specific gene fragment encoding a putative cell-wall associated protein, homologous to *wapA* of *Bacillus subtilis.* However, the functional roles of WapA in *B. subtilis* remain poorly characterized (37).

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CHAPTER II Materials and Methods

2.1 Bacterial strains and growth media

The bacterial strains used in the studies described in chapter V and VI are listed in Table 1. Bacterial strains used in chapter III and IV are listed, separately. *Escherichia coli* strain S17-1 or SM10 were used as a host cell that could introduce plasmid pCON-1 to *L. monocytogenes* by conjugation. The liquid medium used for growing *Listeria* was brain heart infusion (BHI) (BD, Sparks MD) broth or tryptic soy broth with 0.7% yeast extract (TSBYE) (BD). The agar medium was BHI with 1.5% agar, or tryptic soy agar (TSA) (BD) supplemented with yeast extract (0.7%) (TSAYE). The soft agar medium used for motility assays was TSA+0.7%YE with 0.4% agar. Hemolytic activity was determined on Blood Agar (Remel, Lenexa KS USA). The bacteria were grown at 35-37 ºC or room temperature (20-25 ºC). Bacterial strains were preserved in BHI liquid broth with 20% glycerol at –70 ºC. Antibiotics used for *Listeria* were chloramphenicol (6 µg/ml), nalidixic acid (20 µg/ml) and streptomycin (1200 µg/ml). *Escherichia coli* strains were grown in LB broth or agar medium, 100 µg/ml of ampicillin was added if necessary. They were preserved in LB (Luria-Bertani) liquid medium with 20% glycerol at –70 °C.

Table 1: Bacterial strains used in chapter V and VI

Species and strains	Serotype	Relevant genotype and features	Source and reference
Escherichia coli			
S17-1		Conjugation donor; pro *hsdR recA* (with integrated plasmid RP4 Tc : : Mu-Km : : Tn7)	
SM10		Conjugation donor; F^-thi-1 thr-1 leuB6 recA tonA21 lacY1 supE44 (Mu_C^+) λ^- [RP4-2(Tc::Mu)] Km^r Tra^+	
E-pCONIΔ*wap*		Temperature sensitive shuttle vector pCON-1 carrying flanking upstream and downstream DNA fragment of *wap*, gene encoding wall-associated protein in F2365, pCONIΔ*wap*/S17-1	
E-pCONIIΔwap		Temperature sensitive shuttle vector pCON-1 carrying flanking upstream and downstream DNA fragment of *wap*, gene encoding wall-associated protein in H7550, pCONIIΔ*wap*/S17-1	
E-pCONIΔ18R		Temperature sensitive shuttle vector pCON-1 carrying flanking upstream and downstream DNA fragment of region "18" in F2365, pCONIΔ18R/S17-1	
E-pCONIIΔ18R		Temperature sensitive shuttle vector pCON-1 carrying flanking upstream and downstream DNA fragment of region "18" in H7550, pCONIIΔ18R/S17-1	
E-pPL2		Integration vector, pPL2/S17-1	
E-pPL2_II*wap*		Integration vector carrying gene encoding wall-associated protein in H7550, pPL2_II*wap*/S17-1	This study
E-pPL2_*gtcA*P		Integration vector carrying promoter region of *gtcA* in H7550, pPL2*gtcA*P /S17-1	This study
E-pPL2_*gtcA*P_II18R		Integration vector carrying promoter region of *gtcA* followed by the gene cassette flanked by *wap* and *inlA* (region "18") in H7550, pPL2_*gtcA*P_II18R /S17-1	This study

Table 1: Bacterial strains used in chapter V and VI (cont'd)

Listeria monocytogenes			
F2381		Food isolate from Jalisco cheese outbreak (1985)	
F2381L	4b	F2381, StrR derivative of	This study
F2381L-Phi-R	4b	F2381L, c74.22-negative Laboratory variant	This study
F2365	4b	Food isolate from Jalisco cheese outbreak (1985)	This study
H7596	4b	Food isolate from hot dog outbreak (1998-1999)	This study
H7550	4b	Clinical isolate from hot dog outbreak (1998-1999)	This study
H7550S	4b	H7550, Str^R derivative	This study This study
ECI_ pCONIΔ*wap*		F2381-Phi-R containing recombinant plasmid pCONIΔ*wap* (Transconjugants)	This study
ECII_ pCONIIΔwap		H7550 containing recombinant plasmid pCONIIΔ*wap* (Transconjugants)	This study
ECI_pCONIΔ18R		F2381-Phi-R containing recombinant plasmid pCONIΔ18R (Transconjugants)	This study
ECII_pCONIIΔ18R		H7550 containing recombinant plasmid pCONIIΔ18R (Transconjugants)	This study
F2365_ pCONIΔ*wap*		F2365 containing recombinant plasmid pCONIΔ*wap* (Transconjugants)	This study
F2365_ pCONIΔ18R		F2365 containing recombinant plasmid pCONIΔ18R (Transconjugants)	This study
ECIΔ*wap*		F2381-Phi-R with deletion of *wap*	This study
ECIΔ18R		F2381-Phi-R with deletion of the gene cassette flanked by *wap* and *inlA*	This study
F2365Δ*wap*		F2365 with deletion of *wap*	This study
F2365Δ18R		F2365 with deletion of the gene cassette flanking by *wap* and *inlA*	This study
ECIIWT		H7550 without pLM80	This study
ECIIΔ*wap*		H7550 with deletion of *wap* (loss of pLM80)	This study
ECIIΔ*wap*:pPL-2		ECIIΔwap, $tRNA^{Arg}$: pPL2	This study
ECIIΔ*wap*:*wap*		ECIIΔwap, $tRNA^{Arg}$: pPL2_II*wap*	This study
H7550Δ*wap*		H7550 with deletion of *wap* (presence of pLM80)	This study
H7596Δ*wap*		H7596 with deletion of *wap*	This study

Table 1: Bacterial strains used in chapter V and VI (cont'd)

ECIIΔ18R	H7550 with deletion of the gene cassette flanking by *wap* and *inlA*	This study
ECIIΔ18R:*gtcA*P	ECIIΔ18R, $tRNA^{Arg}$: pPL2_*gtcA*P	This study
ECIIΔ18R:*gtcA*P18R	ECIIΔ18R, $tRNA^{Arg}$: pPL2_*gtcA*P_18R	This study

2.2 DNA hybridization analysis

2.2.1 Isolation of genomic DNA of *Listeria monocytogenes*

A single colony was inoculated in 5 ml TSB+ 0.7%yeast extract, and incubated at 37 ºC overnight. Five milliliters of the culture were collected by centrifugation (13k rpm, 1 minute) in a 1.5 ml microcentrifuge tube. The pellet was resuspended in 180 µl enzymatic lysis buffer (10 ml lysis buffer: 200 µl 1M Tris-HCl pH7, 40 µl 0.5 M EDTA, 120 µl Triton x 100, 400 mg lysozyme, 9.64 ml diH_2O) and incubated at 37 ºC for at least 6 hours, followed by the addition of 25 µl of proteinase K (Qiagen) and 200 µl of buffer AL, and incubating at 70 ºC for 30 minutes. The DNeasy tissue kit (Qiagen Valencia, CA) was used to extract and isolate DNA from lysed cells according to the vendor's instructions. Genomic DNA was resuspended in 80-100 µl diH_2O. Twenty microliters of DNA was diluted with 980 µl diH_2O. Absorbance at 260 nm was determined using spectrophotometer (Biorad, SmatSpec™ 3000 CA USA). The value of A_{260nm} was converted into the concentration of DNA on the basis of the equation (1 A_{260nm} = 50 µg/ml × dilution factor). Genomic DNA was stored at 4 ºC.

2.2.2 Probe labeling with DIG-dUTP

DNA probes were labeled with digoxigenin (Genius kit; Roche) by following the manufacturer's instructions. Briefly, 10 ng-1 µg DNA was added to autoclaved diH_2O to a final volume of 15 µl. The DNA was denatured by heating in a boiling water bath at 95-100

ºC for 10 minutes, and immediately placed on ice. Subsequently, 2 µl 10× Hexanucleotide Mix, 2 µl 10× DIG DNA labeling mix and 1 µl Klenow (5U / µl) were mixed with the denatured DNA and was incubated at 37 ºC overnight. The reaction was stopped by adding 2 µl of 0.2 M EDTA (pH 8.0). The labeled DNA was stored at -20 ºC.

2.2.3 Digestion of DNA with restriction enzymes and agarose gel electrophoresis

The most frequently used enzymes in this study were *Eco*RI, *Hin*dIII, *Sau*3AI, *Bam*HI etc (New England, Biolabs, Beverly, MA). Digestion conditions were used as suggested by the vendor. Genomic DNA was completely digested by the restriction enzyme at appropriate temperature for at least 6 hours. The total volume of the restriction mixture was 20 µl (1 µg genomic DNA, 1 µl enzyme *Eco*RI, 2 µl 10× Buffer (supplied by the vendor), H_2O to 20 µl). The whole volume (20 µl) of completely digested genomic DNA was separated in 0.8% agarose gel and 1 ×TBE buffer (10× stock solution: 108g Tris base, 55 g Boric acid, 40 ml 0.5 M EDTA, pH 8.0, H_2O to 1 liter).

2.2.4 Southern transfer

After electrophoresis, the gel was placed in a glass or plastic container and soaked in 250 mM HCl with shaking for no longer than 10 min (depurination), denatured by alkaline solution (0.5 N NaOH, 1.5 M NaCl) for 1 hour and neutralized with neutralization solution (0.5 M Tris-HCl, pH 8.0, 1.5 M NaCl) for 1 hour. A nylon membrane (OSMONICS INC., MagnaGraph, Nylon Transfer Membrane MA) was pre-wetted in H_2O for 5 minutes, then transferred to 2 × SSC (dilution from 20 × SSC, 20 × SSC: 175. 3 g NaCl, 88. 2 g NaCitrate (Citric Acid Trisodium Salt), pH7.0, total volume 1L, autoclave) for several minutes. The DNA was transferred onto nylon membrane in 10× SSC by capillary action, overnight. The

fragmented DNA-bearing nylon membranes were treated with a UV crosslinker on automatic setting (STRATAGENE, UV Strata linker 1800) to fix the DNA on the membrane.

2.2.5 Hybridization

The membrane was prehybridized with the prehybridization solution (5× SSC, 0.1% of N-lauroylsarcosine, 0.02% of SDS, 2% of blocking reagent (Roche), and 50% of formamide) for 3 hours and hybridized with the DIG-labeled DNA probe overnight. Both prehybridization and hybridization were performed in a rolling hybridization bottle (HYBAID MICRO-4) at 42 ºC.

2.2.6 Detection

Before detection, the membrane was washed at room temperature with 2× SSC (containing 0.1% SDS) for 20 minutes, 1× SSC (containing 0.1% SDS) for 20 minutes, and 0.5× SSC (containing 0.1% SDS) for 20 minutes. The membrane was then blocked in blocking buffer (0.15 M NaCl, 0.1 M Tris-HCl, pH 7.5, 2% blocking reagent purchased) with gentle shaking for 1 hr, and incubated with anti-DIG-alkaline phosphatase (dilute 1:10,000, in blocking buffer) for 30 minutes. The membrane was washed with Maleic acid buffer (0.1 M Maleic Acid, 0.15 M NaCl, pH 7.5) for 20 minutes twice. CSPD (Chemiluminescent substrate, Roche) (dilute 1:100 ml in detection buffer 0.1 M Tris, 0.1 M NaCl, pH 9.5) was used as the substrate for alkaline phosphatase. X-ray film (Fuji) was exposed to the chemiluminescent light resulting from hybridization of DNAs with the probe. The DNA on the nylon membrane can be re-probed 2-3 times with little loss of the hybridization signal.

For reprobing, previous probe was removed (“stripped”) by incubating the membrane in alkaline probe stripping solution (0.2 N NaOH, 0.1% SDS) at 42 ºC with rolling in

hybridization bottles for 25 minutes twice, and then washing thoroughly in 6 ×SSC. The membranes then were used in prehybridization and hybridization procedures as described above. After detection, the membranes were soaked in Genius buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), sealed in a plastic bag and stored at 4 ºC. The digoxigenin-labeled DNA probes and the hybridization solutions were kept at -20 ºC. Such stored probes did not lose much activity for 2-3 months.

2.3 RNA extraction and reverse-transcriptase polymerase chain reaction (RT-PCR)

2.3.1 RNA extraction

A single colony of bacteria was inoculated in 3 ml TSB+ 0.7%yeast extract, and incubated at 37 ºC overnight. All 3 ml of the cells were recollected by centrifugation (11.9k rpm, 3 minutes) in a 1.5 ml microcentrifuge tube. and the pellet was suspended in 180 µl enzymatic lysis buffer and incubated at 37 ºC for 4 hours. Twenty five microliter of proteinase K (Qiagen) was added and incubated at room temperature for 30 minutes. 1 ml TRIzol (Invitrogen, Carlsbad, CA) (1 ml of TRIzol per 1.0 $\times 10^7$ cells to homogenize) was added and vortexed for 3 seconds. The mixture was incubated at room temperature for 2-3 minutes, and then was centrifuged (11400 rpm, 4 ºC, 15 minutes). The aqueous phase (the upper colorless phase) was gently transferred to a new 1.5 ml centrifuge tube, avoiding the pipette tip to touch the white middle phase (the protein phase), and the bottom red phase (the DNA phase). Five hundred microliters of isopropyl alcohol was added and mixed by hand shaking. The mixture was incubated at room temperature for 10 minutes and centrifuged (11400 rpm, 4 ºC, 10 minutes). The supernatant was discarded and the RNA pellet was resuspended in 1 ml 75% ethanol, centrifuged (8900 rpm, 4 ºC, 5 minutes), and air dried for

approximately 10 minutes. The RNA was dissolved in 50-100 µl DEPC water (0.1% Diethylpyrocarbonate in diH_2O, kept at room temperature overnight and then autoclaved), and incubated at 55-60 ºC for 10 min. Five microliter RNA was diluted with 995 µl 10 mM Tris-HCl. (pH7.5). Absorbance at 260 nm was determined using spectrophotometer (Biorad, SmartSpec™ 3000). The value of A_{260nm} was converted into the concentration of RNA on the basis of the equation (1 A_{260nm} = 40 µg/ml × dilution factor). RNA was stored at -70 ºC for later use.

2.3.2 Reverse transcriptase-polymerase chain reaction (RT-PCR)

Genomic DNA that might be contaminating the RNA preparation was destroyed by DNase I (Ambion Inc.). ImProm-II™ Reverse Transcription System (Promega, Madison, WI) was used to prepare complementary DNA (cDNA). For RT-PCR, treating 4 µl DNA-free RNA and 1 µl antisense primer at 70 ºC for 5 min. then RNA template and primer mix. was added to the 15 µl reverse transcription mixture (Table 2). In negative control of reaction mixture, reverse transcriptase was replaced by same amount of water. Reverse transcription proceeded by annealing at 25 ºC for 5 min and extend first strand for 60 minutes at 37-42 ºC. The Reverse transcriptase was heat-inactivated at 70 ºC for 15 minutes. Subsequently, 10 µl of the first strand reaction mixture or negative control was used as template for PCR amplification in 50 µl reaction mixture (95 ºC, 5 min; 95 ºC, 1 min; 54 ºC, 1 min; 72 ºC, 2 min; for a total 29 cycles). The components for 50 µl-PCR were listed in Table 3.

Table 2: Reverse transcription Mix.

DEPC H_2O	3.1 µl
ImProm-II™ 5× Reaction Buffer	4 µl
$MgCl_2$, 25 mM	2.4 µl
dNTP mix, 10 mM	4 µl
Recombinant RNasin® Ribonuclease Inhibitor	0.5 µl
Improm-II™ Reverse Transcriptase	1 µl
Total	15 µl

Table 3: PCR amplification

DEPC H_2O	20.7 µl
10× thermophilic polymerase reaction buffer (without $MgCl_2$)	4 µl
$MgCl_2$, 25 mM	2.8 µl
dNTP mix, 10 mM	2 µl
Upstream Primer (10 µM)	5 µl
Downstream Primer (10 µM)	5 µl
Taq DNA Polymerase (5 U/ µl)	0.5 µl
Total	40 µl

2.4 Construction of in-frame deletion mutants

2.4.1 Generation of upstream and downstream fragments using PCR

Two pairs of primers were designed based on the DNA sequence of the fragments flanking both sides of the target gene or target gene cassette. A certain restriction site was incorporated into the 5' end of each primer. Upstream fragment and downstream fragment of target gene were amplified by PCR using *L. monocytogenes* strain F2381 or H7550 genomic DNA as template. PCR products were purified using PCR purification kit (Qiagen Valencia, CA).

2.4.2 Construction of recombinant plasmid

2.4.2.1 Enzyme digestion and ligation

Purified PCR products (upstream fragment and downstream fragment) and vector pCON-1 were digested with restriction enzymes according to the vendor's instruction. Two digested PCR fragments were ligated into digested temperature-sensitive shuttle vector pCON-1 at room temperature overnight (FIG.1). The recombinant plasmid was transformed into competent cells of *E. coli* S17-1 using electroporation.

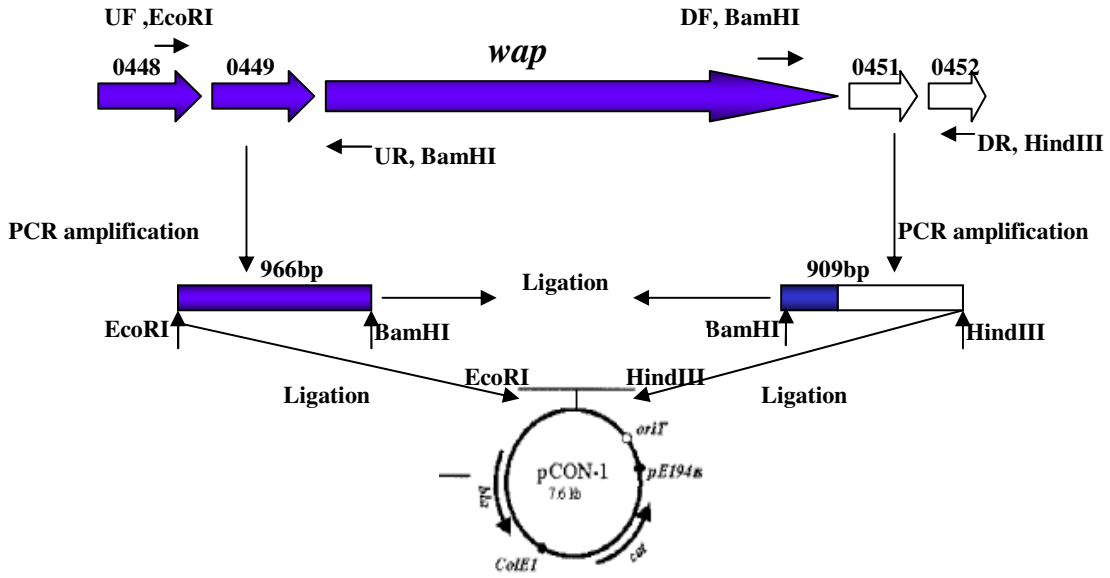

FIG.1: Construction of recombinant plasmid

2.4.2.2 Electroporation

The plasmids were transformed into competent cell *E. coli* SM10 by electroporation. 1.2 µl of the ligation reaction was gently mixed with 50 µl competent cells using a pipette, and the transformation for each recombinant plasmid was done in duplicate. Following electroporation (voltage max: 2.5; resistance: 200; capacitance: 25; time const: 4-5 ms), 1 ml

LB was added to resuspend the mixture in a 15-ml tube, and the cell suspension was incubated with shaking at 37 ºC for about 1hour. One milliliter of transformation mixture was plated on four LB agar with ampicillin (100 mg/l) and incubated at 37 ºC overnight.

2.4.2.3 Screen positive transformants

Colonies of transformants were restreaked on LB agar with ampicillin (100 mg/l). For initial large-scale screenings**,** a small amount of bacteria from 4 to 5 colonies were pooled with sterile plastic inoculation loop into 1 ml BHI. Colony PCR was done using 2 µl of pooled mixture as template, and a combination of forward primer for upstream fragment and reverse primer for downstream fragment. Positive pools were further screened using individual colony PCR for confirmation of single positive colony. The positive transformants carrying the recombinant plasmid were chosen for conjugation to introduce the recombinant plasmid into *L. monocytogenes*.

2.4.3 Bacterial conjugation

2.4.3.1 Conventional method

Donor was *E. coli* S17-1 carrying the recombinant plasmid, and ampicillin resistant. Recipient was *L. monocytogenes*. *L. monocytogenes* strain was naturally nalidixic acid resistant. Both donor and recipient were grown overnight at 37 ºC. Then, 3 ml of donor overnight culture and 1 ml of recipient overnight culture were transferred into micro-centrifuge tubes separately and centrifuged for 1min at maximum speed to pellet cells. The resulting pellet was resuspended in 1 ml BHI and washed by pipetting up and down. The culture was centrifuged for 1 min at maximum speed, and the pellet was resuspended in 1 ml BHI and washed one more time. The pelleted cells of recipient and donor were resuspended

in 1 ml of BHI and combined into one microcentrifuge tube. The pellet of mixture was washed in 1ml BHI twice.

Fifty microliters of the mixture was applied on the nitrocellulose filter (Millipore, 0.45 µm-pore-size HA-type filter) placed on BHI plate supplemented with penicillin G (0.5 mg/l) (Sigma), and incubated at 30 ºC overnight. The nitrocellulose filter with the bacteria was placed in 1 ml BHI, and 50-100 µl of the suspension was spread on BHI plates with nalidixic acid (20 mg/l) and chloramphenicol (6 mg/l). The plates were incubated at 30 ºC for 2 to 3 days. Transconjugants were picked and streaked on the BHI plates with nalidixic acid (20 mg/l) and chloramphenicol (6 mg/l). Transconjugants were further screened by colony PCR using a combination of forward primer for upstream fragment and reverse primer for downstream fragment.

2.4.3.2 Modified procedure for conjugation

An alternative procedure for conjugation was used when efficiency of the conventional method is low. Bacterial stains were grown to mid-log phase ($O.D._{600nm}$= ~0.55) with shaking at 30 ºC. *E. coli* donor strains were grown in LB supplemented with chloramphenicol (25 µg/ml), and *L. monocytogenes* recipient strains were grown in BHI. Overnight recipient culture was treated at 45 ºC for 10 min in water bath. Donor culture (3.0 ml) was mixed with 1.5 ml of heat-treated recipient culture and filtered onto 0.45-µm-pore-size HA-type filters (22 mm; Millipore; 10 ml syringe, BD). The filter was washed once with 10 ml of BHI. The filter was transferred onto a BHI plate without antibiotics, and incubated for 2 h to overnight at 30 ºC. The bacterial cells on the filter were resuspended in 2.5-4.0 ml of BHI, and 25 µl and 50 µl of resuspended bacterial cells were plated on BHI plates supplemented with 6 mg of chloramphenicol per liter and 20 mg of nalidixic acid per liter. Plates were incubated at 30

ºC for 2 to 3 days. In parallel, 50 µl of resuspended bacterial cells was transferred into 30 to 50 ml BHI broth supplemented with chloramphenicol (6 mg/l) and nalidixic acid (20 mg/l) and grown at 30 ºC overnight. Fifty microliter of overnight culture was plated on BHI plates with nalidixic acid and chloramphenicol and incubated at 30 ºC for few days.

2.4.4 Plasmid integration

As a shuttle vector, pCON-1 can also replicate in *L. monocytogenes*. The chloramphenicol resistant gene on pCON-1 is expressed. Therefore the transconjugants will be resistant to both chloramphenicol and nalidixic acid. Plasmid pCON-1 is also temperature-sensitive and can not replicate in the host cell at 42 ºC. Double antibiotic resistant transconjugants were streaked on BHI + nalidixic acid (20 mg/l)+ chloramphenicol (6 mg/l) agar, and incubated at 30 ºC overnight. The colonies were confirmed by PCR using a combination of forward primer for upstream fragment and reverse primer for downstream fragment. Two confirmed transconjugants were inoculated in 5 ml BHI + chloramphenicol (5 mg/l), respectively, incubated at 30 ºC overnight, diluted (1:100 or 1:1000) in BHI, 50 µl was plated on BHI+ chloramphenicol (5 mg/l) agar, and incubated at 42 ºC for 48 hours. Incubation at 42 ºC in medium with chloramphenicol (6 mg/l) facilitates to select the integration of the plasmid onto the chromosome since free pCON-1 plasmid can not replicate at this non-permissive temperature. If necessary, integration of recombinant plasmid could be confirmed by Southern blots.

2.4.5 Deletion construction by double crossover

Three colonies were streaked on BHI with chloramphenicol (5 mg/l) agar, and incubated at 42 ºC for 24 to 48 hours. Two colonies from these plates were inoculated in 5 ml BHI, and

incubated at 30 ºC overnight. This sub-culture was repeated for three more times (total 8-day incubation period). 300 µl of the culture from the fourth subculture was inoculated in 5ml BHI, incubated at 42 ºC overnight, diluted (10^{-5}-10^{-6}), plated (50 µl) on BHI agar, and incubated at 37 ºC overnight. 90 isolated colonies from this plate were tested for chloramphenicol resistance by replica plating on BHI agar and BHI + chloramphenicol (5 mg/l) agar, and incubated at 30 ºC 24-48 hours.

2.4.6 PCR screening and Southern blots to identify deletion mutants

Chloramphenicol sensitive colonies from the previous screen were examined by PCR using a combination of forward primer for upstream fragment and reverse primer for downstream fragment, as well as the primers targeting the deleted gene or gene cassette. Subsequently, colonies of deletion mutant were confirmed by Southern blots and DNA sequencing.

2.5 Construction of complemented strains

2.5.1 PCR amplification and construction of recombinant plasmid

Primers were designed based on the DNA sequence information, the fragment within these two primers included entire target gene and upstream promoter region and downstream transcriptional terminator region. The specific sequences of restriction sites were artificially incorporated at 5' end on each primer for cloning purposes. A regular PCR amplification was performed. PCR product was directly digested by restriction enzymes and further purified using PCR purification kit (Qiagen Valencia, CA). Purified DNA fragment was ligated to enzyme-digested and dephosphorylated site-specific phage integration vector pPL2. The

recombinant plasmid was transformed into *E. coli* S17-1 by electroporation. Positive colonies were confirmed by PCR and restriction enzyme digestion.

2.5.2 Bacterial conjugation

E. coli S17-1 carrying recombinant plasmid was mated into corresponding deletion mutant strain of *L. monocytogenes* as described in section of 2.4.3.2. Transconjugants were picked from the BHI plates with 20 mg/l of nalidixic Acid and 6 mg/l of chloramphenicol, and confirmed by colony PCR and Southern blots.

2.6 Phenotypic characterization of wild type vs deletion mutant strains

2.6.1 Colony immunoblot with monoclonal antibody specific to *L. monocytogenes* serotype 4 (c74.22)

L. monocytogenes strains were grown on nitrocellulose membranes (OSMONICS INC., NitroBind, Cast, Pure Nitrocellulose, MA) placed on the TSA with 0.7% yeast extract plates for 36 to 48 hours at room temperature. The following procedure was performed at room temperature:

1) Bacterial cells on nitrocellulose membranes were washed off with the Towbin transfer buffer (3.03 g Tris, 14.4 g glycine, 200 ml methanol, dH_2O to 1000 ml, adjusted to pH 8.3, with 5-10 ml of 10 % SDS added), with shaking;

2) The membrane was washed twice in TTBS buffer for 5-10 min (TTBS, Tris-buffered saline with Tween-20: 2.42 g Tris base, 8 g NaCl, 3.8 ml 1M Hydrochloric acid, diH_2O to 1000 ml, adjusted to pH 7.6, with 0.1% Tween-20 added);

3) The membrane was incubated in blocking solution (5% non-fat milk powder in TTBS) with shaking for 1 hour;

4) The membrane was then incubated with primary antibody (blocking solution + primary antibody 1:1000; in this study, undiluted primary antibody was used because the reactivity of antibody is getting pretty low), with shaking, for 1 hour;

5) The membrane was washed in TTBS: 2 quick rinses followed by three 5 min washes;

6) The membrane was then incubated with secondary antibody in blocking solution (1:10000 Horseradish Peroxidase (HRP)-conjugated goat-anti mouse immunoglobulin specific polyclonal antibody, BD Pharmingen™; In my study, 1:500 dilution was used to obtain good signal because of low reactivity of primary antibody) for one hour;

7) The membrane was washed in TTBS: 2 quick rinses followed by three 5 min washes;

8) The membrane was incubated in freshly prepared substrate solution (1.2 g of 4-Chloro-1-Naphthol in 40 ml ethanol, 65 ml of 0.01 M Tris-HCl, pH 7.5, and 750 µl of 3% of H_2O_2) for 5 to 30 minutes, with a cover to avoid light until color was fully developed.

9) After color was developed, the membrane was rinsed and soaked in diH_2O for 5 minutes, dried in air, and kept in dark.

Positive colonies stained purple-blue in the immunoblot, while the negative ones were colorless.

2.6.2 *Listeria* phage infection

Listeria-specific phage Φ20422-1 was isolated by our laboratory (Jay-Won Kim, unpublished data) and propagated in F2365 (ECI). A500 was gift from R. Calendar and propagated in F2381 (ECI). Phage was diluted in SM buffer (0.1 M NaCl, 0.015M $MgSO_4$, 0.01% gelatin, and 0.05 M Tris-HCl [pH7.5]). Phage (100 µl) was mixed with host bacteria (200 µl) of an overnight culture grown at 37 ºC in brain heart infusion broth and incubated at 37 ºC for 30 min. Three milliliter of melted softer agar (LB broth (BD) with 0.7% agar with

addition of 10 mM $MgSO_4$) was added to the phage-bacteria mixture, briefly mixed, and immediately poured over modified LB agar plates (LB agar with addition of 10 mM CaCl2 and 10 mM $MgSO_4$). Plates were incubated at 37 ºC overnight.

2.6.3 Surface attached growth and microtiter plate biofilm production assay

Overnight cultures diluted 1000 fold in PBS were grown on nitrocellulose membranes placed on the agar plates (Tryptic Soy Broth [TSB] with 1.2% agar) at room temperature for 36 h. For biofilm production assay, 100 µl of an overnight culture was transferred into the wells of a 96-well plate and incubated for 24 h at 37 ºC. Planktonic bacteria were then removed, and the wells were washed twice with 200 µl of PBS. Wells were air-dried for 20 minutes and heat-fixed at 80 ºC for 30 minutes. Each well was stained with crystal violet (0.1%, wt/vol). A decolorizing solution (ethanol-acetone [80:20]) was added, and absorption of the eluted stain was measured at 595 nm using a microtiter plate reader (TECAN SAFIRE A5082, Austria).

2.6.4 Phenotype Microarrays (PMs)

Phenotypic Microarrays (Biolog, Hayward, CA) can be used to evaluate carbon (PM1-2), nitrogen (PM3), and phosphorus/sulfur (PM4) metabolism of several bacterial species (http://www.biolog.com/accesstoPMTech.html). *L. monocytogenes* wild type strain and deletion mutant strain were streak on BUG+B Agar (Biolog, Hayward, CA) 37ºC or blood agar (Remel, Lenexa KS USA) overnight (two blood agar plates per strain). The following stock solutions were prepare for later use: 100×stock solution of solution D (menadione sodium bisulfite [5.524 mg Sigma Cat# M5750] + 320.2 mg sodium salicylate [Sigma Cat# S3007] in 10 ml diH_2O), 100× stock solution E (2.2 g sodium pyruvate [Sigma Cat# P2256]

+ 570.5 mg sodium thioglycollate [Sigma Cat# T0632] + 24.42 mg uridine [Sigma Cat# U3750] in 10 ml of diH_2O). The stock solutions were filter sterilized, and stored in a refrigerator in 50-ml tubes wrapped in aluminum foil. Solution D was added to Inoculation Fluid (IF)-0 for PM1 and PM2 plates, whereas solution E was added to IF-0 for PM3 and PM4 plates. 150 µl of the 100× stock solution was added to 15 ml of IF-0 inoculating fluid right before preparation of the cell suspensions. A dry sterile cotton swab or a sterile plastic inoculation loop was used to remove a small amount of cells from the agar surface and to completely resuspend the cells in inoculating fluid (IF-0 with solution D/E). Turbidity was adjusted to OD_{590nm}=0.2 (Biorad, SmartSpec™ 3000) and each cell suspension was poured into a sterile reservoir basin and was transferred into the PMs 96-well plates by multiple channels pipetting 100 µl per well. The PMs 96-well plates were incubated at 37 ºC for 24 hr (PM1 and PM2) or for 36-48 hr (PM3 and PM4), and the plates were scanned at wavelength of 590 nm and 595 nm, respectively, using microtiter plates reader(TECAN SAFIRE A5082, Austria). The formation of purple color indicated utilization of the C, N, P, or S source in a particular well. The A-1 well serves as a negative reference well. Any well with more color than the reference well was considered "positive".

2.6.5 Hemolytic activity

Wild type and deletion mutant strains of *L. monocytogenes* were streaked on sheep blood agar plates (Remel, Lenexa KS USA), and were incubated at 37 ºC for 1 day, 25 ºC for 2 days, and 4 ºC for 30 days.

2.6.6 Motility

Wild type and deletion mutant strains of *L. monocytogenes* were grown in TSB+0.7% yeast extract at 37 ºC overnight. Five microliters of an overnight culture was spotted (in triplicate for each strain) on a soft agar plate (TSB+0.7% yeast extract, with 0.4% agar), and incubated at 37 ºC, 25 ºC for 36-48 hr.

2.6.7 Growth on agar plates at 37ºC, 25ºC

Wild type and deletion mutants of *L. monocytogenes* were inoculated in TSB+0.7% yeast extract, and incubated at 37 ºC overnight. Dilutions (10^{-6}) were made in TSB+0.7% yeast extract and OD_{600nm} value was determined with the spectrophotometer (Biorad, SmartSpec™ 3000) to get an almost equal OD_{600nm} value for each culture. 10 µl of each culture was plated onto TSB+0.7% yeast extract agar, and was incubated at 37 ºC for 1.5 days, 25 ºC for 3 days, and 4 ºC for 35 days (done in duplicate).

2.6.8 Cell morphology

Wild type and deletion mutant strains of *L. monocytogenes* were grown in TSB+0.7% yeast extract at 37 ºC for overnight, 25 ºC for 48 hours (observed every 5 hours), and 4 ºC for 30 days. Bacteria were observed with phase contrast microscopy (Leica, Germany).

2.6.9 Survival of wild type vs deletion mutant strains at 42 ºC

Wild type and deletion mutant strains of *L. monocytogenes* were grown at 37 ºC overnight. Ten microliters of overnight culture was transferred into TSB+0.7% yeast extract broth and incubated at 42 ºC for 4 days. The survival of the bacteria was quantified on daily basis by plate counting in duplicate. The experiment was performed twice independently.

2.6.10 Comparative growth fitness

Streptomycin resistant derivative of wild type (Str^R) and deletion mutant strain (Str^S) of *L. monocytogenes* were grown at 37 ºC overnight. Serial dilutions of each overnight culture were plated on TSAYE plates to enumerate the number of cells for inoculation. Equal aliquot (25 µl) of overnight culture of both wild type and deletion mutant strain was transferred into 5 ml fresh TSBYE and incubated at 37 ºC or 42 ºC for 4 days . The survival of the bacteria was quantified on daily basis by plating serial dilutions of mixed culture on TSAYE and TSAYE supplemented with streptomycin (1200 mg/l) in duplicate. The experiment was performed twice independently.

Chapter III

Absence of serotype-specific surface antigen in laboratory variants of epidemic-associated *Listeria monocytogenes* strains

3.1 Abstract

Most foodborne outbreaks of listeriosis have been linked to a small number of clonal groups ("epidemic clones") of *Listeria monocytogenes* serotype 4b. In the course of genetic investigations involving representatives of two such clonal groups (one strain of Epidemic Clone I and one of Epidemic Clone II) we discovered that the bacteria had undergone unsought-for mutations that abolished reactivity with monoclonal antibody c74.22, specific to the serotype 4b complex (serotypes 4b, 4d and 4e). In addition, the variants from both strains were resistant to the *Listeria* genus-specific phage 20422-1. The Epidemic clone I strain variant (F2381L-Phi-R) was in addition resistant to the serotype 4b-specific phage A500, whereas the variant of the Epidemic Clone II strain (ECIIΔ18RV) remained susceptible to this phage. Genetic complementation experiments with *glt*A-*gltB*, previously shown to be required for reactivity of serotype 4b strains with the monoclonal antibody c74.22, conferred partial restoration of reactivity with c74.22 to ECIIΔ18RV, whereas no restoration could be observed with F2381-Phi-R. These findings suggest that different mutations mediated the c74.22-negative phenotype in these two variants, and suggest the presence of additional, currently identified genes required the expression of serotype 4b-specific surface antigens. More importantly, the findings indicate that such variants can readily arise and become established in the course of routine bacteriologic and genetic studies with serotype 4b strains of both Epidemic Clone I and II. The surface alterations of these variants can have potentially important consequences in virulence and pathogenicity, and may seriously compromise the interpretation of virulence and phage susceptibility of genetic constructs derived from these strains. Since such variants are indistinguishable from their wild type counterparts on the basis of routine morphological criteria, it is recommended

that cultures and genetic constructs are frequently monitored with reagents such as MAb c74.22, or with the *Listeria* genus-specific phage 20422-1.

3.2 Introduction

The gram-positive, facultative intracellular pathogen *Listeria monocytogenes* is capable of causing life-threatening infections (listeriosis) in humans and animals. Human populations at risk include pregnant women, newborns, elderly, and immunocompromised patients. The majority of foodborne outbreaks of listeriosis have been associated with a small number of closely related strains ("epidemic clones") of serotype 4b. Epidemic Clone I (ECI) has been involved in numerous outbreaks in Europe and North America, including those in Nova Scotia, Canada (coleslaw, 1981), California (Mexican-style cheese, 1985), France (pork tongue in aspic, 1992), and others. ECII has been implicated in two multistate outbreaks of listeriosis in the United States, both of which involved contaminated ready-to-eat meat products (4).

Serotype 4b strains harbor specific decorations in the teichoic acid (TA) of the cell wall that appear to be required for reactivity with monoclonal antibodies such as c74.22, specific for strains of the serotype 4b complex (serotype 4b and the relatively rare, but closely similar serotypes 4d and 4e) (10). At least two loci (*gtc*A, and the *glt*A-*glt*B gene cassette) have been shown to be required for reactivity of the bacteria with c74.22 and TA glycosylation (7, 9). Additionally, in *L. monocytogenes* serotype 1/2a TA glycosylation appears to be essential for susceptibility of the bacteria to both serotype-specific and genus-specific phage (12). Furthermore, *gtcA* mutants are deficient in invasion of several mammalian cell lines and appear to be impaired for their virulence in murine models of listeriosis (C. Zuprynski, B. Neudeck and S. Kathariou, unpublished findings).

A population-level survey of epidemic-associated strains from three outbreaks (the New England, Nova Scotia, and California outbreaks) revealed several naturally occurring c74.22-negative strains. Such strains were found to indeed harbor alterations in the sugar moieties on the TA of the cell wall, and were resistant to the serotype 4b-specific phage 2671(1). Although these surface antigen variants could arise in the course of the infection, representing an immune system evasion strategy on the part of the pathogen, the possibility also exists that they have arisen in the food vehicle, or during subsequent passages of the bacteria in the laboratory (1).

Although c72.22-negative strains can be generated by transposon mutagenesis in the laboratory (7, 9), their emergence under laboratory conditions has not yet been described. Furthermore, it is not known whether such variants can arise in strains of Epidemic Clone II, which were not recognized prior to the 1998-99 hot dog outbreak. In this study, we demonstrate that such variants can indeed arise both in Epidemic Clone I and Epidemic Clone II strains in the course of laboratory investigations, with significant potential to compromise the interpretation of findings involving this serotype 4b, epidemic-associated strains.

3.3 Materials and Methods

3.3.1 Bacterial strains and media

The bacterial strains used in this study are listed in Table 1. *L. monocytogenes* was grown in brain heart infusion (BHI) broth (BD, Sparks MD) at 37ºC unless otherwise specified. *Escherichia coli* strain was grown in Luria-Bertani (LB) (BD) broth. LB agar or broth was used for phage assay. Ampicillin was added to cultures of *Escherichia coli* strain at

100 µg/ml. Antibiotic used for *Listeria* was nalidixic acid (20 µg/ml) and chloramphenicol (6 µg/ml).

Table 1: Bacterial strains used in this study

Strain	Relevant genotype and features	Reference
E.coli		
S17-1	Conjugation donor; pro hsdR recA (with integrated plasmid RP4 Tc : : Mu-Km : : Tn7)	(11)
E-pPL2	Integration vector, pPL2/S17-1	(6)
E-pPL95	*gtcA* integration vector, pPL95/S17-1	This study
E-pPLAB	*gltA-gltB* integration vector, pPLAB/S17-1	This study
L. monocytogenes		
4b1	*Listeria monocytogenes* 4b wild type (sporadic case isolate)	(9)
M44	Tn*916*Δ*E* mutant	(9)
M44:pPL2	M44, $tRNA^{Arg}$: pPL2	This study
M44:pPL95	M44, $tRNA^{Arg}$: pPL95	This study
F2381L	F2381 (ECI), Str^{R} derivative	(9)
F2381L-Phi-R	F2381L, c74.22-negative Laboratory variant	This Study
F2381L-Phi-R:pPL2	F2381L-Phi-R, $tRNA^{Arg}$: pPL2	This study
F2381L-Phi-R;pPL95	F2381L-Phi-R, $tRNA^{Arg}$: pPL95	This study

Table 1: Bacterial strains used in this study (cont'd)

F2381L-Phi-R;pPLAB	F2381L-Phi-R, $tRNA^{Arg}$: pPLAB	This Study
H7550	*Listeria monocytogenes* 4b wild type (ECII)	(2)
ECIIΔ18R	*Listeria monocytogenes* Δ18R derivative of H7550	Unpublished data
ECIIΔ18RV	ECIIΔ18R, c74.22-negative Laboratory variant	This study
ECIIΔ18RV: pPL2	ECIIΔ18RV, $tRNA^{Arg}$: pPL2	This study
ECIIΔ18RV: pPL95	ECIIΔ18RV, $tRNA^{Arg}$: pPL95	This study
ECIIΔ18RV: pPLAB	ECIIΔ18RV, $tRNA^{Arg}$: pPLAB	This study

3.3.2 Reactivity with MAb c74.22

For colony immunoblots with MAb c74.22 , the bacteria were grown at 22 ºC, transfer to nitrocellulose (Millipore, MA), and processed as described previously (8).

3.3.3 Phage infection assay

Infection with phage was done as described previously with slight modification (14). *Listeria*-specific phage Φ20422-1 was isolated by our laboratory (unpublished data) and propagated in F2365 (ECI). A500 was gift from R. Calender and propagated in F2381 (ECI). Phage was diluted in SM buffer (0.1 M NaCl, 0.015M $MgSO_4$, 0.01% gelatin, and 0.05M Tris-HCl [pH7.5]). Phage (100 µl) was mixed with host bacteria (200 µl of an overnight culture grown at 37ºC in brain heart infusion broth for 30 min. 3 millitres of melted softer agar ([LB] broth (BD) with 0.7% agar with addition of 10mM $MgSO_4$) was added to the

phage-bacteria mixture, briefly mixed, and immediately poured over modified LB agar plates (LB agar with addition of 10 mM $CaCl_2$ and 10mM $MgSO_4$). Plates were incubated at 37 ºC overnight.

3.3.4 Construction of pPL95 and pPLAB and complementation

The DNA fragment harboring *gtcA* gene and its promoter region were PCR amplified from genomic DNA of the wild type strain H7550 using primers VCpNP95F (5'-ATAAGCGGCCGCTTCAAAGGGACAGGCAACATG, *Not*I site underlined) and VCpNP95R (5'-ATAACCCGGGGTACTCAGGATGAATTCCAG, *Xma*I site underlined). The PCR amplicon was digested with *Not*I and *Xma*I and was subcloned into *Not*I - *Xma*I digested and dephosphorylated site-specific phage integration vector pPL2. A DNA fragment containing *gltA-gltB* was PCR generated using primers VC2P3 (5'-AGTAGAGCTCGTAACGTCTCATATAGGGAG) and VC-1R5 (5'-CTCTGTCGACG TAGAACAATTGTAGTACCG), which had a *Sac*I site and a *Sal*I site, respectively, at the 5'end. PCR products were digested by *Sac*I and *Sal*I and ligated to *Sac*I-*Sal*I digested and dephosphorylated pPL2. The resulting plasmids, consisting pPL2 with inserts *gtcA* and *gltA-gltB*, were named pPL95 and pPLAB, respectively. pPL95 and pPLAB were transformed into *E. coli* S17-1 and resulting strains were mated into c74.22-negative laboratory variants as previously described (6). Transconjugants were isolated on BHI agar containing 20 µg/ml nalidixic acid and 6 µg/ml chloramphenicol. chloramphenicol –resistant transconjugants were confirmed by PCR for integration at $tRNA^{Arg}$-*attBB'* using primers NC16 (5'-GTCAAAACATACGCTCTTATC) and PL95 (5'-ACATAATCAGTCCAAAGTAGATGC) (6). The primers pair gtcA_C_F and CAT_G+_R amplified a single ~1.5 kb product if a

pPL95 carrying *gtcA* gene was integrated into the chromosome. Primers gltAB-C-F and CAT_G+_R were used to confirm the integration of *gltA-gltB* in the complemented strain.

3.3.5 Strain typing by Pulse Field Gel Electrophoresis (PFGE)

Bacteria were grown in BHI broth at 37 ºC overnight and agarose plugs were prepared as described using Pulsed Field Certified agarose (BioRad, Hercules, CA) (3) . The plugs were digested with *Asc*I or *Apa*I (New England Biolabs) for 16 hours under conditions suggested by the vendor. Electrophoresis was in a CHEF-DRIII (BioRad, Hercules, CA) unit as described. Standards were lambda concatemers (48.5 to 727.5 kb; New England Biolabs). Band patterns were compared using visual analysis and Bionumerics (Applied Maths, St. Martens-Latem, Belgium).

3.4 Results

3.4.1 Identification of laboratory variants negative with serotype-specific MAb c74.22

Spontaneous c74.22-negative variants were first recognized when we attempted to characterize the potential roles of different serotype 4b-specific gene cassettes by constructing in-frame gene deletion mutants in two epidemic clone strains, F2381 (ECI) and H7550 (ECII). Earlier studies showed that MAb c74.22, reacted exclusively with strains of serotype 4b, 4d, and 4e. The MAb appears to recognize surface antigenic determinants associated with sugar substituents on the teichoic acid of the cell wall. In fact, reactivity with c74.22 is one of most typical phenotypic characteristics of sporadic and epidemic strains of serotype 4b, with the notable exception of several c74.22-negative strains from epidemic-associated populations.

In our genetic studies we chose *L. monocytogenes* F2381L (ECI) and H7550 (ECII) as the prototype strains to characterize the potential genetic and functional roles of genes of interest. Strain F2381L is a streptomycin resistant strain derived from F2381 (California outbreak 1985), a representative of ECI strains implicated in numerous outbreaks of listeriosis. Strain H7550 (hot dog multi-state outbreak, 1998-1999) was used as reference outbreak strain for ECII. In order to characterize the ECI-specific genes in F2381L, we pursued constructions of deletion mutants (this work will be described in another publication). Deletion constructs were evaluated for standard bacteriological characteristics, including phage susceptibility and reactivity with c74.22. Unexpectedly, all strains, including the deletion mutants and the parental strain F2381L, were found to be resistant to the 4b-specific phage A500. Based on previous data from our laboratory, we suspected that the resistance of these strains to the 4b-specific phage was due to alteration of sugar substituents on the TA of the cell wall. Subsequent colony immunoblotting with MAb c74.22 confirmed that the parental strain F2381L (later designated F2381L-Phi-R), and any deletion mutants derived from it, lacked reactivity with c74.22. However, the -80 ºC-preserved stock culture of F2381L maintained intact susceptibility to 4b-specific phage and reactivity with MAb c.74.22. The findings related to phage susceptibility and c74.22 reactivity with F2381L-Phi-R and F2381L are summarized in Table 2.

A c74.22- negative variant of strain H7550 deletion mutant (designated ECIIΔ18RV) arose when we sub-cultured a deletion mutant of H7550 (designated ECIIΔ18R) on blood agar once from an original BHI agar plate which was stored in refrigerator for approximately three weeks. However, the -80 ºC stock culture of ECIIΔ18R had intact reactivity with MAb c74.22 (FIG. 1B). Pulsed-field gel electrophoresis (PFGE) fingerprinting with *Asc*I and *Apa*I

revealed that the PFGE profile of the c72.22-negative ECIIΔ18RV was indistinguishable from that of the stock culture of ECIIΔ18R, which was c74.22-positive (data not shown).

The serotype designation of the variants was confirmed with Southern blotting employing probes derived from the gene *gltA*, found in all screened serotype 4b, 4d, and 4c strains but not in other serotypes of *L. monocytogenes*. Epidemic-associated characteristics of each variant were confirmed by digestion of genomic DNA with *Sau3*AI. The genomic DNA from F2381L-Phi-R was resistant to *Sau*3AI. In contrast, ECIIΔ18RV had DNA that could be readily digested by *Sau3*AI. In addition, these c74.22-negative variants had no readily detectable phenotypic differences from its original form in terms of growth at 37 ºC, motility, hemolytic activity, and colony or cellular morphology. The variant appeared to be stable under routine laboratory culture, and no c74.22-positive revertants were identified following repeated passages in the laboratory.

3.4.2 Phage sensitivity of ECIIΔ18RV and F2381L-Phi-R

The cell wall of *L. monocytogenes* contains large amounts of the anionic polymer teichoic acid (TA), which consists of polyribitol phosphate and is covalently linked to peptidoglycan. TA-associated sugar substituents have been shown to serve as receptors for serotype-specific phages in *L. monocytogenes* (13). Sensitivity to the *Listeria*-specific phage Φ20422-1 (provided by Jae-won Kim) and the serotype 4b-specific phage A500 was evaluated with the two variants. F2381L-Phi-R was resistant to both the *Listeria*-specific phage and the serotype 4b-specific phage. ECIIΔ18RV was resistant to *Listeria*-specific phage Φ20422-1, but sensitive to serotype 4b-specific phage A500 (Table 2). A *gtcA* transposon insertion mutant (M44) of strain 4b1 which showed the loss of reactivity with c74.22, loss of galactose and marked reductions in the glucose in the TA of the cell was used

as control in this study. Insertional inactivation of *gtcA* in this mutant conferred resistance to both *Listeria*-specific phage Φ20422-1 and serotype 4b-specific phage A500 (Table 2).

3.4.3 Genetic characterization of F2381L-Phi-R and ECIIΔ18RV by cis-complementation

An *L. monocytogenes* site-specific phage integration vector, pPL-2 (6) was used to introduce a single copy of *gtcA* and its promoter region (300 nt upstream of the start codon of *gtcA*) into F2381L-Phi-R and ECIIΔ18RV by conjugation, as described in detail in Materials and Methods. The recombinant plasmid (pPL95) became integrated at the phage attachment site within the $tRNA^{Arg}$ that is present only once in the genome of *L. monocytogenes*, resulting in F2381L-Phi-R:pPL95 and ECIIΔ18RV:pPL95. Chloramphenicol-resistant integrants were confirmed in both strains by PCR for the presence of PSA-*attBP'* with primers NC16 and PL95. Further confirmation of the integration of *gtcA* at the phage attachment site was carried out with primers gtcA_F and CAT_G^+_R. We also generated the recombinant plasmid pPLAB, which includes not only *gltA* and *gltB* but also the promoter region 219 nt upstream of the start codon of *gltA*, and introduced this plasmid into both variants, resulting in F2381L-Phi-R:pPLAB and ECIIΔ18RV:pPLAB. Sequence analysis of *gtcA* and *gltAB* showed that their coding sequences were highly conserved within serotype 4b strains (data not shown).

Reactivity of ECIIΔ18RV with c74.22 was restored partially by the integrated pPLAB, whereas ECIIΔ18RV harboring the integrated pPL95 and pPL-2 alone remained negative with this MAb (FIG.1B). However, reactivity of F2381L-Phi-R with c74.22 was not recovered by either integrated pPL95 or pPLAB (data not shown). In contrast, reactivity of M44 with c74.22 was fully complemented by pPL95, as expected (FIG.1A).

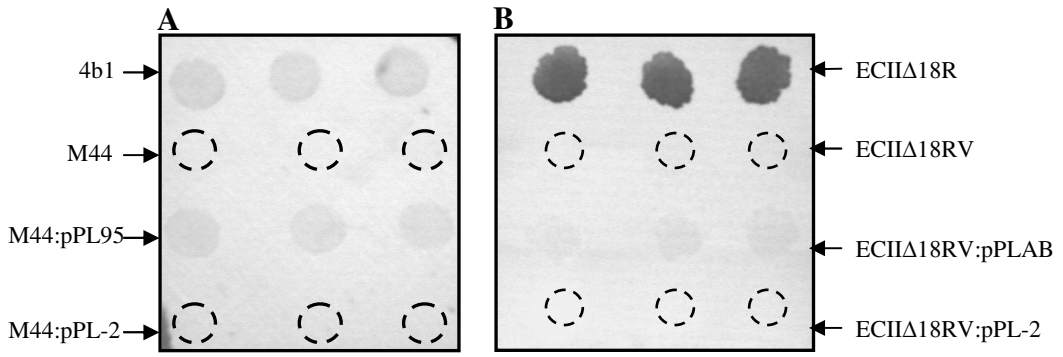

FIG.1: Cis-complementation of surface antigen expression of M44 (A) and ECIIΔ18RV(B) with wild-type *gtcA* (A) or wild-type *gltA-gltB* (B). Colony immunoblotting with MAb c74.22 was done as described in Materials and Methods. Each strain was spotted on the nitrocellulose membrane in triplicate. (A) from top to bottom: 4b1 (wild-type 4b1 strain), M44 (transposon-induced *gtcA* mutant), M44:pPL95 (M44 harboring pLP95), M44:pPL-2 (negative control, M44 harboring cloning vector pPL-2). (B) from top to bottom, ECIIΔ18R (positive control, deletion mutant derived from strain H7550), ECIIΔ18RV (c74.22-negative variant of ECIIΔ18R), ECIIΔ18RV:pPLAB (ECIIΔ18RV harboring pPLAB), ECIIΔ18RV:pPL-2 (negative control, ECIIΔ18RV harboring cloning vector pPL-2). Lack of reaction is indicated as dotted circles.

Phage sensitivity of F2381L-Phi-R to both *Listeria*-specific phage and 4b-specific phage was not recovered by either pPL95 or pPLAB. The pPL95-complemented ECIIΔ18RV strain also did not recover sensitivity to the *Listeria*-specific phage. Surprisingly, the sensitivity of ECIIΔ18RV to *Listeria*-specific phage was not restored by pPLAB, even though weak complementation was observed in its reactivity with c74.22. Phage sensitivity of M44 to both *Listeria*-specific and serotype-specific phage was complemented by pPL95 (Table 2).

Table 2: Phage susceptibility and reactivity of strains investigated in this study

Strain	Reactivity to		
	c74.22	Φ20422-1	ΦA500
4b1	+	S[b]	S
M44	-	R[c]	R
M44: pPL95	+	S	S
F2381L	+	S	S
F2381L-Phi-R	-	R	R
F2381L-Phi-R:pPL95	-	R	R
F2381L-Phi-R:pPLAB	-	R	R
F2381L-Phi-R:pPL-2	-	R	R
ECIIΔ18R	+	S	S
ECIIΔ18RV	-	R	S
ECIIΔ18RV:pPL95	-	R	S
ECIIΔ18RV:pPLAB	W[a]	R	S
ECIIΔ18RV:pPL-2	-	R	S

[a] W: weak; [b] S: sensitive; [c] R: resistant.

3.5 Discussion

This study described two c74.22-negative spontaneous mutants, derived from strains of ECI and ECII, respectively, in the course of laboratory investigations. PFGE data suggested that the c74.22-negative variants have not undergone detectable genomic rearrangements. Most likely, the variants arose by spontaneous mutations of the original strain in the course of sub-cluturing and / or during storage under refrigeration. However, laboratory conditions

that may have favored the establishment of the variants remain to be identified. Generally, the variants are stable after repeated passage in the laboratory. As we discussed above, two variants were recognized when we attempted to characterize specific genes of interest by the construction of deletion mutants. Such variants may result in serious errors in the interpretation of phenotypes of specific, deliberately constructed mutants, as demonstrated by the case of ECIIΔ18R, where the variant arose subsequent to the construction of the mutant. Thus, attributes associated with the spontaneous variant's mutations (phage resistance, lack of reactivity with c74.22, and possibly impact in pathogenicity) could be erroneously attributed to the loss of the gene of interest in the deletion mutant. In addition, this situation may also mislead researchers investigating phage susceptibility and serotype-specific surface antigens. Although the strains investigated here were both associated with outbreaks, and represent well-defined clonal groups, it is conceivable that similar variants can arise and become inadvertently established in other serotype 4b strains, and possibly in strains of other serotypes, as well.

On the other hand, these spontaneous mutants represent valuable reagents and opportunities for better understanding of the genetic basis of serotype 4b-specific surface antigens of *L. monocytogenes.* This is especially needed for Epidemic Clone II strains, which have not been extensively characterized genetically, due to their relatively recent identification. The H7550 variant described here represents the first time that a c74.22-negative mutant derived from an ECII strain has become identified.

Previous studies revealed that serotype-specific genes, including *gtcA* and *gltA-gltB,* were essential for the reactivity with 4b-specific monoclonal antibodies and glycosidic decoration of TA. To prove if the mutation(s) responsible for the c74.22-negative phenotype

of the two variants reside in these previously identified loci, we generated complemented strains by integrating *gtcA* or the *gltAB* cassette at the phage attachment site on the chromosome of variants, by use of site-specific phage integration vector, pPL-2. Full complementation of reactivity with c74.22 by pPL95 in M44 indicated that the integrated *gtcA* was functional, and responsible for the phenotype of M44. Partial complementation of MAb c74.22 reactivity of ECIIΔ18RV by *gltA-gltB* suggests a spontaneous mutation in this locus, in ECIIΔ18RV. The reasons for the low level of complementation are not clear, and similar findings with low-level complementation were obtained in the previously studied mutant XL7, where *gltA* was inactivated by a transposon insertion (7). However, reactivity of F2381L-Phi-R with c74.22 was not complemented by either integration (*gtcA* or *gltA-gltB).* This suggests that the spontaneous mutation in this variant resides neither in *gtcA* gene nor in the *gltA-gltB* cassette. The spontaneous mutation may have occurred in some as-yet-unidentified locus, which can regulate the expression of these genes. An alternative possibility is that the mutation is localized in another, currently unknown gene involved in the expression of surface antigens required for c74.22 reactivity, and phage susceptibility. Further investigations are needed to understand the genetic basis responsible for MAb-negative phenotype of this naturally occurring variant.

TA-associated glycosidic substituents have been shown to be essential for phage infection in *L. monocytogenes* (13). The resistance of two variants and M44 to *Listeria*-specific phage is in agreement with our previous data of *gtcA* mutants of *L. monocytogenes* 4b1 and *L. innocua* (5). Sensitivity of M44 to *Listeria*-specific and serotype-specific phages was restored by pPL95. This may suggest that TA glycosylation is required for proper exposure or conformation of the receptor determinants on the peptidoglycan, since *Listeria*-specific phage

was shown to use peptidoglycan as a receptor in previous study (13). ECIIΔ18RV is sensitive to serotype-specific phage A500, which is in agreement with the results of phage assay of *gltAB* mutant (XL17) of *L. monocytogenes* 4b1 strain. However, phage sensitivity of ECIIΔ18RV was not restored by pPLAB. The low level of complementation in immunoblotting may account for the lack of restoration of phage infection. Thus, c74.22-negative strains appeared to be at least resistant to either or both of *Listeria*-specific and serotype 4b-sepcific phages (Table 3).

Table 3: Reactivity with MAb c74.22 and sensitivity to *Listeria*-specific phage and serotype 4b-specific phage of *L. monocytogenes* strains from different origins

Strain	Reactivity to		
	c74.22	*Listeria*-specific phage	serotype 4b-specific phage
c74.22-negative	-	ND [a]	R[b]
epidemic-associated strains			
XL7(*gltAB* mutant)	-	R	S[c]
M44 (*gtcA* mutant)	-	R	R
F2381L-Phi-R	-	R	R
ECIIΔ18RV	-	R	S

[a] ND, not determined; [b] R: resistant; [c] S: sensitive.

In earlier work, surveys of epidemic and non-epidemic strains with c74.22 MAb showed that epidemic strain populations appear to contain c74.22-negative strains. On the contrary, all tested non-epidemic strains reacted with this antibody. It remains to be determined whether this c74.22-negative predominantly associated with epidemic strain

populations by obtaining data from a larger population of epidemic and nonepidemic isolates. In this study, the isolation of two laboratory variants of epidemic-associated strains may tempt us to speculate that epidemic strains somehow are prone to lose this c74.22-specific antigen, since we never encountered this c74.22-negative spontaneous mutant when we screened numerous other serotype 4b strains in our laboratory. The mechanisms that select for the establishment of the c74.22-negative phenotype in epidemic strain populations, naturally or under laboratory conditions, remain to be elucidated. A possible hypothesis is that c74.22-negative strains became established in the course of infection in certain of host. On the other hand, the phage resistance associated with c74.22-negative phenotype may confer to laboratory variants a selective advantage in their environment.

In conclusion, extensive studies have revealed that c74.22-negative strains deserve special attention because of the potential roles of this c.74.22-specific antigen in pathogenesis and adaptive physiology of *L. monocytogenes*. The isolation of laboratory variants may reflect this potential. As we described here, the c74.22-negative spontaneous mutants could become established under laboratory condition without any known signals. It seems important for researchers to monitor the existence of 4b-specific surface antigens to avoid complication of their findings by the spontaneously acquired absence of the 4b-specific surface antigens. In our opinion, phage susceptibility assays would be an ideal way for most laboratories to monitor maintenance of the 4b-specific antigens in their strains, when 4b-specific monoclonal antibodies are not available.

3.6 References

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CHAPTER IV

DNA probes differentiate Epidemic Clone II from other *Listeria monocytogenes* serotype 4b strains

4.1 Abstract

Listeria monocytogenes strains implicated in the 1998-1999 and the 2002 multistate outbreaks in the USA represent a unique epidemic-associated clonal group, designated Epidemic Clone II (ECII). Comparative genomic analysis has identified an ECII-specific region, designated region-18. PCR primers and DNA probes derived from this ECII-specific region can readily differentiated ECII strains from other serotype 4b strains. A panel of 56 serotype 4b strains isolated from patients, foods and food processing plants were investigated by DNA array-based hybridizations using four ECII-specific DNA probes. All screened ECII or ECII-like strains, including eight from the 1998-99 outbreak and three from the 2002 outbreak, yielded a positive reaction with all four probes, whereas other serotype 4b strains were negative with at least one of the four probes. The ECII-specific probes identified in this study can greatly facilitate the detection and monitoring of strains belonging to the ECII clonal group in foods, clinical samples, and the environment.

4.2 Introduction

Gram-positive food-borne pathogen *Listeria monocytogenes* can cause listeriosis with severe symptoms (meningitis, septicemia and abortions) and relatively high mortality (20-30%) in individuals at risk, primarily pregnant women and their fetuses, the elderly and immuno-compromised patients (5).

The majority of human listeriosis cases involve strains of three serotypes, 1/2a, 1/2b, and 4b (12). In addition, most human outbreaks of foodborne listeriosis have involved a small number of genetically related strains, commonly of serotype 4b. A cluster of closely related strains, designated Epidemic Clone I (ECI), have been found responsible for a number of temporally and geographically distinct outbreaks in North America and Europe. Several genetic and phenotypic markers unique to ECI strains have been identified (8). The genome of strain F2365 (ECI) implicated in the Jalisco cheese outbreak of 1985 in California has been sequenced to closure (10).

In 1998-99, a multistate outbreak of listeriosis traced to contaminated hot dogs was found to involve a different strain type of serotype 4b, with a pulsed-field gel electrophoresis (PFGE) pattern and ribotype rarely encountered before (1, 2). These hot dogs outbreak strains appeared to represent a novel epidemic-associated lineage, designated Epidemic Clone II (ECII) (8). Previous studies have shown that an unusual divergent genomic region, designated “region 18”, appears to be either absent or markedly divergent in the hot dog outbreak strains but conserved among other serotype 4b strains (4). Recent findings suggest that isolates from the 2002 multistate outbreak are closely related to the 1998-99 hot dogs outbreak isolates (9).

It is currently unclear whether prevalence of these epidemic-associated serotype 4b strains in human cases is due to their increased virulence to the human host or to relatively high fitness in foods or food-related environments. Findings based on ribotyping and vitro cytopathogenicity suggested that three epidemic ribotypes (DUP-1042B, DUP-1038B, and DUP-1044A) had increased cytopathogenicity in a tissue culture plaque assay, compared to other ribotypes (6). Overall, however, limited information is available concerning the prevalence of serotype 4b or epidemic-associated strains in foods and food processing plants, and pathogenic differences between epidemic-associated strains and other serotype 4b strains.

Several genetic and phenotypic markers unique to ECI strains have been identified (13-15). Evans et al. reported the identification of genetic fragments unique to non-ECII serotype 4b strains (4). The recently available genome sequence of strain H7858 (implicated in the 1998-99 outbreak, and representative of ECII strains), along with the previous findings resulting from the characterization of ECII isolates make it feasible to identify ECII-specific genetic markers (7). The usefulness of certain ECII-specific DNA probes has been demonstrated in two recent studies, focusing on the detection ECII clonal group in turkey processing plants and on further characterization of strains implicated in the 2002 outbreak (3, 9).

In this study, we evaluated the utility of combined ECII-specific DNA probes for the detection and monitoring of ECII strains from different sources.

4.3 Materials and Methods

4.3.1 Bacterial strains and growth media.

The serotype 4b *L. monocytogenes* strains from different sources used in this study were listed in Table 1. The panel included several confirmed ECII strains from both the 1998-99

and the 2002 outbreaks. One *L. monocytogenes* of 1/2a serotype was included as negative control. *L. monocytogenes* strains were routinely grown in tryptic soy broth with 0.7% yeast extract (TSBYE) (BD) for 18 h at 37 ºC.

Table 1: *L. monocytogenes* strains and DNA array detection results

Strain (serotype)	Year isolated	Origin	Hybridization with probes						
			ECIIinter-WAP [b]	Hsp 01219[a]	H18R P11/12[a]	H18R P9/10[a]	ECIIC-WAP [a]	4bSF18[C]	ECIC-WAP [C]
F2365 (ECI)	1985	Jalisco cheese	+	-	-	-	-	+	+
4b1 (4b)	1962	Clinical	+	-	-	-	-	+	+
266 (4b)	1999	Clinical	+	-	-	-	-	+	+
J2213 (ECI-like[d])	2003	CDC, Clinical	+	-	-	-	-	+	+
J2479 (4b)	2003	CDC, Clinical	+	-	-	-	-	+	-
J2288 (ECI-like)	2003	CDC, Clinical	+	-	-	-	-	+	+
J2422 (4b)	2003	CDC, Clinical	+	-	-	-	-	+	+
82-2a (4b)	2003	Turkey processing plant, floor drain	+	-	-	-	-	+	+
L0228 (ECI-like)	2003	Turkey processing plant, raw product rinse	+	-	-	-	-	+	+
L0328 (ECI-like)	2003	Turkey processing plant, air conditioning unit	+	-	-	-	-	+	+

Table 1: *L. monocytogenes* strains and DNA array detection results (cont'd)

Strain (serotype)	Year isolated	Origin	Hybridization with probes						
			ECIIinter-WAP [b]	Hsp 01219[a]	H18R P11/12[a]	H18R P9/10[a]	ECIIC-WAP [a]	4bSF18[C]	ECIC-WAP [C]
04-471 (4b)	2004	NCDHHS, Clinical	+	-	+	+	-	-	-
04-643 (4b)	2004	NCDHHS, Clinical	+	-	-	-	-	+	+
05-062 (4b)	2005	NCDHHS, Clinical	+	-	-	-	-	+	+
2001-75R (4b)	2003	NCDHHS, Clinical	-	-	-	-	-	+	-
2003-173R (4b)	2003	NCDHHS, Clinical	+	-	-	-	-	+	+
2001-182R (ECI-like,4b)	2001	NCDHHS, Clinical	+	-	-	-	-	+	+
2003-151R (4b)	2003	NCDHHS, Clinical	-	-	-	-	-	+	-
2003-332 (ECI-like, 4b)	2003	NCDHHS, Clinical	+	-	-	-	-	+	+
2004-287 (ECI-like, 4b)	2004	NCDHHS, Clinical	+	-	-	-	-	+	+

Table 1: *L. monocytogenes* strains and DNA array detection results (cont'd)

Strain (serotype)	Year isolated	Origin	Hybridization with probes						
			ECIIinter-WAP [b]	Hsp 01219[a]	H18R P11/12[a]	H18R P9/10[a]	ECIIC-WAP [a]	4bSF18[C]	ECIC-WAP[C]
12LA (4b)	1988	Clinical	+	-	-	-	-	+	+
17LA (4b)	1988	Clinical	+	-	-	-	-	+	+
23LA (4b)	1988	clinical	+	+	+	+	+	-	-
25LA (4b)	1988	clinical	+	-	-	-	-	+	+
32LA (4b)	1988	clinical	+	-	-	-	-	+	+
43LA (4b)	1988	clinical	+	+	+	+	+	-	-
NA [f]									
NA									
NA									
1/2a(255)		Negative control	-	-	-	-	-	-	-
NA									
NA									
NA									
NA									
NA									
NA									

Table 1: *L. monocytogenes* strains and DNA array detection results (cont'd)

Strain (serotype)	Year isolated	Origin	Hybridization with probes						
			ECIIinter-WAP [b]	Hsp 01219[a]	H18R P11/12[a]	H18R P9/10[a]	ECIIC-WAP [a]	4bSF18[C]	ECIC-WAP [C]
H7550 (ECII)	1998-1999	Hotdog outbreak, Clinical	+	+	+	+	+	-	-
H7738 (ECII)	1998-1999	Hotdog outbreak, Clinical	+	+	+	+	+	-	-
H7596 (ECII)	1998-1999	Hotdog outbreak, Food	+	+	+	+	+	-	-
H7762 (ECII)	1998-1999	Hotdog outbreak, Food	+	+	+	+	+	-	-
H7943 (ECII)	1998-1999	Hotdog outbreak, Clinical	+	+	+	+	+	-	-
H7962 (ECII)	1998-1999	Hotdog outbreak, Clinical	+	+	+	+	+	-	-
H7969 (ECII)	1998-1999	Hotdog outbreak, Food	+	+	+	+	+	-	-
H9127 (ECII)	1998-1999	Hotdog outbreak, Clinical	+	+	+	+	+	-	-
J1735 (ECII)	2002	Turkey deli, Clinical	+	+	+	+	+	-	-
J1815 (ECII)	2002	Turkey deli, Environ.	+	+	+	+	+	-	-
J1925 (ECII)	2002	Turkey deli, Food	+	+	+	+	+	-	-

Table 1: *L. monocytogenes* strains and DNA array detection results (cont'd)

Strain (serotype)	Year Isolated	Origin	Hybridization with probes						
			ECIIinter-WAP [b]	Hsp 01219[a]	H18R P11/12[a]	H18R P9/10[a]	ECIIC-WAP [a]	4bSF18[C]	ECIC-WAP [C]
34-6a (ECII-like[e])	2003	Turkey processing plant, floor	+	+	+	+	+	-	-
L0226 (ECII-like)	2003	Turkey processing plant, overhead pipe	+	+	+	+	+	-	-
L0327 (ECII-like)	2003	Turkey processing plant, carcasses	+	+	+	+	+	-	-
L0720 (ECII-like)	2004	Turkey processing plant, floor drain	+	+	+	+	+	-	-
J2206 (ECII-like)	2003	CDC, Clinical	+	+	+	+	+	-	-
J2255 (4b)	2003	CDC, Clinical	+	-	+	+	-	-	-
J2446 (ECII-like)	2003	CDC, Clinical	+	+	+	+	+	-	-
J2621 (4b)	2003	CDC, Clinical	+	-	+	+	-	+	-
03-196R (4b)	2003	NCDHHS, Clinical	+	-	+	+	+	-	-

Table 1: *L. monocytogenes* strains and DNA array detection results (cont'd)

Strain (serotype)	Year isolated	Origin	Hybridization with probes						
			ECIIinter-WAP [b]	Hsp 01219[a]	H18R P11/12[a]	H18R P9/10[a]	ECIIC-WAP [a]	4bSF18[C]	ECIC-WAP [C]
WS1(ECIa, 4b)	2000	CDC, Clinical	+	-	+	+	+	-	-
H6383 (ECII-like)	1996	CDC, Clinical	+	+	+	+	+	-	-
J3033 (ECII-like)	2004	CDC, Clinical	+	+	+	+	+	-	-
J3006 (ECII-like)	2004	CDC, Clinical	+	+	+	+	+	-	-
J2685 (ECII-like)	2004	CDC, Clinical	+	+	+	+	+	-	-
H3396 (ECII-like)	1997	CDC, Clinical	+	+	+	+	+	-	-
J1838 (ECII-like)	2002	CDC, Clinical	+	+	+	+	+	-	-
J3200 (ECII-like)	2004	CDC, Clinical	+	+	+	+	+	-	-
J3238 (ECII-like)	2005	CDC, Clinical	+	+	+	+	+	-	-

Table 1: *L. monocytogenes* strains and DNA array detection results (cont'd)

Strain (serotype)	Year isolated	Origin	Hybridization with probes						
			ECIIinter-WAP [b]	Hsp 01219[a]	H18R P11/12[a]	H18R P9/10[a]	ECIIC-WAP [a]	4bSF18[C]	ECIC-WAP [C]
J2230 (ECII-like)	2003	CDC, Clinical	+	+	+	+	+	-	-
H2444 (ECII-like)	1996	CDC, Clinical	+	+	+	+	+	-	-

[a] ECII-specific probes, Hsp01219: outside of region 18; H18RP11/12, H18RP9/10: derived from region 18 of H7858 (ECII); ECIIC-WAP: derived from C-terminal tail of a gene encoding wall-associated protein (*wap*) in genome of H7858.
[b] ECIIinter-WAP, derived from conserved portion of *wap* detected in both F2365 and H7858 strain.
[c] ECI-specific probe, ECIC-WAP: derived from C-terminal tail of a gene encoding wall-associated protein (*wap*) in genome of F2365. 4bSF18: derived from LMOf2365_0466 (region 18 of F2365, ECI)
[d] ECI-like, isolates with *Sau3*AI-resistant DNA and other ECI-specific genetic markers.
[e] ECII-like, isolates with *Sau3*AI-sensitive DNA and other ECII-specific genetic markers.
[f] NA, not applicable.

Strains with unusual hybridization profile with ECI or ECII-specific probes are shaded in light gray.

4.3.2 Isolation of genomic DNA.

Genomic DNA from *L. monocytogenes* was extracted using DNeasy tissue kit (Qiagen, Valencia, Calif.) from 5 ml of overnight cultures grown at 37 ºC by the procedure suggested by the manufacturer.

4.3.3 Polymerase chain reactions (PCR) and Southern blots.

Primers used in this study are listed in Table 2. Primers were designed based on the whole genome sequences of *L. monocytogenes* H7858 (GeneBank accession number, AADR00000000) and F2365 (GeneBank accession number, AE017262). PCR was performed as previously described. To obtain DNA probes for Southern blotting or DNA arrays, PCR products were amplified from genomic DNA of the serotype 4b strain H7550 (Hotdog Outbreak, 1998-1999) or the ECI strain F2365. The PCR products were purified with QIAquick gel extraction kit (Qiagen) and labeled with digoxigenin (Genius kit, Roche) by following the manufacturer's instructions. Southern blots were performed as described before (11).

Table 2: Primers used for PCR amplification.

Primer	Sequence	Corresponding ORF in genome of strain H7858 or F2365
H18RP7/8F (H18RCYP7)	5'TGGTGGAAGGGGTTTAAAGC 3'	LMOh7858_0484
4bSF18F	5'ACGGGCGTTTTATATTAAATGGG 3'	LMOf2365_0466
4bSF18R	5'AATATCTCGAAAACTCCGAGT 3'	LMOf2365_0466
H18RP7/8R (H18RCYP8)	5'CAACCCACGCATTACCAAT 3'	LMOh7858_0484
H18RP9/10F (H18RCYP9)	5'CTGGATTTGCAGCTTATGAT 3'	LMOh7858_0487
H18RP9/10R (H18RCYP10)	5'CCTATTCTTTCCATAAGTAAT 3'	LMOh7858_0487

Table 2: Primers used for PCR amplification (cont'd)

H18RP11/12F (H18RCYP11)	5'GAATAGGTAGGGATAAATGC 3'	LMOh7858_0488
H18RP11/12R (H18RCYP12)	5'CAGTATAATGACATTAACTC 3'	LMOh7858_0488
H18RP13/14F (H18CYP13)	5'GGAAGCTTGAACAAATTTTGA 3'	LMOh7858_0485
H18RP13/14R (H18CYP14)	5'TTCTAAAATGCCTTTTTCCTG 3'	LMOh7858_0485
H18RP15/16F (H18RCYP15)	5'GCATGGAATTTGGACCAGAG 3'	LMOh7858_0483
H18RP15/16R (H18RCYP16)	5'CATGAGAATGTCCTTCAGTAC 3'	LMOh7858_0483
Hspec01219F	5'GAGGCTATCGAAATTGCTCG 3'	LMOh7858_1168
Hspec01219R	5'AGGATTCGGAATTTCATCCA 3'	LMOh7858_1168
ECIC-WAPF	5'ATGGAAATTGGGCATGGC 3'	LMOf2365_0450
ECIC-WAPR	5'GTAGTTCCAGTGGACATG 3'	LMOf2365_0450
ECIIC-WAPF	5'GGGAACTTTCCATTAGCC 3'	LMOh7858_0479
ECIIC-WAPR	5'TTAAATGGGATATGATGT 3'	LMOh7858_0479
ECIIinter-WAPF	5'AATCCATATGGAAGATGCC AAATCAAACGG 3'	LMOh7858_0479
ECIIinter-WAPR	5'ATTCGGATCCGATGACTTAG GAGAGTGCC 3'	LMOh7858_0479
ECIIWAP_R _R1	5'CATCGTACGTATAACTCG 3'	LMOh7858_0479
ECIIWAP_R_F3	5'ACAGAAGAAGCCAAAGC 3'	LMOh7858_0479
hlyF	5' ACGCGGATGAAATCGATAAG 3'	LMOf2365_0213
hlyR	5' GATTGCGCCGAAGTTTACAT 3'	LMOf2365_0213

4.3.4 DNA arrays

Genomic DNA from *L. monocytogenes* was extracted using DNeasy tissue kit (Qiagen) Concentration of genomic DNA was determined by spectrophotometer at 260nm (Biorad, SmatSpec™ 3000 CA USA) and was adjusted to a concentration of 50 ng/µl using diH_2O. Approximately 30 µl of genomic DNA (≈50 ng/µl) was transferred into 57 wells of a 96-well PCR plate (GeneMate Kaysville UT). For each well, 30 µl of DMSO was added and mixed with the genomic DNA. Genomic DNA (≈20 ng) was spotted onto nylon membranes

by using a VP408 Multi-Blot replicator (V&P Scientific, Inc.). The spotted DNA was denatured by incubating the membrane face up for 15 min on four layers of Whatman paper soaked with denaturing solution (1.5 M NaCl and 0.5 M NaOH). Membranes were neutralized by dipping them in sequentially in two 6 ×SSC baths and one 1×SSC bath. Membranes were treated with a UV crosslinker on automatic setting (STRATAGENE, UV Strata linker 1800) to fix the DNA on the membrane. For hybridization and signal detection, the same procedures used in Southern blots were followed. DNA probes used in this study were listed in Table 1. In addition, DNA probe derived from *hly* (the gene encoding listeriolysin) were used as quality control for genomic DNA isolate from *L. monocytogenes* strains.

4.3.5 Inverse PCR.

Genomic DNA was digested with *Dra*I, purified with QIAquick gel extraction kit (Qiagen), and self-ligated. The circular, self-ligated DNA was used as template for inverse PCR amplification with primers ECIIWAP_R _R1 and ECIIWAP_R_F3 (listed in Table 2) to obtain genomic fragments on either side of DNA fragment of known sequence. Inverse PCR products were purified with PCR purification kit (Qiagen) and sequenced.

4.4 Results

4.4.1 Identification of ECII-specific sequences by comparative genomic analysis.

Evans et al. had identified an unusual diversification in genomic region ("region 18") of ECII, which was otherwise conserved among *L. monocytogenes* of serotype 4b (4). BLAST analysis revealed that several genomic fragments within "region 18" of strain H7858 (ECII) had no detectable homologies in genome sequence of strain F2365 (ECI) (FIG.1). This ECII-

specific genomic region had G+C contents ranging from 25.57% to 35.43%, noticeablely lower than the average G+C content of *L. monocytogenes* (ca. 38%).

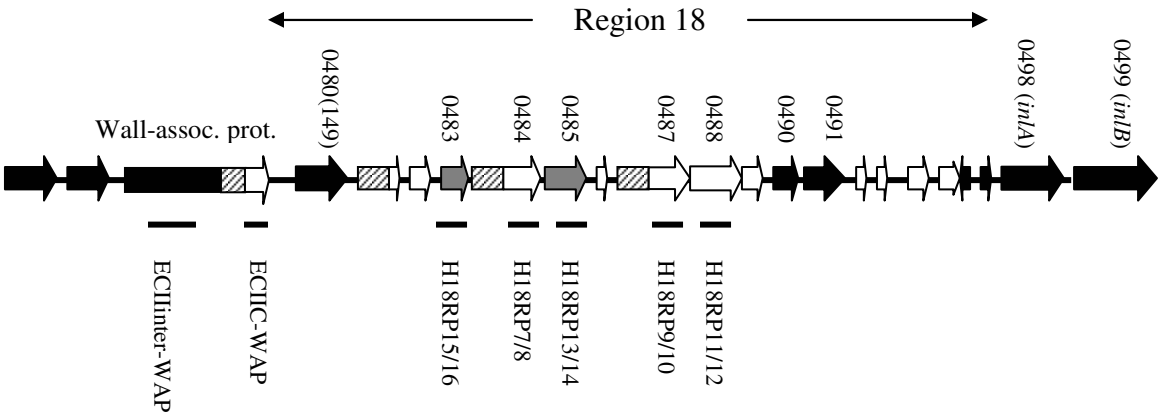

FIG.1: ECII-specific genomic regions within "region 18" of strain H7858 (ECII). Genomic regions specific to H7858 (ECII) (on the basis of genomic comparison between strain F2365 (ECI) and H7858 (ECII)) are indicated in white, and regions sharing 60-70% identity with nucleotide sequences of strain F2365 are indicated in gray. ORFs (Open reading frame) conserved between strain F2365 and H7858 are indicated in black. Genomic regions from which DNA probes were derived are underlined. DNA probes were generated with the primers listed in Table 2.

4.4.2 The presence of ECII-specific genomic regions in isolates from the 1998-99 outbreak and the 2002 outbreak was confirmed by PCR and Southern blots.

To further confirm the presence and conservation of ECII-specific genomic fragments in ECII isolates from the 1998-99 outbreak and the 2002 outbreak, we used PCR with primers derived from the ECII-specific genomic fragments identified by comparative genomic analysis. Primers specific to five of these fragments, H18RP7/8, H18RP9/10, H18RP11/12,

H18RP13/14, and H18RP15/16, generated PCR products of the predicted sizes with all three ECII strains from the 2002 outbreak and both ECII strains from the 1998-1999 outbreak, as well as with a strain (WS1) from the Winston-Salem outbreak of 2000-01. In contrast, no PCR products were produced with an ECI strain (F2381) and two serotype 4b "sporadic" clinical isolates (4b1 and 266). Figure 2 showed the PCR results for a representative panel of isolates with primers derived from H18RP9/10. The PCR results with primers specific for H18RP7/8, H18RP9/10, H18RP11/12, H18RP13/14, and H18RP15/16 are summarized in Table 3.

The failure to produce PCR products with an ECI strain and two "sporadic" strains suggested nucleotide sequence divergence in one or both primer regions. In order to determine whether homologues of these ECII-specific fragments were absent in the genome of the ECI strain and serotype 4b "sporadic" strains, Southern blots was employed using H18RP7/8, H18RP9/10, H18RP11/12, H18RP13/14, and H18RP15/16 as probes. Southern blots with these ECII-specific probes yielded the same hybridization bands with all tested 1998-99 outbreak isolates and the 2002 outbreak isolates, as well as with strain WS1 from the Winston-Salem outbreak, which is in agreement with the PCR results discussed above (FIG.3). Southern blot data are summarized in Table 3.

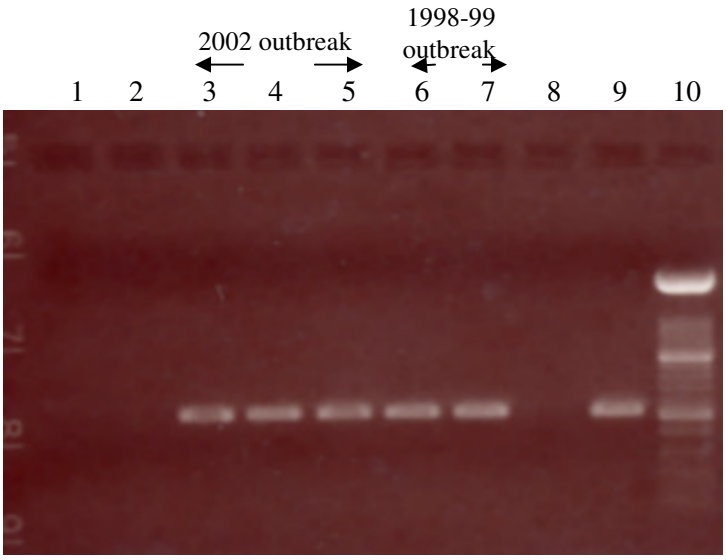

FIG.2: PCR amplification with primers derived from ECII-specific genomic fragment H18RP9/10 in *L. monocytogenes* serotype 4b strains. Lanes: 10, 100-bp molecular size marker XIV (Roche); 1 and 2, 4b1(sporadic) and F2381 (ECI), respectively; lane 3, 4, and 5, 2002 outbreak strains; lane 6 and lane 7, 1998-99 outbreak strains; lane 8, 266 (sporadic); lane 9, WS1, Winston-Salem outbreak.

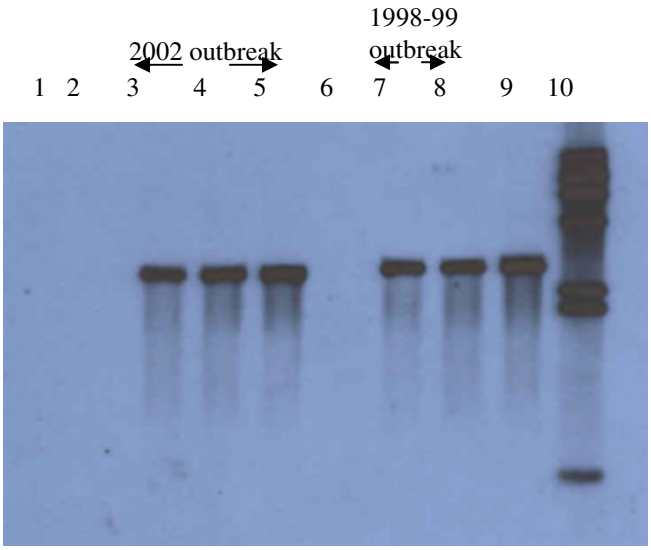

FIG.3: Southern blot of *Eco*RI-digested chromosomal DNA of selected *L. monocytogenes* serotype 4b strains with probe H18RP9/10. Lane 10, λ DNA digested with *Hind*III, used as molecular size markers (fragment sizes, in kilobases, are [from top to bottom] 23, 9.4, 6.5, 4.3, 2.3, 2.03, and 0.56); lane 1 and lane 2, 4b1 (sporadic) and F2381 (ECI), respectively; lane 3, 4, and 5, 2002 outbreak strains (ECII) ; lane 6, 266 (sporadic); lane 7 and lane 8, 1998-99 outbreak strains (ECII) ; lane 9, WS1, Winston-Salem outbreak.

Table 3: ECII-specific genomic fragments in selected *L. monocytogenes* serotype 4b strains

Isolates	Year	Origin	DNA Probe [a] /PCR result [b]				
			H18RP7/8	H18RP9/10	H18RP11/12	H18RP13/14	H18RP15/16
H7550 (ECII)	1998-99	Hotdog outbreak, Clinical	+/+	+/+	+/+	+/+	+/+
H7738 (ECII)	1998-99	Hotdog outbreak, Clinical	+/+	+/+	+/+	+/+	+/+
J1735 (ECII)	2002	Turkey deli, Clinical	+/+	+/+	+/+	+/+	+/+
J1815 (ECII)	2002	Turkey deli, Environ.	+/+	+/+	+/+	+/+	+/+
J1925 (ECII)	2002	Turkey deli, Food	+/+	+/+	+/+	+/+	+/+
WS1		Winston-Salem outbreak	+/+	+/+	+/+	+/+	+/+
266		Sporadic	-/-	-/-	-/-	-/-	-/-
4b1	1962	Sporadic, Clinical	-/-	-/-	-/-	-/-	-/-
F2381 (ECI)	1985	Jalisco cheese outbreak	-/-	-/-	-/-	-/-	-/-

[a] Probes H18RP7/8, H18RP9/10, H18RP11/12, H18RP13/14, and H18RP15/16: derived from ECII-specific genomic fragments of H7550 within region 18. "+" and "–" indicate the presence and absence of a hybridization signal, respectively.

[b] PCR results obtained with primers designed for fragments H18RP7/8, H18RP9/10, H18RP11/12, H18RP13/14, and H18RP15/16. +, expected amplicon obtained; –, no PCR product obtained.

4.4.3 DNA array-based hybridization reveals the ECII-specific genomic regions are conserved among EII strains or ECII-like strains.

To determine whether ECII or ECII-like strains harbor unique chromosomal genetic markers identified in H7858 and possibly unique to ECII, we investigated a panel of 56 serotype 4b isolates, including 9 of ECI or ECI-like isolates, 20 of serotype 4b strains without typical ECI or ECII characteristics, and 27 of ECII or ECII-like strains. In addition, a strain of serotype 1/2a was included as negative control. Probes derived from ECII-specific

genomic fragments within region 18 (H18RP9/10, H18RP11/12) and upstream of region 18 (ECIIC-WAP derived from a gene encoding wall-associated protein), as well as an addition probe derived from ECII-specific sequences in another chromosomal location (Hsp01219) (10) were used in DNA array-based hybridizations. Two F2365 (ECI)-specific probes (4bSF18 and ECIC-WAP) were used in DNA array-based hybridizations. All screened strains of ECII or ECII-like strains, including 8 isolates from the 1998-99 outbreak and 3 isolates from the 2002 outbreak, yielded a positive reaction with all four of probes, whereas the other serotype 4b strains were negative with at least one of four probes (Table 1, FIG.4B). On the other hand, all of ECII or ECII-like strains tested here were negative with ECI-specific probes. All of ECI or ECI-like strains reacted positively with ECI-specific probes (Table 1, FIG.4B). In addition, among the tested strains, J2479, 04-471, 2001-75R, 2003-151R, 03-196R, WS1, J2255 and J2621 appeared to have atypical hybridization profile with ECII-specific or ECI-specific probes (Table 1).

All ECII-like strains harbored the ECII-specific C-terminal tail nucleotide sequence of a gene encoding wall-associated protein (*wap*), whereas, with four exceptions (strains J2479, 04-471, J2255 and J2621), other serotype 4b strains contained the C-terminal tail present in F2365. Strains J2479, 04-471, J2255 and J2621 appeared to harbor neither ECI-specific nor ECII-specific C-terminal tail of deduced Wap. In agreement with previous data, J2255 and J2621 hybridized with ECII-specific probes derived from region 18 but not with ECII-specific probe Hsp01219. Furthermore, isolates J2255 and J2621 do not have typical ECII-specific C-terminal tail of *wap*. Strain 03-196 and an *Mbo*-I resistant strain WS1 shared common hybridization profile with ECI or ECII-specific probes. They are positive with three of ECII-specific probes (H18RP9/10, H18RP11/12 and ECIIC-WAP) and negative with

Hsp01219. Two non-epidemic associated isolates (2001-75R, 2003-151R) are only positive with ECI-specific probe 4bSF18, while they remained negative with the other six probes listed in Table 1. These two isolates will not be further discussed in this communication. Without considering these two isolates, all of the other strains positively reacted with probe ECIIinter-WAP, which was derived from a conserved portion of *wap* detected in both F2365 and H7858 strains. The negative control remained negative with all ECI or ECII-specific DNA probes but was positive with the probe derived from *hly*.

A

	1	2	3	4	5	6	7	8	9	10	11	12
A	F2365	4b1	266	J2213	J2479	J2288	H7550	H7738	H7596	H7762	H7943	H7962
B	J2422	82-2a	L0228	L0328	04-471	04-643	H7969	H9127	J1735	J1815	J1925	34-6a
C	05-062	2001-75R	2003-173	2001-182R	2003-151R	2003-332	L0226	L0327	L0720	J2206	J2255	J2446
D	2004-287	12LA	17LA	23LA	25LA	32LA	J2621	03-196R	WS1	H6383	J3033	J3006
E	43LA				1/2a negative control		J2685	H3396	J1838	J3200	J3238	J2230
F							H2444	L0327				
G												
H												

B **C**

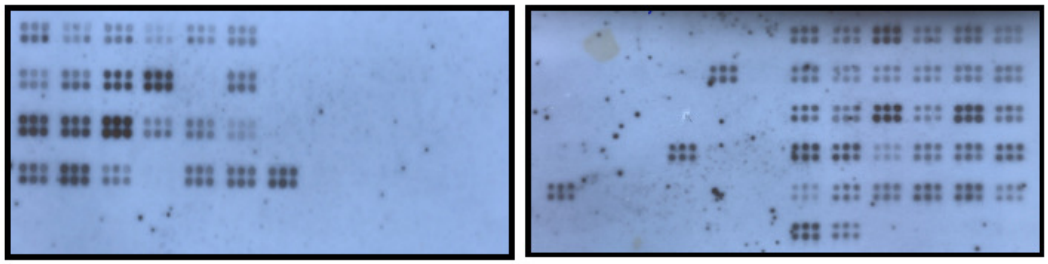

FIG.4: DNA array-based hybridizations of chromosomal DNA of *L. monocytogenes* strains with probes H18RP11/12 and 4bSF18. (A) ID of Genomic DNA spotted on nylon membrane. (B)Hybridization signal detected by probe 4bSF18. (C) Hybridization signal detected by probe H18RP11/12.

4.4.4 DNA sequencing revealed a high divergence of C-terminal tail of *wap* in strains J2479, 04-471, J2255 and J2621.

DNA array-based hybridization analysis revealed that a high divergence of *wap* C-terminal tail was present in serotype 4b strain J2479, 04-471, J2255, and J2621, although the internal portion of *wap* is conserved across these strains (Table 1). DNA sequence analysis of *wap* C-terminal tail derived by inverse PCR showed strain 04-471, J2255, and J2621 shared a common C-terminal tail of *wap* (~472-bp) with 100% identity (data not shown). The deduced amino acid sequence of *wap* C-terminal tail in strain 04-471, J2255, and J2621 was distinct from that of *wap* C-terminal tail in either strain F2365 or H7858 (FIG.5). In contrast, partial sequencing data revealed that J2479 has a unique nucleotide sequence of *wap* C-terminal tail, which is distinct to those discussed above (data not shown).

```
H7858_Wap       WGNVLKNTASTEEAKANPYGYAGYTYDKEIEQYYLMARYYEPEQGVFTAYDPDPGDEDDP 2040
F2365_Wap       WGNVLKNTASTEEAKANPYGYAGYTYDKEIEQYYLMARYYEPEQGVFTAYDPDPGDEDDP 2040
J2255_Wap       ------------------------TYDKEIEQYYLMARYYEPEQGVFTAYDPDPGDEDDP 36
                                        **********************************

H7858_Wap       QTMNGYNYANNNPVMMVDPDGNFPLAIPAVYWG-VSGAIAAAPFVGYGIG----AAGTKI 2095
F2365_Wap       QTMNGYNYANNNPVMMVDPDGNWAWLIPAAMAAGKFIYKFRKPIAKYGK------KGAKW 2094
J2255_Wap       QTMNGYNYANNNPVRYVDPDGHWVWLAINAGFAAYDGYKAYKKTKSWKSAGWAAAKGAVG 96
                ************** *****::      .  .            :         *:

H7858_Wap       WNNTKKVNRGYKFLWNNRKQ---IARKVYKST--RIKWGHIKNRHSPKSSIKKKGKFRNN 2150
F2365_Wap       VG--KTAKKGAKWVGKTAKKGAKWARSRYNSK--KKEFTVTKNWKVGLPKKSQGGRIFAI 2150
J2255_Wap       GGKFKGGKKAYRFIKKAYIKTKKWNRERKWSKGTFNSSRASLNYHYNKHVVKKGSNINKS 156
                 .  *  ::. ::: :   :     *.   *.    .     * :      .: ..:

H7858_Wap       RTLKRTTRATLRSGKARPGEKGRTILEKTFKERVGVNRDGDPSYRVRVIRAPNGKVITSY 2210
F2365_Wap       INRKKGRLFALDYGKVGGNQK----------KYLHVHWNYKPKHYIIYPRKGRGK----- 2195
J2255_Wap       QYTKEAVKLKRKNRKSRTKAN----------FKVGKGYKIKNGKYFGYYTRG-GKIITYG 205
                   *.         *    :           :    . .    .       **::

H7858_Wap       PI- 2212
F2365_Wap       ---
J2255_Wap       KNR 208
```

FIG.5: Multiple sequence alignment (ClustalW) of deduced amino acid sequences of *wap* C-terminal tail in strain F2365, H7858, and J2255. Amino acid sequences of *wap* C-terminal tails are underlined.

4.5 Discussion

Previous studies have shown that the hot dog outbreak strains (ECII) were of serotype 4b and possessed a common ribotype and closely related PFGE patterns. The unusual PFGE profile and ribotype of the strains, the sensitivity of their genomic DNA to *Sau3*AI digestion and the lack of ECI-specific genetic markers suggested that this clonal group of strains represented a novel epidemic-associated lineage (ECII). More recently, molecular subtyping (PFGE), genotyping with ECII-specific probes, and DNA macroarray-based genomic analysis have revealed the 2002 multistate outbreaks involved closely related members of the same clonal group (ECII) (9), which had not been identified in outbreaks of listeriosis prior to 1998. The characterization of the ecology, evolution, and virulence of Epidemic Clone II strains of *L. monocytogenes* remains limited. To date, only one ECII-specific RFLP has been identified, which is able to differentiate ECII from other serotype 4b strains (13). A further characterization of ECII strains revealed that ECII isolates had an unusual diversification in a genomic region ("region 18"), which was otherwise conserved among other serotype 4b strains.

In this study, several genetic markers unique to ECII were identified on the basis of the genome sequences of strains representing ECI (F2365) and ECII (H7858). Five ECII-specific probes were derived from region 18 of strain H7858 (Table 3). Our Southern blot data showed that all five ECII-specific genomic regions were conserved in both tested isolates from the 1998-99 outbreak and all three tested isolates from the 2002 outbreak. Furthermore, PCR using primers based on these five ECII-specific fragments amplified the expected DNA fragments from the 1998-99 and 2002 outbreak isolates tested here but not those from ECI strain and sporadic strains, suggesting sequence divergence at least in the primer region.

The findings triggered the further investigation of the conservation of these ECII genetic markers among a larger population of ECII or ECII-like strains and possible applications for the ECII-specific probes in the development of DNA-based tools to specifically detect strains of the ECII clonal group. In DNA array-based hybridization analysis, we employed two ECII-specific probes derived from region 18 (H18RP9/10, H18RP11/12) and two additional ECII-specific probes, one derived from a genomic fragment next to region 18 of H7858 (ECIIC-WAP) and the other from a sequence (Hsp01219) in another chromosomal location of H7858. Our data suggested a promising potential application of these ECII-specific probes for differentiating ECII or ECII-like isolates from other serotype 4b strains. For instance, using only one ECII-specific probe, Hsp01219, we can differentiate members of the ECII clonal group from other serotype 4b strains by Southern blots. This determination could be further confirmed by positive reactions with other ECII-specific probes (H18RP9/10, H18RP11/12, and ECIIC-WAP) in Southern blot. In addition to Southern blots, the probes can be used to detect ECII strains by means of simpler technical formats with automation potential, such as DNA arrays (e.g. oligo microarrays). Furthermore, the identification of a hybridizing band of the same apparent size with ECII-specific probes derived from region 18 suggested that region 18 was conserved in the genomes of ECII isolates tested here. The hybridization data based on DNA-arrays with ECII-specific probes also indicated that the genomic regions from which ECII-specific probes were derived were conserved among serotype 4b isolates with typical ECII-characteristics.

All ECII or ECII-like isolates harbored common ECII C-terminal tail sequence of *wap*, whereas an ECI C-terminal tail sequence of *wap* was present in most non-ECII isolates, including a ECI strain (Table 1). Surprisingly, four isolates (strains J2479, 04-471, J2255,

and J2621) had neither the ECI C-terminal tail nor the ECII C-terminal tail in *wap*. DNA sequencing analysis revealed a common C-terminal tail sequence of *wap* shared by strains 04-471, J2255 and J2621. Similarly, isolates 04-471, J2255, and J2621 appeared to have the same hybridization profile with ECII-specific probes, whereas J2479 did not hybridize with any of ECII-specific probes. The mechanisms that have driven this diversification of C-terminal tail sequences of *wap* among serotype 4b strains remain unknown at this time. However, the fact that all tested ECII or ECII-like strains harbored a common C-terminal tail of *wap* may reflect the high genetic relatedness of ECII and ECII-like isolates. Further investigation of the ECII-specific genomic region in strains without typical ECI or ECII hybridization profile with ECI-specific or ECII-specific probes (WS1, 03-196R, 04-471, J2255, and J2621) will extend our understanding of the evolutionary relationship between epidemic clone strains and other serotype 4b strains.

In conclusion, ECII-specific probes identified in this study will provide a useful epidemiologic tool with high discriminatory power to enhance the specific detection and monitoring of these strains in foods, clinical samples, and the environment.

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CHAPTER V

Genetic characterization of gene encoding a putative wall-associated protein in *Listeria monocytogenes* Epidemic Clones I and II

5.1 Abstract

A cluster of closely related strains of *Listeria monocytogenes* (designated Epidemic Clone I) have been implicated in numerous outbreaks in Europe and North America, including the California outbreak of 1985. *L. monocytogenes* strains implicated in the 1998-1999 and the 2002 multistate outbreaks in the USA represent another unique epidemic-associated clonal group, designated Epidemic Clone II (ECII). Comparative genome sequence analysis revealed a large (ca. 6.6 kb) gene encoding a putative cell wall-associated protein (*wap*) immediately flanking a region that has undergone significant divergence between ECII and other serotype 4b strains. Within *L. monocytogenes*, *wap* was unique to serotype 4b. The deduced Wap protein harbored 15 YD-dipeptide containing RHS repeats, implicated in carbohydrate binding in various prokaryotic and eukaryotic proteins. To characterize the functional role of this gene in ECI and ECII strains, *wap* deletion mutants of stains F2365 (ECI) and H7550 (ECII) were constructed by integration-excision procedure. Both deletion mutants showed intact reactivity with the serotype 4b-specific monoclonal antibody c74.22. There were no noticeable differences between the *wap* mutant and the parental wild-type strain in terms of hemolytic ability, motility, phage susceptibility, cell shape, colony size, and growth rate on complex media. However, surface adherence and growth of the *wap* mutant of the ECII strain H7550 on nitrocellulose appeared to be strongly impaired. A decreased biofilm production, compared to the parental wild-type strain, was also observed in the ECII strain *wap* mutant, but not the *wap* mutant of the ECI strain. The results suggest that *wap* may be involved in specific environmental adaptations such as surface adherence and possibly biofilm formation in ECII strains.

5.2 Introduction

Listeria monocytogenes is the etiological agent of the foodborne illness listeriosis with severe symptoms (septicemia, meningitis, and abortions) and relative high mortality (20-30%) in individuals at risk, primarily pregnant women and their fetuses, the elderly, and immunocompromised patients (10, 27). Three serotypes, 1/2a, 1/2b and 4b, account for more than 95% of clinical isolates. Furthermore, serotype 4b is implicated in about 40% of sporadic cases and in the majority of epidemics of foodborne outbreaks of listeriosis reported in Europe and North America during the past 20 years (27). Many epidemic-associated serotype 4b strains implicated in several geographically and temporally unlinked outbreaks appear to be generically closely related. One clonal group, Epidemic Clone I (ECI) has been implicated in numerous outbreaks of listeriosis in Europe and North America, including those in Nova Scotia, Canada (coleslaw, 1981), California (Mexican-style cheese, 1985), France (pork tongue in aspic, 1992), and others. F2365 (a cheese isolate from the 1985 Jalisco cheese outbreak in California) (10, 15, 27), a representative of ECI, has been sequenced to closure (25). A new strain type of *L. monocytogenes* serotype 4b was implicated in the 1998-1999 and the 2002 multistate outbreaks in the USA, both of which involved contaminated processed meats, and has been designated Epidemic Clone II (17). The genome of one of the isolates (H7858) from the 1998-99 outbreak has been sequenced (25).

In earlier studies we found that a genomic region (region 18) had undergone pronounced divergence between ECII and other serotype 4b strains (9). This region was flanked on one side by a large (ca. 6.6 kb) gene encoding a putative cell wall-associated protein (*wap*) in ECII and other serotype 4b strains. On the other side, the region was flanked

by *inlA* and *inlB*, implicated in virulence of *L. monocytogenes* (9). Analysis of genome sequencing information suggested that, within *L. monocytogenes*, *wap* was specific to serotype 4b, being absent from the genomes of EGD-e and F6854 (serotype 1/2a). In *Bacillus subtilis* a gene (*wapA*) with homology to the *L. monocytogenes wap* has been identified and found to be involved in two-component systems, which may indicate a role of *wapA* in sensing environmental changes (7, 28). The protein encoded by *wapA* belongs to a super-family of surface-associated proteins involved in various cellular processes, including surface hydrophobicity, wall metabolism, secretion, pathogenicity, immunogenicity and cell adhesion (31).

The functional role of *wap* in *L. monocytogenes* has not yet been described, in spite of the intriguing association of this gene with serotype 4b, and its genomic localization next to a region of genetic divergence in serotype 4b strains. In this study, we have constructed and characterized deletion mutants of *wap* in both the ECI strain F2365 and the ECII strain H7550.

5.3 Materials and Methods

5.3.1 Bacterial strains and growth conditions

The ECII strain H7550 was a clinical isolate from the 1998-99 hot dog outbreaks (1, 2). Strain F2365, which was implicated in the 1985 Mexican-style cheese outbreak in California, was included as representative strain of ECI (23). Bacteria were grown at the indicated temperature in brain heart infusion broth (BHI, BD NJ) or tryptic soy broth supplemented with yeast extract (0.7%) (TSBYE, BD NJ) as described previously (9). Agar cultures were grown in BHI with 1.5% agar or tryptic soy agar supplemented with 0.7% yeast extract (TSAYE, BD NJ). When appropriate, antibiotics used for *Listeria* were streptomycin (1,200

µg/ml) and chloramphenicol (6 µg/ml). Antibiotics used for *Escherichia coli* were ampicillin (100 µg/ml), nalidixic acid (20 µg/ml), and chloramphenicol (25 µg/ml). All genome sequences were derived from DDBJ/EMBL/GeneBank. The genome sequences of strains F2365 and H7858 are under accession number AE017262 and AADR00000000, respectively. The genome sequences of *Listeria innocua* CLIP11262 and *Listeria welshimeri* serovar 6b str. SLCC5334 are under accession number AL592102 and AM263198, respectively.

5.3.2 Sequence analysis and database searches

Homology searches at the nucleotide or at the amino acid level were carried out using BLASTN2.0.12 and BLASTX2.1.1 (http://www.ncbi.nlm.nih.gov/BLAST/). Sequence alignments were performed using the ClustalW algorithm (http://www.ebi.ac.uk/clustalw/) and output was displayed with BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html). Conserved domains were identified with Pfam 20.0 at the Pfam database (http://pfam.wustl.edu/hmmsearch.shtml). Both SignalP 3.0 (5) and iPSORT (3) were used to detect putative signal peptides in the deduced Wap of both F2365 and H7858. Subcellular localization of Wap was predicted by PSORTb v 2.0. Specifically, in the Localization Scores area, the confidence values for each of the localization sites are given. If one of the sites has a score of 7.5 or greater, this site and its score are returned in the “Final Prediction” section. If two sites have high scores, a flag of "This protein may have multiple localization sites" is also returned in the “Final Prediction” field (13). TMHMM Server v. 2.0 was used to identify putative transmembrane helices in the proteins (19). For RNA secondary structure analysis, we used RNA folding (mfold) (http://bioweb.pasteur.fr/seqanal/interfaces/mfold.html)

5.3.3 Reverse transcriptase PCR (RT-PCR)

RNA for Northern blots was prepared from 3 ml of overnight cultures. Following treatment with lysozyme and proteinase as for DNA extraction, 1 ml TRIzol (Invitrogen, Carlsbad, CA) was added. Extraction and precipitation of RNA were done as described in chapter II. To prepare cDNA for RT-PCR, 4 µl DNA-free RNA and 1 µl antisense primer were incubated at 70ºC for 5 min, then RNA template and primer mix were added to the 15 µl reverse transcription mixture containing 1 µl ImProm-II™ reverse transcriptase (Promega, Madison, WI) . In negative control reactions, the reverse transcriptase was replaced by same amount of water. Reverse transcription proceeded by annealing at 25 ºC for 5 min and extension of the first strand for 60 minutes at 37-42 ºC. The reverse transcriptase was heat-inactivated at 70 ºC for 15 minutes. Subsequently, 10 µl of the first strand reaction mixture or negative control was used as template for PCR amplification in 50 µl reaction mixture (95 ºC, 5min; 95 ºC, 1min; 54 ºC, 1min; 72 ºC, 2min; for a total 29 cycles). Antisense primers RTCYP36 and RTCY18RH22 (Table 1) were used for cDNA synthesis for strains F2365 and H7550, respectively. The subsequent PCRs were performed by using primers listed in Table 1. RTCYP8 and RTCYP9 were used for strain F2365 in RT-PCR. RTCY18RH1, RTCY18RH2, RTCY18RH3, RTCY18RH6, RTCY18RH7, RTCY18RH9, RTCY18RH11, RTCY18RH13, RTCY18RH14, RTCY18RH16, and RTCY18RH18 were used for strain H7550 in RT-PCR.

5.3.4 Construction of the wap deletion mutant strains

Primers used for construction of deletion mutants are listed in Table 1. To clone a DNA fragment harboring the desired deletion of F2365 *wap*, a DNA fragment containing 966-bp of the sequence upstream of *wap* was amplified using primers ECIWAPUF and ECIWAPUR

(with *Eco*RI and *Bam*HI at 5' side respectively), and a DNA fragment containing 909-bp of the sequence downstream of *wap* was amplified using primers ECIWAPDF and ECIWAPDR (with *Bam*HI and *Hind*III at 5' side respectively). After digestion, the two fragments were ligated into the corresponding sites of the conjugative temperature sensitive vector pCON-1(4) , resulting in pCONIΔ*wap*. Similarly, to clone a DNA fragment harboring the desired deletion of H7550 *wap,* an upstream fragment of 966-bp was amplified with primers ECIIWAPUF and ECIIWAPUR, and a downstream fragment of 859-bp was generated using primers ECIIWAPDF and ECIIWAPDR. The upstream fragment was digested with *Eco*RI and *Bam*HI. The downstream fragment was digested with *Bam*HI and *Xba*I. Subsequently, two fragments were cloned into pCON-1 vector, leading to pCONIIΔ*wap*. pCONIΔ*wap* or pCONIIΔ*wap* was introduced into *E. coli* S17-1 (29) by electroporation, using Gene Pulser (Bio Rad, Hercules, CA). Positive colonies on ampicillin plates were confirmed by PCR with primers ECIWAPUF and ECIWAPDR (for F2365) or ECIIWAPUF and ECIIWAPDR (for H7550). The two recombinant plasmids, pCONIΔ*wap* and pCONIIΔ*wap* were introduced into F2365 and H7550, respectively, by conjugation as previously described (20) with slight modifications. Briefly, the bacterial strains were grown at 30ºC with shaking overnight. *E.coli* S17-1 donor strains were grown in LB supplemented with chloramphenicol (25 µg/ml), and *L. monocytogenes* recipient strains were grown in BHI. The donor *E. coli* S17-1 culture (3 ml) was mixed with 1.5 ml of recipient *L. monocytogenes* culture and filtered onto a sterile 0.45-µm-pore-size HA-type filters (20 mm, Millipore, MA). The filter was washed once with 10 ml BHI, transferred to a BHI plate without antibiotics, and incubated overnight at 30 ºC. The bacterial cells were suspended in 4 ml BHI, and 50 µl aliquots of mixture were plated on BHI containing 20 µg/ml of nalidixic acid (to which *L. monocytogenes* is naturally resistant,

but *E. coli* is susceptible) and 6 µg/ml of chloramphenicol. Chloramphenicol-resistant transconjugants were confirmed by PCR. Subsequently, selection for loss of vector sequences and replacement of *wap* by the fragment harboring the *wap* deletion was performed as previously described (21), yielding strains F2365Δ*wap* and H7550Δ*wap*, respectively.

Table 1: Primers used for amplification in this study

ID	Primer[1] Sequence and restriction sites (5'-3')	Location
ECIWAPUF	AATGAATTCACAGTAGGTCCAACCAGCC (*Eco*RI)	LMOf2365_0448 (nt 718-nt736)
ECIWAPUR	CATGGATCCAGCTATCAGGCTCACTAAG (*Bam*HI)	LMOf2365_0450 (nt 30-nt48)
ECIWAPDF	ATAGGATCCGTCCACTGGAACTACAAACC (*Bam*HI)	LMOf2365_0450 (nt 6526-6545)
ECIWAPDR	TCAAAGCTTGTTCTACACTGCCGCC (*Hind*III)	LMOf2365_0452 (nt 70-nt85)
ECIIWAPUF	AATGAATTCACAGTAGGTCCAACCAGCC (*Eco*RI)	LMOh7858_0476 (nt718-nt736)
ECIIWAPUR	CATGGATCCAGCTATCAGGCTCACTAAG (*Bam*HI)	LMOh7858_0479 (nt 30-nt 48)
ECIIWAPDF	CGGGGATCCCAGAGCTATAGAGTAAGAG (*Bam*HI)	LMOh7858_0479 (nt6576-6652)
ECIIWAPDR	CCGAGTCTAGAAAGCTGGACAATGTTG (*Xba*I)	LMOh7858_0480 (nt 762-nt 777)
RTCYP36	AATCGACCTTCGTCATCCAC	LMOf2365_0452
RTCYP9	ACAGAAGAAGCCAAAGCCAA	LMOf2365_0450
RTCYP8	AGGAAGCCCTACCTTCCAAT	LMOf2365_0450
RTCY18RH22	GCCGATGAATTCCATATCCA	LMOh7858_0489
RTCY18RH1	AGAGAAAGGCCGGACAATTT	LMOh7858_0479
RTCY18RH2	GCCCTAATTGTGCTATGGGTA	LMOh7858_0479
RTCY18RH3	GGACGAATTGTTGAGTGTGG	LMOh7858_0480
RTCY18RH6	GCTAAAGCCAATTCATGGAGA	LMOh7858_0483
RTCY18RH7	CATGGAATTTGGACCAGAGG	LMOh7858_0483
RTCY18RH9	ATTCGGTTTCAGACGGGAGT	LMOh7858_0484
RTCY18RH16	AGGCAATAGCCCATAATCCA	LMOh7858_0485
RTCY18RH11	TGCTTCTGGAGGAAAGAGTG	LMOh7858_0485
RTCY18RH14	TCCCTTTGACCACTTTCGTT	LMOh7858_0487
RTCY18RH13	CGAAAGTGGTCAAAGGGAAC	LMOh7858_0487
RTCY18RH18	AAAGCTCTCATCAGCCAAGC	LMOh7858_0488

[1]Underlined sequences represent recognition sites for the indicated restriction enzymes

5.3.5 Surface-attached growth and microtiter plate biofilm production assay

Overnight cultures diluted 1000 fold in PBS were grown on sterile nitrocellulose membranes placed on the agar plates (Tryptic Soy Broth [TSB] with 1.2% agar) at room temperature for 36 h. For biofilm production assay, 100 µl of an overnight culture in Tryptic Soy Broth with 0.7% Yeast Extract (TSBYE) was transferred into eight PVC microtiter plate (Costar, Corning Inc., NY) wells per strain using a multichannel pipette (Eppendorf, Germany). Plates were made in duplicate and incubated for 24 h at 37 ºC. Planktonic bacteria were then removed, and the wells were washed twice with 200 µl PBS. Wells were air-dried for 20 minutes and biofilms were heat-fixed at 80 ºC for 30 minutes. Each well was stained with crystal violet (Sigma) (0.1%, wt/vol). A decolorizing solution (ethanol-acetone [80:20]) was added (30), and A_{595nm} measurements of the eluted stain were obtained with a microtiter plates reader (TECAN SAFIRE A5082, Austria).

5.3.6 Colony immunoblots

Overnight cultures were grown on nitrocellulose membranes placed on TSB agar plates at room temperature for 36 h. The bacterial colonies were washed off with Towbin transfer buffer. The nitrocellulose membrane was then processed using monoclonal antibody c74.22, as described previously (26).

5.3.7 Phenotype Microarrays (PM)

The PM [1-4] kit (Biolog Inc., Hayward, CA) was used in this study. Wild type and deletion mutant strains were streaked onto BUG+B Agar Plates (Biolog Inc.) and were grown overnight at 37 ºC. A small amount of cells was removed from the agar surface and suspended in inoculating fluid (Biolog) to adjust the cell density to an absorbance of 0.2 at

590 nm with 1 cm cuvette using spectrophotometer (SmartSpec™ 3000, Biorad, CA). The suspensions were then inoculated into each PM plate at a volume of 100 µl/well. The PMs were incubated at 37 ºC for 24 hrs (PM1, PM2) or 36-48 hrs (PM3, PM4). All PM data were obtained using the microtiter plate reader (TECAN SAFIRE A5082, Austria) at 590 nm and the PMs were also examined visually at the end of incubation, for independent verification of results.

5.3.8 Statistical analysis

Comparisons of biofilm production between *wap* deletion mutant and parental strains were analyzed statistically by *t*-tests using StatCrunch (http://www.statcrunch.com/)

5.4 Results

5.4.1 Bioinformatics analysis

5.4.1.1 Comparison of *wap* sequence of F2365 (ECI) and H7858 (ECII)

The genome organization of the *wap* region in strains F2365 and H7858 is shown in FIG.1A. The largest portion of the DNA sequence of *wap* (nts 1-2062) was highly conserved (98% identity) between F2365 and H7858. The deduced amino acid sequence of *wap* in F2365 (2195 aa) shares 95% identity (over 2066 aa) with the deduced product of *wap* in H7858 (2212 aa). In addition, *wap* was absent in *L. monocytogenes* EGD-e genome, while it was detected in the sequenced genomes of two nonpathogenic strains, *L. innocua* (91% identity over 2167 aa) and *L. welshimeri* (89% identity over 2192 aa) (FIG.1A). Even though the first 2062 residues of the deduced Wap polypeptide were highly conserved among the ECI and ECII strains, the C-terminal tail sequence appeared to be highly divergent (boxed in FIG.1 A and B). Furthermore, this divergence in the C-terminal tail was noted among the

non-pathogenic listeriae (FIG.1B). Interestingly, homologies of the carboxyl terminus of Wap (aa 1907 to aa 2081) were found in several polypeptides within the region 18 of H7858, with 70% to 90% identity to N-terminus of deduced polypeptides encoded by LMOh7858_0481, LMOh 7858_0484, and LMOh 7858_0487 (FIG.1A and FIG.2). In contrast, no homology of C-terminus of Wap was detected with the region 18 of F2365.

Homology was also detected between *wap* of *L. monocytogenes* and the gene encoding wall-associated protein precursor in several *Bacillus* spp. The deduced Wap of *L. monocytogenes* was similar to proteins from *B. thuringiensis* (46%, 2141 aa/2171 aa), *B. cereus* (47%, 2050 aa/2046 aa), and WapA of *B. subtilis* (36%, 2203 aa/2230 aa).

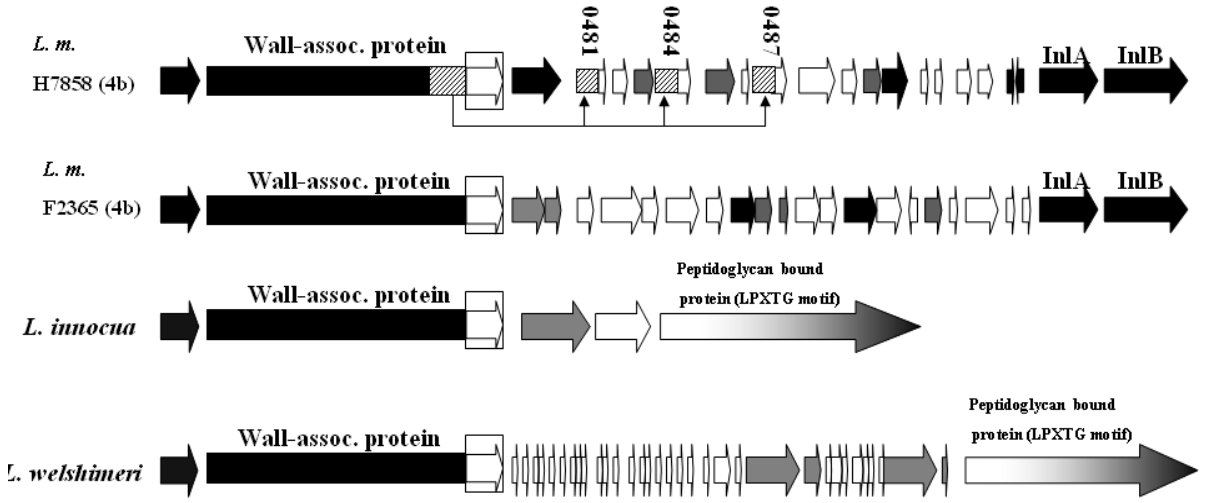


FIG.1: (A) The Genome organization of the *wap* region in *L. innocua*, *L. welshimeri*, *L. monocytogenes* H7858 and *L. monocytogenes* F2365. Genes are indicated by arrows showing their relative orientation (not drawn to scale). ORFs (Open reading frame) conserved across the genomes are indicated in black. Genomic regions specific to each genome are indicated in white. Divergent C-terminal tail sequences in the genomes are boxed. Sequences of homology between a segment of *wap* (near the 5' end of the gene, corresponding to aa 1907 to aa 2081, with the complete Wap being 2212 amino acids) and other ORFs in region 18 of strain H7858 are highlighted with diagonal lines and are also indicated with arrows.

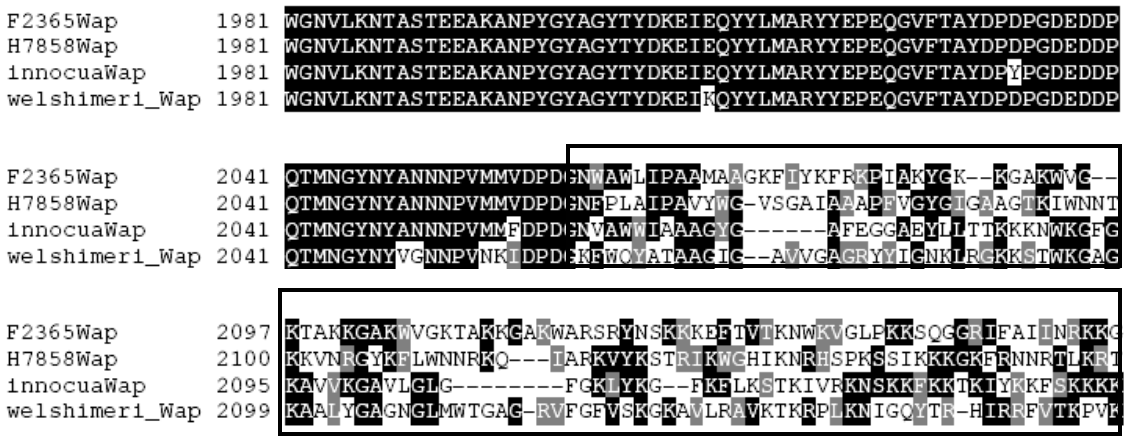


FIG.1: (B) Amino acid sequence alignment of deduced Wap in *L. innocua*, *L. welshimeri*, *L. monocytogenes* H7858 and *L. monocytogenes* F2365. Identical residues are shaded. The divergent C-terminal tail sequences of the deduced Wap polypeptides are boxed.

Using PSORTb v 2.0, Wap in Both F2365 and H7858 was predicted to have a signal peptide (residues 1 to 30). The prediction reveals that Wap may have multiple location sites, with a location score of 5.44 in cell wall, as well as a location score of 4.56 in extracellular sites. A match was found with a wall-associated protein precursor in *B. subtilis* (GI: 586256) in the Gram-positive subset of the current PSORTdb dataset. However no cell wall binding motifs such as LPXTG or GW motifs were detected in the deduced amino acid sequences of *wap* of either F2365 or H7858. In the deduced Wap of F2365, one transmembrane helix (residues 9-31) was detected by TMHMM Server v. 2.0. In contrast, two transmembrane helices were predicted in Wap of H7858 (residues 9-31, and residues 2069-2091, located in the C-terminal variable segment of the protein) (FIG. 2). Interestingly, Wap in both *L. innocua* and *L. welshimeri* was predicted to have the same transmembrane features as F2365, with only one transmembrane helix at residues 9-31(data not shown).

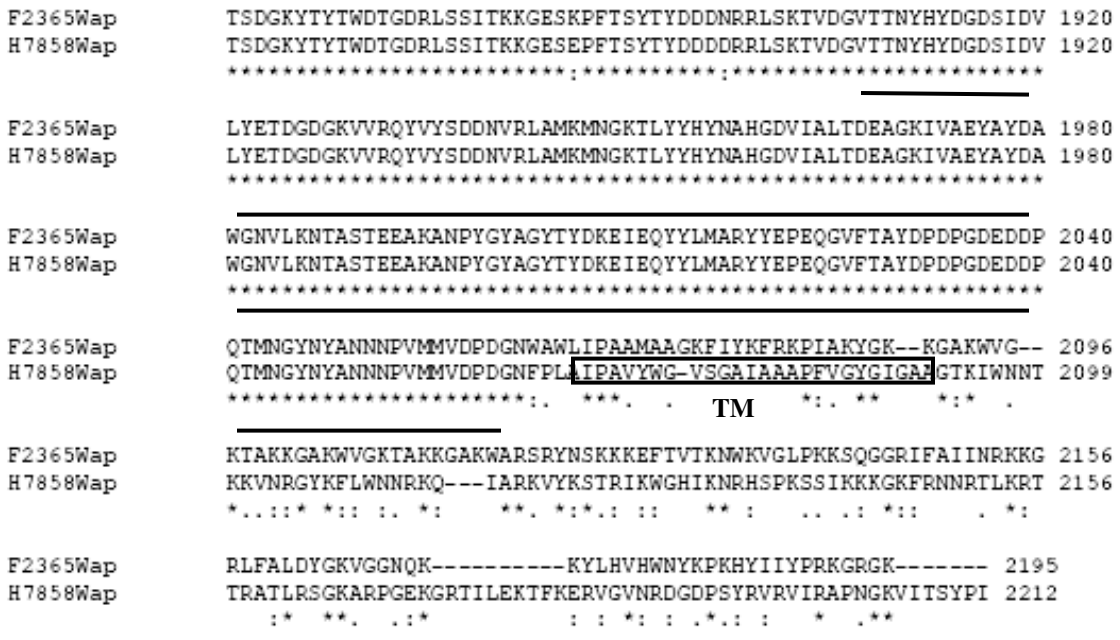


FIG.2: Amino acid sequence alignment of deduced C-terminus of Wap in F2365 (F2365Wap) and H7858 (H7858Wap). Numbers indicate residue positions in the amino acid sequence. The second transmembrane helix (residues 2069-2091) in H7858Wap is boxed. The homologous sequences also found in deduced polypeptides from LMOh7858_0481, LMOh7858_0484, and LMOh7858_0487 within region 18 of H7858 is underlined.

Multiple RHS (rearrangement hot spot) domains PF05593) were detected using Pfam. These domains contain two tandem copies of a 21-residue repeats with YD-dipeptide (YDxxGxxxxxxxxxxxxxxxxYDxxGxxxxxxxxxxxxxxxxx), that appear in general to be involved in binding carbohydrate (11, 24) (FIG.3A and B). In addition, a carbohydrate binding domain (PF02018) was detected by pfam (FIG.3A). The same features were observed in the deduced Wap of strain F2365, since the region harboring these domains is highly conserved between ECI and ECII strains (data not shown).

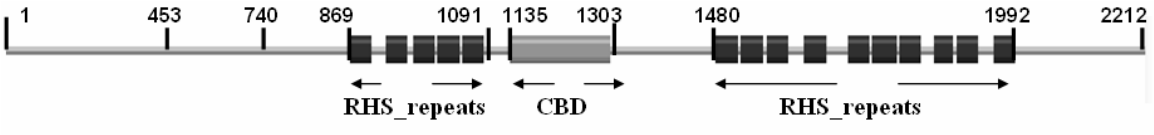


FIG.3**:** (A) Conserved domains in deduced Wap of ECII strain H7858 are identified by pfam. Numbers indicate residue positions in the amino acid sequence. RHS repeats are indicated by black rectangles. CBD (Carbohydrate binding domain) is indicated by gray rectangle.

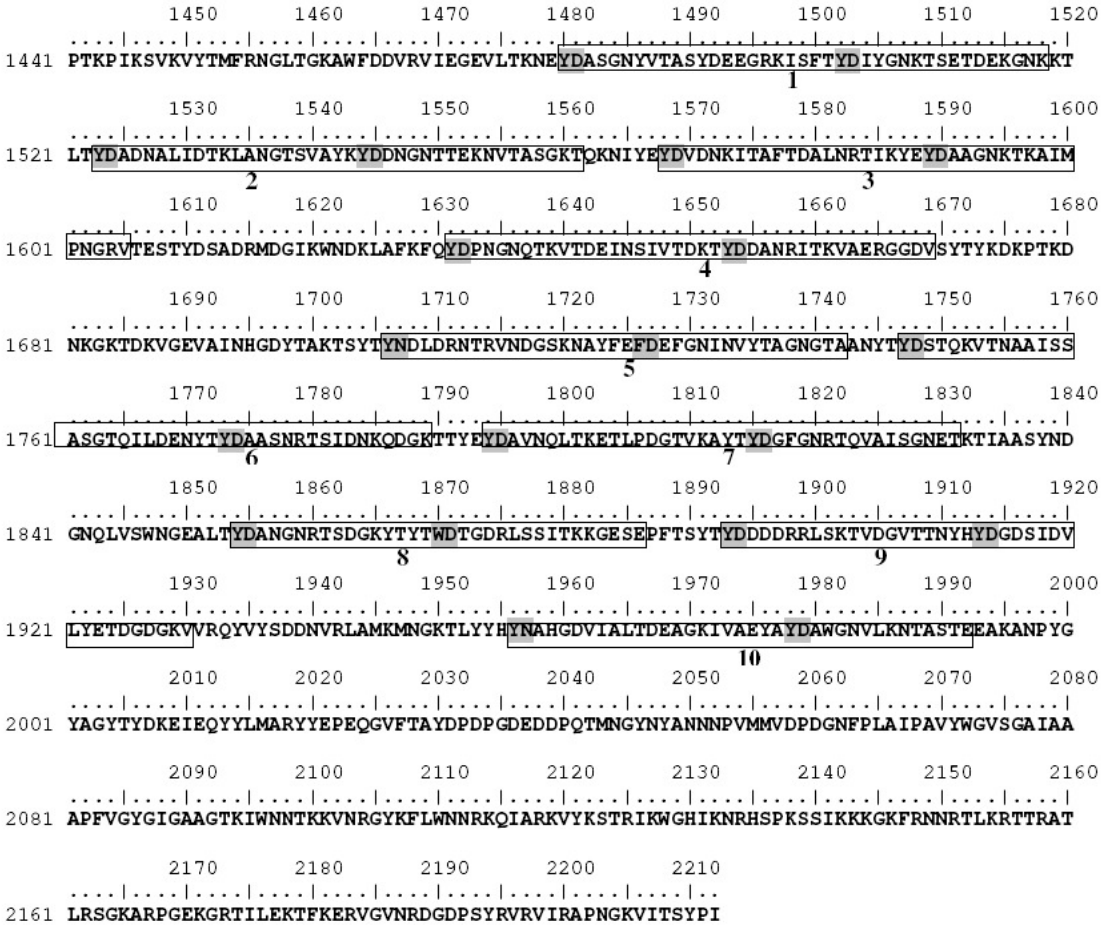


FIG.3: (B) Region (residue 1480-1992) containing two tandem copies of 21-residue repeats with YD-dipeptide (YDxxGxxxxxxxxxxxxxxxYDxxGxxxxxxxxxxxxxxxxx) in deduced Wap of ECII strain H7858 (YD- dipeptide repeat are shaded and consensus sequences containing YD-dipeptide repeat are boxed).

5.4.1.2 Comparative analysis of amino acid sequence between Wap of *L. monocytogenes* H7858 and WapA of *B. subtilis*

In *B. subtilis*, the N-terminal half of WapA contains three direct repeats of a 102-amino acid sequence responsible for cell-wall binding, with 40% identity among the three repeats (12). The amino acid sequence alignment of the ECII Wap with WapA of *B. subtilis* suggests that the first and third cell-wall binding direct repeats are present, while the second one is absent in N-terminus of ECII Wap (FIG.4). The same features were observed in ECI Wap, since the N-terminus of Wap is highly conserved between ECI and ECII strains.

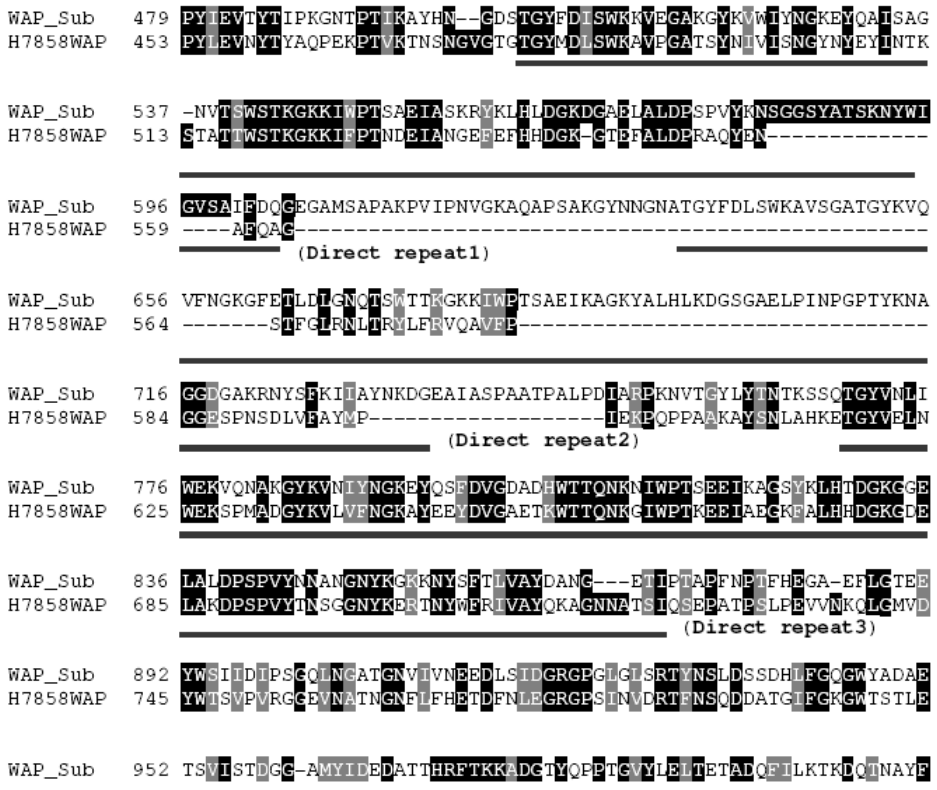


FIG.4: Amino acid sequence alignment of ECII Wap (H7858WAP) and *B. subtilis* 168 WapA (WAP_Sub) (Numbers indicate residue positions in the amino acid sequence. Identical residues are shaded. The putative cell-wall binding regions are underlined).

5.4.2 Transcriptional analysis of *wap* in ECI strain F2365 and ECII strain H7550

RT-PCR was used to determine whether *wap* was cotranscribed with its downstream genes. Our preliminary RT-PCR data suggested that in both ECI strain F2365 and ECII strain *wap* was not cotranscribed with its upstream ORFs (unpublished data). In this study, the transcriptional relationship of *wap* with its downstream ORFs was investigated. The results of RT-PCR were summarized in FIG.5. RT-PCR results suggested that *wap* was cotranscribed with two neighboring downstream ORFs (LMOf2365_0451 and LMOf2365_0452) in ECI strain F2365. In a agreement with this experimental result, a putative *rho*-independent transcriptional terminator (estimated free energy of formation, -21.3 kcal/mol) was predicted using mfold between LMOf2365_0452 and LMOf2365_0453 (FIG.5). In ECII strain 7550, *wap* appeared to be cotranscribed with a cluster of downstream ORFs, including three ORFs which share high identity with C-terminus of deduced Wap (FIG.5). However, no putative *rho*-independent transcriptional terminator with significant free energy value was identified by mfold between LMOh7858_0489 and LMOh7858_0490.

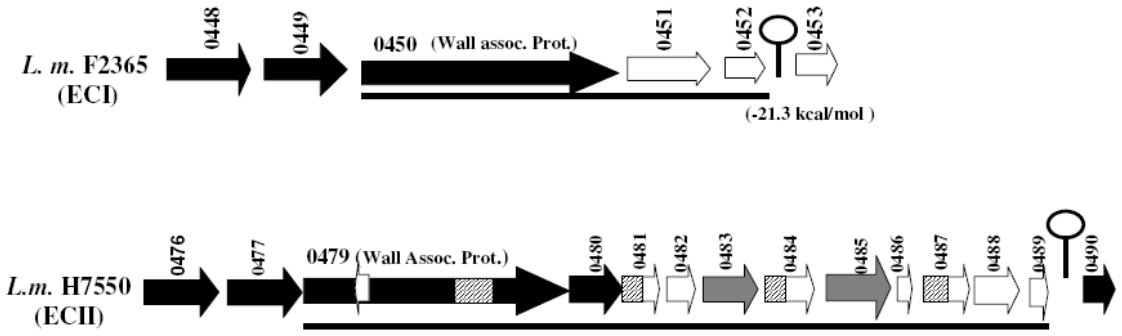


FIG.5: Transcriptional organization of the *wap* genomic region determined by RT-PCR. Arrows indicate the direction of transcription. Lollipops represent putative transcription terminators. Transcriptional units are underlined.

5.4.3 Construction of deletion mutant of *wap* in F2365 and H7550

A 6477-bp deletion (position 49 to 6525) of coding sequence for *wap* (6588 bp) was introduced into the chromosome of F2365, resulting in strain F2365Δ*wap*. The deletion was confirmed by PCR using the ECIWAPUF and ECIWAPDR primers to produce an approximately 1.8-kb band. A 6527-bp (position 49 to 6575) of coding sequence for *wap* (6639 bp) in H7550 was also deleted from chromosome, leading to a deletion mutant strain H7550Δ*wap*. PCR with ECIIWAPUF and ECIIWAPDR confirmed the presence of the deletion. Subsequently, Southern blot analysis and sequencing were used to confirm the structure of the *wap* deletion mutant strains F2365Δ*wap* and H7550Δ*wap* (data not shown). Manual examination of sequences indicated that the presence of deletion and the adjacent sequences of the deletion remained intact.

5.4.4 Phenotype Microarrays (PM) analysis

PM assays were performed on the *wap* deletion mutants and their wild-type counterparts, in a set of four 96-well microtiter plates, including PM1-2, containing various carbon sources, and PM3-4, containing nitrogen and phosphorus/sulfur sources. As shown in Table 2, PM results indicate that F2365Δ*wap* had lower growth using carbon sources such as β-methyl-D-glucoside, D-trehalose, and maltotriose than its parental strain (F2365), while it had an increased growth in the presence of other carbon sources such as adenosine, D-glucosamine, dextrin**,** and arbutin. There were also differences for the utilization of nitrogen sources, including xanthine and guanosine (Table 2). However, no noticeable changes in the utilization of carbon sources, nitrogen sources and phosphorus/sulfur sources were detected by PM comparisons between H7550Δ*wap* and its parental strain (H7550) (data not shown).

Table 2: PM comparison between F2365 and *wap* deletion mutant (tetrazolium formation was monitored with microtiter reader at 590 nm)

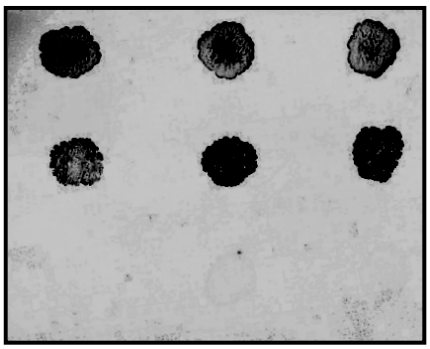
	PM1			
	(β-Methyl-D-Glucoside)	(D-Trehalose)	(Maltotriose)	(Adenosine)
F2365	0.3285	0.4167	0.3154	0.0873
F2365Δ*wap*	0.1712	0.157	0.1901	0.1922
	PM2			
	(D-Glucosamine)	(Dextrin)	(Arbutin)	
F2365	0.5665	0.0861	0.4054	
F2365Δ*wap*	0.9208	0.3048	0.5901	

Table 2: PM comparison between F2365 and *wap* deletion mutant (tetrazolium formation was monitored with microtiter reader at 590 nm) (cont'd)

	PM3	
	(Xanthine)	(Guanosine)
F2365	0.3863	0.1209
F2365Δ*wap*	-0.0004	1.306

5.4.5 Reactivity of *wap* deletion mutants with 4b serotype specific monoclonal antibody (c74.22)

Previous data from our laboratory have shown that the reactivity of serotype 4b, 4d, 4e-specific monoclonal antibody c74.22 is associated with glycosylated wall teichoic acid (26). Colony immunoblots revealed that the reactivity with c74.22 remains intact in H7550 *wap* deletion mutant (FIG.6) and F2365 *wap* deletion mutant (data not shown). This result suggests that, unlike other serotype specific genes that have been characterized in our laboratory, this wall associated protein is not required for serotype-specific glycosylation of wall teichoic acid, which is responsible for the reactivity with the serotype 4b-specific monoclonal antibody c74.22.



H7550

H7550Δ*wap*

ECIIΔ18RV (negative control)

FIG.6: Colony immunoblots with serotype 4b-specific monoclonal antibody C74.22. Top, H7550, wild type; Middle, *wap* deletion mutant of ECII strain H7550; Bottom, negative control (ECIIΔ18RV, a spontaneously derived C74.22-negative variant of H7550 that has been isolated and characterized in our laboratory).

5.4.6 Surface adherence and biofilm production of *wap* deletion mutants

H7550Δ*wap* consistently had decreased growth on nitrocellulose membranes compared with its parental wild type counterpart (FIG.7). In contrast, no difference in growth on nitrocellulose membranes was observed between F2365Δ*wap* and its parental wild type strain (data not shown). Biofilm production on wells of PVC microtiter plates following incubation at 37 ºC for 24 hours was quantified by crystal violet staining as described in Materials and Methods. A significantly lower level of biofilm production was noted in the H7550 *wap* deletion mutant than in the parental wild type strain ($p<0.05$) (FIG.8). This decreased biofilm production could also be detected visually. However, we could not obtain adequate biofilm production by the F2365 strain or its *wap* deletion, in spite of repeated efforts. Thus, the impact of the *wap* deletion on biofilm formation in this strain is not known.

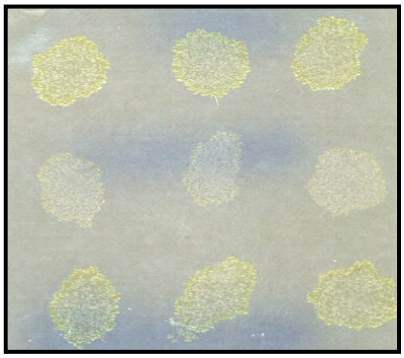


FIG.7: Surface-attached growth on nitrocellulose membrane in H7550 *wap* deletion mutant. Parental strain H7550 served as positive control. H7550 (wt*) represents a phenotypically wild type strain which was regenerated by two-step integration-excision procedures employed to isolate the deletion mutant.

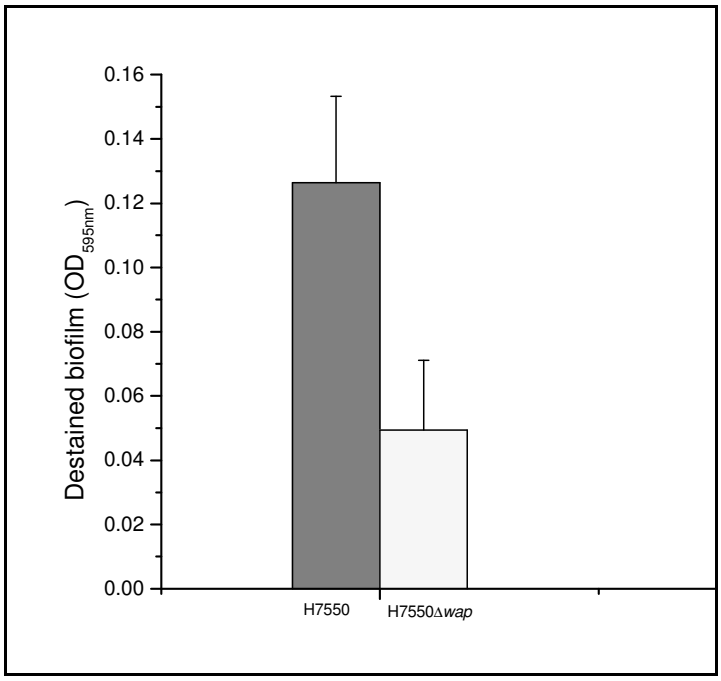


FIG.8: Decreased biofilm production (OD_{595nm}) in H7550 *wap* deletion mutant, determined in a microtiter plate biofilm assay.

5.4.7 Other phenotypic assays

Deletion of *wap* gene in F2365 and H7550 did not cause detectable changes in motility, phage susceptibility, morphology, and growth rate on complex media at different temperatures 4 ºC (FIG.9), 25 ºC, 37 ºC(data not shown).

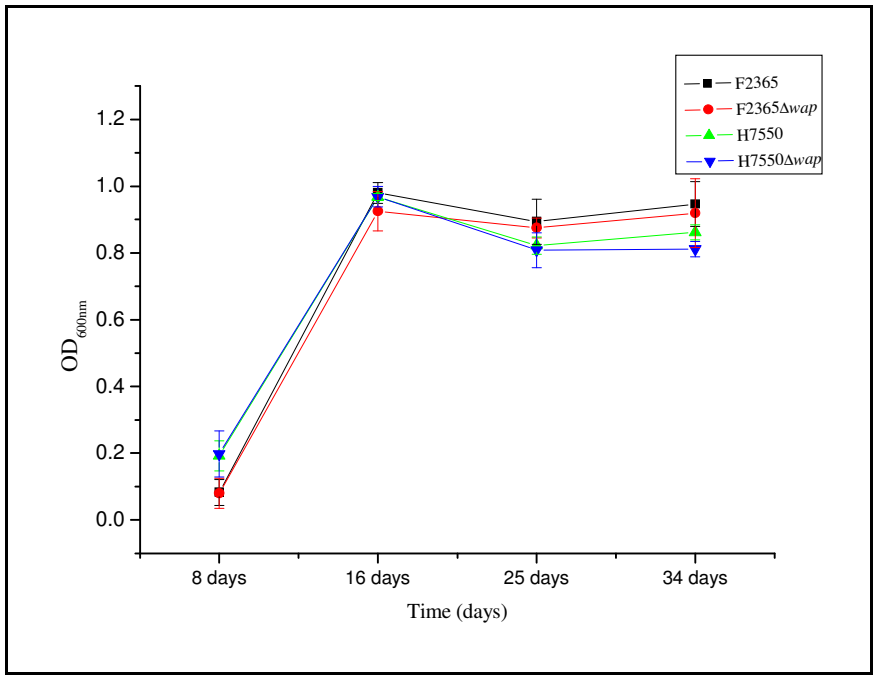


FIG.9: Growth of *wap* deletion mutants at 4 ºC in liquid media (BHI broth)

5.5 Discussion

Comparative genomic analysis revealed that within *L. monocytogenes* a large gene (*wap*, ca. 6.6 kb) encoding a putative cell wall-associated protein, is present in serotype 4b strains, but is absent in *L. monocytogenes* EGD-e (1/2a). Interestingly, *wap* with high similarity to

the one of serotype 4b is detected in both nonpathogenic strains for which the complete genome has been sequenced, *L. innocua* and *L. welshimeri*, suggesting that *wap* is not sufficient for virulence. This speculation is supported by the fact that F2365Δ*wap* appears to have the same virulence as its parental strain in an oral mouse model of infection (Chunk Czuprynski, Ying Cheng and S. Kathariou, unpublished data). The conservation of *wap* between different species may imply that *wap* was present in an ancestral strain preceding the diversification of *Listeria* into the different species, but, within *L. monocytogenes*, it has been maintained only in strains of serotype 4b.

Even though most of the Wap sequence is conserved among the different strains that harbor the gene, a high divergence was observed in the C-terminal tail of Wap of different strains. Mechanisms which have driven this diversification remain unclear. Sequence analysis suggests that Wap is an exported protein, since it harbors a signal peptide at the N-terminus. In addition, an extra TM helix was predicted in the C-terminal tail of H7858 Wap but not in that of F2365 Wap, suggesting a membrane retention signal at C-terminal tail of H7858 Wap (but not in F2365 Wap). Prediction of subcellular localization of Wap by PSORTb v.2.0 is in a good agreement with the results derived from the sequence alignment of ECII Wap (H7858WAP) and *B. subtilis* 168 WapA (WAP_Sub), indicating the association with cell-wall. Additionally, Wap was also predicted to be released extracellularly, which has been experimentally demonstrated for WapA of *B. subtilis*.

Pfam search detected RHS domains containing two tandem copies of 21-residue repeats that harbor YD-dipeptide (YDxxGxxxxxxxxxxxxxxxxYDxxGxxxxxxxxxxxxxxxxxx). The RHS domain has been shown to be implicated in binding carbohydrate or other ligands (24) The YD-repeats are most similar to the core of the *rhs* elements of *Escherichia coli* (11). The

functional roles of these *rhs* elements in *E.coli* are not determined yet. They are clearly not essential for cell growth, since *E. coli* strains can contain none or several of these elements. More distantly related YD-repeats found in toxin A of *Clostridium difficile* have been shown to bind to carbohydrates (18, 32). A previous study reported that recombinantly expressed proteins containing the YD dipeptide-repeats of teneurin-1 were involved in interacting with carbohydrates (24). The identification of a CBD domain in Wap also implies the potential role of Wap in carbohydrate binding. At this point, the region (residue 1480-2030 in Wap of strain H7550) harboring 20 of YD dipeptide- repeats has been cloned into pET-22b(+) which provides a 6 His-tag at the N-terminus of the protein. In future studies, the expression of the polypeptide containing YD-dipeptide repeats in *E.coli* and further purification of polypeptide will help us to experimentally address the role of polypeptide containing YD-dipeptide repeats.

In this study, we initiated the functional characterization of Wap by constructing the in-frame deletion mutants of *wap* in both ECI and ECII strain. No noticeable differences were observed between the *wap* mutant and the parental wild-type strain in terms of hemolytic ability, motility, phage susceptibility, cell shape, colony size, and growth rate on complex media at different temperatures (37, 25, 4 ºC). However, we detected changes in carbon metabolism of F2365Δ*wap*, compared to its parental strain (F2365). We also observed the F2365Δ*wap* is defective in the utilization of β-methyl-D-glucoside, D-trehalose, and maltotriose. These findings may suggest Wap of F2365 is implicated in the initial binding and further transportation of certain carbohydrates. However, at this point, it is necessary to confirm these observations in independent growth studies. Surprisingly, there are no noticeable differences in the utilization of carbon, nitrogen, phosphorus and sulfur sources

between H7550Δ*wap* and its parental strain. This discrepancy can probably be attributed to the distinct subcellular localization of Wap, or the presence of functional redundancy of the internal *wap* domain in ECII strains (i.e. homologies of C-terminus of Wap with several other genes in region 18 of ECII strains). Additionally, our RT-PCR data also suggested that the transcriptional unit containing *wap* was distinct in ECI strain F2365 and ECII strain H7550. This different transcription status may also contribute to the phenotypic differences between F2365Δ*wap* and H7550Δ*wap* that we observed here.

As we discussed earlier, since Wap is specific to serotype 4b strains and possibly located at cell wall, it is worthwhile to investigate whether Wap is related to expression of serotype 4b-specific antigenic determinants. Intact reactivity of either F2365Δ*wap* or H7550Δ*wap* with serotype-4b specific antibody (c74.22) suggested Wap was not involved in serotype-specific glycosylation of wall teichoic acid, which was supported by evidence from phage assays. Both *wap* deletion mutants appear to be sensitive to genus-specific or serotype-specific phages (data not shown). However, the possible role of Wap in expression of other serotype 4b-specific surface antigens remains unknown.

The ability of *L. monocytogenes* to form biofilm on food preparation surfaces could serve as a source of product contamination. It is known that biofilm formation is a complex process. In Gram-positive bacteria, it is not unusual that surface proteins are involved in surface attachment and biofilm formation. A number of cell surface structures have been described as being important in biofilm development in Streptococci, including cell surface adhesins and autolysins (8, 14, 16, 22). A previous study in *Staphylococcus aureus* has identified a cell wall associated protein of 2,276 amino acids (Bap) which is involved in biofilm formation (6). In this study, we compared the biofilm formation between *wap* deletion mutant and its

parental wild type strain. A *wap* deletion in H7550 (ECII) appeared to result in impaired surface-attached growth on nitrocellulose membranes and marked decreased biofilm formation, compared with parental wild type. Thus, Wap may play a part in adaptation to specific environmental stress (i.e. surface attachment, biofilm formation) in ECII strains. A modified biofilm assay for ECI strain is needed to increase the biofilm production of this strain and further determine whether similar roles may be played by *wap* in ECI strains. In addition, a complemented strain of H7550 *wap* deletion mutant has been recently constructed to further confirm that the deletion of *wap* in ECII strain is responsible for the impaired attachment and biofilm production.

To date, several studies on *wap*A in *Bacillus subtilis* suggested that *wap*A was positively or negatively regulated by two-component systems. Dartois et al. (1998) reported that repression of *wap*A under high salt stress was mediated by the DegS-DegU two-component system (7). Thus, it was hypothesized that WapA increased cell wall permeability by functioning as a sieve, or an ion pore or channel, and that its repressed synthesis at high salt concentration may prevent excessive exchanges between the outside medium and the cytoplasmic compartment. Recently, comparative DNA microarray analyses revealed that *wap*A in *B. subtilis* was positively regulated by the YvrGHb two-component system (28), which is possibly implicated in maintaining homeostasis of the state of the cell surface under normal culture conditions. The tight regulation of *wapA* expression by different two-component systems suggests that WapA is most likely involved in sensing environmental changes. These findings on WapA of *B. subtilis* may shed light on our on-going functional characterization of Wap in *L. monocytogenes.*

Further investigation on the potential role of Wap in helping *L. monocytogenes* serotype 4b to survive environmental stressors such as elevated temperature, oxidative and salt stress, will be important to further characterize the functions of Wap and to understand the physiological characteristics that this protein may confer on ECI and ECII strains of *L. monocytogenes*.

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CHAPTER VI

Genetic characterization of a diversified genomic region (region-18) in *Listeria monocytogenes* Epidemic Clones I and II

6.1 Abstract

An earlier study has identified a genomic region (region-18) which appeared to be either absent or markedly divergent in the ECII strains but conserved among other serotype 4b strains. In serotype 4b strains, region-18 is flanked by a large gene encoding a putative cell-wall associated protein (*wap*) on one side, and a well-known virulence gene, internalin A (*inl*A), on the other side. *L. monocytogenes* strains of other serotypes lack region-18, or *wap*. Comparative genomic analyses across five genomes from different *L. monocytogenes* isolates and *Listeria* species suggested that region-18 in serotype 4b strains may have been acquired by horizontal gene transfer, which is consistent with previous data. Mutational and functional analyses showed the deletion of region-18 had no effect on hemolytic ability, phage susceptibility, cell shape, or colony size, in either ECI strain F2365 or ECII strain H7550. However, Phenotype Microarray data revealed the deletion mutant of region-18 in ECI strain F2365 had a defect in the utilization of certain carbon sources. In contrast, enhanced death rate during post-stationary phase incubation at 42 ºC was observed in the deletion mutant of region-18 in ECII, suggesting that in ECII strains region-18 may be implicated in post-stationary phase stress responses.

6.2 Introduction

Listeria monocytogenes, a facultative intracellular pathogen of humans and animals, is the etiological agent of epidemic and sporadic food-borne listeriosis with severe symptoms (septicemia, meningitis, abortions) and relatively high mortality (20-30%) (6, 19). The ability of *L. monocytogenes* to grow at refrigeration temperature and survive under extreme conditions makes this pathogen particularly difficult to control as well as to regulate. Outbreaks of listeriosis have been commonly associated with the consumption of cold-stored ready-to-eat (RTE) foods, especially deli meat and unpasteurized dairy products. Most human listeriosis cases are caused by strains of three serotypes, 1/2a, 1/2b, and 4b. Furthermore, most outbreaks have involved a small number of genetically related strains of serotype 4b. Among these strains two major clonal groups have been recognized, and have been designated Epidemic Clone I (ECI) and Epidemic Clone II (ECII) (11). ECI has been implicated in numerous outbreaks in Europe and North America, including the California outbreak of 1985. In contrast, ECII represents a novel epidemic clone, which was not identified until the 1998-99 multistate outbreak in the United States.

Currently, the advancement of DNA sequencing technology has made it feasible to obtain the whole genome sequences of different strains of the species. To date, two nonpathogenic strains, *Listeria innocua* CLIP 11262 (serotype 6a) and *Listeria welshimeri* serovar 6b str. SLCC5334 have been sequenced (7, 9). Strain EGD-e (serotype 1/2a) was the first *L. monocytogenes* to have its genome sequenced (7). Representatives of the two major genomic lineages of *L. monocytogenes* as represented by strains of serotype 1/2a and 4b were chosen for whole genome sequencing in 2004. The genome of serotype 4b strain F2365, a representative of ECI strains which was implicated in the Jalisco cheese outbreak of 1985 in

California (1), has been sequenced to closure. The genome of strain H7858, implicated in the 1998-99 outbreak (2) and representing ECII has been sequenced as well (16). Based on the whole genome sequences, data generated from DNA microarrays, and whole-genome comparisons have provided considerable insight on the ecology, evolution and virulence of *L. monocytogenes*.

Genomic comparisons between serotype 4b and serotype 1/2a and DNA macroarray data, along with genomic subtraction data have identified several 4b-specific genomic regions (4, 10). An earlier study identified a genomic region (region-18) which appeared to be either absent or markedly divergent in the ECII strains but conserved among other serotype 4b strains (5). Interestingly, this region is flanked on one side by a large gene encoding a putative cell-wall associated protein (*wap*). On the other side the region was flanked by a well-known virulence gene, *inl*A, encoding Internalin. Comparative genomic analysis has suggested that this region may have been acquired by horizontal transfer from another unidentified source since the G+C content of most Open Reading Frames (ORFs) in this region of ECII or other 4b strains was noticeably lower than the average for the genome (5).

Although the mechanisms that have driven this genomic diversification between ECII and other serotype 4b strains remain unknown, one should nevertheless consider the possibility that the existence of different subsets of genes in this region confers additional physiological and virulence traits. In this study, in order to assess the possible functions of the genes in this region in ECII and ECI strains, we constructed and characterized in-frame deletion mutants of region-18 in the ECI strain F2365, as well as the ECII strain H7550.

6.3 Materials and Methods

6.3.1 Bacterial strains and growth conditions

The ECII strain H7550 was a clinical isolate from the 1998-99 hot dog outbreak, and has been described before (2). Strain F2365, which was implicated in the 1985 Jalisco cheese outbreak in California, was included as representative strain of ECI. *E. coli* SM10 was used as conjugation donor in deletion construction (20)(described below). Bacteria were grown at the indicated temperature in brain heart infusion broth (BHI, BD NJ) or tryptic soy broth supplemented with yeast extract (0.7%) (TSBYE, BD NJ) as described previously (5). Agar cultures were grown in BHI with 1.5% agar or tryptic soy agar supplemented with 0.7% yeast extract (TSAYE, BD NJ). When appropriate, antibiotics used for *Listeria* were streptomycin (1,200 µg/ml), nalidixic acid (20 µg/ml), and chloramphenicol (6 µg/ml). Antibiotics used for *Escherichia coli* were ampicillin (100 µg/ml) and chloramphenicol (25 µg/ml). Isolates of *L. monocytogenes* were preserved and revived as described elsewhere (8).

6.3.2 Nucleotide sequence analysis

BLAST algorithms (http://www.ncbi.nlm.nih.gov/BLAST/) were used for DNA and protein database searches and analyses. Sequence alignments were performed using the ClustalW algorithm (http://www.ebi.ac.uk/clustalw/). For RNA secondary structure analysis, we used RNA folding (mfold) (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/). All genome sequences were derived from DDBJ/EMBL/GeneBank. The genome sequences of strains F2365 and H7858 under accession number AE017262 and AADR00000000, respectively. The genome sequences of *Listeria innocua* CLIP11262 and *Listeria welshimeri* serovar 6b str. SLCC5334 are under accession number AL592102 and AM263198, respectively.

6.3.3 Reverse transcriptase PCR (RT-PCR)

Primers used for RT-PCR were listed in Table 1. Procedures for RNA extraction from *L. monocytogenes*, amplification of cDNA, and reverse transcription PCR (RT-PCR) were as described elsewhere (18). Antisense primer RTCYP1 was used for cDNA synthesis for strain F2365 and strain H7550. RTCYP1, RTCYP3 and RTCYP5 were used for subsequent PCR reactions in strain F2365. RTCYP1, RTCYP5, RTCY18RH17 and RTCY18RH20 were used for PCR reactions in strain H7550.

6.3.4 Construction of the deletion mutants of region-18

To clone a fragment harboring the F2365 region-18 deletion, a 934-bp upstream fragment encompassing ORF LMOf2365_0450 and LMOf2365_0451 was amplified using primers ECIΔ18RUF and ECIΔ18RUR (Table 1). *Eco*RI and *Bam*HI sites were incorporated into the 5' end of each primer. A 794-bp downstream fragment encompassing ORF LMOf2365_0469 and LMOf2365_0471 was amplified using primers ECIΔ18RDF and ECIΔ18RDR (Table 1). *Bam*HI and *Xba*I were introduced into the 5' end of each primer. After digestion with the indicated enzymes, the two fragments were ligated into the corresponding sites of the temperature-sensitive shuttle vector pCON-1(3), resulting in pCONIΔ18R.

To clone a fragment harboring the H7550 region-18 deletion, two DNA fragments of 485-bp and 511-bp were generated using genomic DNA of strain H7550 in PCR amplification, with primers ECIIΔ18RUF and ECIIΔ18RUR used to amplify the 485-bp upstream fragment, and primers ECIIΔ18RDF and ECIIΔ18RDR (Table 1) used to amplify the 511-bp downstream fragment. The fragments were digested with the indicated enzymes and cloned into pCON-1, yielding deletion-harboring fragment, pCONIIΔ18R. pCONIΔ18R or pCONIIΔ18R was introduced into *E. coli* SM10 by electroporation. Positive colonies were

confirmed by PCR and restriction enzyme digestion. The two recombinant plasmids, pCONIΔ18R and pCONIIΔ18R were introduced into F2365 and H7550, respectively, by conjugation as previously described with slight modification (13). Briefly, the bacterial strains were grown at 30 ºC with shaking overnight. *E. coli* donor strains were grown in LB supplemented with chloramphenicol (25 µg/ml), and *L. monocytogenes* recipient strains were grown in BHI. The donor culture (3 ml) was mixed with 1.5 ml of recipient culture and filtered onto 0.45-µm-pore-size HA-type filters (20 mm, Millipore). The filter was washed once with 10 ml BHI, transferred to a BHI plate without antibiotics, and incubated overnight at 30 ºC. The bacterial cells were suspended in 4 ml BHI, and 50 µl aliquots of mixture were plated on BHI containing 20 µg/ml of nalidixic acid (to which *L. monocytogenes* is naturally resistant, but *E. coli* is susceptible) and 6 µg/ml of chloramphenicol. Chloramphenicol-resistant transconjugants were confirmed by PCR. Subsequently, integration-excision procedure of recombinant plasmid in strains F2365 and H7550 was performed as previously described (14). Finally, chloramphenicol sensitive colonies were identified by replica plating on BHI agar and BHI supplemented with chloramphenicol (6 µg/ml) agar, and incubated at 30 ºC for 24-48 hours. Chloramphenicol sensitive (CM^s) colonies from the previous screen were examined by PCR using a combination of a forward primer for the upstream fragment and a reverse primer for the downstream fragment, as well as the primers targeting the deleted gene or gene cassette, thus confirming the construction of strains F2365Δ18R and ECIIΔ18R, respectively. Theoretically, after integration-excision procedure, the ratio of regenerated wild type colonies to deletion mutant colonies should be 1:1.

Table 1: Primers used for PCR amplification in this study[1]

Primer	Sequence
ECIΔ18RUF	5'-GACGAATTCCGAGTTATACGTACGATGAT-3'
	*Eco*RI
ECIΔ18RUR	5'-CGAGGATCCTTTGTCAAGCATTATTTTCCCC-3'
	*Bam*HI
ECIΔ18RDF	5'-ATAGGATCCCCAGCAACGCATACAAAG-3
	*Bam*HI
ECIΔ18RDR	5'-TTGGTCTAGATCTGTTTGCGAGACCG-3'
	*Xba*I
ECIIΔ18RUF	5'-ATCGAATTCCAGCTGCGCCATTTGTTG-3'
	*Eco*RI
ECIIΔ18RUR	5'-CATGGATCCAGTACTTCTCCTCCAGTTTC
	*Bam*HI
ECIIΔ18RDF	5'-ATAGGATCCCCAGCAACGCATACAAAG-3'
	*Bam*HI
ECIIΔ18RDR	5'-TGCTCTAGAGGATTTTCTCCTGCATCC-3'
	*Xba*I
gtcA_P_F	5'-ATAAGGTACCTTCAAAGGGACAGGCAACATG-3'
	*Kpn*I
gtcA_P_R	5'-GCGACCCGGGAATAACTTCTTTCTATGTG-3'
	*Sma*I
ECII18RCF4	5'-GTAACCCGGGCTATAGAGTAAGAG-3'
	*Sma*I
ECII18RCR2	5'- CTATACTAGTTAACACATAAGGATGTCG-3'
	*Spe*I
NC16	5'-TCAAAACATACGCTCTTATC-3'
PL95	5'-ACATAATCAGTCCAAAGTAGATGC-3'
ECIIWAPDR	5'-CCGAGTCTAGAAAGCTGGACAATGTTG-3'
RTCYP1	5'- ATCCATACTCCACTGCCAAA-3'
RTCYP5	5'- GATTTGCATGGTTGAATTCCT-3'
RTCYP3	5'- AAGGAAAAAGCGGAAGTGGT-3'
RTCY18RH17	5'- AAGAAAGCGGACAAAAAGAAT-3'
RTCY18RH20	5'- TGTATGCGTTGCTGGTTGAT-3'

[1]. Underlined sequences correspond to recognition sequences for the indicated restriction enzymes

6.3.5 Construction of complemented strains for ECIIΔ18R

For complementation, the site-specific phage integration vector pPL-2 (13) was used to construct recombinant plasmids. To ensure the transcription of the first gene within region-18, the promoter of the serogroup 4-specific gene *gtcA*, previously characterized in our laboratory (18) was PCR-amplified with primers gtcA_P_F (*Kpn*I) and gtcA_P_R (*Sma*I) (Table 1) and cloned into pPL-2. The resulting plasmid pPL2_*gtcA*P was used for the subsequent subcloning of region-18. The entire region-18 were amplified by One Shot LA PCR™ Mix kit (TaKaRa, BIO INC., Otsu, Shiga, Janpan) using primers ECII18RCF4 (*Sma*I) and ECII18RCR2 (*Spe*I) (Table 1). PCR products were *Sma*I-*Spe*I digested and ligated into pPL2_*gtcA*P digested by *Sma*I-*Spe*I, resulting in pPL2_*gtcA*P_II18R. The construction was verified by PCR and restriction enzyme digestions. Recombinant plasmids were electroporated into *E. coli* SM 10 for conjugation into the region-18 deletion mutant of *L. monocytogenes* H7550 strain (strain ECIIΔ18R). Conjugation of pPL2_*gtcA*P_II18R from *E .coli* SM 10 into ECIIΔ18R was performed as described above. Chloramphenicol-resistant transconjugants were screened by PCR. The primer pairs NC16 and PL95 are expected to amplify a 499-bp product in strains that contain the integration vector at $tRNA^{Arg}$-*attBB'* (13). Primers gtcA_P_F and ECIIWAPDR were used to confirm the presence of region-18 in the complemented strain.

6.3.6 Colony immunoblots

Overnight cultures were grown on nitrocellulose membranes placed on TSB agar plates at room temperature for 36 h. The bacterial colonies were washed off with Towbin transfer buffer. The nitrocellulose membrane was then processed using monoclonal antibody c74.22, as described previously (18).

6.3.7 Phenotype Microarray (PM) analysis

The PM [1-4] kit (Biolog Inc., Hayward, CA) was used in this study. Wild type and deletion mutant strains were streaked onto BUG+B Agar Plates (Biolog) and were grown overnight at 37 ºC. A small amount of cells was removed from the agar surface and suspended in inoculating fluid (Biolog) to adjust the cell density to an absorbance of 0.2 at 590 nm with 1 cm cuvette using spectrophotometer (SmartSpec^{TM} 3000, Biorad, CA). The suspensions were then inoculated into each PM plate at a volume of 100 µl/well. The PMs were incubated at 37 ºC for 24 hrs (PM1, PM2) or 36-48 hrs (PM3, PM4). All PM data were obtained using the microtiter plate reader (TECAN SAFIRE A5082, Austria) at 590 nm and the PMs were also examined visually at the end of incubation, for independent verification of results.

6.3.8 Enumeration of region-18 deletion mutants in single and mixed-culture

In order to assess growth and survival of the deletion mutants at 42 ºC, the deletion mutant strain and its parental wild type counterpart were grown in BHI at 37 ºC overnight, and 50 µl was transferred into 5 ml of fresh BHI, and incubated at 42 ºC. Presence of equivalent numbers of CFU/ml for inoculation was confirmed by serial dilution and plate counting in duplicate. The growth and survival of the bacteria at 42 ºC was monitored on a daily basis by plating serial dilutions of the cultures on TSAYE. The experiment was performed twice independently.

For mixed-culture experiments, a streptomycin resistant derivative of H7550 (H7550Str^{R}) and the region-18 deletion mutant ECIIΔ18R were grown in BHI at 37 ºC overnight. Serial dilutions of each overnight culture were plated on TSAYE plates to enumerate the number of cells for inoculation, and equal aliquots (25 µl) of overnight cultures of both H7550Str^{R} and

deletion mutant ECIIΔ18R were transferred into 5 ml fresh TSBYE and incubated at 37 ºC or 42 ºC for 4 days . Bacteria were enumerated on a daily basis by plating serial dilutions of the mixed culture on TSAYE and TSAYE supplemented with streptomycin (1200 mg/l) in duplicate. Colonies on TSAYE supplemented with streptomycin were expected to represent H7550Str^R . The experiment was performed twice independently.

6.4 Results

6.4.1 Comparative genomic analyses

In an earlier study, Evans et al. (2004) showed that ECII strains harbored an unusually diversified genomic region (designated "region-18"), which was otherwise serotype 4b-specific and conserved among other serotype 4b strains. A genomic comparison across *L. monocytogenes* 4b ECI (F2365), *L. monocytogenes* EGD-e, and *L. innocua* suggested region-18 may have been introduced into serotype 4b by an insertion in the genome between *wap* and *inlA* (5). More recently, two additional genomes have been sequenced, which makes us able to extend the genomic comparison among the five sequenced genomes. An inspection of genomic organization in region-18 revealed that a gene (*wap*) coding for the cell-wall associated protein and two small ORFs upstream of *wap*, had an apparently conserved genomic organization in serotype 4b strains, *L. innocua* and *L. welshimeri*. In contrast, *wap* and the upstream neighboring small ORF were absent from *L. monocytogenes* EGD-e, which lacked the entire region-18 (8-kb) flanked with *wap* and *inlA* (FIG.1). A gene (*lmo435*) coding for a putative peptidoglycan-bound protein harboring a LPXTG motif shared homologies among *L. monocytogenes* EGD-e, *L. innocua* (*lin0457*) and *L. welshimeri* (*Lew043*1)but was absent in serotype 4b strains (FIG.1). Furthermore, ORFs in region-18 of ECI strain F2365 and in the ECII strain H7858 had G+C contents ranging from 19.44% to

35.77% (in F2365) and from 22.38% to 32.75% (in H7550), noticeably lower than the average G+C content of *L. monocytogenes* (38%). The counterpart of region-18 in *L. welshimeri* has lower-than-average G+C contents as well (FIG.2). Comparative sequence analysis between the genome of ECI strain (F2365) and that of ECII strain (H7858) indicates that region-18 has undergone divergence between ECII and other serotype 4b strains (FIG.1).

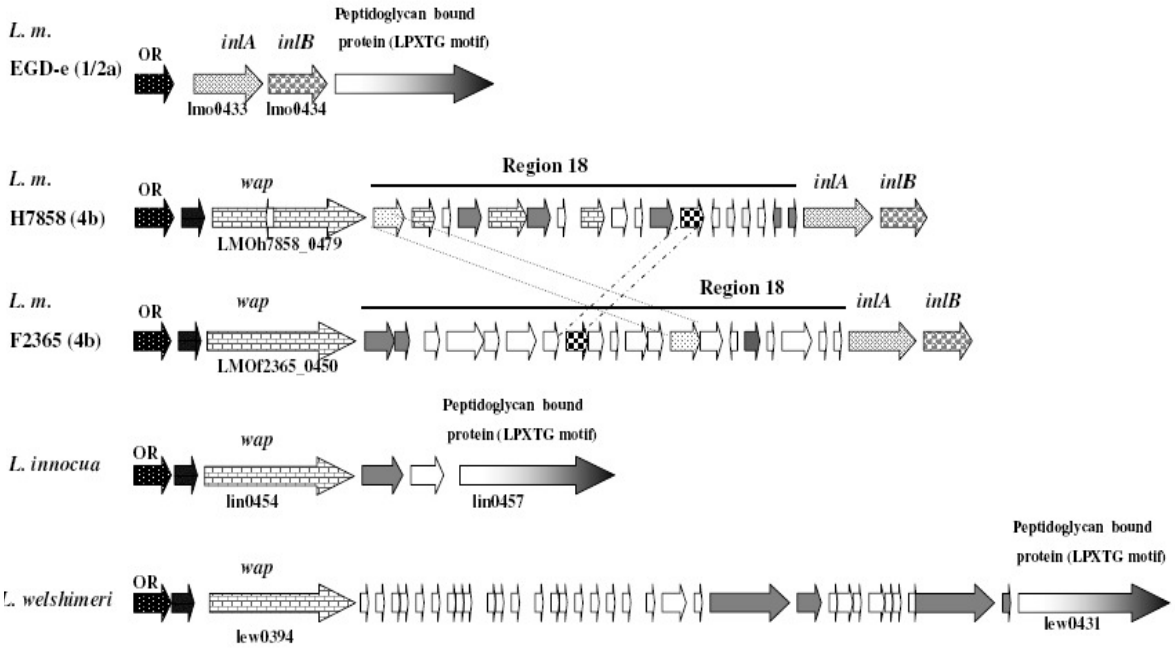

FIG.1: Organization of region-18 in genomes of *L. monocytogenes*, *L. innocua* and *L. welshimeri* strains. Representation of the alignment between genomes of *L. monocytogenes* EGD-e, *L. innocua*, *L. welshimeri*, *L. monocytogenes* H7858 (serotype 4b) and *L. monocytogenes* F2365 (serotype 4b) in region-18 is illustrated. Genes are indicated by arrows showing their relative orientations (not drawn to scale). Arrows with same fill pattern indicate homologous genes. ORFs conserved between strains H7858 and F2365 (LMOf2365_0458 and LMOf2365_0463) are linked by diagonal lines. OR: Oxidoreductase; *wap*: wall-associated protein; *inlA*: internalin A; *inlB*: internalin B.

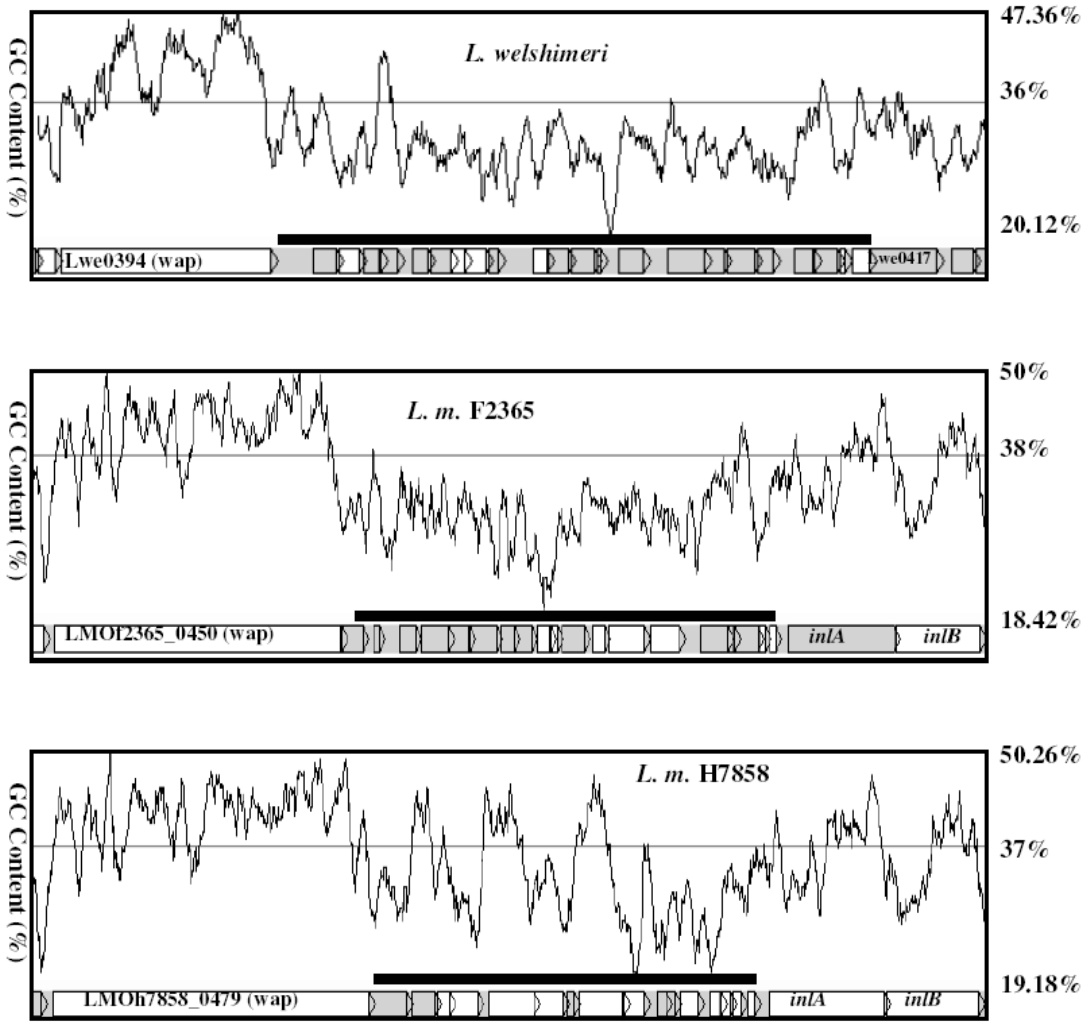

FIG.2: G+C contents of region-18 in *L. monocytogenes* strain F2365 (ECI) and H7858 (ECII), and the counterpart of region-18 in *L. welshimeri*. From top to bottom, *L. welshimeri, L. monocytogenes* strain F2365, and *L. monocytogenes* strain H7858. ORFs are indicated by arrows (not drawn to scale). Black bars indicate region-18 or its counterpart.

Although region-18 is highly divergent, two ORFs (LMOf2365_0458 and LMOf2365_ 0463 in F2365) are conserved between ECII and ECI strains, even though their relative location in region-18 differs between the two types of strains (FIG.1). Interestingly, an ECII-specific feature of region-18 is that three ORFs (LMOh7858_0481, LMOh 7858_0484, and LMOh 7858_0487) encoding cell surface proteins were detected within region-18 in the genome of ECII strain H7858. The locations of these three ORFs within region-18 are shown schematically in FIG.3. Nucleotide sequence identity between these ORFs ranged from 80 to 90% identity. The deduced amino acid sequences of these three ORFs not only shared high identity with each other (81-90 %), but also showed 70-85 % homology to C-terminal sequence of wall-associated protein (Wap) immediately flanking to region-18 (Table 2, FIG.4). At the nucleotide sequence level, the *wap d*omain had 79 to 92 % identity with the three homologous ORFs. Region-18 in F2365 lacked such putative surface proteins.

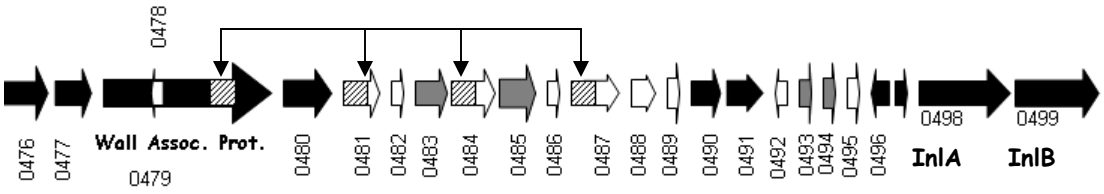

FIG.3: Distribution of ORFs harboring homologies of Wap C-terminus within region-18 of strain H7858. Homologies are indicated by arrows and upward diagonal pattern.

Table 2: Identity scores generated by ClustalW alignment of LMOh7858_0481, LMOh 7858_0484, LMOh 7858_0487 and C-terminus of Wap.

SeqA	Name	Len(aa)	SeqB	Name	Len(aa)	Score
1	WAP_C_term	175	2	0481	134	70
1	WAP_C_term	175	3	0484	177	79
1	WAP_C_term	175	4	0487	189	85
2	0481	134	3	0484	177	90
2	0481	134	4	0487	189	73
3	0484	177	4	0487	189	81

FIG.4: Multiple sequence alignment (CLUSTAL) of the deduced sequences of LMOh7858_0481 (aa 1 to aa 134 of 151 amino acids), LMOh7858_0484 (aa 7 to aa 183 of 300 amino acids), LMOh7858_0487 (aa 2 to aa 190 of 306 amino acids) and a segment of the C-terminus of Wap (aa 1907 to aa 2081 of 2212 amino acids).

6.4.2 Transcriptional analysis of ORFs immediately upstream to *inlA* in ECI strain F2365 and ECII strain H7550

RT-PCR was used to determine whether ORFs in region-18 were cotrascribed with downstream gene *inlA*. RT-PCR results suggested that ORFs in region-18 were not cotranscribed with the neighboring downstream gene *inlA* in either ECI strain F2365 or ECII strain H7550. A putative *rho*-independent transcriptional terminator (estimated free energy of formation, -12.3kcal/mol) was predicted using mfold immediately upstream to the promoter region of *inlA* in both ECI strain F2365 and ECII strain H7858 (FIG.5).

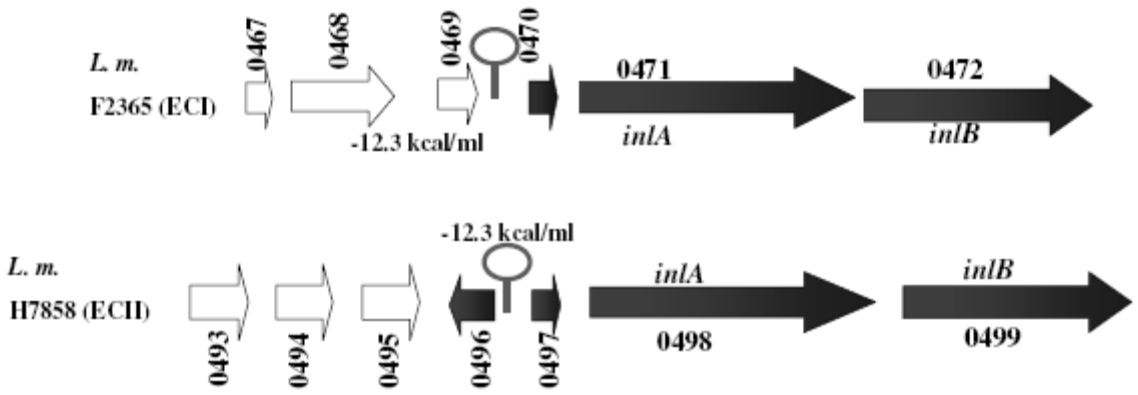

FIG.5: Transcriptional relationship of ORFs in region-18 with *inlA* in ECI strain F2365 and ECII strain H7858 determined by RT-PCR. Arrows indicate the direction of transcription. Lollipops represent transcription terminator determined by RT-PCR.

6.4.3 Construction of deletion mutant of region-18 in strains F2365 and H7550

6.4.3.1. Region-18 deletion mutant in strain F2365

A deletion of region-18 (9376-bp, from ORF LMOf2365_0451 to LMOf2365_0469) was constructed. In this deletion mutant, designated F2365Δ18R, *wap* is adjacent to *inlA*. The deletion was confirmed by PCR using the ECIΔ18RUF and ECIΔ18RDR to produce an approximately 1.7-kb band. Subsequently, Southern blot analysis and sequencing were used to confirm the deletion of the region-18 in mutant. The DNA sequence of the deletion mutant was manually examined to ensure that a putative transcriptional terminator and promoters upstream to *inlA* were intact (data not shown).

6.4.3.2 Region-18 deletion mutant in strain H7550

A deletion of region-18 (7797-bp, from ORF LMOh7858_0480 to LMOh7858_0496) was also pursued in strain H7550. An isogenic deletion mutant (ECIIΔ18R) was expected to be generated by two-step integration-excision procedures as described in Materials and Methods. However, despite repeated attempts to introduce deletion of region-18 into strain H7550 by two-step integration-excision procedures, no deletion mutant was detected among 184 chloramphenicol-sensitive colonies that were picked for screening. Therefore, we examined the integration of pCONIIΔ18R in the chromosome of integrants selected by incubation at 42 ºC on BHI agar plates containing chloramphenicol (6 µg/ml) using Southern blots. After integration of pCONIIΔ18R was confirmed in the chromosome of integrants, we started another screening with these integrants. Eventually, 1 of 28 screened chloramphenicol-sensitive colonies was confirmed to harbor the expected deletion by PCR with ECIIΔ18RUF and ECIIΔ18RDR primers. In addition, Southern blots as well as sequencing confirmed that the deletion mutant harbored the expected deletion (data not

shown). Manual examination of DNA sequence was done to ensure that a putative transcriptional terminator and promoters upstream to *inlA* were intact.

6.4.4 Construction of complemented strains of ECII Δ18R

A *L. monocytogenes* site-specific phage integration vector, pPL-2 was used to introduce a single copy of promoter region of *gtcA* and the entire region-18 of ECII strain H7550 (8033-bp) into ECIIΔ18R by conjugation as described in Materials and Methods. Recombinant plasmid pPL2_*gtcA*P_II18R carrying promoter region of *gtcA* and entire region-18, integrated at the phage attachment site of PSA prophage within the $tRNA^{Arg}$ that is present only once in the genome of *L. monocytogenes*, resulting in ECIIΔ18R:*gtcA*P18R. Those chloramphenicol-resistant integrants were confirmed by PCR for the presence of PSA-*attBP*' with primers NC16 and PL95. The confirmation of the integration of promoter region of *gtcA* and entire region-18 at the phage attachment site was carried out with primers gtcA_P_F and ECIIWAPDR as well. Furthermore, Southern blots using DNA probe derived from genomic fragment internal to region-18 of ECII strain H7550 was employed to confirm the presence of region-18 in complemented strain ECIIΔ18R:*gtcA*P18R (data not shown).

6.4.5 Reactivity of region 18 deletion mutants with serotype 4-specific monoclonal antibody (c74.22)

Previous data from our laboratory have shown that reactivity of serotype 4b, 4d, 4e-specific monoclonal antibodies (MAb) c74.22 is associated with glycosylated wall teichoic acid (12, 18). We tested reactivity of deletion mutant strains F2365Δ18R and ECIIΔ18R with serotype 4b-specific MAb since region-18 was initially identified as a serotype 4b-specific region. Colony immunoblots revealed that reactivity with c74.22 remains intact in both

deletion mutants ECIIΔ18R and F2365Δ18R (FIG.6). This result suggests that, this region either in ECI strain F2365 or in ECII strain H7550 is not required for serotype-specific glycosylation of wall teichoic acid, which is responsible for reactivity with the serotype 4b-specific monoclonal antibody c74.22.

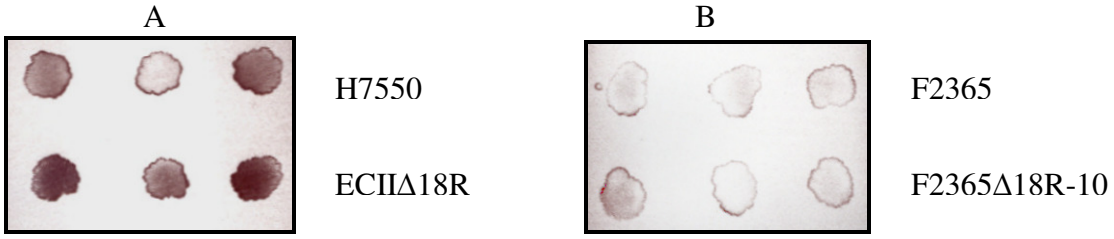

FIG.6: Reactivity of region-18 deletion mutants with serotype 4b-specific monoclonal antibody C74.22. (A) Top, positive control (wild type strain H7550 [ECII]); Bottom, region-18 deletion mutant of strain H7550. (B) Top, positive control (wild type strain F2365 [ECI]); Bottom, region-18 deletion mutant of strain F2365.

6.4.6 Phenotype Microarrays (PM) analysis

In this study, we employed phenotype microarrays (PM) as a screening platform to provide a starting point for further functional characterization of genomic region-18. PM assays were performed on the deletion mutant, compared with the wild-type strain, in a set of four 96-well microtiter plates, with PM1-2 containing various carbon sources and PM3-4 containing nitrogen and phosphorus/sulfur sources. As shown in Table 3, PM results indicated that strain F2365Δ18R showed lower growth using carbon sources such as β-

methyl-D-glucoside, D-trehalose, maltose and maltotriose than its parental strain (F2365). However, no noticeable changes in the utilization of carbon sources, nitrogen sources and phosphorus/sulfur sources were detected by PM comparison between ECIIΔ18R strain and its parental strain (H7550) (data not shown).

Table 3: PM comparison between F2365 and region-18 deletion mutant (tetrazolium formation was monitored with microtiter reader at 590 nm).

	PM1			
	(β-Methyl-D-Glucoside)	(D-Trehalose)	(Maltose)	(Maltotriose)
F2365	0.3285	0.4167	0.4378	0.3154
F2365Δ18R	0.1222	0.2123	0.1918	0.1876

6.4.7 Fitness of region-18 deletion mutant of ECII strain H7550 in single and mixed-cultures

When grown alone, the wild type strain H7550 and ECIIΔ18R grew to equivalent levels after overnight incubation at 42 ºC (log10 CFU/ml are 8.76 ± 0.33 and 8.77 ± 0.24, respectively). However, during prolonged incubation at 42 ºC, ECIIΔ18R had substantially enhanced death rates compared to the wild type strain (FIG.7).

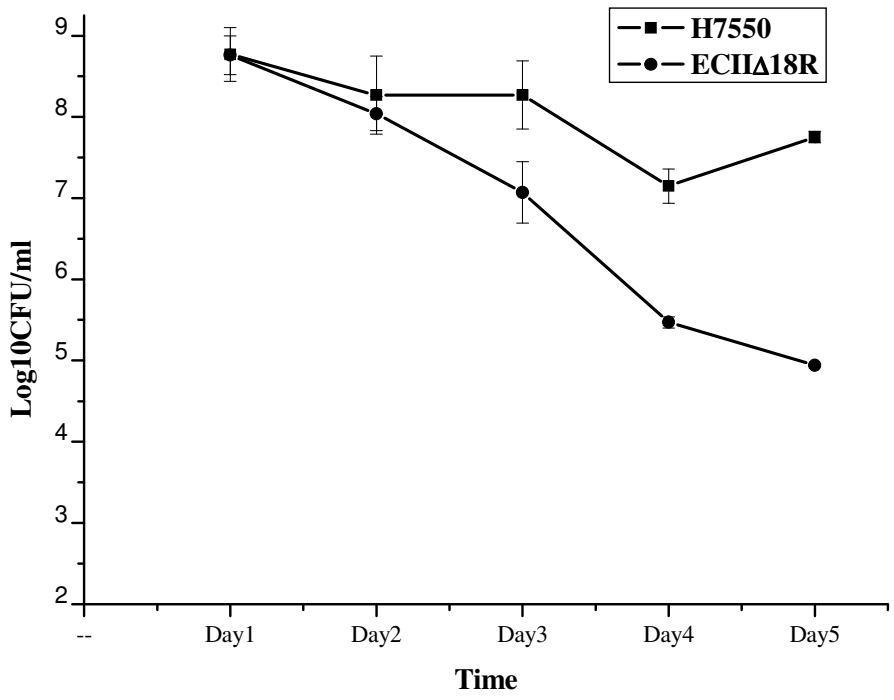

FIG.7: Post-stationary phase survival of ECIIΔ18R strain during incubation at 42 ºC. H7550 and ECIIΔ18R were grown individually and incubated for 5 days at 42 ºC. Bacteria were enumerated on a daily-basis as described in Materials and Methods. The error bars indicate the standard deviations from the means of duplicate experiments

To determine competitive fitness in mixed cultures, a streptomycin resistant mutant of H7550 (H7550str^R) was co-cultured with ECIIΔ18R. When co-cultured with H7550, H7550str^R was found to have comparable growth and survival at 37 ºC (FIG.9A). At 42 ºC, H7550str^R grew well, but at levels somewhat lower than those of H7550 (FIG.8A).

Surprisingly, when ECIIΔ18R was co-cultured with H7550str^R, at either 37 ºC or at 42 ºC, H7550str^R failed to grow. In such mixed cultures ECIIΔ18R consistently accounted for nearly 100% of the mixed overnight culture (FIG.8B and FIG.9B). Thus, a comparison of survival rates between strains ECIIΔ18R and H7550str^R in the mixed-culture during

prolonged incubation at these temperatures is compromised by the failure of H7550str^R to grow under these circumstances. Nonetheless, the behavior of ECIIΔ18R in the 42 ºC mixed culture (FIG.8B) simulates that of ECIIΔ18R grown alone (FIG.7), confirming the mutant's marked drop in CFU/ml during prolonged stationary phase at this temperature. Such a decrease in CFU/ml of ECIIΔ18R was not noted when the organism was co-cultured with H7550str^R at 37 ºC (FIG. 9B), suggesting that the mutant did not experience a marked loss of CFU/ml upon prolonged incubation at this temperature. Detailed survival curves of ECIIΔ18R and H7550 at 37 ºC and 42 ºC are needed to confirm that the deletion mutant has a more pronounced loss of viability upon prolonged incubation at the higher temperature, than its wild-type counterpart.

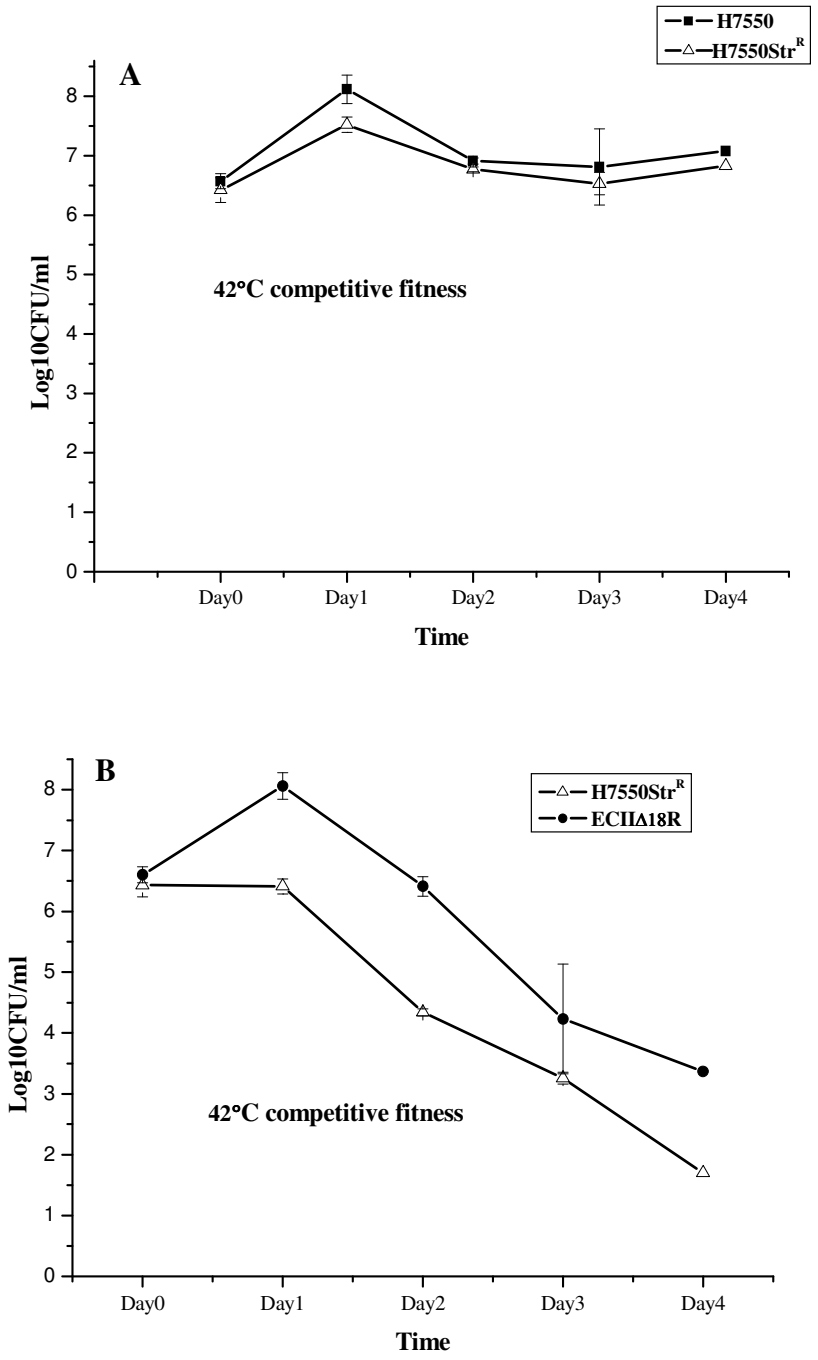

FIG.8: Post-stationary phase survival of ECIIΔ18R and H7550strR during incubation at 42 ºC. H7550str^R and ECIIΔ18R were co-cultured and incubated at 42 ºC (B) for 4 days. Bacteria were enumerated on a daily-basis as described in Materials and Methods. H7550 and H7550str^R were grown in mixed cultures as control (A). The error bars indicate the standard deviations from the means of duplicate experiments

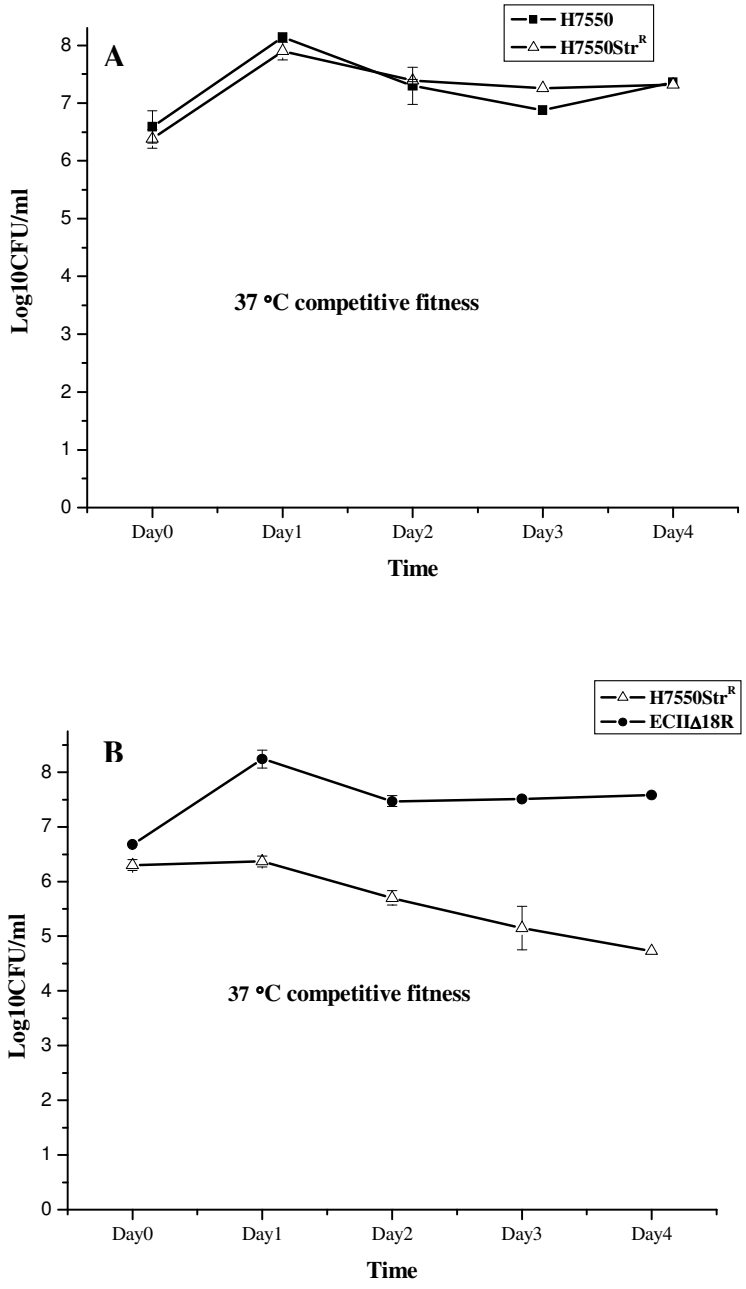

FIG.9: Post-stationary phase survival of ECIIΔ18R and H7550strR during incubation at 37 ºC. H7550str^R and ECIIΔ18R were co-cultured and incubated at 37 ºC (B) for 4 days. Bacteria were enumerated on a daily-basis as described in Materials and Methods. H7550 and H7550str^R were grown in mixed cultures as control (A). The error bars indicate the standard deviations from the means of duplicate experiments

6.5 Discussion

L. monocytogenes serotype 4b strains are responsible for most major outbreaks of foodborne listeriosis in Europe and North America since 1981. To date, two epidemic clonal groups of serotype 4b have been identified, and have been designated ECI and ECII. Genome sequence data and preliminary characterization of epidemic-associated strains have provided important insights into the understanding of the ecology, evolution, and virulence of epidemic-associated strains of *L. monocytogenes*. However, the question of whether indeed there is a molecular basis for the increased pathogenic potential of epidemic *L. monocytogenes* strains remains unanswered. Characterization of ECI and ECII strains by Southern hybridization and genome subtraction identified a number of serotype 4b-specific genomic fragments (10), which opened the way to study the possible association of epidemic prevalence with additional pathogenic traits conferred by epidemic-associated genes. In this study, we focused on a serotype 4b-specific region (region-18) which is either divergent in or absent from the genome of ECII strains. Comparative genomic analysis between serotype 4b strains and EGD-e revealed that this region is adjacent to the internalin A (*inlA*) and internalin B (*inlB*) genes, which are extensively characterized virulence determinants of *L. monocytogenes*.

Sequence analysis of region-18 of serotype-4b strains (F2365 and H7858) in comparison to the corresponding genomic regions of *L. innocua* and *L. welshimeri* showed a conservation of genomic organization upstream of region-18. A large gene encoding a putative wall-associated protein (*wap*) was commonly present in serotype 4b strains, *L. innocua* and *L. welshimeri,* but absent in *L. monocytogenes* strain EGD-e. As expected, the *inlAB* locus was absent in the corresponding region of the non-pathogenic species *L. innocua* and *L.*

welshimeri. Recently, analysis of the whole-genome sequence of *L. welshimeri* revealed that the counterpart of region-18 in *L. welshimeri* was also highly divergent, compared to that in *L. innocua*. In *L. innocua*, ORF lin0454 (encoding a wall-associate protein) was close to ORF lin0457. In *L. welshimeri*, lwe0394 (homolog of lin0454) and lwe0431 (homolog of lin0457) were separated by a large insertion of ~ 48-kb which most likely was acquired by horizontal gene transfer, suggesting that this genomic region is "hot spot" region for horizontal gene transfer and gene rearrangement (9) (FIG.1). In non-pathogenic *Listeria*, genes encoding surface–associated proteins containing LRR (leucine-rich repeat) and LPXTG motifs (i.e. internalin genes) appear to have been selectively lost (9). Thus, most likely, *inlA* and *inlB* have been present in an ancestral *L. monocytogenes* lineage and lost from the non-pathogenic species *L. innocua* and *L. welshimeri*. Taken together, these findings based on comparative genomic analyses have provided important insights into our understanding of the evolution of region-18 in serotype 4b *L. monocytogenes*. Therefore, it is more likely that *wap* flanking region-18 in *L. monocytogenes* of serotype 4b may represent an ancestral sequence that has become eliminated from other *L. monocytogenes* lineages. For instance, *L. monocytogenes* EGD-e may have experienced the loss of the gene encoding wall-associated protein flanking one side of region-18, while maintaining the two virulence genes *inlA* and *inlB*. *L. innocua* and *L. welshimeri* probably evolved from the loss of virulence genes *inlA* and *inlB*, leading to the generation of non-pathogenic species.

The origin of the region-18 genes in serotype 4b strains remains unclear. Previous studies have suggested that region-18 was introduced into serotype 4b by an insertion between *wap* and *inlA*. In agreement with these studies, the lower-than-average G+C content of most ORFs in region-18 in *L. monocytogenes* strains F2365 and H7858, or its counterpart

in *L. welshimeri*, indicates that this region is a hot spot region for horizontal gene transfer. It is not unlikely that most ORFs in this region were acquired from another unidentified source as an intact gene cluster.

A close inspection of region-18 in the genomes of strains F2365 and H7858 revealed an unusual diversification in this region, which is in agreement with results derived from Southern hybridization (5). However, the mechanisms that have driven these genomic differences between ECII and other serotype 4b strains are still not clear. It could be speculated that ECII strains and other 4b strains acquired region-18 was from different sources independently. Alternatively, region-18 in ECII strains was diversified from ancestral genomic sequences, which are still maintained in other serotype 4b strains. This genomic diversification could be driven by a genomic rearrangement facilitated by recombination events. A segment of the C-terminus sequence of *wap* was found repeated in triplicate (LMOh7858_0481, LMOh7858_0484, and LMOh7858_0487) within region-18 in ECII strain H7858, which may suggest that recombination events occurred.

With the exception of the aforementioned genes coding for wall-associated proteins and LMOf2365_0468 (coding for PBS lyase HEAT-like repeat domain protein), no information on the functions of these ORFs in region-18 of either ECII or ECI is available, and no significant homologies were detected in the nucleotide and protein databases. RT-PCR data and mfold prediction suggested that ORFs immediately upstream to *inlA* were not cotranscribed with *inlA*. In order to investigate whether serotype 4b-specific genes and ECII-specific genes within this highly divergent region confer additional pathogenic traits to strains prevalent in epidemics, we constructed in-frame deletion mutants which harbor a deletion of the entire region-18 either in ECI strain F2365 or ECII strain H7550, since most

ORFs in this region are likely acquired by horizontal gene transfer as a cluster. Two mutant strains (F2365Δ18R and ECIIΔ18R) were characterized phenotypically.

No detectable differences were observed between deletion mutant strains and their parental wild type strain in terms of hemolytic ability, phage susceptibility, cell shape, colony size, in either strain F2365 or H7550 background. The findings probably suggest that region-18 is not implicated in cell division and hemolytic activity in these strains. Furthermore, our data indicated that even though region-18 was serotype 4b-specific, the region was not required for serotype-specific glycosylation of wall teichoic acid or for phage infection.

Our preliminary data from PM suggested that region-18 genes in non-ECII serotype 4b strains may be implicated in the utilization of carbon sources such as β-methyl-D-glucoside, D-trehalose, maltose and maltotriose. However these results need to be confirmed by independent growth studies using these carbohydrates as single carbon sources. In contrast, ECII-specific region-18 appeared not to be related to the utilization of carbon, nitrogen, phosphorus and sulfur sources.

ECIIΔ18R appeared consistently more susceptible to prolonged incubation at elevated temperature (42 ºC) than its wild type counterpart, although the underlying mechanisms remain unclear at this point. In order to elucidate whether ECIIΔ18R is susceptible to elevated temperatures, post-stationary phase survival of ECIIΔ18R needs to be further assessed at this and other temperatures, including 37 ºC. Furthermore, currently a complemented strain of ECIIΔ18R has been constructed and will be used to confirm that the deletion of region-18 in strain H7550 is responsible for the observed phenotypes of ECIIΔ18R strain. As we described earlier, several runs of screening for ECIIΔ18R colonies with chloramphenicol-sensitive phenotype were unsuccessful (none of 184 chloramphenicol-

sensitive colonies). Eventually, one deletion mutant colony was obtained among 28 screened chloramphenicol-sensitive colonies. This observation may suggest that the deletion confers some growth defect or fitness disadvantage. Otherwise, bacteria that contain the mutation of interest within their chromosomal sequences generally represent about 50% of the recovered population. Surprisingly, when strain ECIIΔ18R was co-cultured with a streptomycin derivative of the wild type strain (H7550str^R), in overnight mixed cultures, the deletion mutant appeared to outgrow greatly H7550str^R. This finding may suggest that deletion of region-18 confers some advantages on bacterial growth in the mixed-culture. Similar findings were observed in *Ralstonia* sp. TFD14 and *Escherichia coli* K-12 (15, 17). A growth advantage was attributed to a deletion of a 2.4-kb genomic fragment that results in a defect in the quorum sensing circuit, which allows bacteria to grow to unusually high cell density. However, more evidence will be needed to support this conclusion. A detailed growth rate comparison between the deletion mutant strain and the wild type strain in mixed cultures during exponential phase will be necessary to confirm the growth defect of wild type. Comparative fitness analysis of strain F2365Δ18R and its parental wild type will also be helpful to further elucidate whether the stress response phenotypes of ECIIΔ18R are attributed to ECII-specific genes or to serotype-4b specific genes within region-18.

In conclusion, comparative genomic analysis of different *L. monocytogenes* strains and *Listeria* species provided more evidence to support that region-18 might be acquired by horizontal gene transfer. Diversification of region-18 in ECII strains could be attributed to gene rearrangement and duplication driven by recombination events. Region-18 in strain F2365 or in H7550 was not related to expression of serotype-specific surface antigens and teichoic acid (TA) glycosylation, which is responsible for the reactivity with the serotype 4b-

specific monoclonal antibody c74.22. Region-18 in strain F2365 may be involved in the utilization of some carbon sources. On the other hand, region-18 in strain H7550 probably plays a role in response to environmental stress during post-stationary phase (i.e. elevated temperature, starvation). Complementation studies will be necessary to confirm these observed phenotypes of the deletion mutants. On-going virulence tests in murine models will provide more insight into functional roles of this region in ECI and ECII strains. This study will provide a starting point for further characterization of individual genes in this region.

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