7

Genomics and Proteomics Features of *Listeria monocytogenes*

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1. Introduction

Foodborne illness has become the major concern for the public health sector due to the significant rise of the globalization of food trade, leading to the consumption of contaminated foods every day. With the change in the lifestyle, humans are more prone to consumption of minimally processed, contaminated, ready to eat convenience foods. In most of the cases, pathogenic bacterial contamination has played the eminent role in epidemic outbreaks of foodborne illness. *Listeria monocytogenes* has been distinguished as one of the major foodborne pathogens since the early 1980s, causing several outbreaks of foodborne listeriosis with high mortality in immunocompromised hosts. The presence of *L. monocytogenes* as pathogenic bacteria for animals has been reported by Murray et al. (1926), where it was first isolated from rabbits, causing "circling disease". *L. monocytogenes* is a small, Gram-positive, non-sporulating, catalase-positive, facultative anaerobe, flagellated, rod-shaped bacterium and is mainly classified in the Firmicutes division (Donnelly 2001, Donnelly and Diez-Gonzalez 2013). Genus *Listeria* consists of both pathogenic and nonpathogenic species, such as *L. monocytogenes, L. innocua, L. seeligeri, L. welshimeri, L. grayi, L. ivanovii* (subspecies *ivanovi* and subspecies *londoniensis*), *L. marthii* and *L. rocourtiae* (Seeliger 1986, Boerlin 1992, Leclercq 2010, Tham and Danielsson-Tham

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2013). The differentiation between various species of genus *Listeria* is made based on the biochemical reactions, such as beta hemolysis, reduction of nitrates to nitrites, production of acid from mannitol, L-rhamnose, and D-xylose, etc. Out of the twenty species of *Listeria* genus, *L. monocytogenes* is considered as a potential candidate for causing illness in humans. While very few reports of disease outbreaks caused by *L. seeligeri* are available, *L. innocua* and *L. welshimeri* were reported as being incapable of causing illness in humans or animals (Donnelly and Diez-Gonzalez 2013). These nonpathogenic *Listeria* species were used as a species of interest instudying the detailed pathogenesis of *Listeria* infection. Due to the presence of diversified genomic characteristics and intricate surface proteome, *Listeria* is capable of surviving diverse environmental conditions including food and the cytosol of eukaryotic cells. Of the 13 serovars of *L. monocytogenes* (i.e., 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, 4ab and 7), only 3 (i.e., 1/2a, 1/2b, and 4b) are responsible for 98% of human listeriosis outbreaks (Roche et al. 2008). Listeriosis, a life-threatening disease caused by *L. monocytogenes*, favourably affects the host with compromised immune conditions, leading to meningitis, meningoencephalitis, and fetus infection, mostly due to the consumption of contaminated foods. Although fewer cases of listeriosis outbreaks have been reported, it has a higher mortality rate than cases of *Clostridium botulinum* (Huang et al. 2014*)*. A recent investigation of the total genome of *L. monocytogenes* has reported the prediction of a total of 2,853 proteins of which 133 are surface proteins. These surface proteins are classified according to their different anchoring systems with several structural domains (Bierne and Cossart 2007). Additionally, the presence of these exclusive proteomic features made *L. monocytogenes* capable of having a protective cell-mediated immune response in mammalian host cells (phagocytes). The role of different genes and proteins involved in the pathogenesis of *L. monocytogenes*, along with their regulation, is discussed in the following sections. Furthermore, possible techniques for the detection of *L. monocytogenes* in contaminated food are also discussed.

2. Listeriosis

L. monocytogenes is the primary causative organism of human listeriosis, which follows an intracellular mode of infection. The incidence of *L. monocytogenes* as a foodborne pathogen is lower as compared to other notorious pathogens such as *Campylobacter* and *Salmonella*, but the food is the primary root of infection for *Listeria*. The contamination may occur at any stage of food processing and eventually, the pathogen could acquire a sporadic or an epidemic stage. *Listeria* produces enzymes like superoxide dismutase that are responsible for the catalytic dismutation of superoxides produced by phagocytes, which in turn favours the survival of the pathogen in the host system (Vasconcelos and Deneer 1994). The pathogen adapts to a wide range of environmental conditions and is known to affect both healthy and immunodeficient population, although the symptoms and conditions differ. *Listeria* shows an invasive mechanism in affecting dendritic cells and macrophages, the immune cells which provide the primary defence against foreign bodies and are responsible for presenting processed antigens to T-cells in order to generate a secondary response to infection (Drevets and Bronze 2008). The non-invasive pathway causes feverish gastroenteritis (Schlech 1997) which is associated with symptoms like headaches, nausea, fatigue

and diarrhea, to name a few. The non-invasive infection manifests the disease symptoms within hours as the incubation period for the pathogen, in this case, is around 18–20 hours, whereas the invasive infection has an incubation period of 30 days and shows more acute symptoms such as conjunctivitis, meningitis, pneumonia and cardiac lesions (Bundrant et al. 2011).

Upon ingestion of the contaminated food product, the pathogen must first survive through the physiological conditions in the body before finally reaching the incubation period which may vary from days to months, altogether depending on the number of colonies initially taken in with the food. *Listeria,* after entering into the body, sustains itself through the unfavourable conditions of the alimentary canal, e.g., low pH conditions of the stomach and high salinity in the gastrointestinal (GI) tract. Once the pursuit of survival is over, the pathogen colonizes the GI tract and multiplies until it permeates through the intestinal layer barrier and seeps into the circulatory and the lymphatic system, eventually reaching other vital organs where the systemic phase of infection takes over, leading to the diseased condition. The virulence factors responsible for the pathogenesis of *L.monocytogenes* are present on a *prfA*-dependent region, located on the chromosome which comprises of a cluster of genes. The product of *prfA* is an upregulator of the virulence genes present in the gene cluster. The virulence genes *plcA* and *plcB* code for certain phospholipases which function in vacuole escape from macrophages and epithelial cells, whereas the product of *mpl* gene, which is a metalloprotease, ensures the cell to cell spreading of the bacteria by facilitating localization to the host surfaces from the cytosol (Bhunia 2018). Also, listeriolysin O, a hemolysin present in *Listeria*, is an important factor responsible for virulence. Phagosomes are lysed by listeriolysin O and, as a result, *Listeria* is released into the cytoplasm. Hence, the bacteria proliferate causing further infection (Geoffroy et al. 1987). Loss of functioning of any of these factors results in loss of pathogenicity. Other species of *Listeria*, which include *L. ivanovii* and *L. seeligeri*, possess a special virulence factor which helps them utilize the phospholipids that are available in erythrocytes of ruminant animals (Barbuddhe et al. 2008).

3. Routes of Listeriosis

3.1 Gastrointestinalmode of Infection-Survival and Pathogenesis

L. monocytogenes has to overcome many stress conditions within the body of the host in order to ensure that it survives until it elicits its pathogenic properties. For the pathogen to begin colonizing the GI tract before it invades the system, it must first sustain the acidic conditions of the stomach, the variations in osmotic pressure in different regions of the alimentary canal and also the bile salts that are released into the small intestine. The survival of *Listeria* under low pH conditions is to be credited to the glutamate decarboxylase (GAD) system. Uptake of glutamate through antiporters, and its decarboxylation to gamma-aminobutyrate, is catalyzed by three enzymes of *Listeria*'s GAD system (Cotter et al. 2001). This decarboxylation reaction consumes the cytoplasmic protons of the bacteria and, hence, the pH of cytoplasm increases. This mechanism is of paramount importance to the survival of *Listeria* under acidic conditions and works efficiently only when the surrounding environment provides a sufficient amount of glutamate. Hence, glutamate-rich foods are a boon for the survival

and passage of *Listeria* through the stomach. There is an increase in osmolarity of GI tract as the region has high salinity. This increase in osmotic pressure is known to instigate the expression of genes important for colonization and further proliferation of *Listeria*. Membrane transporters like BetL (Sleator et al. 1999) and Gbu (Ko and Smith 1999) that are present in *Listeria* are associated with the uptake of osmolytes, which in turn maintain the water level in the cytoplasm. *Listeria* escapes the pressure of bile salts in the small intestine with the help of an operon which is homologous to the osmoprotectant uptake (Opu) system present in other Gram-positive bacteria (Sleator et al. 1999). The products of this gene play a role in excluding the bile salts from the system.

The exact mechanism of the pathogenic manifestation of *Listeria* in GI tract is not yet known, but the organism tends to effect villi responsible for nutrient uptake and secretions. Persons under medication for gastric acid problems are usually at greater risk. The early symptoms are experienced within 24 hours of incubation, these may include a mild fever along with vomiting and diarrhoea (Bortolussi 2008).

3.2 Listeriosis Linked to Disorders in Pregnancy

Pregnant women have a decreased cell-mediated immune response in order to prevent the rejection of the developing fetus. As a result of this natural immunosuppressive action of the system, the body is left vulnerable to several infections and diseases, listeriosis being one of them. Early symptoms may include mild fever, but prolonged sustenance may lead to severe outcomes such as the child being born with the infection, and there are even cases of stillbirths and spontaneous abortion (Gibbs 2002). The localisation of the bacterium to the placenta is relatively less likely to occur but if even a single bacterium finds its way it would find a suitable environment and begin to proliferate, thereby spreading the infection to the mother's body. Immediate abortion followed by infection is very likely a defence mechanism of the body to ensure the survival of the mother.

3.3 Systemic Listeriosis

Listeria has been known to infect immunocompromised individuals. The individuals who are already in a diseased condition are more susceptible to the infection. In systemic infection, the bacterium attaches to and colonizes the intestine for a short period and then infects other major organs by crossing the intestinal layer and spreading through the blood circulatory system and the lymphatic system. The liver, which is the major drug metabolising organ, receives around 90% of the bacterial population. In the liver, the virulence factor Internalin (InlB) is responsible for the entry of the pathogen into the hepatocytes (Parida et al. 1998). Thus, the hepatic cycle of infection begins, which gives rise to a condition called liver abscess. The systemic mode of infection of *Listeria* also includes organs like the spleen, lymph nodes, and gall bladder which are infected by a comparatively smaller population of the bacterium. *Listeria* continues to spread through the system until it crosses the blood-brain barrier (Lecuit 2005) and infects the brain, which leads to meningitis and encephalitis of the brain stem. Fever, bacteremia and ataxia are symptoms associated with the systemic spread of listeriosis.

4. Mechanism of Pathogenesis

Of the 13 serovars of *L. monocytogenes* (i.e., 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, 4ab and 7), only 3 (i.e., 1/2a, 1/2b, and 4b) are responsible for 98% of human listeriosis outbreaks (Roche et al. 2008). It is an intracellular pathogen that causes gastroenteritis and fever in addition to β-hemolysis. Its pathogenicity is brought about by several virulence factors which are either surface bound or secreted proteins (Table 1) aiding in its entry, persistence, spread and evasion. This is a complex two-stage process, involving an intestinal and systemic phase of infection. In the intestinal phase, it colonizes the intestine and subsequently breaches the epithelial barrier for transportation by the blood or lymph (Bhunia 2018). During the systemic dissemination, it is translocated by dendritic cells and macrophages to the liver, lymph nodes, spleen, brain and placenta (in case of pregnancy). The crucial virulence genes, in its 3 Mb chromosome, include a major virulence gene cluster (*vgc*) in the *Listeria* pathogenicity island 1 (LIPI1; 9 Kb; *prfA-plcA-hly-mpl-actA-plcB*), internalin genes (*inlA* and *inlB*), *hpt*, *bsh* and *bilE*, which are regulated by *prfA* gene, the major regulator of *Listeria* virulence gene expression (Fig. 1). The last five genes along with *gadA* are co-regulated by an alternative sigma factor, Sigma B (*σB*).

Upon consumption, GAD (glutamate decarboxylase) helps in acid resistance of *L. monocytogenes* during the gastric passage, while BilE, BSH and OpuC impart bile salt and osmotic tolerance in the intestinal lumen. Then it is translocated from the intestinal lumen to the basolateral side of the mucosal epithel[illegible] via three possible ways (Fig. 2): (1) through phagocytic M-cells (Microfold ce[illegible]und in the gut-associated lymphoid tissue (GALT) of the Peyer's patches in the small intestine (passive process), (2) through dendritic cells and macrophages (in the lamina propria of intestinal mucosa, are the antigen-presenting cells which either present antigens to T-cells of the immune system, or, help in systemic spread) (passive process), and (3) through enterocytes (intestinal absorptive cells found in the small intestine) (active process through internalin/E-cadherin pathway). In the case of the active processes,

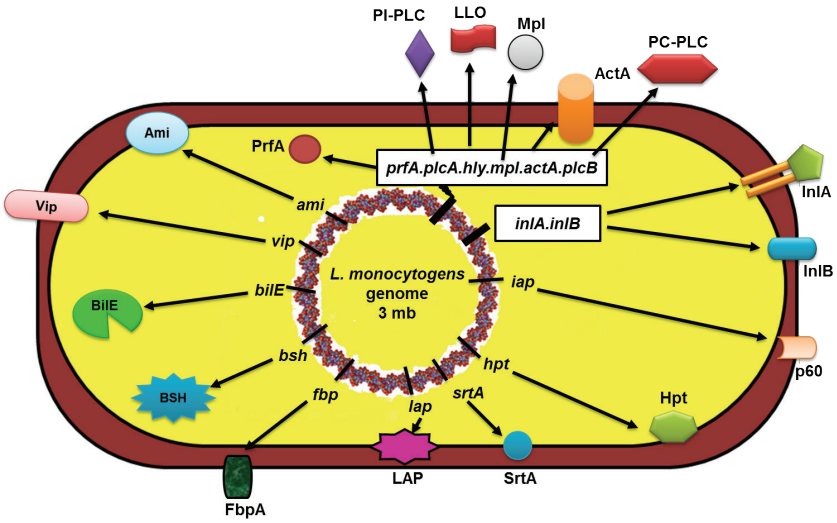

Fig. 1. Arrangement of *L. monocytogenes* virulence genes in its chromosome and the location of their gene products (Adapted and revised from Bhunia 2018).

Table 1. Major virulence factors responsible for *L. monocytogenes* pathogenesis (Bhunia 2018, Cabanes et al. 2011, Bierne and Cossart 2007, Lebreton et al. 2011, Prokop et al. 2017, Roche et al. 2008).

Protein	Name	Gene	Receptor	Function
1. Surface proteins				
Lmo0433	Internalin A (InlA)	*inlA*	E-cadherin	Adhesion and invasion of enterocytes and placenta
Lmo0434	Internalin B (InlB)	*inlB*	Met (tyrosine kinase), gC1q-R/p32	Invasion of hepatocytes and endothelium
Lmo2821	Internalin J (InlJ)	*inlJ*	E-cadherin	Invasion of enterocytes
Lmo0320	Virulence-associated invasion protein (Vip)	*vip*	Gp96 (chaperone protein)	Invasion of enterocytes
Lmo1634	*Listeria* adhesion protein (LAP)	*lap*	Hsp60 (heat shock protein)	Adhesion to enterocytes
Lmo2558	Autolysin amidase (Ami)	*ami*	Peptidoglycans	Adhesion to host cells
Lmo0582	p60 (a cell wall hydrolase, invasion-associated protein)	*iap*	Peptidoglycans	Adhesion and invasion of host cells
Lmo0204	Actin polymerization protein (ActA)	*actA*	Heparan sulphate	Actin tail formation for movement in host cell cytoplasm
Lmo0929	Sortase A (SrtA)	*srtA*	-	InlA, InlJ and Vip anchoring on *Listeria* surface
Lmo1829	Fibronectin-binding protein (FbpA)	*fbpA*	Fibronectin	Adhesion to host cells
Lmo1847	lipoprotein promoting entry (LpeA)	*lpeA*	-	Invasion of host cells and metal transport
Lmo2196	OppA (surface bound protein of ABC transporter system)	*oppA*	-	Low temperature tolerance and oligopeptide transport
Lmo1076	Autolysin (Auto)	*aut*	-	Invasion of host cells
-	Hexose phosphate translocase (Hpt)	*hpt*	-	Multiplication in host cells
2. Secreted and intracellular proteins				
Lmo0200	Protein regulatory factor (PrfA)	*prfA*	-	Central virulence regulator
Lmo0895	Sigma B (σB)	*sigB*	-	Stress transcription factor
Lmo0202	Listeriolysin O (LLO)	*hly*	Cholesterol	Hemolysin aiding lysis of phagosomes
Lmo0203	Zinc metalloprotease (Mpl)	*mpl*	-	Maturation of PC-PLC
Lmo0201	Phosphatidylinositol-specific phospholipase C (PI-PLC)	*plcA*	-	Lysis of phagosome membrane
Lmo0205	Phosphatidylcholine-specific phospholipase C (PC-PLC)	*plcB*	-	Lysis of double membrane vacuole (cell-to-cell spread)
-	Bile exclusion system (BilE)	*bilE*	-	Bile salt tolerance (gut)
Lmo2067	Bile salt hydrolase (BSH)	*bsh*	-	Bile salt tolerance (gut)
Lmo1426	OpuC	*opuC*	-	Osmotic tolerance (gut)
Lmo0206	OrfX	*orfX*	-	Nucleomodulin (interacts with RybP)
Lmo0438	Listeria nuclear targeted protein (LntA)	*lntA*	-	Nucleomodulin (targets the chromatin repressor BAHD1)

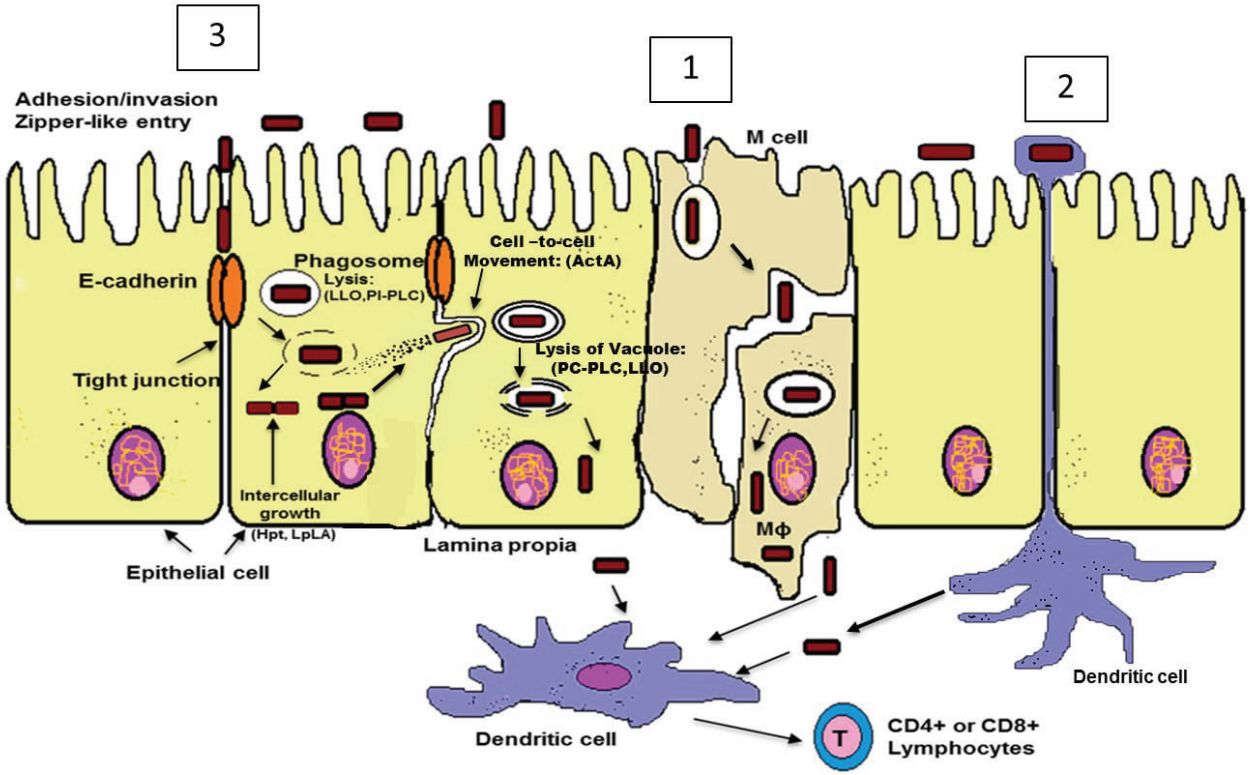

Fig. 2. Three possible translocation pathways of *L. monocytogenes* through the intestinal epithelial lining and its cellular mechanism of pathogenesis (1) through M-cells, (2) by dendritic cells, and (3) active invasion through epithelial cells by E-cadherin signalling pathway (Adapted and revised from Bhunia 2018).

bacteria interact with the receptors on the host cell surface, giving rise to a signalling cascade which facilitates the host cell invasion (Bhunia 2018). The cellular mechanism of *L. monocytogenes* infection cycle encompasses the following four fundamental steps (Fig. 2).

4.1 Attachment and Invasion of Host Cell

Adhesins are specialized surface proteins which recognize specific receptors on the host cell surface, thereby mediating bacterial adhesion. These host receptors are the components of extracellular matrix, like structural proteins (collagen and elastin), specialized proteins (fibrillin, fibronectin, and laminin), proteoglycans and mucins, which play a crucial role in bacterial adhesion to the epithelium (Bierne and Cossart 2007). Several adhesion factors act in unison for the attachment of *L. monocytogenes* bacteria onto the host cells. InlA interacts with host cell receptors, called epithelial cadherin (E-cadherin), and initiates the intestinal and placental infection. InlB interacts with Met/gC1q-R/proteoglycan (GAGs) receptors in order to instigate hepatic and endothelial infection. Other major virulence factors involved are InlJ, Vip, LAP, FbpA, Ami, p60, LpeA and Auto (Bhunia 2018).

4.1.1. InlA. Encoded by *inlA* and a part of the internalin multigene family, it shares the same locus with *inlB*. InlA (88 kDa, 800 amino acids) is a surface protein bound by covalent anchoring to cell wall peptidoglycan (*meso*-diaminopimelic acid residues) through its C-terminal LPXTG (Leu-Pro-X-Thr-Gly) sorting motif-mediated by transpeptidase sortase (SrtA). Presence of N-terminal leucine-rich repeats (LRRs) in InlA is critical for the invasion of intestinal and placental cells subsequent to interaction with the receptor, E-cadherin (Ecad, a glycoprotein, cell adhesion molecule which maintains the integrity of adherens junction of polarized epithelium and stays connected to cytoskeletal proteins). The Pro residue at position 16 in human Ecad is responsible for InlA recognition (Bierne and Cossart 2007). This leads to actin cytoskeleton rearrangement for bacterial entry though the recruitment of β- and α-c in, vezatin and myosin VIII. A full-length and un-mutated InlA is necessary for Lis pathogenicity and crossing of the blood-brain barrier (Bhunia 2018).

4.1.2. InlB. Encoded by *inlB* and part of the internalin multigene family, InlB (65 kDa, 630 amino acids) is a surface protein bound by non-covalent interaction with the lipoteichoic acid of the cell wall through its C-terminal GW module (three repeats of 80 amino acid long clusters, each starting with GW, i.e., Gly-Trp repeats). It also has N-terminal LRRs which help wit entry into hepatocytes and endothelial cells via interaction with the hepatocytes growth factor receptor, Met (a tyrosine kinase) and a coreceptor, gC1q-R/p32 (globular part of complement cascade protein C1q, it interacts with GW module). This leads to actin cytoskeletal rearrangement for bacterial entry through the recruitment of Cb1, Shc, Gab1, which in turn activates PI-3 kinase, Rac (a GTPase) and Arp2/3 (actin nucleator protein) (Bhunia 2018).

4.1.3. InlJ. Encoded by *inlJ* and part of internalin multigene family, InlJ (92 kDa, 851 amino acids) is a surface protein bound by covalent anchoring to cell wall peptidoglycan through its C-terminal LPXTG sorting motif (similar to InlA) mediated by transpeptidase sortase (SrtA). Presence of N-terminal leucine-rich repeats (LRRs)

in InlJ that are similar to InlA is critical for the invasion of enterocytes subsequent to interaction with the receptor, E-cadherin (a glycoprotein) (Cabanes et al. 2011).

4.1.4. Vip. Vip (43 KDa, 399 amino acids) is a surface protein bound by covalent anchoring to cell wall peptidoglycan through its C-terminal LPXTG sorting motif-mediated by transpeptidase sortase (SrtA). Its N-terminal signal sequence helps in the invasion of enterocytes subsequent to interaction with the receptor, endoplasmic reticulum resident chaperone Gp 96 (a 96 kDa chaperone protein belonging to the Hsp 90 family) (Cabanes et al. 2011).

4.1.5. LAP. LAP (104 kDa, 866 amino acids) is a bifunctional membrane-bound enzyme having two major domains, i.e., the N-terminal acetaldehyde, dehydrogenase, and the C-terminal alcohol, dehydrogenase. It is secreted and translocated onto the cell surface for interaction with the Hsp60 receptor on the host cell in order to enhance its affinity towards the epithelium (Bhunia 2018).

4.1.6. FbpA. FbpA (55.3 kDa, 570 amino acids) is a novel multifunctional virulence factor of *L. monocytogenes*. It binds to fibronectin receptor (450 kDa glycoprotein in plasma, extracellular fluid and cell surface of the host), thereby contributing in adherence and colonization of the epithelium. It also behaves as a chaperone protein or escort protein for LLO and InlB, the two key virulence factors (Barbuddhe et al. 2008). It does not possess a characteristic surface-exposed domain. Rather, it is exported via the auxiliary secretory protein (SecA2)—dependent secretion pathway (Bierne and Cossart 2007).

4.1.7. Ami. Ami is a surface-associated protein bound to the lipoteichoicacid of bacterial cell wall via non-covalent interaction with the GW modules present in its C-terminal domain (Barbuddhe et al. 2008). It is an autolytic enzyme also serving as an adhesin. Its size varies between the serovars, in 1/2a it is 917 amino acids long while in 4b it has 770 amino acids. Ami has three domains, the N-terminal alanine amidase, followed by a 30 amino acid long signal sequence, and the C-terminal cell wall anchoring (CWA) domain containing GW modules. This CWA domain helps it in anchoring to the cell wall (here it exerts autolytic action), as well as in adherence to the host cell surface. This CWA domain is highly variable among the two serovars, 1/2a contains eight GW modules while 4b consists of six GW modules (Bhunia 2018).

4.1.8. p60. Autolysin p60 (60 kDa, 484 amino acids) is a major extracellular protein, better known as cell wall hydrolase. It contains LysM (lysine motif) domains responsible for its non-covalent anchoring to the cell wall (here it exerts murein hydrolysis for proper cell division). They are secreted out of the bacteria cell via SecA2-dependent secretion pathway (Barbuddhe et al. 2008). It is an invasion-associated protein that helps with entry into hepatocytes and macrophages.

4.1.9. LpeA. LpeA belongs to a family of surface-exposed proteins called the lipoprotein-associated antigen I (LraI). It is a lipoprotein and a component of the ABC transporter system. It also consists of a metal binding site composed of His-67, His-139, Glu-205 and Asp-280, thus helping in metal transport (Zn/Mn ions). It is necessary for adherence and invasion into epithelium and hepatocytes. LpeA might act

directly through interaction with cell receptor or indirectly being an ABC transporter, thereby influencing the signalling cascade mediated invasion (Bierne and Cossart 2007).

4.1.10. Auto. Auto encoded by *aut* gene is a surface protein having autolytic activity. It is necessary for invasion of host cells by controlling the bacterial surface architecture or by peptidoglycan hydrolysis modulating host immune response (Cabanes et al. 2011). Auto contains an N-terminal autolysin domain and a C-terminal CWA domain comprising of four GW modules (Barbuddhe et al. 2008).

4.2 Lysis of Phagosomes

In the process of invasion into the host cell, *L. monocytogenes* gets trapped inside primary single membrane vacuoles, called the phagosomes, by the process called phagocytosis. Before being digested by the hydrolytic enzymes from lysosomal fusion, *L. monocytogenes* escapes by disrupting the phagosomes with its enzymes, namely listeriolysin O (LLO) and phosphatidylinositol-specific phospholipase C (PI-PLC) (Barbuddhe et al. 2008).

4.2.1. LLO. LLO (60 kDa) is a sulfhydryl (SH)-activated and cholesterol-binding pore-forming hemolysin. Multiple numbers of LLO molecules (monomers) oligomerize in the vacuole membrane and lyse it by pore formation. The maximum lytic activity of LLO is seen at pH 5.5, same as that inside the phagosomes, thereby aiding in its lysis and release of *L. monocytogenes* into host cell cytoplasm. LLO has a PEST-like sequence (Pro-Glu-Ser-Thr), which helps the bacteria to persist in the host cell cytoplasm by acting as the target for degradation. LLO lacking this sequence induces host cell apoptosis and death, thereby hampering bacterial spread. Therefore, LLO plays a key role in both phagosomal lysis as well as cell-to-cell spread during the *L. monocytogenes* infection-cycle. LLO is also known to kill erythrocytes, hepatocytes, dendritic cells and B-lymphocytes by membrane lysis, intracellular release of hydrolytic enzymes and DNA degradation (Bhunia 2018).

4.2.2. PI-PLC. Encoded by *plcA*, PI-PLC (36 kDa) works synergistically with LLO to cause phagosomal lipid-bilayer membrane disruption thereby aiding in the escape of *L. monocytogenes*. Phosphatidylinositol acts as the substrate for PI-PLC, and it has absolutely no activity on phosphatidylcholine (Bhunia 2018).

4.3 Intracellular Growth and Multiplication

After escaping into the cytoplasm, *L. monocytogenes* replicates rapidly inside the host cell by fuelling its organelles using energy from the host cell-mediated via Hpt and lipoate protein ligase (LpLA1) (Bhunia 2018).

4.3.1. Hpt. Hpt helps *L. monocytogenes* exploit hexose phosphates from the host cell as a source of carbon and energy to fuel their growth and multiplication in a host cell. Hpt scavenges host-derived glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate and mannose-6-phosphate from host cell cytoplasm. Hpt is a translocase which, upon entering the host cell, induces a set of virulence factors required for

virulence and intracellular proliferation. Hpt is the first virulence factor specifically involved in the replication phase of pathogenesis (Barbuddhe et al. 2008).

4.3.2. LpLA1. *L. monocytogenes* produces LpLA1 in order to scavenge lipoic acid from the host cell cytoplasm. It ligates this lipoic acid to the E2 subunit of the pyruvate dehydrogenase (acts as a cofactor for the enzyme activity) forming E2-lipoamide, having an important role in the aerobic metabolism of bacteria (Bhunia 2018).

4.4 Cell-to-Cell Systemic Spread

4.4.1. ActA. ActA (90 kDa, 639 amino acids) endows *L. monocytogenes* with an actin-assembly capability in order to steer intracellular bacterial movement (actin-based motility) in the host cell cytoplasm (Barbuddhe et al. 2008). It is a bacterial cell surface protein anchored to the cell membrane bilayer via hydrophobic tail of the protein present at the C-terminal (a carboxy-terminal stretch of hydrophobic residues followed by a few charged residues serving as a stop-transfer signal) (Bierne and Cossart 2007). ActA along with PC-PLC initiates the process of cell-to-cell spread via actin polymerization forming structures called actin-comet tails, which propel *L. monocytogenes* through the cytoplasm towards the cell membrane to infect the adjacent cells. Then, *L. monocytogenes* is enveloped by a filopodia-like protruding structure which is engulfed by the adjacent cell, thereby forming a secondary double-membrane vacuole facilitating cell-to-cell bacterial spread. The outer membrane is from the newly infected cell and the inner membrane is from the previously infected cell. Finally, this secondary vacuole is lysed by LLO and PC-PLC in order to aid in bacterial escape, which in turn initiates a new infection-cycle (Roche et al. 2008). ActA is the first protein identified as actin nucleation-promoting factor. It comprises of 3 domains, the N-terminal domains which interact with Arp2/3 complex thereby initiating actin accumulation. The proline-rich domain located at the centre interacts with a special family of proteins called Enabled (Ena)/ vasodilator-stimulated phosphoprotein (VASP), thereby accelerating directional actin assembly for actin-based motility. The C-terminal domain aids in its anchorage to the *Listeria* cell wall, as already discussed. Detection of actin filaments on *Listeria* surface attracts the microfilament crosslinking proteins (α-actinin, villin and fimbrin) around the bacteria, in addition to the association of other proteins like tropomyosin, talin, vinculin, profilin and plastin (Bhunia 2018).

4.4.2. PC-PLC. PC-PLC (29 kDa) exhibits both lecithinase and sphingomyelinase activity around neutral pH. Phosphatidylcholine acts as the substrate for PC-PLC,and it shows a weak hemolytic activity. The *plcB* gene encodes a 33 kDa proenzyme precursor protein, which then requires a zinc metalloprotease, Mpl (encoded by *mpl* gene of lecithinase operon), for its maturation in order to form the active PC-PLC enzyme (needs zinc as the cofactor) (Bhunia 2018).

5. General Features of the *L. monocytogenes* Genome

L. monocytogenes have a circular chromosome of 3 Mb length and low G+C content (around 38%). The genome sequence encodes approximately 2900 protein-coding genes. There are around 517 polycistronic operons coding for 1719 genes. A large

number of genes encode transport systems, transcriptional regulators, surface-bound proteins and secreted proteins associated with colonization of *L. monocytogenes* on a wide range of niches. 331 genes are reported to encode transport proteins, out of which 39 were supposed to be phosphotransferase sugar-uptake systems (Cabanes et al. 2011), while 209 genes (7% of total genome) encode transcriptional regulators responsible for coordinated gene expression for bacterial adaptation and virulence. Also, 133 surface proteins and 86 secreted proteins are encoded by the *L. monocytogenes* genome. Among the 133 surface proteins, 41 proteins belong to the LPXTG family (Bierne and Cossart 2007). Several studies have reported three distinct phylogenetic lineages of *L. monocytogenes*, based on their ribopatterns and association with outbreaks. Lineage I strains have highest pathogenic potential,and they are involved in most epidemic outbreaks in humans (1/2b, 4b, 3b, 4d and 4e). Lineage II strains show intermediate pathogenicity and are responsible for sporadic outbreaks in humans (1/2a, 1/2c, 3c and 3a). Lineage III strains comprise mostly of animal pathogens (4a and 4c). Of these serovars 1/2a, 1/2b and 4b are responsible for 98% of the disease outbreaks in humans, while 4b is the most virulent (Bhunia 2018).

6. Regulation of Virulence-Associated Genes

Expression of the *L. monocytogenes* genome is orchestrated by a complex regulatory network, where PrfA-σB interplay holds the central role. While PrfA is the major virulence regulator, σBplays a crucial role in the intestinal adaptation of *L. monocytogenes*. PrfA is the main switch of a regulon comprising of several virulence-associated loci scattered all over the bacterial chromosome, including the internalin-multigene family (Barbuddhe et al. 2008). The genes coding for the virulence factors is mainly concentrated in the vgc in the LIPI1. The vgc comprises of three transcriptional units, where the *hly* monocistron occupies the central position coding for a pore-forming hemolysin LLO (vital virulence factor). Upstream from *hly*, there lies the *plcA-prfA* bicistronic operon, where the *prfA* gene is placed immediately downstream of *plcA,*and they are cotranscribed (Roche et al. 2008). *plcA* encodes PI-PLC which acts synergistically with LLO and PC-PLC (encoded by *plcB*is a zinc-metalloprotease) in lysis of the primary and secondary vacuoles in the infection cycle. Downstream from *hly*, there lies the *mpl-actA-plcB* operon. Where, *mpl* (coding for Mpl) is responsible for maturation of PC-PLC, while actA endows *L. monocytogenes* with actin-driven motility inside the host cells (Barbuddhe et al. 2008). PrfA-dependent genes are induced only when *L. monocytogenes* grows in the host cell cytoplasm. Transcription of some virulence genes, such as *inlA* and *inlB*, are co-regulated by the stress-responsive σB. Regulators of two-component systems such as VirR and DegU are also associated with the pathogenesis of *L. monocytogenes*. Moreover, the synthesis of some virulence factors, like PrfA, InlA, ActA, and LLO, is post-transcriptionally regulated (Sabet et al. 2008). Some of the major virulence regulators are described in the subsequent paragraphs.

6.1 PrfA

It is the direct, best characterized and pleiotropic transcriptional regulator of *L. monocytogenes* virulence. It belongs to the Crp/Fnr family of transcriptional activators (Sabet et al. 2008). *prfA* gene contains three separate transcription binding

sites in its promoter region responsible for its expression. First is *prfA1*, which is a 14-bp palindromic sequence (i.e., -TTAACANNTGTTAA-) called PrfA-box. The PrfA protein activates most of the virulence genes by recognizing and binding to the PrfA-box located upstream of their transcription start site. All the *prfA* regulated virulence genes contain the PrfA-box. Second is *prfA2* (i.e., TTGTTACT-N_{14}-GGGTAT), which highly resembles the consensus sequence of σB-dependent promoters. Thus, in addition to its self-regulation, the *prfA* gene is also partially regulated by σB via this *prfA2* (Roche et al. 2008). The two promoters *prfA1* and *prfA2* are present immediately upstream of the *prfA* coding region. While the third promoter, i.e., *prfA3*, is located upstream of the *plcA* gene. Thus, self-regulation of *prfA* is mediated by the activation of *plcA* transcription (Bhunia 2018). Different genes are regulated differently by PrfA regulator, and they are divided into three groups accordingly. Group I consists of 11 positively regulated genes which are preceded by PrfA box. Group II consists of eight genes which are negatively regulated, while Group III is comprised of 53 genes (only two having PrfA-box). Therefore, PrfA can either up-regulate or down-regulate different genes and can directly or indirectly activate different genes by associating with different sigma factors (Cabanes et al. 2011).

AQ: TTAACANNTGTTAA is not strictly palindromic, a palindrome must be the same when read backwards and forwards(as far as I know), e.g. AABBCCCCBBAA. Please review this with the author.

6.2 σ^B

Upon ingestion, *L. monocytogenes* has to first withstand the host's proteolytic enzymes, high acidity, bile stress, and inflammatory immune response in the stomach and intestine. A protein subunit of RNA polymerase (RNAP), i.e., alternate sigma factor σ^B (encoded by *sigB*), is responsible for its survival during passage through the stress environment (Roche et al. 2008). The alternative sigma factor σ^B regulates stress response genes, such as those necessary for growth during oxidative and osmotic stress, low pH stress, low temperature and carbon starvation. It regulates 55 genes, including several virulences and stress response genes like *bsh*, *gadA*, *opuC*, *inlA* and *prfA* (Bhunia 2018). Both σ^B and PrfA are critical for transcription of *inlA* and *inlB*. There are 105 positively regulated genes, while 111 genes are negatively regulated by σ^B. The σ^B operon comprises of σ^B itself, along with seven regulators of σ^B genes (*rsb*) (Barbuddhe et al. 2008). The σB regulon consists of genes coding for solute transporters, novel cell wall proteins, universal stress proteins, transcriptional regulators, and those involved in osmoregulation, carbon metabolism, virulence, motility, niche-specific survival and adaptation, and chemotaxis (Cabanes et al. 2011).

6.3 VirR

A novel virulence regulatory factor VirR (encoded by *virR*) is a response regulator of a two component system. It is a second key virulence regulon after the *prfA* regulon, and it controls the virulence by regulating surface component modifications. These modifications may affect the interaction of *L. monocytogenes* with host cells and components of the immune system. There are 12 VirR-regulated genes like *dltA* and *mprF* (Cabanes et al. 2011).

6.4 DegU

DegU (encoded by *degU*) is a pleiotropic response regulator involved in the expression of virulence genes and motility at low temperature. Two *L. monocytogenes* operons are positively regulated by DegU and the flagella-specific genes, and monocistronically transcribed *flaA* genes are expressed only at low temperature (Cabanes et al. 2011).

7. Detection of *Listeria monocytogenes*

7.1 Enrichment and Enumeration

For the prevention of sporadic outbreaks of listeriosis caused by *L. monocytogenes*, early detection is one of the major concerns for the food processing industries. Detection of *Listeria* contaminants in various food products or the food processing environments is achieved by the application of various standard and rapid microbiological procedures. Among the different detection procedures, the most widely used protocols for the detection of *Listeria* in food products, such as poultry, meat, dairy products, vegetables, fruits, and seafood products, are accomplished by United States Department of Agriculture-Food Safety Inspection Service (USDA-FSIS) and Food and Drug Administration (FDA). In European countries, an extensively accepted method for the detection of *Listeria* in food products is the Netherlands Government Food Inspection Service (NGFIS), developed by Van Netten et al. Additionally, along with the classical conventional microbiological techniques for *Listeria* detection, there are different, widely-applied, rapid techniques available which have been recognized for regulatory screening.

L. monocytogenes can be easily cultured from most of the clinical samples as they can be identified easily in the sterile sites, such as in blood or cerebrospinal fluid. *Listeria* is usually capable of growing on the unselective media such as blood agar plates, following incubation at 35–37°C for 24–48 h. However, in comparison with the clinical samples, detection of *Listeria* in food and faecal samples is more difficult due to the complex matrix and competing for microflora present in the food and faecal samples. Generally, most of the food testing protocol especially for the detection of *Listeria* spp. includes the use of an enrichment broth, where samples are completely mixed with the broth in order to prepare a homogenized solution following with the incubation for 24–48 hours. After incubation with the enrichment broth, a small portion of the sample is mixed with enrichment broth for further incubation, following this, it is plated in a selective agar medium which is specific for *Listeria* spp. The composition of the enrichment broth may vary with the specific strains of *Listeria* species, which consists of a different combination of antimicrobial agents. In specific cases, antimicrobial agents, to which *monocytogenes* are resistant, are used and can provide the detection of *L. monocytogenes* by restricting the growth of other unwanted microflora.

The most commonly-used antimicrobial agents include nalidixic acid, cycloheximide and acriflavin. Specific agar media are used for isolation of *L. monocytogenes* through direct plating methods, although agar media with less selective property have also been utilized successfully. According to the FDA

protocol, buffered *Listeria* enrichment broth (BLEB) is used as an enrichment broth for mixing the food samples followed with the plating onto specific agar, such as Oxford, MOX or PALCAM agar plates. Additionally, the USDA method employs the use of University of Vermont medium (UVM) as an enrichment medium in the initial step, with simultaneous transfer to MOX medium and slightly modified BLEB as the secondary step after 24 hours of incubation. The USDA-FSIS protocol verifies the quality of the hazardous analysis and critical control point (HACCP) systems used by the food processing industries, such as poultry and meat products, by collecting the samples from ready to eat food products for pathogenic microorganisms like *L. monocytogenes*. Although the current protocols rely on highly specific enrichment medium for identification of *L. monocytogenes* in food samples, these media are less sensitive towards the low level of *L. monocytogenes* contamination at the initial stage (Donnelly and Diez-Gonzalez 2013). Additionally, food products consisting of low levels of *L. monocytogenes* in early stages can outgrow in multiplied numbers during storage conditions, leading towards the outbreaks of listeriosis in humans. Hence, enrichment of the contaminated samples alone for the detection of the low level of *L. monocytogenes* contamination is not sufficient. Regarding enumeration, the described procedures in FDA's Bacteriological Analytical manual consist of two different methodologies: (1) direct plating method and (2) most probable number (MPN) technique. The MPN method implies the use of modified BLEB medium in a nine-tube series for the enumeration process with the higher efficacy and sensitivity towards the low level of *L. monocytogenes* contamination detecting 100 CFU/g or less. On the other hand, the direct plating method involves the use of UVM as the primary diluent of the food samples for preparation of a homogenized solution, followed by plating it directly on a selective agar media such as modified Oxford medium (MOX). Additionally, the direct plating method has been considered as less selective and sensitive as compared to MPN method, due to its inferior sensitivity in detecting the actual amount of contaminant (pathogenic bacterial cell) present in food samples. Hence, direct plating methods are possibly considered for those samples containing a high level of *L. monocytogenes* contamination. Additionally, newly developed FDA protocol provides application of MPN filter and DNA probed colony hybridization techniques for enumeration methodologies. The addition of chromogenic substances in agar media can immensely improve the ability to discriminate among *Listeria* spp. Based on the identification of single virulence factor, such as phosphatidylinositol-specific phospholipase C, few commercial chromogenic substances are capable of detecting *L. monocytogenes* in food samples (Donnelly and Diez-Gonzalez 2013).

7.2 Advanced Molecular Techniques

Rapid advancement in the molecular techniques used for the detection and quantification of pathogenic microbial contaminants in food samples has been proved as a promising technology in the aspect of food safety in food processing industries. There are several commercial methodologies available for the detection of *L. monocytogenes.* Polymerase chain reaction (PCR)-based techniques have gained a considerable amount of attention due to their ability to utilize primer-based technology targeting the virulence and non-virulence based factors, such as invasion-associated protein (*iap*), hemolysin (*hly*) and 16S rRNA genes in the case of *Listeria* detection (Tham and Danielsson-Tham 2013). Additionally, real-time PCR or quantitative real-

time PCR (qPCR), which helps to quantify the amount of DNA or RNA amplified during the PCR reaction with the help of some fluorescence molecules, has reduced the time required for acquiring the results of the *Listeria* detection by taking only 2 days, as compared to the conventional technologies which used to take 7 days (Postollec et al. 2011). Detection of *L. monocytogenes* using enzyme-linked immunosorbent assay (ELISA) in food samples such as poultry, processed meat and ready to eat food products has been standardized. A comparison between two different methodologies, such as ELISA (specific for *L. monocytogenes* detection) and ISO 11290-1:1996, for isolation and identification of *L. monocytogenes* contamination in food samples produced an immense concordance index (Portanti et al. 2011).

Conclusions

Understanding of the molecular mechanisms of the pathogenesis is of critical importance for the development of prevention and detection strategies of a pathogen. This chapter attempted to highlight the proteins involved in the pathogenesis of *L. monocytogenes*, along with the genes coding them and their regulation. With the use of advanced techniques like comparative genomics, proteomics, transcriptomics and phenotypic arrays, it will be possible to predict the genes and proteins of different strains of *L. monocytogenes*, which are essential in encountering the stresses in different food environments and pathogenicity.

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