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MINIREVIEW

Molecular Determinants of *Listeria monocytogenes* Pathogenesis

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INTRODUCTION

Listeria monocytogenes is a rapidly growing, gram-positive, food-borne human and animal pathogen responsible for serious infections in immunocompromised individuals and pregnant women (17). The murine model of listeriosis has received enormous attention over the years because of the utility of *L. monocytogenes* as a model pathogen to study cell-mediated immunity. In fact, much of our current understanding of cell-mediated immunity, such as the concept of the activated macrophage, has its roots in the study of murine listeriosis (21, 35, 44). The beauty of the murine model is that it provides a highly reproducible system for the quantitation of *L. monocytogenes* virulence. However, until relatively recently almost nothing was known about the cell biology of intracellular growth or bacterial determinants of pathogenicity.

A number of technical advances have led to the recent renaissance in the study of *L. monocytogenes*. The first was the development of tissue culture models of infection in a variety of primary cells and cell lines (15, 22, 28, 48). The second technical advance was the use of the transposable elements Tn*1545*, Tn*916*, and derivatives of Tn*917* to generate mutants (4, 6, 16, 23, 25, 28a, 48a, 52). Other genetic tools that have been developed include transformation of plasmid DNA (4, 6, 47, 57), the use of vectors which permit complementation (6, 32), allelic exchange and site-specific plasmid integration (25, 39, 42, 57), and lastly, the use of *Bacillus subtilis* (2, 12) and *Listeria innocua* (14) as hosts for the expression of *L. monocytogenes* genes.

By using tissue culture models of infection, the cell biology of *L. monocytogenes* infection has been characterized at the morphological level (7, 15, 43, 53, 54) and is summarized as follows. Subsequent to internalization, bacteria escape from host vacuoles and enter the cytoplasm, where rapid growth ensues. Shortly thereafter, the bacteria appear to mediate the nucleation of host actin filaments which rearrange to form a tail consisting of short actin filaments and actin-binding proteins. By use of video microscopy, the bacteria have been observed moving through the cytoplasm at rates of up to 1.5 μm/s (7), and it is hypothesized that actin polymerization is directly required for the movement, as cytochalasin D causes immediate cessation of movement. Some of the bacteria move to the surface of the cell and are extruded from the cell in pseudopodlike structures. The pseudopods are apparently recognized by the neighboring cell and phagocytosed, whereupon the bacteria have to escape from the resulting double-membrane vacuole in order to enter the cytoplasm once again. This model provides a cell biological explanation for the classic observation that antibody plays little or no role in immunity to *L. monocytogenes*. It is our goal to dissect this system at the molecular level.

In this minireview, we will summarize the current knowledge of *L. monocytogenes* virulence genes and report an agreed-upon nomenclature for these genes.

L. MONOCYTOGENES DETERMINANTS OF PATHOGENESIS

***hly* (previously called *hlyA* and *lisA*).** The *L. monocytogenes* hemolysin, listeriolysin O (LLO), is without a doubt the best-characterized determinant of *L. monocytogenes* pathogenesis. It is a member of a family of sulfhydryl-activated pore-forming cytolysins of which streptolysin O is the prototype (51). The essential role of LLO was documented by the isolation of nonhemolytic transposon mutants which were completely avirulent; i.e., the 50% lethal dose increased approximately 5 logs (6, 16, 23, 48). Absolute proof for its role in pathogenicity was provided by the introduction of the cloned gene on a plasmid into a strain containing a structural gene mutation followed by the restoration of virulence (6). Moreover, a study of isogenic mutants affected in single-amino-acid positions in LLO established a direct correlation between hemolytic activity and virulence (42). The most likely role for LLO is to mediate lysis of bacterium-containing vacuoles, as LLO-negative mutants are usually found residing in host vacuoles and are consequently unable to grow intracellularly (15, 28, 48, 54).

For direct analysis of the role of LLO, the structural gene, *hly*, was cloned into the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible SPAC cassette and transformed into an asporogenic mutant of *B. subtilis*, which then expressed and secreted LLO (2). Following internalization by the J774 macrophagelike cell line, the hemolytic *B. subtilis* lysed the phagosomal membrane and grew rapidly and extensively in the host cell cytoplasm. These results strongly support the hypothesis that the role of LLO is to lyse the host vacuole. In addition, it suggests that the eucaryotic cytoplasm can serve as a growth medium for *B. subtilis*. However, as will be discussed below, there are other *L. monocytogenes* determinants, in addition to LLO, which may contribute to the lysis of the host vacuole both initially and during cell-to-cell spread.

***plcA* (previously also called ORFU and *pic*).** Adjacent to *hly* and transcribed divergently (Fig. 1) is a gene which encodes a phosphatidylinositol-specific phospholipase C (PI-PLC) (3, 31, 38, 52). *plcA* was identified by both DNA sequencing and

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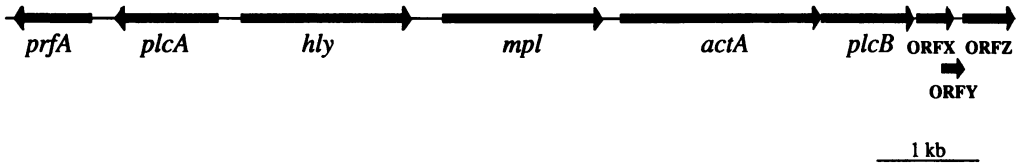

FIG. 1. *L. monocytogenes* listeriolysin gene (*hly*) and the two adjacent operons: the *plcA-prfA* operon and the lecithinase operon. *prfA* encodes a positive regulatory factor, *plcA* encodes a phosphatidylinositol-specific phospholipase C, *mpl* encodes a metalloprotease, *actA* encodes a surface protein necessary for actin assembly, and *plcB* encodes a lecithinase.

subsequent amino acid homology analysis and during a screen for *L. monocytogenes* mutants which formed small plaques in monolayers of mouse fibroblasts (52). Verification of secreted PI-PLC activity encoded by the *plcA* gene was shown by multiple assays, and it was shown that PI-PLC hydrolyzes both PI and PI-glycan. However, complete biochemical characterization awaits purification and further studies.

The *plcA* sequence predicts a protein with approximately 30% amino acid identity to a *Bacillus thuringiensis* and *Bacillus cereus* PI-PLC. Interestingly other gram-positive bacteria such as *Staphylococcus aureus*, *Clostridium novyi*, and *Bacillus anthracis* also secrete PI-PLC activity (34). Some of these enzymes have been used as reagents to identify and characterize eucaryotic membrane proteins anchored to the cell surface by PI-glycan (33), but the role of these enzymes in vivo has not been determined. *plcA* insertion mutants of *L. monocytogenes* are clearly of reduced virulence (3, 38), but the polar effect of these mutations on the downstream regulatory gene *prfA* (39) (see below) makes a definitive assignment of a role for PI-PLC premature. Identification of the biologically relevant substrates for the listerial PI-PLC and its precise role(s) in pathogenesis await the construction and characterization of an in-frame deletion mutation within *plcA*. Nevertheless, it should be noted that only pathogenic species in the genus *Listeria* secrete PI-PLC activity (31, 38, 45).

Lecithinase operon. Downstream from *hly* lies an operon that encodes the *L. monocytogenes* lecithinase (see below). This operon comprises the genes *mpl*, *actA*, and *plcB* and three open reading frames of unknown function, ORFX, -Y, and -Z (Fig. 1) (55).

***plcB* (previously called *prtC*).** *L. monocytogenes* isolates produce one or both of two distinct types of reaction on egg yolk agar, either a faint halo or a very dense zone of opacity surrounding the colony (13). The former reaction may be due to the PI-PLC, as it is absent in *plcA* mutants, while the latter reaction is due to the secretion of a broad-spectrum phospholipase C which hydrolyzes phosphatidylcholine (lecithin), hence its designation as a lecithinase (18, 29). Strains of *L. monocytogenes* which express high amounts of lecithinase activity secrete polypeptides of 29 and 32 kDa, both of which exhibit lecithinase activity on egg yolk overlays of renatured sodium dodecyl sulfate-polyacrylamide gels (24). The sequence of the gene encoding this enzyme, *plcB* (55), predicts a polypeptide of 289 amino acids with sequence similarity to the phosphatidylcholine-phopholipases C of *B. cereus* and *Clostridium perfringens* (alpha-toxin), with a signal sequence of 25 amino acids and by analogy with the *B. cereus* enzyme, a putative propeptide of 26 amino acids. *plcB* mutants have been constructed by interruption of the gene through the use of thermosensitive plasmids (25). These mutants express no lecithinase activity and make small plaques on 3T3 fibroblast monolayers. Electron microscopic analysis of these *plcB* mutants suggest that the lecithinase might be involved in lysis of the double-membrane vacuole which is formed during cell-to-cell spread (55).

***mpl* (previously also called ORFD and *prtA*).** The first gene of the lecithinase operon (55) encodes a protein which contains significant amino acid homology to a family of metalloproteases, of which thermolysin is the prototype (8, 40). A polypeptide corresponding to the mature form of the metalloprotease was detected with antiserum raised against thermolysin, but proteolytic activity has yet to be conclusively demonstrated (8). Mutants with transposon insertions in *mpl* are of reduced virulence and are reduced in lecithinase production (40, 48a). Interestingly, these mutants express only the 32-kDa form of the lecithinase polypeptide, suggesting that the metalloprotease may proteolytically process the lecithinase. Transposon insertions in *mpl* exert a partial polar effect on the expression of *plcB* because transcription in this operon also proceeds from a second promoter located downstream of *mpl* (55).

***actA* (previously called *prtB*).** *actA* is the second gene of the lecithinase operon (55). The nucleotide sequence predicts a 639-amino-acid protein with a signal sequence and a membrane anchor. *actA* mutants do not express lecithinase, do not form plaques in monolayers of mouse fibroblasts, and do not nucleate the polymerization of actin filaments (9, 25). Gene disruption of *plcB* and transformation of the *actA* mutant strain with *actA* on a plasmid have shown that the *actA* gene product is a surface protein necessary for *L. monocytogenes* actin assembly (9, 25). These data also indicate that the mature gene product is a 610-amino-acid protein with an apparent molecular mass of 90 kDa. Whether *actA* encodes an actin nucleator or another function is not yet known.

***prfA*.** A spontaneous nonhemolytic mutant of *L. monocytogenes* was shown to have a deletion in a region downstream of *plcA* (20, 30). These deletions interrupted a gene, *prfA*, encoding a protein of 237 amino acids with no homology to any known protein (32, 39). These mutants expressed barely detectable levels of *hly* mRNA, suggesting that *prfA* was a positive regulatory factor for *hly*. Complementation of the spontaneous deletion mutant with a plasmid carrying *prfA* dramatically increased not only *hly* transcription (32) but also that of *plcA*, *mpl*, and *plcB* (39), demonstrating that *prfA* is an activator of at least four genes. In addition, mutants with transposon and site-specific integration mutations in the *prfA* gene or its promoter region were defective in the expression of the *plcA*, *plcB*, *hly*, and *mpl* gene products (5, 39). *prfA* is the second gene of an operon and can be expressed either from its own promoter located in the *plcA-prfA* intergenic region or from the *plcA* promoter, suggesting that *prfA* regulates its own synthesis (39). Whether the *prfA* gene product acts directly on all of the genes under its control has not been demonstrated, but in *B. subtilis*, the *prfA*-encoded gene product directly activates

the transcription of *hly* (12). In addition, it was hypothesized that PrfA may recognize a 14-bp palindromic sequence found in the −35 region of the promoters for *hly*, *plcA*, and *mpl* (41), suggesting that this palindrome may be the target site for PrfA-mediated activation. Indeed, a single-base-pair change in the 14-bp palindrome upstream of *hly* abolishes recognition by *prfA* in *B. subtilis* (12). Lastly, the *prfA* gene is present in all serovars of the pathogenic species *L. monocytogenes* (56), and expression of *prfA*-regulated genes is thermoregulated (31a).

***inlAB* operon.** *L. monocytogenes* transposon mutants unable to invade cultured epithelial cells resulted from a transposon insertion upstream from a locus named *inl* (14). Introduction of *inlA*, the first gene of the operon, into *L. innocua* confers upon this noninvasive *Listeria* species the ability to invade epithelial cells. The sequence of *inlA* predicts a protein, internalin, of 80 kDa. Two-thirds of internalin is made up of two regions of repeats, and the carboxy terminus is similar to those of surface proteins from gram-positive cocci, such as the *Streptococcus pyogenes* M protein. This was the first sequenced *L. monocytogenes* membrane protein and the first example of a gram-positive rod with the carboxyl-terminal motif LPTXGD thought to play a role in membrane attachment in gram-positive cocci (11). The gene *inlA* was shown to belong to a gene family in *L. monocytogenes*. A second gene homologous to *inlA*, *inlB*, is present immediately downstream from *inlA*, but its function is unknown.

***iap*.** All isolates of *L. monocytogenes* secrete a protein of 60 kDa as a major extracellular product (27). The sequence of the cloned gene predicts a basic protein of 484 amino acids consisting of a 27-amino-acid signal sequence and an extended repeat region consisting of 19 threonine-asparagine units (26). Spontaneous rough mutants of *L. monocytogenes* show reduced expression of p60 and form long chains which possess double septa between the individual cells. Interestingly, rough strains spontaneously revert to smooth isolates and express normal amounts of p60. Rough mutants show a decrease in invasiveness (27) but are relatively normal in intracellular growth and polymerization of actin filaments (52).

***lmaBA* operon.** The product of the *lmaA* gene was demonstrated to encode a 20-kDa protein capable of inducing a specific delayed hypersensitivity reaction in *L. monocytogenes*-immune mice (19). Cell fractionation studies and immunoblotting experiments have located the *lmaA* gene product in the cell wall fraction. Furthermore, the *lmaA* sequence was found to be uniquely present in *L. monocytogenes* isolates. Subsequent nucleotide sequence analysis has demonstrated that the *lmaA* gene is part of an operon and is preceded by the *lmaB* gene, which encodes a 14-kDa polypeptide. The role of the *lmaBA* operon remains to be determined.

DISCUSSION

The cell biology of *L. monocytogenes* infection can be divided into four broad stages: internalization, escape from a vacuole, nucleation of actin filaments, and cell-to-cell spread. Genes involved in each of these steps have now been identified, namely, *inlA*, *hly*, *actA*, and *plcB*, respectively. The next few years should lead to the biochemical characterization of the gene products and a precise assignment of the role of each in pathogenesis.

There are a number of questions which should be answered in the near future. (i) What are the precise roles of LLO and the two distinct phospholipses C in escape from a vacuole and cell-to-cell spread? (ii) Does *actA* encode an actin nucleator, and how many other genes are required to mediate intracellular movement? (iii) How does *L. monocytogenes* regulate vacuolar versus cytoplasmic gene expression, and more specifically, what is the precise role of *prfA* and what other regulatory proteins are involved? (iv) What are the functions of the different *inl* genes?

Lastly, it should be pointed out that the cell biology of *L. monocytogenes* infection is highly reminiscent of the intracellular behavior of *Shigella flexneri* (1, 36, 37, 46, 49) and *Rickettsia tsutsugamushi* (10, 50). However, there is no obvious relatedness between these species. It is interesting to ponder whether this represents an example of convergent or divergent evolution or horizontal transfer.

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