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Advances in *Listeria* Genomics : New Insights into Gene Regulation, Biodiversity and Evolution

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Listeria monocytogenes is the etiologic agent of listeriosis, a severe food-borne disease. Immuno-compromised individuals, elderly people, pregnant women, neonates and occasionally healthy people are at risk. One striking property of *L. monocytogenes* is its capacity to survive and even to multiply in the extreme conditions encountered in the food chain, such as high salt concentrations, extreme pHs and extreme temperatures (20). These characteristics are shared by non-pathogenic *Listeria* species such as *L. innocua* which is generally associated with *L. monocytogenes* in food and the environment.

The clinical features of listeriosis include meningitis, meningoencephalitis, septicemia, abortion, perinatal infections and, as recently shown, gastroenteritis (1, 9). Following the ingestion of contaminated food, *Listeria* disseminates from the intestinal lumen to the central nervous system and the foeto-placental unit. The mechanism underlying the crossing of the intestinal barrier was recently elucidated (19, 23). However, it is still not known how *Listeria* crosses the blood-brain barrier or the foeto-placental barrier. Recovery from infection and protection against secondary infection relies on a rapid and efficient T cell response. This may explain why few individuals develop listeriosis even though the general population is frequently exposed to *L. monocytogenes*. *L. monocytogenes* infection is an extensively used model to study cellular immunity against intracellular parasites (17).

The cell biology of the infectious process has been investigated in detail. *L. monocytogenes* has the capacity to enter and to reside in non-phagocytic cells. After internalization, *L. monocytogenes* escapes from the vacuole and moves intra- and inter-cellularly by a mechanism involving the recruitment and polymerization of cellular actin. Genetic and molecular studies have identified some of the genes involved in these processes processes (7, 26). They include the two invasion genes *inlA* and *inlB* and genes encoding proteins that promote escape from the phagocytic vacuole (LLO, PlcA and PlcB), intracellular actin-based motility and cell-to-cell spread (ActA). Most of these genes are clustered with their common regulator, *prfA*, on a 7.5 kb "virulence locus", which is absent from the non-pathogenic species *L. innocua* (5). A few other genes have also been shown to play roles in virulence, albeit at an unknown level (3, 7, 8, 12, 13, 18, 21, 24, 25). To provide an insight into the specific properties of *L. monocytogenes*, we sequenced the genomes of *L. monocytogenes* and *L. innocua* and compared them. Both encode an exceptionally high number of surface proteins, transporters, secreted proteins and transcription regulators and also contain species-specific genes. The 272 *L. monocytogenes* genes that are not found in *L. innocua* are scattered in

100 islets on the 2.94-megabase chromosome suggesting that the specific attributes of this virulent species result from multiple lateral gene transfer and deletion events.

This genome comparison revealed an important diversity between the two *Listeria* species. However, not all strains of *L. monocytogenes* seem to be equally capable of causing disease in humans. Isolates from four (1/2a; 1/2c; 1/2b; 4b) of the 13 serovars (sv) identified within this species are responsible for over 98% of the human listeriosis cases reported (15). Furthermore, all major food-borne outbreaks of listeriosis, as well as the majority of sporadic cases, have been caused by serovar 4b strains suggesting that strains of this sv may possess unique virulence properties. A number of different typing and population genetic studies suggested that different genetic divisons/lineages exist within the species *L. monocytogenes,* which correlate with serovars (2, 4, 14, 22). Hereafter we will designate sv 1/2a, 1/2c, and 3c strains as lineage I, sv 4b, 1/2b and 3b strains as lineage II, and sv 4a and 4c strains as lineage III. Genetic analyses using multilocus sequence typing of virulence-associated genes, RFLP analysis and ribotyping suggested that epidemic strains are mostly found in lineage II and sporadic strains in lineage I and II, while lineage III strains are extremely rare and mostly animal pathogens (16, 27). However, these methods are unable to further characterize the genetic basis for this observed variability.

In order to address questions regarding epidemiological and evolutionary relationships between pathogenic and non-pathogenic *Listeria* and to define characteristics of particularly successful clonal pathovariants in causing disease, we partially sequenced an epidemic isolate of *L. monocytogenes* sv 4b. We identified an unexpected genetic divergence between the two strains, as about 8% of the sequences were sv 4b specific. These sequences included seven genes coding for surface proteins, two of which belong to the internalin family, or three genes coding for transcriptional regulators, all of which might be important in different steps of the infectious process. Based on the sequence information, we then characterized the gene content of 113 *Listeria* strains with a newly designed *Listeria*-array containing the 'flexible part' of the sequenced *Listeria* genomes. Hybridization results showed that all the previously identified virulence factors of *L. monocytogenes* were present in the 93 *L. monocytogenes* strains tested. However, distinct patterns of presence or absence of other genes were identified among the different *L. monocytogenes* sv and *Listeria* species. These results allow new insights into the evolution of *L. monocytogenes*, suggesting that early divergence of ancestral *L. monocytogenes* 1/2c from 1/2b strains led to two major phylogenetic lineages, one of them including the serogroup 4 strains which branched off the 1/2b ancestral lineage leading, mostly by gene loss, to the species *L. innocua*. The identification of 30 *L. monocytogenes* specific and several sv specific marker genes like three *L. monocytogenes* sv 4b specific surface protein-coding genes, should prove powerful for rapidly tracing listeriosis outbreaks, but also represents a fundamental basis for the functional study of virulence differences between *L. monocytogenes* strains. The correlation of genomic, phylogenetic and epidemiological properties of the strains allowed to identify linage specific marker genes and to propose new evolutionary relationships. The results open new avenues for the development of rapid typing tools as well as for functional analysis of species and serovar-specific genes to understand their role in pathogenicity.

A second approach to identify virulence genes and to better understand pathogenicity of *L.*

monocytogenes was based on the global analysis of the PrfA regulon. The only regulatory factor identified to date, necessary for the regulation of the expression of most of these virulence genes, is PrfA. PrfA activates all genes of the so called virulence gene cluster of *L. monocytogenes* (*prfA, plcA, hly, mpl, actA* and *plcB)* as well as the expression of *inlA* and *inlB*, which encode two invasion proteins (InlA and InlB) (10), *inlC*, which encodes a small secreted internalin-like protein (InlC) (11) and *hpt*, a gene encoding an UhpT-related sugar phosphate transporter that mediates rapid intracellular proliferation (6). PrfA is the major regulator of *Listeria* virulence gene expression. This protein is a member of the Crp/Fnr family of transcription regulators. To gain a deeper understanding of the PrfA regulon, we constructed a whole genome array based on the complete genome sequence of *Listeria monocytogenes* strain EGDe, and evaluated the expression profiles of the wild type EGDe and a *prfA-deleted mutant (EGDe ΔprfA)*. Both strains were grown at 37°C in Brain Heart Infusion broth (BHI) and BHI supplemented with either activated charcoal, a compound known to enhance virulence gene expression, or cellobiose, a sugar reported to downregulate virulence gene expression in spite of full expression of PrfA. We identified three groups of genes that are regulated differently. Group I comprises, in addition to the ten already known genes, two new genes, *lmo2219* and *lmo0788,* both are positively regulated and preceded by a putative PrfA box. Group II comprises eight genes negatively regulated: *lmo0278* is preceded by a putative PrfA box, the remaining seven genes (*lmo0178-lmo0184*) are organised in an operon. Group III comprises 53 genes, of which only two (*lmo0596* and *lmo2067*) are preceded by a putative PrfA box. Charcoal addition induced upregulation of group I genes but abolished regulation by PrfA of most group III genes. In the presence of cellobiose all of the group I genes were downregulated while group III genes remain fully activated. Group II genes were repressed in all conditions tested. A comparison of the expression profiles between a second *L. monocytogenes* strain (P14), its spontaneous mutant expressing a constitutively active PrfA variant (P14*prfA**) and its corresponding *prfA*-deleted mutant (P14Δ*prfA*) and the EGDe strain, revealed interesting strain specific differences. Sequences strongly similar to a sigma B dependent promoter were identified upstream of 22 group III genes. These results suggest that PrfA positively regulates a core set of 12 genes preceded by a PrfA box and probably expressed from a sigma A dependent promoter. In contrast a second set of PrfA regulated genes lack a PrfA box and are expressed from a sigma B dependent promoter. This study reveals that PrfA can act as activator or as repressor and suggests that PrfA may activate directly or indirectly different sets of genes in association with different sigma factors.

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