J Microbiol Immunol Infect.
2007;40:4-13

Review Article

Listeria — review of epidemiology and pathogenesis

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Received: February 20, 2006 Revised: December 22, 2006 Accepted: January 22, 2007

Listeria monocytogenes (commonly called *Listeria*) is a Gram-positive facultatively intracellular foodborne pathogen often found in food and elsewhere in nature. It can cause a rare but serious disease called listeriosis, especially among pregnant women, the elderly or individuals with a weakened immune system. In serious cases, it can lead to brain infection and even death. *Listeria* is more likely to cause death than other bacteria that cause food poisoning. In fact, 20 to 30% of food borne listeriosis infections in high-risk individuals may be fatal. Recent technological developments have increased the ability of scientists to identify the cause of foodborne illnesses. *L. monocytogenes* has been used as a model organism for the study of intracellular parasitism. Whilst the basic mechanisms of cellular pathogenesis have been elucidated by a series of elegant studies, recent research has begun to focus upon the gastrointestinal phase of *L. monocytogenes* infection. Epidemiological studies of outbreaks of human disease now demonstrate that the pathogen can cause gastroenteritis in the absence of invasive disease and associated mortality. Elucidation of whole genome sequences and virulence determinants have greatly contributed to understanding of the organism and its infection pathways.

Key words: Epidemiology; Food contamination; Genomics; *Listeria* infections; *Listeria monocytogenes*

Introduction

Listeria monocytogenes (*Listeria*) is a pathogenic, Gram-positive foodborne bacterium that causes listeriosis. It is frequently overlooked as a possible cause of illness due to its unique growth capabilities. First, it is somewhat difficult for laboratories to grow, and when they do so, *Listeria* can be confused with common harmless contaminants and disregarded. Second, most bacteria grow poorly when temperatures fall below 4°C, while *Listeria* survives in temperatures from below freezing (–7°C) to body temperature and it grows best at –18 to 10°C, a temperature range including that used for refrigeration. As a result, *Listeria* may be transmitted in ready-to-eat foods that have been kept properly refrigerated. Its ability to grow in such diverse environments is just one of the many challenges presented by this dangerous bacterium [1]. *Listeria* infection is relatively uncommon. However, the fatality rate can be as high as 30% among at-risk people. It is estimated that *Listeria* causes approximately 1600 cases of listeriosis annually, resulting in 400 to 500 deaths [2].

L. monocytogenes is a facultative intracellular pathogen that induces its own uptake into non-phagocytic cells and spreads from cell to cell using an actin-based motility process. This review covers both well established aspects and recent advances in the characterization of *L. monocytogenes* virulence determinants and comparative genomics and their role in the pathophysiology of listeriosis.

Background: Occurrence and Clinical Features

There are many opportunities for contamination with *Listeria* during the process of food production, because *L. monocytogenes* is ubiquitous in the environment [1,2]. For example, it can be grown from wild and domestic

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animals, birds, insects, soil and waste water, and vegetation. As it is found in soil and vegetation, *L. monocytogenes* is easily contracted and transmitted by herd animals. The bacterium is often isolated in cattle, sheep, and fowl, and is also found in dairy products, fruits, and vegetables. *Listeria* is found in grazing areas, stale water supplies and poorly prepared animal feed. It can live in the intestines of humans, animals, and birds for long periods of time without causing infection and has been found in up to 5% or more of normal healthy people, usually in the gut. For this reason, exposure to the bacterium is unavoidable. It has the unusual characteristic of being able to grow, albeit slowly, at temperatures as low as 0°C. Several subtypes can be distinguished by laboratory tests. An example is *Listeria innocua*, which is very similar to *L. monocytogenes* but apparently does not cause disease in man.

L. monocytogenes can cause a variety of diseases, including infections in pregnancy, ranging from a mild chill to a severe illness which may precipitate premature birth or miscarriage, and meningitis in newborn children. Septicemia and meningitis occur in adults [1], whose immunity to infection is impaired, such as those suffering from cancer or leukaemia or transplant patients. Infection does occur in otherwise healthy adults and children, although this is extremely rare. Immunocompromised individuals, such as those being treated for cancer, those with organ transplants, and those with acquired immunodeficiency syndrome are at increased risk [3]. The fetus, newborn and pregnant women are particularly susceptible to *L. monocytogenes*. The infection may be treated with antibiotics but in about one-third of cases the disease is fatal [3]. *Listeria* infection is not statutorily notifiable, but microbiology laboratories which grow *L. monocytogenes* from infected patients notify either the Public Health Laboratory System Communicable Disease Surveillance Centre or, in Scotland, for example, the Scottish Centre for Infection and Environmental Health. It is unlikely that many serious cases of diagnosed infection go unreported. The number of cases in England and Wales between 1983 and 1986 ranged from 115 to 149 cases per year. However, in 1987, there was a sharp increase in cases to 259, in 1988 there were 291 cases and in 1989, 250 cases [2]. The numbers dropped to between 102 and 130 cases per year during the years 1990 to 1994. The relatively sharp increase between 1987 and 1989 has been associated with a newly recognized subtype of *L. monocytogenes* which was isolated at the same time from a large number of retail samples of meat pate.

Since May 2000, 29 illnesses caused by a strain of *L. monocytogenes* have been identified in 10 states of the United States: New York (15 cases); Georgia (three); Connecticut, Ohio, and Michigan (two each); and California, Pennsylvania, Tennessee, Utah, and Wisconsin (one each) [3]. When subtyped, the *L. monocytogenes* isolates from these cases were indistinguishable by pulsed-field gel electrophoresis (PulseNet pattern numbers, GX6A16.0014 by Asc1 and GX6A12.0017 by Apa1) and ribotyping (DUP-1053). Eight perinatal and 21 non-perinatal cases were reported. The median age of the 21 non-perinatal patients was 65 years (range, 29-92 years); 13 (62%) were female. The 29 cases have been associated with four deaths and three miscarriages/stillbirths [3].

Epidemiology and Infection Process

Listeria is found widely in nature. In domestic animals, listeriosis is primarily foodborne. Diseases in animals are characterised by septicemia and the formation of multiple visceral abscesses andd meningoencephalitis. Most human infections are also foodborne. *L. monocytogenes* can be isolated transiently in the stool of 1-10% of the population. Cabbage fertilised by manure from sheep and subsequently stored at 4°C was implicated in one outbreak [3]. Other outbreaks have been associated with contaminated cheese and milk. Rarely, contacts with infected cows have caused skin infections in veterinarians.

Anecdotal evidence has long suggested that *L. monocytogenes* can be transmitted by the foodborne route [2]. However, in 1979 the first documented outbreak of foodborne listeriosis occurred in 23 patients in a Boston hospital [2]. Epidemiological evidence strongly suggested an association with consumption of contaminated vegetables prepared within the hospital. A further outbreak due to consumption of contaminated coleslaw occurred in 1981 in the Maritime Provinces, Canada [3]. Epidemiological follow-up of this outbreak showed that the *L. monocytogenes* isolate found in contaminated coleslaw was identical to that isolated from infected patients thus providing, for the first time, definitive proof of foodborne transmission [3].

Subsequent outbreaks of epidemic listeriosis have been reviewed elsewhere [4,5]; however, certain features of these epidemics are worth noting. For instance, the foods implicated in disease outbreaks have varied from vegetable products in the early 1980s to dairy products in the mid-1980s and early 1990s [4]. Recently, contaminated ready-to-eat meat and poultry products

have been associated with disease transmission, including a large multistate outbreak of listeriosis due to consumption of contaminated hot dogs that affected 101 individuals with 21% mortality [5]. In addition, whilst all major outbreaks of invasive disease have been caused by a specific serotype of *L. monocytogenes* (serotype 4b), some studies have indicated that this serotype may be relatively rare within raw foods [6,7].

Recent studies have determined that initial outbreaks of listeriosis were caused by a cluster of highly related strains designated Epidemic Clone I (ECI), whilst the recent hot dog outbreak was caused by a divergent clone (ECII) that is missing genetic information found in other serogroup 4b strains [8-10]. In addition, in an analysis of 300 clinical *L. monocytogenes* isolates, 96% expressed a full-length, presumably functional copy of internalin A, a protein required for invasion of host cells. In contrast, only 65% of food isolates expressed the full-length form of the internalin protein [9]. Other studies have shown that *L. monocytogenes* strains exist in three separate lineages, with lineage I containing epidemic clones, lineage II containing clones associated with sporadic human disease and lineage III containing none of the 20 human isolates tested [10].

An emerging hypothesis from these genetic analyses of *Listeria* isolates suggests that distinct subsets of strains are associated with human disease and that a smaller discrete group of clones are responsible for epidemics of invasive listeriosis. Experimental evidence suggests that serotype 4b strains may have increased virulence for mice when compared with other serotypes [11]. However, despite evidence that serotypes differ significantly at the genome level [12], functional genetic examination of the pathogenesis of epidemic clones is still lacking. Thus, the fundamental basis of the epidemic potential of ECI and ECII strains remains unclear [13].

Another interesting feature of the evolution of epidemic listeriosis is the recent documentation of epidemics in which febrile gastroenteritis rather than invasive disease is the predominant symptom of infection [14]. These epidemics are characterized by their appearance in otherwise healthy people (rather than immunocompromised individuals), causation by serotype 1/2 as well as 4b strains, short incubation periods and the presence of very high levels of *L. monocytogenes* in implicated foods. Whilst evidence of gastrointestinal symptoms associated with listeriosis has long been apparent, it was Dalton et al in 1997 [15] who definitively determined that gastroenteritis may occur in the absence of more serious symptoms following foodborne infection with *L. monocytogenes*. This study established that chocolate milk was the source of infection in otherwise healthy individuals during an outbreak of gastroenteritis in 1994. Furthermore, it was established that the *L. monocytogenes* strain involved was serotype 1/2b in the milk product. It was estimated that the average median dose that individuals consumed was in the region of 2.9×10^{11} colony-forming units of *Listeria* [15].

Another outbreak of *L. monocytogenes* gastroenteritis occurred in Italy in 1997 and was traced to consumption of a contaminated maize and tuna salad. A total of 1566 persons reported symptoms that predominately included headache, abdominal pain and fever; however, there was little evidence of invasive disease and no fatalities ensued. The *L. monocytogenes* isolate in this case was serotype 4b [16]. The outbreaks outlined above provide evidence that *L. monocytogenes* can be present at very high levels in foods and yet cause gastroenteritis rather than severe invasive disease. It is evident that much further research is necessary to determine the bacterial and/or host factors that influence the outcome of foodborne *L. monocytogenes* infection [17].

Listeriosis is clinically defined when the organism is isolated from blood, cerebrospinal fluid, or an otherwise normally sterile site. The manifestations of listeriosis include septicemia, meningitis or cervical infections in pregnant women. Diarrhoea is occasionally an early symptom and may accompany or precede bacteremia and meningitis. Human infection most commonly (60%) involves the central nervous system, where meningitis is usually present. Rarely, encephalitis occurs which is characterised by the presence of multiple abscesses in the brain stem. In the United States, *L. monocytogenes* is the fifth most common form of bacterial meningitis [10]. The other major forms of listeriosis include primary bacteremia, focal infections and perinatal sepsis. Infections in pregnant women may lead to abortion, stillbirths, or premature birth [8]. Transplacental infections result in the dissemination abscesses or granulomas in multiple organs [8]. Perinatal infection (through maternal bacteremia or acquisition during vaginal delivery) may result in bacteremia and neonatal meningitis.

For laboratory diagnosis, the organism grows well on blood or nutrient agar and in conventional blood culture broths. On blood agar, the colonies are usually

surrounded by a narrow band of beta (β)-hemolysis resembling that of β-streptococci. It can be differentiated from β-streptococci by Gram stain and by motility testing at 20-25°C and at 37°C. *L. monocytogenes* ferments glucose, producing principally lactic acid without gas. It elaborates catalase, hydrolyzes esculin, and produces acetoin (Voges-Proskauer test). Instillation into the conjunctival sac of a rabbit produces a purulent conjunctivitis followed by a keratitis (Anton test). On the basis of somatic (O) and flagellar (H) antigens, 17 serotypes have been described. Serotypes 1a, 1b, and 4b account for more than 90% of clinical isolates. Serological and phage typing can be helpful in the investigation of common source outbreaks. The methods for analysis of food are complex and time-consuming. The present United States Federal Drug Administration (FDA) method requires 24 and 48 h of enrichment, followed by a variety of other tests [16]. Total time for identification is 5 to 7 days. Ampicillin or penicillin is the drug of choice. A synergistic combination of a β-lactam with gentamicin is sometimes used in immunocompromised or neutropenic patients.

Listeria Food Contamination

Although infection occurs via contact with animals and neonatal spread occurs, the consumption of contaminated food is believed to be the principal route of infection. A wide range of food types has been implicated in transmission, including meat, dairy, fish, shellfish and vegetable products [16]. The laboratory methods used to detect *L. monocytogenes* in food have improved in recent years and the organism has been found, but usually in small numbers, in many foods including raw fish, shellfish and fish products; raw meat, poultry and their products, including hot dogs and pate; raw and processed vegetables; ripened soft cheeses; ice cream; retail cook-chill meals; salads including coleslaw; raw and inadequately pasteurized milk as well as raw and liquid egg.

In response to the problem, the food manufacturing industry has implemented hazard analysis and codes of practice, and indeed during the 1990s the number of foods contaminated with *L. monocytogenes* types was reduced. Although the organism is killed by proper cooking and pasteurization, it has been found occasionally in some cheeses made from pasteurized as well as unpasteurised cow's and goat's milk and the presence of the organism in cooked foods indicated inadequate cooking or contamination after cooking. This is clearly undesirable and the food industry and trade are reported to be taking steps to eliminate it.

Retail chilled meals carry instructions to reheat before consumption and if this is done correctly *L. monocytogenes* should be killed. Similarly, the organism should be eliminated from food taken from cook-chill catering units when the food is properly reheated. Conventional ovens should cause no problems, but microwave cookers do not always reheat food uniformly and bacteria may survive in cold spots.

Listeriosis has been recognized as an important public health problem in the United States, even to the present date. Scientific information from the FDA's risk assessment outlines measures that industry, retailers and consumers can take to dramatically reduce the risk of infection [2]. On the basis of the number of reported cases in England and Wales, the incidence of listeriosis in pregnancy is estimated to be approximately 1 in 30,000 live and stillbirths. Because of the risks of listeriosis, it is recommended that pregnant women should avoid eating certain ripened soft cheeses such as Brie, Camembert and the blue veined types such as Danish Blue, Stilton or Gorgonzola as these may contain high levels of the *Listeria* organism. However, hard cheeses such as the Cheddar and Cheshire types, soft fresh cheeses such as cottage cheese, cream cheese and Fromage Frais as well as processed cheese products in sealed packages (such as cheese spreads) have not given cause for concern when eaten fresh. In addition, pregnant women should avoid eating any type of meat-based pate as these may also contain high levels of the *Listeria* organism.

Although the number of organisms found in other foods, such as cooked chilled meals and ready-to-eat poultry is usually low, these foods should be reheated thoroughly until they are 'piping hot', rather than eaten cold by pregnant women. Pregnant women should not help with lambing, milk ewes that have recently given birth or touch the after-birth or come into contact with newborn lambs, as these are also a potential source of *Listeria*. People with deficient immunity are advised to take the same precautions as pregnant women, but the risk of infection in healthy people over the age of four weeks is regarded as too low to warrant any dietary changes. Keeping ready-to-eat foods cold is the key to reducing listeriosis [2].

The FDA and the Centers for Disease Control and Prevention (CDC) advise that the most important things consumers can do to reduce the risk of illness are: store ready-to-eat foods at 4°C or lower — use a refrigerator

thermometer to check the temperature; use perishable and ready-to-eat foods as soon as possible; and clean the refrigerator regularly [5]. The CDC's FoodNet program has recorded a decrease of over 40% in its incidence during the past five years. The results of the risk assessment reinforce past studies that found that, even though foodborne listeriosis is rare and declining, it remains a public health concern. The CDC estimates that in the United States, 3500 people become seriously ill with listeriosis each year, and of these 500 to 600 die.

Pathogenesis and Epidemiology

Immunocompromised individuals are particularly vulnerable to this intracellular pathogen. About 70% of patients with *Listeria* infection have underlying immunosuppression [18]. Other groups of individuals at increased risk include those on drugs which reduce gastric acidity, patients with cirrhosis, hemochromatosis, and chromic renal failure patients requiring frequent transfusions.

Clinical manifestations of invasive listeriosis are usually severe and include abortion, sepsis, and meningoencephalitis. Listeriosis can also manifest as a febrile gastroenteritis syndrome. In addition to humans, *L. monocytogenes* affects many vertebrate species, including birds. *Listeria ivanovii,* a second pathogenic species of the genus, is specific for ruminants. The current view of the pathophysiology of listeriosis was derived largely from studies with the mouse infection model. Pathogenic *Listeria* enters the host primarily through the intestine. The liver is thought to be the first target organ after intestinal translocation. In the liver, *Listeria* actively multiply until the infection is controlled by a cell-mediated immune response [17]. This initial, subclinical step is thought to be common due to the frequent presence of *L. monocytogenes* in food. In normal individuals, the continual exposure to listerial antigens probably contributes to the maintenance of anti-*Listeria* memory T cells. However, in debilitated and immunocompromised patients, the unrestricted proliferation of *Listeria* in the liver may result in prolonged low-level bacteremia, leading to invasion of the preferred secondary target organs (the brain and the gravid uterus) and to overt clinical disease [18]. *L. monocytogenes* and *L. ivanovii* are facultative intracellular parasites able to survive in macrophages and to invade a variety of normally non-phagocytic cells, such as epithelial cells, hepatocytes, and endothelial cells. In all of these cell types, pathogenic *Listeria* go through an intracellular life cycle involving early escape from the phagocytic vacuole, rapid intracytoplasmic multiplication, bacterially induced actin-based motility, and direct spread to neighboring cells, in which they re-initiate the cycle. In this way, *Listeria* disseminate in host tissues sheltered from the humoral arm of the immune system. Over the last 15 years, a number of virulence factors involved in key steps of this intracellular life cycle have been identified [19].

A number of *L. monocytogenes* strains of different origins were evaluated for in vitro invasion capacity for various human cell types (monocytic THP-1, enterocytic Caco-2, and hepatocytic HepG2 cells) and for expression levels of specific virulence genes. For THP-1 cells, no differences between clinical and non-clinical *L. monocytogenes* strains in invasion capacity or in production of the proinflammatory cytokine interleukin-8 (IL-8) were observed, whereas for the Caco-2 and HepG2 cells, significant differences in invasion capacity were noticed [18]. On average, the clinical strains showed a significantly lower invasion capacity than the non-clinical *L. monocytogenes* strains. Furthermore, it was shown that the clinical strains induce lower IL-8 levels in HepG2 cells than do the non-clinical strains. This observation led to study of mRNA expression levels of internalin genes *inlA*, *inlB*, and *ami*, important virulence genes mediating adhesion and invasion of eukaryotic cells, by real-time reverse transcription-polymerase chain reaction (PCR) for 27 clinical and 37 non-clinical *L. monocytogenes* strains [10]. Significant differences in *inlA* and *inlB* expression were observed, with clinical strains showing a lower expression level than non-clinical strains. These observations were in accordance with in vitro invasion of Caco-2 and HepG2 cells, respectively. Differential expression levels of *inlA* and *inlB* possibly play a role in the virulence capacities of *L. monocytogenes* strains. The lower capacity of clinical strains to invade HepG2 cells and to induce IL-8 is possibly a mechanism of immune evasion used by specific *L. monocytogenes* strains [4].

The secreted cholesterol-dependent cytolysin listeriolysin O (LLO) mediates phagosomal escape and allows bacterial growth in the cytosol of infected cells. In order to identify new LLO determinants participating in bacterial pathogenesis, research was focused on a major target of LLO proteolytic cleavage in vitro, the cytotoxic T lymphocyte epitope region (residues 91-99). Mutations were generated by site-directed mutagenesis in the epitope or in the two clusters of positive charges

flanking the epitope [20]. Two LLO mutants (a single mutation K103A and a double mutation R89G, K90G) were normally and stably secreted by *L. monocytogenes*. In contrast, a mutant carrying four amino acid substitutions in the epitope itself (Y92K, D94A, E97K, Y98F) was highly susceptible to proteolytic degradation. While these three LLO mutant proteins showed reduced hemolytic activity, they all promoted efficient phagosomal escape and intracellular multiplication in different cell types, and were non-cytotoxic.

The deletion of the epitope (delta [Δ]91-99), as well as the substitution of two, three or four of the four lysine residues (K103 to K106) by alanine residues did not lead to the production of a detectable protein. These results confirm the lack of correlation between hemolytic activity and phagosomal membrane disruption. These reports revealed the importance of the 91-99 regions in the production of a stable and functional LLO. Fifty-percent lethal dose (LD_{50}) determinations in the mouse model suggested a possible link between LLO stability and virulence [5].

The cell wall envelopes of Gram-positive bacteria represent a surface organelle that not only functions as a cytoskeletal element but also promotes interactions between bacteria and their environment [21]. Cell wall peptidoglycan is covalently and non-covalently linked with teichoic acids, polysaccharides and proteins. The sum of these molecular properties provides bacterial envelopes with species- and strain-specific properties that are ultimately responsible for bacterial virulence, interactions with host immune systems, and the development of disease symptoms or successful outcomes of infections. Surface proteins typically carry two topogenic sequences, N-terminal signal peptides and C-terminal sorting signals. Sortases catalyze a transpeptidation reaction by first cleaving a surface protein substrate at the cell wall sorting signal. The resulting acyl enzyme intermediates between sortases and their substrates are then resolved by the nucleophilic attack of amino groups, typically provided by the cell wall cross bridges of peptidoglycan precursors [6]. The surface protein linked to peptidoglycan is then incorporated into the envelope and displayed on the microbial surface.

Bovine lactoferrin (BLf) and its derivative peptide lactoferricin B (LfcinB) are known for their antimicrobial activity towards several pathogens, including *L. monocytogenes* [7]. To add further information on the antibacterial effects of these compounds, the influence of BLf, LfcinB and the antimicrobial centre of LfcinB, the hexapeptide $LfcinB_{4–9}$, on the invasive behaviour of *L. monocytogenes* was analyzed in interferon-gamma-activated human macrophagic cells (THP-1). Significant inhibition of bacterial entry in THP-1 cells was observed at LfcinB concentrations that were unable to produce any bacteriostatic or bactericidal effect, compared with BLf and $LfcinB_{4–9}$ peptide. This inhibition occurred when LfcinB was incubated during the bacterial infection step and was not only due to competition for common glycosaminoglycan receptors. Assays performed through a temperature shift from 4 to 37°C showed that inhibition of invasion took place at an early post-adsorption step, although an effect on a different step of intracellular infection could not be ruled out.

French scientists have described how *L. monocytogenes* commandeers cellular transport machinery to invade cells and elude the immune system. The research was conducted by Pascale Cossart, a Howard Hughes Medical Institute international research scholar, and her colleague Esteban Veiga at the Institut Pasteur in Paris. According to Veiga and Cossart, when *InlB* interacts with the hepatic growth factor receptor Met, the cell responds by adding a chemical tag to Met that flags it for protein recycling or degradation [22]. *Listeria* is transported into the cell along with transport tagged Met, by endocytosis. By manipulating the gene expression of the cells *Listeria* was invading, Veiga and Cossart showed that specific molecules known to be involved in endocytosis were essential for successful invasion by *Listeria* [22]. Similarly, they found that an enzyme that tags proteins for recycling was also required [22].

Genetic Studies

The genome sequence (2.94 megabases) of strain EGD-e of *L. monocytogenes* has been completed by a consortium of 10 laboratories coordinated by Pascale Cossart. The completed genome sequences of other bacteria of the genus *Listeria* have been reported: the complete sequence of *L. innocua* (a non-pathogenic bacterium closely related to *L. monocytogenes*), a partial sequence *of L. monocytogenes* 4b (an epidemic strain), and — in collaboration with the German consortium "Pathogenomics" — the nearly complete sequence of *L. ivanovii* (an animal pathogen). Specific features of *L. monocytogenes* EGD-e, deduced from the analysis of its genome, have also been described [8]. The organism contains a large number of proteins of the following families: surface proteins containing an

LPXTG motif, internalins, sugar transport systems, proteins showing similarities to *Bacillus subtilis* competence proteins, and regulators of the Crp/Fnr family. Comparison of these genome sequences with the help of the software "Find Target" is used to identify potential virulence genes, and more generally, to understand the pathogenicity of *L. monocytogenes* and its ability to contaminate food. Furthermore, characterization of clinical and environmental isolates of *Listeria* by use of a comparative genomics approach based on DNA arrays is in progress.

For the characterization of the biodiversity of the genus *Listeria*, and in particular that of the species *L. monocytogenes*, a comparative genomics approach with macroarrays has been found to show remarkable achievements in gene expression studies [9]. Specific genes for *L. innocua*, for the strains EGD-e and 4b (CLIP80459) of *L. monocytogenes* have been identified. In collaboration with the Laboratoire de Référence des *Listeria* (Institut Pasteur), PCR fragments corresponding to these specific genes have been deposited on high density membranes and hybridized with total DNA from other isolates of *Listeria*: two additional strains of *L. innocua*; two strains of *L. ivanovii*; two strains of *Listeria seeligeri*, *Listeria welshimeri* and *Listeria grayi*; as well as eighty strains of *L. monocytogenes*, isolated from patients, the food chain or the environment. It was possible to find a correlation between epidemiology and genomic data, in order to distinguish between potentially virulent and non-virulent strains.

The two *L. monocytogenes* strains EGD and National Collection of Type Cultures (NCTC) 7973 display different regulation patterns of their PrfA-dependent genes. All PrfA-dependent genes from *L. monocytogenes* NCTC 7973 are much more efficiently transcribed in brain-heart infusion medium than those from strain EGD. Transcription of these genes in EGD is, however, induced after shift into minimal essential medium (MEM) to a level that is comparable to that of strain NCTC 7973. Expression of the *inlA* is also influenced by PrfA, but only one (P2) out of three mapped promoters is strictly dependent on PrfA [23].

In contrast to the other PrfA-regulated genes, transcription of *inlA* (even from the P2 promoter) is reduced in both strains after shift into MEM. The prfA deletion mutant SLCC 53 complemented with multiple copies of prfA synthesizes large amounts of monocistronic prfA transcript, but there is no concomitant increase in the transcripts of the PrfA-dependent genes. However, upon a shift into MEM, transcription of the PrfA-dependent genes (with the exception of the *inlA* gene) is highly induced even in the absence of de novo protein synthesis. The PrfA proteins of the two studied *L. monocytogenes* strains differ in their ability to activate PrfA-dependent genes. This difference is probably the result of amino acid exchange(s) in the C-terminal part of these proteins. Strain EGD supplemented with multiple copies of prfA-7973 shows a similar regulation of the PrfA-dependent genes as strain NCTC 7973, whereas multiple copies of prfA-EGD introduced into strain EGD barely change the rate of transcription of the PrfA-dependent genes [10].

Expression of the *iap* gene of *L. monocytogenes* in the *L. monocytogenes* rough mutant RIII and in *B. subtilis* DB104 caused the disruption of the cell chains which these two strains normally form under exponential growth conditions. The p60 protein produced by *L. monocytogenes* and *B. subtilis* DB104 also exhibited bacteriolytic activity detected in denaturing polyacrylamide gels containing heat-killed *Micrococcus lysodeikticus*. Purification of the p60 protein led to aggregation of p60 and loss of the cell chain disruption and bacteriolytic activities. A cysteine residue in the C-terminal part of p60 which is conserved in all p60-like proteins from the other *Listeria* species seems to be essential for both activities [11]. The *iap* gene could not be inactivated without a loss of cell viability, indicating that p60 is an essential housekeeping protein for *L. monocytogenes* and probably also for other *Listeria* species. A new tool may be at hand for subtyping strains of *L. monocytogenes* which cause foodborne illness. Subtyping determines the strain affiliation of *Listeria* specimens isolated in the laboratory. This is a critical tool for tracing outbreaks to their source, as well as to government and industry efforts to safeguard food supplies through environmental monitoring, disinfection, sanitation and other measures.

Eight spontaneously occurring rough mutants of *L. monocytogenes* were investigated for their ability to express two previously reported autolysins, p60 and MurA [24]. All mutants lack of MurA expression and showed strongly reduced levels of extracellular p60. One rough strain harbors a variant of the p60 protein with a partially truncated catalytic domain. In seven cases there were shifts in the localization of p60 to the membrane fraction. Mutations within the *secA2* gene, encoding an auxiliary protein secretion system paralog, were previously shown to be involved in the smooth-rough phenotypic variation seen with *Listeria* strains. An isogenic Δ*secA2* EGD-e deletion strain displayed a

strong pleiotropic reduction of p60 and MurA, in addition to a large number of secreted and surface proteins [25]. However, no apparent SecA2 dysfunction was observed in several of the investigated strains as determined by direct sequencing of the *secA2* gene and complementation of the ΔsecA2 mutant with the respective allele cloned from the rough mutant. To determine the gene products required for the smooth-rough transition, researchers created mutants lacking the individual *iap* and *murA* genes as well as a Δiap ΔmurA double mutant. The double mutant displayed a rough phenotype and exhibited many of the properties seen with the ΔsecA2 mutant and the results implicated p60 and MurA as important determinants in controlling the cell shape of *L. monocytogenes* [12].

The luciferase-based vector pPL2*lux* is a derivative of the listerial integration vector pPL2 and harbors a synthetic *luxABCDE* operon encoding a fatty acid reductase complex (LuxCDE) involved in synthesis of the fatty aldehyde substrate for the bioluminescence reaction catalyzed by the LuxAB luciferase. A pPL2*lux* derivative was constructed in which the *secA* and *hlyA* promoters were translationally fused to *luxABCDE* and integrated as a single copy into the chromosome of *L. monocytogenes* EGD-e. Growth experiments revealed that *hlyA* was expressed predominantly in the stationary phase in Luria Bertani medium buffered at pH 7.4, whereas *secA* expression could be detected in the exponential growth phase [15]. Moreover, the correlation between luciferase activity and transcription levels, as determined by reverse transcriptase PCR, was confirmed using conditions known to lead to repression and activation of hemolysin expression (addition of cellobiose and activated charcoal, respectively). Furthermore, hemolysin expression could be monitored in real time during invasion of an intact monolayer of C2Bbe1 (Caco-2-derived) cells. Finally, hemolysin expression could be detected in the livers, spleens and kidneys of mice 3 days post-infection. These experiments clearly established the effectiveness of pPL2*lux* as a quantitative reporter system for real-time, noninvasive evaluation of gene expression in *L. monocytogenes* [15].

A successful transition of *L. monocytogenes* from the extracellular to the intracellular environment requires a precise adaptation response to conditions encountered in the host milieu. Although many of the key intracellular steps in the pathogenesis are well characterized, knowledge about the factors required for cytosolic proliferation is still rather limited. DNA microarray and real-time reverse transcriptase PCR analyses were used to investigate the transcriptional profile of intracellular *L. monocytogenes* following epithelial cell infection. Approximately 19% of the genes were differentially expressed by at least 1.6-fold relative to their level of transcription when grown in brain heart infusion medium, including genes encoding transporter proteins essential for the uptake of carbon and nitrogen sources, factors involved in anabolic pathways, stress proteins, transcriptional regulators, and proteins of unknown function [21].

In order to validate the biological relevance of the intracellular gene expression profile, a random mutant library of *L. monocytogenes* was constructed by insertion-duplication mutagenesis and screened for intracellular-growth-deficient strains [21]. By interfacing the results of both approaches, evidence was provided that *L. monocytogenes* can use alternative carbon sources such as phosphorylated glucose and glycerol and nitrogen sources such as ethanolamine during replication in epithelial cells and that the pentose phosphate cycle, not glycolysis, is the predominant pathway of sugar metabolism in the host environment.

L. monocytogenes is a model organism for the study of intracellular bacterial pathogens. The publication of its genome sequence and that of the non-pathogenic species *L. innocua* initiated numerous comparative studies and efforts to sequence all species comprising the genus. The Proteome Database LEGER (http://leger2.gbf.de/cgi-bin/expLeger.pl) was developed to support functional genome analyses by combining information obtained by applying bioinformatics methods and from public databases to improve the original annotations. LEGER offers three unique key features: 1) it is the first comprehensive information system focusing on the functional assignment of genes and proteins; 2) integrated visualization tools, Kyoto Encyclopedia of Genes and Genomes pathway and Genome Viewer, alleviate the functional exploration of complex data; and 3) LEGER presents results of systematic post-genome studies, thus facilitating analyses combining computational and experimental results [16]. Moreover, LEGER provides a membrane proteome analysis of *L. innocua* and visualizes total experimentally-validated information about the subcellular localizations of 789 different listerial proteins.

In recent reports, researchers used fluorescently labeled probes to document the various interactions between the bacteria and intestinal cells [17,20]. They found that the microbe can invade cells only at the sites where the epithelium is ejecting dead cells — a constant

process in this layer of the intestine. The living epithelial cells briefly loosen their connection with neighboring cells so they can cast out dead ones. This transient breach of the epithelial barrier exposes a protein that *Listeria* attaches to, thus being transported to the other side.

Conclusion

Raw or contaminated food is the most common mode of *L. monocytogenes* infection in humans; soft cheeses, delicatessen meats and raw and smoked fish are the usual culprits. The bacteria that survive the acidity of the stomach may enter the small intestine, crossing the epithelial barrier and then spreading to the liver, spleen, central nervous system and, in pregnant women, the fetus. According to the CDC, about 2500 cases of listeriosis are reported in the United States annually, with about 500 deaths. The ability of *Listeria* to cause severe, invasive disease depends on its ability to cross the intestinal epithelium. By learning more about how it does this, researchers may devise better ways to protect against *Listeria*. Currently, the only preventive measures are thorough cooking and cleaning of food and pasteurizing of dairy products. Antibiotics are the primary treatment for listeriosis. Future research may contribute to eventual eradication of clinical *Listeria* infection.

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