Listeria Pathogenesis and Molecular Virulence Determinants

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INTRODUCTION

The genus *Listeria* consists of a group of gram-positive bacteria of low G+C content closely related to *Bacillus, Clostridium, Enterococcus, Streptococcus*, and *Staphylococcus. Listeria* spp. are facultative anaerobic rods of 0.4 by 1 to 1.5 μm that do not form spores, have no capsule, and are motile at 10 to 25°C (98, 552, 579). *Listeria* spp. are isolated from a diversity of environmental sources, including soil, water, effluents, a large variety of foods, and the feces of humans and animals. The natural habitat of these bacteria is thought to be decomposing plant matter, in which they live as saprophytes. Domesticated ruminants probably play a key role in the maintenance of *Listeria* spp. in the rural environment via a continuous fecal-oral enrichment cycle (178, 318, 420, 559, 682, 687, 692). The genus *Listeria* currently includes six species: *L. monocytogenes, L. ivanovii, L. seeligeri, L. innocua, L. welshimeri*, and *L. grayi*. Two of these species, *L. monocytogenes* and *L. ivanovii*, are potentially pathogenic. The infectious disease caused by these bacteria is known as listeriosis. *L. monocytogenes* causes serious localized and generalized infections in humans and a variety of other vertebrates, including domesticated and wild birds and mammals. The official discovery of *Listeria* microorganisms dates back to 1924, when E. G. D. Murray, R. A. Webb, and M. B. R. Swann isolated *L. monocytogenes* as the etiological agent of a septicemic disease affecting rabbits and guinea pigs in their laboratory at Cambridge in England (458). The first cases of human listeriosis were reported in 1929 in Denmark (481). However, the first recorded culture of *L. monocytogenes* dates from 1921, with the bacterium isolated in France by Dumont and Cotoni from a patient with meningitis (159, 604). *L. ivanovii* (formerly known as *L. monocytogenes* serotype 5) was first isolated in Bulgaria in 1955 from lambs with congenital listeriosis (299). Human cases of *L. ivanovii* infection are rare (116), the vast majority of reported isolations of this species being from abortions, stillbirths, and neonatal septicemias in sheep and cattle (4, 90, 134, 529, 610, 693). A third species, *L. seeligeri*, is considered nonpathogenic (555), although it has been implicated in at least one case of human listeriosis (556).

For many years, clinical *Listeria* isolates were a mere laboratory rarity, and the epidemiology of the disease was an unresolved mystery (603, 604). However, at the end of the 1970s and the start of the 1980s, the number of reports on *Listeria* isolations begun to increase, and from 1983 onwards, a series of epidemic outbreaks in humans in North America and Europe clearly established listeriosis as an important food-borne infection (46, 180, 386, 421, 572). The foods most frequently implicated are soft cheeses and dairy products, pâtés and sausages, smoked fish, salads, "delicatessen," and in general industrially produced, refrigerated ready-to-eat products that are eaten without cooking or reheating (176, 425–427, 551, 572). In ruminants, *Listeria* infection is transmitted by consumption of spoiled silage, in which these bacteria multiply readily, resulting in herd outbreaks (178, 666, 671, 693). *Listeria* organisms

are widely disseminated in the rural environment and, consequently, contaminate the raw materials used in the preparation of industrially processed foods and the production plants as well (237). These bacteria are well equipped to survive food-processing technologies. For example, they tolerate high concentrations of salt and relatively low pHs, and worst of all, they are able to multiply at refrigeration temperatures (145, 362, 393, 426). This makes *Listeria* microorganisms a serious threat to food safety and ranks them among the microorganisms that most concern the food industry.

Researchers in immunology were interested in *L. monocytogenes* long before its importance as a risk to public health and food safety was recognized, because an infection highly reminiscent of human listeriosis was easily reproducible in laboratory rodents and protection could be transferred in syngeneic mice through spleen cells. Since the pioneering work of Mackaness in the early 1960s, which demonstrated that *L. monocytogenes* is able to survive and multiply in macrophages, this bacterium has been used in immunological research as a prototype intracellular parasite (401). For decades, the experimental model of *Listeria* infection in the mouse has made a significant contribution to our understanding of the cellular immune response (615). For example, key concepts such as the inability of antibodies to protect against infections produced by intracellular pathogens, the importance of activated macrophages in the elimination of intracellular parasites, and that the T cell is the macrophage-activating element required for cell-mediated immunity were established based on studies with the murine model of listeriosis (405, 406, 443, 478, 479).

In addition to the emergence of *L. monocytogenes* as a major food-borne pathogen, the 1980s also marked the start of investigations into the molecular mechanisms underlying *Listeria* virulence. The attention of researchers was first drawn to hemolytic activity, classically considered a virulence marker because it was present in the pathogenic species but not (with the exception of *L. seeligeri*) in the nonpathogenic species. These studies led between 1986 and 1989 to the discovery of the hemolysin gene, *hly*, and to elucidation of the key role that hemolysin plays in escape from destruction inside phagosomes, a prerequisite for intracellular bacterial proliferation. So, a decade ago, hemolysin became not only the first *Listeria* virulence factor to have its gene characterized, but also the first bacterial gene product to which a function critical to the survival of a parasite within the cells of the eukaryote host was attributed.

L. monocytogenes has since become not only an important paradigm for immunological investigation but also an important model system for analysis of the molecular mechanisms of intracellular parasitism (107). The identification of the *hly* gene was rapidly followed by a succession of discoveries resulting in complete characterization of the genetic locus to which this gene maps. This locus is a 9-kb virulence gene cluster that is involved in functions essential to intracellular survival. The internalin locus, *inlAB*, was also identified and characterized at

this time (193). This locus encodes the first invasin described in a gram-positive bacterium, implicated in internalization by cells that are not usually phagocytic, such as epithelial and endothelial cells and hepatocytes. The progress made in the molecular characterization of listerial virulence factors during this period was summarized in a minireview published in 1992 (517). Many advances have since been made that have significantly increased our understanding of the molecular mechanisms of *Listeria* intracellular parasitism. The sequencing of the genomes of *L. monocytogenes* and *L. innocua* is currently finished (European *Listeria* Genome Consortium, unpublished data), with the expected outcome that very soon the panorama of research into the molecular pathogenesis of *Listeria* spp. will change dramatically.

The aim of this article is to review the knowledge gathered during the pregenomic era of *Listeria* research about the pathogenesis of listeriosis and the molecular virulence determinants involved. Readers interested more specifically in immunopathogenesis and the immune response should consult reference 497a. Those interested in issues related to taxonomy, clinical diagnosis and disease management, molecular typing, epidemiology, ecology, and food safety are also referred to other publications (145, 176, 289, 395, 550, 551, 573, 594, 605). For a historical perspective on *Listeria* spp. and listeriosis, we recommend the book *Listeriosis* by the pioneer listeriologist Heinz P. R. Seeliger (602) and the classical review, "*Listeria monocytogenes* and listeric infections," by Gray and Killinger (238).

PATHOPHYSIOLOGY OF *LISTERIA* INFECTION

Clinical Features

The clinical signs of *L. monocytogenes* infection are very similar in all susceptible hosts. Two basic forms of presentation can be distinguished: perinatal listeriosis and listeriosis in the adult patient. In both instances, the predominant clinical forms correspond to disseminated infection or to local infection in the central nervous system (CNS). Listeriosis is usually a very severe disease—in fact, one of the most deadly bacterial infections currently known—with a mean mortality rate in humans of 20 to 30% or higher despite early antibiotic treatment (422, 423, 554, 594).

Fetomaternal and neonatal listeriosis. This form of presentation mainly results from invasion of the fetus via the placenta and develops as chorioamnionitis. Its consequence is abortion, usually from 5 months of gestation onwards, or the birth of a baby or stillborn fetus with generalized infection, a clinical syndrome known as granulomatosis infantiseptica and characterized by the presence of pyogranulomatous microabscesses disseminated over the body and a high mortality (336a) (Fig. 1). The infection is usually asymptomatic in the mother or may present as a mild flu-like syndrome with chills, fatigue, headache, and muscular and joint pain about 2 to 14 days before miscarriage. Less frequently (10 to 15% of perinatal cases), late neonatal listeriosis is observed. It generally occurs 1 to 8 weeks postpartum and involves a febrile syndrome accompanied by meningitis and, in some cases, gastroenteritis and pneumonia. It presumably results from aspiration of contaminated maternal exudates during delivery, although hospital-acquired cases involving horizontal transmission by fomites or medical personnel in neonatal units have been reported (177,

554, 628). The mortality of late-onset neonatal listeriosis is lower (10 to 20%), but like early-onset neonatal listeriosis, it may have sequelae such as hydrocephalus or psychomotor retardation (173). *L. monocytogenes* is one of the three principal causes of bacterial meningitis in neonates (391, 554, 594, 645).

Listeriosis in adults. The listerial infection most frequently reported in nonpregnant adults is that affecting the CNS (55 to 70% of cases). Pure meningeal forms are observed in some cases, but infection normally develops as a meningoencephalitis accompanied by severe changes in consciousness, movement disorders, and, in some cases, paralysis of the cranial nerves (Fig. 1). The encephalitic form, in which *Listeria* organisms are isolated with difficulty from the cerebrospinal fluid (CSF), is common in animals (Fig. 1A to D) but rare in humans (see below). Its course is usually biphasic, with an initial subfebrile phase lasting 3 to 10 days in which there may be headache, vomiting, visual disorders, and general malaise, followed in a second phase by the onset of severe signs of rhombencephalitis. The mortality rate for CNS infection is around 20% but may be as high as 40 to 60% if associated with concurrent, underlying debilitating disease. It has been estimated that *L. monocytogenes* accounts for 10% of community-acquired bacterial meningitis. Due to effective vaccination against *Haemophilus influenzae*, *L. monocytogenes* is now the fourth most common cause of meningeal infection in adults after *Streptococcus pneumoniae*, *Neisseria meningitidis*, and group B streptococci. However, in certain high-risk groups, such as cancer patients, *L. monocytogenes* is the most common cause of bacterial meningitis (391, 473, 593). Another frequent form of listeriosis (in some series of patients reported, even more frequent than CNS infection) is bacteremia or septicemia (15 to 50% of cases), with a high mortality rate (up to 70%) if it is associated with severe underlying debilitating conditions (391) (see below). There are other atypical clinical forms (5 to 10% of cases), such as endocarditis (the third most frequent form), myocarditis, arteritis, pneumonia, pleuritis, hepatitis, colecystitis, peritonitis, localized abscesses (e.g., brain abscess, which accounts for about 10% of CNS infections by *Listeria* spp.), arthritis, osteomyelitis, sinusitis, otitis, conjunctivitis, ophthalmitis, and, in cows, mastitis (48, 176, 199, 201, 390, 391, 395, 422–424, 550, 628).

An association between clinical episodes of invasive listeriosis and a history of gastrointestinal symptoms, including diarrhea, vomiting, and fever, was noticed some time ago (286, 542, 599). Investigations of recent food-borne outbreaks have provided compelling evidence that a febrile gastroenteritis syndrome may indeed be the main clinical manifestation of *L. monocytogenes* infection (19, 118, 442, 577, 628). The lesson to be learned from these epidemics is that *L. monocytogenes* should be sought as a possible etiologic agent in cases of diarrheagenic disease in humans. The potential enteropathogenicity of *L. monocytogenes* has also been recognized in animals, with outbreaks of diarrhea and gastroenteritis having been reported in sheep (52, 219, 395).

There is also a primary cutaneous form of *Listeria* infection characterized by a pyogranulomatous rash. This form occurs sporadically among farmers and veterinarians and is contracted by direct contact with the genital tract or placenta from cows having had a miscarriage due to *Listeria* infection (6, 428).

FIG. 1. Clinical and pathological characteristics of *Listeria* infection in animals and humans. (A to D) Neuromeningeal listeriosis in sheep. (A) Typical aspect of circling disease, first described by Gill in 1933 in New Zealand (215, 216) and the most characteristic clinical manifestation of listeriosis in ruminants; the syndrome is characterized by involuntary torticollis and walking aimlessly in circles as a result of brainstem lesions. (B) In a further step of the infectious process, animals lie on the ground with evident signs of uncoordination (paddling movements) and cranial nerve paralysis (strabismus, salivation, etc.). (C) Section of the medulla oblongata of a sheep with listerial rhombencephalitis, showing clear inflammatory lesions in the brain tissue. (D) Microscopic preparation of the brainstem showing extensive parenchymal inflammatory infiltration and typical perivascular cuffing (one is indicated by an arrowhead) (panels C and D copyright M. Domingo, Barcelona, Spain). (E and F) Stillborn with generalized *L. monocytogenes* infection, a clinical condition also known as granulomatosis infantiseptica and characterized by the presence of military-disseminated pyogranulomatous lesions on the body surface (E) and internal organs (F, liver of the fetus in E) and a high mortality. (G and H) Extensive pyogranulomatous hepatitis in a lamb experimentally infected with *L. ivanovii*, with multiple necrotic foci in the liver surface (G) and the parenchyma (H, hematoxylin/eosin-stained cut from the liver in G, showing two subcapsular pyogranulomes [magnification, ×60]).

Pathogenesis

The pathophysiology of *Listeria* infection in humans and animals is still poorly understood. Most of the available information is derived from interpretation of epidemiological, clinical, and histopathological findings and observations made in experimental infections in animals, particularly in the murine model. As contaminated food is the major source of infection in both epidemic and sporadic cases (176, 512), the gastrointestinal tract is thought to be the primary site of entry of pathogenic *Listeria* organisms into the host. The clinical course of infection usually begins about 20 h after the ingestion of heavily contaminated food in cases of gastroenteritis (118), whereas the incubation period for the invasive illness is generally much longer, around 20 to 30 days (386, 542). Similar incubation periods have been reported in animals for both gastroenteric and invasive disease (138, 219, 671).

Ingestion of *L. monocytogenes* is likely to be a very common event, given the ubiquitous distribution of these bacteria and the high frequency of contamination of raw and industrially processed foods. However, the incidence of human listeriosis is very low, normally around 2 to 8 sporadic cases annually per million population in Europe and the United States (176, 301, 554, 649). Higher incidence rates have been reported for sporadic listeriosis (for example, 10.9 cases per million population per year in Barcelona, Spain [476], and 14.7 in France [234]), but these are exceptional. In epidemic situations, the incidence in the target population increases by a factor of 3 to 10 (46, 386, 587, 599). Thus, *L. monocytogenes* seems to have a lower pathogenic potential than other food-borne pathogens. This is consistent with the relatively high 50% lethal dose (LD_{50}) values reported for mice infected experimentally by the oral (10^9 [18, 485]) or parenteral (10^5 to 10^6 [18, 333, 553]) routes. The minimum dose required to cause clinical infection in humans has not been determined, but the large numbers of *L. monocytogenes* bacteria detected in foods responsible for epidemic and sporadic cases of listeriosis (typically 10^6) suggest that it is high. However, these data should be interpreted with caution, given the long incubation period of invasive listeriosis and the time normally elapsed between diagnosis and analysis of the food eaten, during which *Listeria* organisms can have multiplied in the patient's refrigerator. It therefore cannot be excluded that low doses may be able to cause infection, at least in immunosuppressed persons, old people, and pregnant women. Indeed, levels of contamination as low as 10^2 to 10^4 *L. monocytogenes* cells per g of food have been associated with listeriosis in humans (176, 425). Obviously, the infectious dose may vary depending upon the pathogenicity and virulence of the *L. monocytogenes* strain involved and the host risk factors.

Pathogenicity of *L. monocytogenes*. Heterogeneity in the virulence of *L. monocytogenes* has been observed in several in vivo (mice) and in vitro (cell culture) studies (68, 129, 550, 663), but in most cases a clear correlation between the level of virulence and the origin or type characteristics of the strain could not be established. However, a recent study has found statistically significant differences in virulence between strains of food and clinical origin, the latter having slightly lower lethal doses (477). On the other hand, there is ample circumstantial evidence for an association between antigenic composition and pathogenicity in *Listeria* spp. The clearest example is provided by the *L. ivanovii*-specific serovar 5, which is recovered almost exclusively from ruminants, especially sheep. In these animals, serovar 5 strains cause perinatal infections but not encephalitis, the most typical clinical manifestations of ovine listeriosis (134, 397, 606, 610, 693) (Fig. 1A to D). Further evidence comes from the fact that only 3 of the 12 known serovars of *L. monocytogenes*, 1/2a, 1/2b, and 4b, account for more than 90% of human and animal cases of listeriosis (176, 397, 594), although other serovars, such as 1/2c, are often found as food contaminants (172, 176). Among the listeriosis-associated serovars, 4b strains cause over 50% of listeriosis cases worldwide, but strains of antigenic group 1/2 (1/2a, 1/2b, and 1/2c) predominate in food isolates (52, 172, 550, 557, 591). This suggests that serovar 4b strains are more adapted to mammalian host tissues than strains from serogroup 1/2. A phenotypically and genomically closely related group of serovar 4b isolates was found to be responsible for major outbreaks of food-borne human listeriosis in California in 1985 (386), Switzerland from 1983 to 1987 (46), Denmark from 1985 to 1987 (580), and France in 1992 (233), supporting the notion that some clones of *L. monocytogenes* may be particularly pathogenic (302, 510).

A number of observations suggest that there may be differences in pathogenic tropism between *L. monocytogenes*' strains. In humans, for example, serovar 4b strains have been found to occur more frequently in fetomaternal cases than in cases not associated with pregnancy (424). In sheep, the two major clinical forms of *L. monocytogenes* infection, meningoencephalitis and abortion, do not tend to occur simultaneously in the same flock (13, 397, 671). Molecular epidemiological evidence for such pathogenic tropism has been presented recently by Wiedman et al. (696). These authors found a correlation between the three serovar-related evolutionary branches into which *L. monocytogenes* isolates can be grouped (44, 510, 531, 678) and the pathogenic potential for humans and animals. Thus, one group of strains (serovars 1/2b and 4b) contained all isolates from human food-borne epidemics and isolates from sporadic cases in humans and animals, another (serovars 1/2a, 1/2c, and 3a) contained strains from both human and animal cases but no isolates from human food-borne epidemics, while a third group (serovar 4a) contained only animal isolates. A specific ribotype in the first group comprised all the serovar 4b strains associated with human food-borne epidemics but less than 10% of the ruminant isolates, suggesting a possible pathogenic tropism for humans (696). This possible tropism for humans of certain clones of *L. monocytogenes* serovar 4b may explain, for example, the observations made in Scotland, where during the same time period serovar 4b was most commonly isolated from human cases, whereas most ruminant cases were due to serovar 1/2a strains (397). There are also indications of adaptation to cause a particular clinical form in *L. monocytogenes* serovar 4b strains, as deduced from the analysis of two food-borne outbreaks of human listeriosis in France in 1992 and 1993, each associated with a different phage type of this serovar. In these outbreaks, the target population was similar but there was a significant difference in the percentage of fetomaternal cases (33 versus 80%) (557, 558).

Host risk factors. Host susceptibility plays a major role in the presentation of clinical disease upon exposure to *L. monocytogenes*. Thus, most listeriosis patients have a physiological or
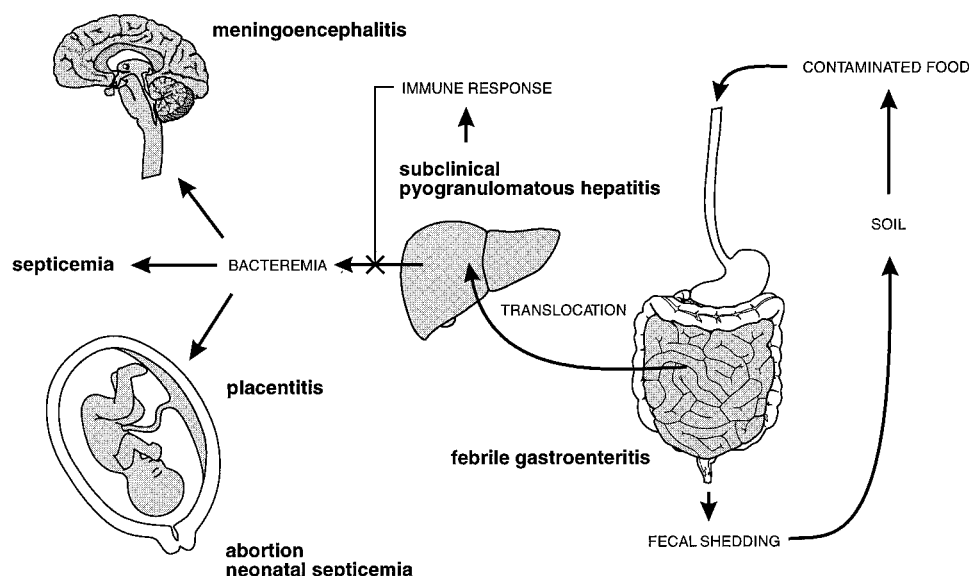
FIG. 2. Schematic representation of the pathophysiology of *Listeria* infection. See text for details.

pathological defect that affects T-cell-mediated immunity. This justifies the classification of *L. monocytogenes* as an opportunisitic pathogen. The groups at risk for listeriosis are pregnant women and neonates, the elderly (55 to 60 years and older), and immunocompromised or debilitated adults with underlying diseases. Listeriosis in nonpregnant adults is associated in most cases (>75%) with at least one of the following conditions: malignancies (leukemia, lymphoma, or sarcoma) and antineoplastic chemotherapy, immunosuppresant therapy (organ transplantation or corticosteroid use), chronic liver disease (cirrhosis or alcoholism), kidney disease, diabetes, and collagen disease (lupus) (176, 423, 551, 554, 594).

Human immunodeficiency virus (HIV) infection is also a significant risk factor for listeriosis. AIDS is the underlying predisposing condition in 5 to 20% of listeriosis cases in nonpregnant adults. It has been estimated that the risk of contracting listeriosis is 300 to 1,000 times higher for AIDS patients than for the general population. Nevertheless, listeriosis remains a relatively rare AIDS-associated infection, probably due to the preventive dietary measures taken by HIV-infected patients (avoidance of high-risk foods), the antimicrobial treatments that they receive regularly to treat or prevent opportunistic infections, and the fact that HIV infection does not significantly reduce the activity of the major effectors of immunity of *Listeria* spp. (innate immune mechanisms and the CD8$^+$ T-cell subset [266]) (35, 306, 316, 592).

The health status of the patient greatly influences the outcome of listeriosis. Immunocompetent patients usually survive listeriosis, whereas those with underlying debilitating diseases often succumb to the infection (mean mortality rate for this group, >30 to 40%) (235, 627). Although most listeriosis cases are associated with underlying risk factors, there are also a few adult patients for whom no obvious predisposing condition can be identified (177, 233, 235). This shows that *L. monocytogenes* has the potential to infect immunocompetent individuals. In some epidemics, all the nonpregnant adult patients had pre-existing illnesses or immunosuppresive conditions (180, 386),

whereas in others no such predisposing factors were found in any of the nonperinatal cases (587). This may be due to differences in the bacterial load present in the contaminated food involved or to the degree of virulence of the corresponding strains of *L. monocytogenes*.

Entry and colonization of host tissues. (i) Crossing the intestinal barrier. Before reaching the intestine, the ingested *Listeria* organisms must withstand the adverse environment of the stomach (Fig. 2). Oral infective doses are lower for cimetidine-treated experimental animals than for untreated animals (586a), and the use of antacids and H2-blocking agents has been reported to be a risk factor for listeriosis (286, 592). This indicates that gastric acidity may destroy a significant number of the *Listeria* organisms ingested with contaminated food.

There is controversy concerning the point of entry and the mechanism of intestinal translocation used by *L. monocytogenes*. In an early study by Racz et al. with guinea pigs infected intragastrically with 10^{10} *L. monocytogenes*, detailed histological analyses revealed that all the animals developed enteritis (526). In the initial stages, bacteria were detected mostly in the absorptive epithelial cells of the apical area of the villi, whereas in later phases most were inside macrophages of the stroma of the villi, suggesting that *L. monocytogenes* penetrates the host by invading the intestinal epithelium (526). This is consistent with the observation that *L. monocytogenes* is able to penetrate the apical surface of polarized, differentiated human enterocyte-like Caco-2 cells with a brush border (322) (see Fig. 3B and C). In other studies using mice inoculated per os with 10^8 to 10^9 bacteria, no invasion of the intestinal villous epithelium was observed; instead, there was colonization of the Peyer's patches (400, 410, 411), suggesting that *L. monocytogenes* uses the M-cell epithelium as an entry portal, as reported for other bacterial pathogens (623). Direct evidence that *L. monocytogenes* may indeed penetrate the host via the M cells overlying the Peyer's patches has been provided by a recent study using a murine ligated-loop model and scanning electron microscopy (308). However, listerial penetration at this level appears not

to be very efficient, because only small numbers of bacteria are observed in association with the follicle-associated epithelium overlying Peyer's patches (411).

Intestinal translocation of pathogenic listeriae occurs without the formation of gross macroscopic or histological lesions in the gut of mice (411). This suggests that an epithelial phase involving bacterial multiplication in the intestinal mucosa is not required by *L. monocytogenes* for systemic infection. Indeed, a recent study using a rat ileal loop model of intestinal infection (523) has shown that *Listeria* organisms are translocated to deep organs very rapidly (within a few minutes), demonstrating that crossing of the intestinal barrier occurs in the absence of prior intraepithelial replication. This study also showed that defined mutants defective in virulence factors known to be involved in epithelial cell invasion (InlA and InlB), intracellular survival (Hly), and cell-to-cell spread (ActA) (see below), and even the nonpathogenic species *L. innocua*, translocated at the same rate as wild-type *L. monocytogenes*. Translocation was dose dependent, and the presence of Peyer's patches in ligated loops did not affect the rate of translocation, levels of uptake being similarly low for Peyer's patches and villous intestine (50 to 250 bacteria per cm^2 of tissue after inoculation of the loop with 10^9 bacteria) (523). These findings favor the view that listerial translocation is a passive, nonspecific process similar to that observed for other bacteria (36), at least in mice and rats. However, *L. monocytogenes* proliferated in the intestinal wall, whereas *L. innocua* did not. The preferential site for bacterial replication was the Peyer's patches, and the essential listerial virulence factor Hly (hemolysin) was indispensable for this process, showing that *L. monocytogenes* establishes an active local infection in these lymphoid structures of the intestine. The foci of infection consisted of pyogranulomatous reactions in the subepithelial follicular tissue, with bacteria visible inside permissive mononuclear cells, presumably nonactivated resident macrophages and dendritic cells (522a, 523). Thus, antigen presentation events probably already take place in the intestine during the early phases of host colonization, and this may play an important role in the acquired resistance to subsequent reinfection that develops after primary oral exposure to *L. monocytogenes* (394, 400). A recent study has shown that invasion of intestinal epithelial cells by *L. monocytogenes* induces the activation of NF-κB and the subsequent upregulation of interleukin-15 (IL-15), and that at early stages of an oral infection with *L. monocytogenes* in mice and rats, intestinal intraepithelial lymphocytes become activated and produce Th1-type cytokines. This suggests that the interaction between intestinal epithelial and lymphoid cells may be relevant in the local host defence against listerial infection in the gut (706).

Experimental observations made with the mouse and rat models of intestinal translocation do not, however, explain how *L. monocytogenes* causes enteritis. The association of gastroenteric symptoms with fever is consistent with invasive intestinal disease, as observed by Racz in the guinea pig model (526). Pathogenic *Listeria* organisms pass directly from cell to cell by a mechanism involving host cell actin polymerization (see below). Therefore, regardless of the mechanism of entry used, the bacteria that penetrate the intestinal wall might then invade neighboring enterocytes by basolateral spread, leading to enteritis. This is consistent with in vitro experimental data

showing that *L. monocytogenes* enters polarized Caco-2 cells predominantly via the basolateral surface (196). Gross intestinal lesions develop in experimental animals only if large oral doses of *L. monocytogenes* are given (400, 523). Similarly, episodes of listerial gastroenteritis in humans occur in the form of outbreaks with very short incubation periods and high attack rates among immunocompetent adults (118, 577), consistent with the ingestion of a very high dose of bacteria (as high as 2.9×10^{11}, as estimated for one of these oubreaks, caused by the consumption of heavily contaminated chocolate milk [118]). Thus, intestinal invasion and the ensuing febrile gastroenteritis syndrome probably result from extensive exposure of the intestine to pathogenic *Listeria* organisms. However, the possibility that some *L. monocytogenes* strains have a greater enteropathogenic potential and cause intestinal damage at a lower dose cannot be ruled out.

(ii) Multiplication in the liver. The *Listeria* organisms that cross the intestinal barrier are carried by the lymph or blood to the mesenteric lymph nodes, the spleen, and the liver (411, 523) (Fig. 2). As stated above, this initial step of host tissue colonization by *L. monocytogenes* is rapid. The unusually long incubation period required by *L. monocytogenes* for the development of symptomatic systemic infection after oral exposure in relation to that for other food-borne pathogens is therefore puzzling and indicates that listerial colonization of host tissues involves a silent, subclinical phase, many of the events and underlying mechanisms of which are unknown.

Experimental infections of mice via the intravenous route have shown that *L. monocytogenes* bacteria are rapidly cleared from the bloodstream by resident macrophages in the spleen and liver (99, 112a, 405). Most (90%) of the bacterial load accumulates in the liver, presumably captured by the Kupffer cells that line the sinusoids. These resident macrophages kill most of the ingested bacteria, as shown by in vivo depletion experiments (161), resulting in a decrease in the size of the viable bacterial population in the liver during the first 6 h after infection. Kupffer cells are believed to initiate the development of antilisterial immunity by inducing the antigen-dependent proliferation of T lymphocytes and the secretion of cytokines (243). Not all *Listeria* cells are destroyed by tissue macrophages, and the surviving bacteria start to grow, increasing in numbers for 2 to 5 days in mouse organs (93, 99, 128, 181, 381, 408, 448).

The principal site of bacterial multiplication in the liver is the hepatocyte (100, 112a, 197, 241, 241a, 563). This finding has led to the dismissal of the long-held idea that the major host niche for the parasitic life of *L. monocytogenes* is the macrophage population. There are two possible ways for *L. monocytogenes* to gain access to the liver parenchyma after its intestinal translocation and carriage by the portal or arterial bloodstream: via Kupffer cells, by cell to cell spread, or by the direct invasion of hepatocytes from the Disse space after crossing the fenestrated endothelial barrier lining the sinusoids. *L. monocytogenes* has been shown to efficiently invade hepatocytes in vitro (149, 701).

Electron microscopy of hepatic tissue from infected mice suggests that *L. monocytogenes* goes through the complete intracellular infectious cycle in hepatocytes (197, 621), including actin-based intercellular spread (see below). Direct passage from hepatocyte to hepatocyte would lead to the formation of

infectious foci in which *L. monocytogenes* disseminates through the liver parenchyma without coming into contact with the humoral effectors of the immune system. This may explain why antibodies play no major role in anti-*Listeria* immunity (516).

During the early steps of liver colonization, polymorphonuclear neutrophils are recruited at the sites of infection, forming discrete microabscesses. Neutrophils have been shown to play an important role in controlling the acute phase of *Listeria* infection (561) and in mediating the destruction of *Listeria*-infected hepatocytes in vivo (99). Hepatocytes respond to *Listeria* infection by releasing neutrophil chemoattractants and exhibiting an increase in adhesion to neutrophils, resulting in microabscess formation (560). They also respond by initiating an apoptosis program that may be critical for removing *Listeria*-infected cells from the liver tissue at early stages of infection (448a, 560). Using transgenic mice expressing a degradation-resistant IκB transgene, it has been shown recently that NF-κB activation in mouse hepatocytes is essential to clear *L. monocytogenes* from the liver, possibly by coordinating local innate immune response (368). Two to four days after infection, neutrophils are gradually replaced by blood-derived mononuclear cells together with lymphocytes to form the characteristic granulomas (283, 408) (Fig. 1G and H). These granulomas are the histomorphological correlate of cell-mediated immunity and presumably act as true physical barriers that confine the infectious foci, impeding further bacterial dissemination by direct cell-to-cell passage (516).

Between days 5 and 7 postinfection, *L. monocytogenes* bacteria start to disappear from mouse organs until their complete clearance as a result of gamma interferon (IFN-γ)-mediated macrophage activation and the induction of an acquired immune response primarily mediated by CD8$^+$ lymphocytes, which together destroy *Listeria*-infected cells (241a, 268, 328, 441). These cytotoxic T lymphocytes (CTL) are directed against listerial epitopes present in secreted virulence-associated proteins, such as Hly (59, 71, 266, 281, 625, 677), the metalloprotease Mpl (79), and the surface protein p60 (203, 204, 267, 624), and possibly also in somatic housekeeping proteins such as superoxide dismutase (280). Protection against *L. monocytogenes* also involves a vigorous Th1-biased CD4$^+$ T-cell response (125, 203) and innate mechanisms such as the activation of IFN-γ-producing natural killer (NK) cells in response to IL-12 and tumor necrosis factor alpha (TNF-α) secretion by infected macrophages (125, 166, 268, 290). Hepatocytes may contribute to protection by becoming less permissive to intracellular proliferation of *Listeria* organisms upon exposure to IFN-γ (244), especially if costimulated with other cytokines (646).

The above course of events is accelerated in immune animals, resulting in rapid elimination of *L. monocytogenes* from the liver (Fig. 2). This is probably the most common outcome of *L. monocytogenes* infection in humans and animals in normal conditions, given the potentially high frequency of exposure to the pathogen via contaminated food and the relatively rare occurrence of clinical disease. Indeed, T lymphocytes directed against *Listeria* antigens are commonly found in healthy individuals (457), probably due to chronic stimulation of the immune system by *L. monocytogenes* antigens that are presumably continuously delivered to the immune system via food. A recent study has shown that the nonpathogenic species *L. in-*nocua can enhance a previously established *L. monocytogenes*-specific T-cell memory via recognition of cross-reactive p60 epitopes shared by the two species (204). *L. innocua* is commonly found in food (573), and as stated above, it is translocated to the deep organs of mice as efficiently as *L. monocytogenes*. Therefore, repeated contact with *L. innocua* may boost protective immunity against pathogenic *Listeria* spp., possibly explaining in part the rare occurrence of listeriosis in the general population.

If the infection is not controlled by an adequate immune response in the liver, as may occur in immunocompromised individuals, unlimited proliferation of *L. monocytogenes* in the liver parenchyma may result in the release of bacteria into the circulation (Fig. 2). *L. monocytogenes* is a multisystemic pathogen that can infect a wide range of host tissues, as indicated by its capacity to cause septicemia involving multiple organs and by the variety of potential sites of localized *Listeria* infection (see above). However, the principal clinical forms of listeriosis clearly show that *L. monocytogenes* has a pathogenic tropism towards the gravid uterus and the CNS (Fig. 2).

(iii) Colonization of gravid uterus and fetus. Abortion and stillbirth due to *Listeria* spp. have been reproduced experimentally by intravenous, oral, and respiratory inoculation in naturally susceptible gestating animal hosts, such as sheep, cattle, rabbits, and guinea pigs, as well in pregnant mice and rats (1, 238, 239, 329, 450, 487, 488, 586). This shows that *L. monocytogenes* gains access to the fetus by hematogenous penetration of the placental barrier (Fig. 2). In pregnant mice, the blood-borne bacteria first invade the decidua basalis and then progress to the placental villi, where they cause diffuse inflammatory infiltration and necrosis (1, 535, 536). Macrophages appear to be excluded from the murine placenta, neutrophils acting as the main antilisterial effector cell population (251a). Using homozygous mutant mice, it has been shown recently that colony-stimulating factor-1 is required for the recruitment of neutrophils to the infectious foci in the decidua basalis. This occurs via induction of neutrophil chemoattractant synthesis by the trophoblast (251a). In humans, placental infection is characterized by numerous microabscesses and focal necrotizing villitis (502, 582). Colonization of the trophoblast layer followed by translocation across the endothelial barrier would enable the bacteria to reach the fetal bloodstream, leading to generalized infection and subsequent death of the fetus in utero or to premature birth of a severely infected neonate with miliary pyogranulomatous lesions (the above-mentioned granulomatosis infantiseptica) (Fig. 1E and F).

The depression of cell-mediated immunity during pregnancy (686) presumably plays an important role in the development of listeriosis. In laboratory rodents, pregnancy reduces resistance to *L. monocytogenes* (58, 399) and significantly prolongs the course of primary infection in the liver (1). The T-cell-dependent elimination of *L. monocytogenes* from the organs of pregnant mice is delayed in late pregnancy. This correlates with a failure in the production of IFN-γ and results in listerial invasion of placental and fetal tissues (463). Abnormally high physiological levels of estrogenic hormones, such as those that occur during late pregnancy, may account for the disruption of T-cell-mediated resistance to infection, as treatment of mice with steroids inhibits the proliferative response to *L. monocytogenes* of splenic T cells and increases susceptibility to *Listeria*

infection (524). This correlates with a decrease in the production of IL-2 (524), a cytokine that stimulates resistance to *Listeria* spp. in mice (255). The intracellular killing function of macrophages has also been shown to be decreased by β-estradiol, which is associated with inhibition of both IL-12 and TNF-α secretion in vivo (578). Local depression in the cellular immune response in the placenta, which physiologically is important for preventing rejection of the fetus, may also contribute to the higher susceptibility to uterine infection by *L. monocytogenes* (398, 535, 536). It is not known why mothers suffering *Listeria*-induced miscarriage never develop CNS infection or overt septicemic disease.

(iv) Invasion of the brain. In humans, CNS infection by *Listeria* spp. presents primarily in the form of meningitis. This meningitis, however, is often associated with the presence of infectious foci in the brain parenchyma, especially in the brain stem (391, 473), suggesting *L. monocytogenes* has a tropism for nerve tissue. The neurotropism and special predilection of *L. monocytogenes* for the rhombencephalon are shown most clearly in ruminants, in which listerial CNS infection, in contrast to the situation in humans, develops mainly as primary encephalitis. In these animals, infectious foci are restricted to the pons, medulla oblongata, and spinal cord (Fig. 1C). Although there is inflammatory lymphocyte or mononuclear cell infiltration of the meninges, this condition occurs as an extension of the brain process, and macroscopic lesions may not even be evident or may be restricted to basal areas, midbrain, and cerebellum. Unilateral cranial nerve paralysis is a characteristic of listerial rhombencephalitis in ruminants, leading to the well-known circling disease syndrome (92, 103, 215, 216, 312, 534, 666, 693) (Fig. 1A and B). In humans, primary nonmeningeal brain infection is seldom observed. However, as in ruminants, it develops as cerebritis involving the rhombencephalon (15, 20, 69, 390, 391, 473).

Brain lesions in listerial meningoencephalitis are typical and very similar in humans and animals. They consist of perivascular cuffs of inflammatory infiltrates composed of mononuclear cells and scattered neutrophils and lymphocytes (Fig. 1D). Bacteria are generally absent from these perivascular areas of inflammation. Parenchymal microabscesses and foci of necrosis and malacia are also typically present (Fig. 1D). Bacteria are relatively abundant in these lesions, within phagocytes or free in the brain parenchyma around the necrotic areas (92, 103, 312, 315, 412, 489). Depletion experiments in mice using a neutrophil-specific monoclonal antibody have shown that neutrophils play a critical role in eliminating *L. monocytogenes* from infectious foci in the brain (389a). Less commonly, bacteria are observed within neurons in both natural (412, 489) and experimentally induced (588) infections. This is consistent with in vitro data showing that the invasion of cultured neurons is a relatively rare event (151, 505). However, neurons are efficiently invaded in vitro by direct cell-to-cell spread from infected macrophages or microglial cells (151). A recent study (146) has shown that *L. monocytogenes* can efficiently invade sensory neurons of rat dorsal root ganglia but not hippocampal neurons, suggesting that there may be differences in the susceptibility to infection among different types of neurons. The relevance of neuronal invasion to the pathogenesis of *Listeria* encephalitis is subject to speculation, as discussed below.

The mechanisms by which *L. monocytogenes* infects the CNS

are still largely unknown. Numerous attempts have been made to reproduce listerial meningoencephalitis in susceptible animal hosts and laboratory rodents using various routes of inoculation, with variable success. Intravenous delivery of the inoculum does not usually result in CNS infection in sheep (103). This, and the usual absence of visceral involvement in natural cases of ovine listerial encephalitis, seems to argue against a hematogenous route of infection. The topographic distribution of the lesions in sheep, strictly confined to the brainstem, and the frequent unilateral involvement of the trigeminal nerve, with neuritis affecting distal portions of a single or a few fasciculi in only one of the nerve branches, led to the hypothesis that *L. monocytogenes* invades the brain by centripetal migration along cranial nerves (17, 92, 661). To test this hypothesis, Asahi and coworkers (17) scarified oral, nasal, and labial mucosae of mice with *L. monocytogenes*. Most mice developed neurological signs, such as torticollis, rolling movements, and paralysis of the hindlegs, 5 days after infection. These signs correlated with the presence of mononuclear and round cell infiltrations along the trigeminal nerve, in the corresponding nerve ganglion, and in the medulla oblongata, suggesting retrograde bacterial ascension along the cranial nerve to the brainstem. In another experiment, 4 of 11 goats inoculated in the lip developed listerial encephalitis 17 to 28 days after inoculation, with microscopic lesions identical to those found in natural cases of listeriosis, with ganglioneuritis of the trigeminal nerve (17). In spontaneous cases of ovine listerial encephalits, bacteria have been observed in linear arrays in individual nerve fibers, inside axons, and with actin tails (91, 92, 489), suggesting active intra-axonal movement. Experiments using a double-chamber cell culture system and dorsal root ganglia neurons provided evidence that *L. monocytogenes* can infect axons and spread along them towards the nerve cell body (146).

Experimental evidence has been obtained that intra-axonal bacterial transport may indeed occur in vivo. Otter and Blakemore (489) developed a mouse model in which *L. monocytogenes* was injected distally into the sciatic nerve. Paralysis of the leg into which the bacteria were injected occurred 7 to 12 days later, and examination of the spinal cord revealed lesions very similar to those found in the brains of naturally affected sheep. Bacteria were seen in these lesions in neutrophils and, in some cases, in axons (489). The feasibility of ascending intra-axonal transport via the termini of the trigeminal nerve was tested by experimentally injecting *L. monocytogenes* directly into the dental pulp of sheep through a hole drilled in the tooth crown (22). Six of 21 sheep developed neurological signs 20 to 40 days after challenge, and most animals showed histological encephalitis and trigeminal ganglioneuritis on the side of inoculation. These observations favor the view that *L. monocytogenes* may directly invade exposed sensory terminal ramifications of the cranial nerve in the mouth and reach the brain by centripetal spread. They also demonstrate that cases of mild CNS invasion by *Listeria* organisms may occur without overt clinical signs. This is important because it provides one possible explanation for the relatively small number of cases with neurological manifestations that are observed among animals exposed to contaminated silage in a herd affected by listerial encephalitis (and, by extension, to humans exposed to *Listeria*-contaminated food). However, in another study in which brains from

natural cases of sheep with meningoencephalitis were analyzed, inflammatory infiltration was observed only in the intracranial portion of the trigeminal tract, always associated with meningitis adjacent to proximal parts of cranial nerves. The authors were of the opinion that cranial nerve invasion was only secondary to a hematogenous brain infection and occurred by centrifugal spread from the brainstem (103). The above-mentioned experiments with the two-chamber model (146) suggested that both retrograde and anterograde transport of *L. monocytogenes* may occur inside infected neurons.

Although intravenous inoculation only occasionally gives positive results, intracarotid inoculation consistently results in encephalitis in sheep (103, 314, 486). This supports the notion that *L. monocytogtenes* may indeed reach the brain via the blood. Strong evidence that meningoencephalitis in ruminants has a vascular basis is provided by the nature of the brain lesions themselves, with extensive perivascular infiltrates and mononuclear microabscesses and necrotic foci developing in close proximity to brain capillaries (103) (Fig. 1D). In contrast to intravenous inoculation, intracarotid delivery provides the inoculum direct access to the vascular system of the brain, enabling bacteria to bypass the clearance mechanisms of organs such as the spleen and liver. In an immunocompetent host, these clearance mechanisms should be sufficient to eliminate rapidly a single-dose intravenous inoculum, preventing the bacteria from invading the brain. Intracarotid inoculation in sheep causes CNS lesions involving the choroid plexus and the ependyma of the cerebral ventricles and aqueduct, resulting in primary meningitis and secondary lesions in the adjoining cerebral tissues (17). These brain structures are only rarely affected in natural cases of listerial meningoencephalitis in ruminants (103). The reason for these differences between natural and experimental lesion patterns probably resides in the concentration of bacteria in the blood, as this is an important factor affecting the mode by which bacterial pathogens gain access to the CNS (659, 660). This concentration would reach an extraordinarily high peak in the brain after intracarotid delivery, and high-grade bacteremia is more likely to cause meningitis due to massive bacterial penetration across the epithelium lining the choroid plexus, which has an exceptionally high rate of blood flow. In contrast, low-grade bacteremia, such as that resulting from the release of bacteria into the blood from infectious foci located in distant organs (e.g., the liver), tends to be associated with multiple foci in the brain parenchyma due to individual penetrations along the extensive microvascular endothelial bed (660).

Experimental infections in small laboratory animals also provide evidence for a hematogenous route of CNS invasion for *L. monocytogenes*. Intravenous inoculation in mice results in meningoencephalitis in a significant proportion of animals that survive the initial systemic phase of infection. Lesions include choroiditis, meningitis, and the characteristic perivascular cuffing and parenchymal pyogranulomatous foci in the brainstem (31, 103). No brain invasion is detected in mice challenged with low infective doses that induce only transient bacteremia, even if there is significant bacterial multiplication in the spleen and liver. However, brain invasion is achieved with high infective doses causing severe systemic disease and prolonged bacteremia (31). This suggests that the persistence of large numbers of bacteria in the blood is essential for the

induction of meningoencephalitis by *L. monocytogenes*. This conclusion is consistent with a recent study by Blanot and coworkers (47), who adapted a model of otitis media in gerbils to study listerial CNS invasion. With a low infective dose of *L. monocytogenes* (10^3/ear), these authors induced persistent low-level bacteremia (10^2 bacteria/ml) which was associated with the development of rhombencephalitis in gerbils mimicking that observed during human listerial meningoencephalitis, with leptomeningitis, parenchymal foci, and perivascular sheaths. The number of bacteria in the liver and spleen of gerbils decreased by day 5 postinoculation, but growth in brain tissue was unrestricted (47), suggesting that nerve tissue is a privileged niche for the multiplication of *L. monocytogenes*. CNS invasion was also regularly observed in mice after oral or subcutaneous inoculation (9, 17, 522). These infection routes imply systemic involvement and, clearly, also lymphohematogenous dissemination of bacteria to the brain.

In experiments with orally or subcutaneously infected mice, parenchymal brain lesions are irregularly observed, but marked inflammatory lesions in the meninges and ventricular system (choroid plexus, lateral, third and fourth ventricles, and aqueduct) are consistently recorded (9, 522). This striking affinity of *L. monocytogenes* for mouse ventricular structures was also noted after direct intracerebral inoculation (588), which prevents primary blood-borne exposure of the ependyma via the choroid plexus. In contrast, no involvement of the ventricular system was noticed in the gerbil model (47). These observations suggest that there may be anatomical and physiological differences in the brains of the various animal species that affect *L. monocytogenes* neuropathogenesis. Indeed, this may account for the differential characteristics of listerial CNS infection in humans and ruminants. However, it cannot be excluded that the peculiar characteristics of listerial meningoencephalitis in ruminants result from the fact that an alternative primary nonvascular mechanism of CNS invasion, which may perfectly coexist with hematogenous dissemination, has specifically evolved in these animals. Ruminants eat plant material, the physical characteristics of which may lead to small breaches of the oral mucosa. If animals are fed contaminated silage, these small wounds are repeatedly exposed to *L. monocytogenes* during rumination, favoring invasion of the trigeminal nerve terminals and subsequent intraneural, direct spread to the brain stem.

Bacterial pathogens such as *H. influenzae, N. meningitidis, S. pneumoniae*, and *Streptococcus suis* cross the blood-brain barrier (BBB) primarily via the choroid plexus. This enables them to reach the CSF and create purulent meningitis by spreading through the subarachnoid space (525, 659, 660, 697). Although in human listerial infections of the CNS, purulent meningitis also occurs and bacteria may also be detected in the CSF, it is clear that *L. monocytogenes*, unlike other meningitis-causing bacterial pathogens, tends to affect brain parenchymal tissue. This may reflect a tropism for the microvascular endothelium, in particular that lining the capillary bed of the rhombencephalon, resulting in significant or preferential (in ruminants) crossing of the BBB at this point. Direct uptake by endothelial cells of bacteria circulating free in the blood is one possible mechanism by which *L. monocytogenes* may cross the microvascular BBB. Evidence for this mechanism is provided by an electron microscopic study of human brainstem tissue, in which

bacteria have been observed within endothelial cells or adhering to the luminal face of the microvascular endothelium (335). This mechanism is also consistent with in vitro data showing that *L. monocytogenes* is able to invade cultured human brain microvascular endothelial cells (BMEC) (245, 246). The listerial surface protein InlB, of the internalin family (see below), has been shown to be required for invasion of endothelial cells in vitro (246, 498), indicating that specific bacterial molecules are actively involved in the interaction with the BBB. *L. monocytogenes* efficiently replicates for long periods of time within brain microvascular cells without causing any evident damage, creating heavily infected foci from which bacteria spread to neighboring cells by actin-based motility (246). In this way, *L. monocytogenes* may reach and disseminate easily into the protected spaces of the CNS.

L. monocytogenes infection induces a potent response in cultured endothelial cells which is accompanied by the upregulation of endothelial adhesion molecules (P- and E-selectin, ICAM-1, and VCAM-1) (see below), leading to an increase in the binding of polymorphonuclear leukocytes to infected endothelial cells (152, 247, 330, 600, 618). Heterologous plaque assays using infected macrophages as vectors have demonstrated that *L. monocytogenes* can spread efficiently from macrophages into endothelial cells (157, 246). Therefore, the recruitment of phagocytes to the infected endothelium, primarily a host defense response, may also be used by *L. monocytogenes*, in addition to direct invasion, to cross the BBB. Evidence for a significant role in vivo for such a Trojan horse mechanism of CNS invasion, presumably involving direct bacterial cell-to-cell spread from infected phagocytes carried by the blood after adhesion to endothelial cells, has been provided recently by Drevets (154). This author showed that phagocyte-associated *L. monocytogenes* accounted for 30% of the total bacterial population circulating in the bloodstream after intravenous inoculation. These entrapped bacteria spread to endothelial cells in vitro, and *Listeria*-infected peripheral blood leukocytes successfully established brain colonization in vivo (154). It is unknown whether trafficking of infected phagocytes across the BBB, as part of the inflammatory influx induced by endothelial and underlying brain tissue infection, occurs in vivo during CNS invasion by *L. monocytogenes*.

Hypothetical scenario. From the information presented above, the following hypothetical scenario for the pathogenesis of listeriosis can be proposed. Clinical outcome of *Listeria* infection depends on three major variables: (i) the number of bacteria ingested with food, (ii) the pathogenic properties of the strain, and (iii) the immunological status of the host. In immunocompetent individuals with no predisposing conditions, ingestion of low doses of *L. monocytogenes* will probably have no effect other than the development or boosting of antilisterial protective immunity. In contrast, oral exposure to large doses is likely to result in an episode of gastroenteritis and fever and, depending on the virulence of the strain, possible invasive disease. Immunocompromised and debilitated individuals, however, cannot mount an immune response strong enough to control bacterial proliferation in the liver, the primary target organ of *L. monocytogenes*, and are therefore susceptible to invasive disease following the ingestion of a lower inoculum. Inefficiently restricted growth of *L. monocytogenes* in the hepatocytes in these individuals is likely to result

in an increase in the critical mass of bacteria and their release into the bloodstream. The ensuing prolonged bacteremia will result in local infections in secondary target organs (particularly the brain and placenta) or in septicemic disease in severely immunocompromised hosts. Figure 2 summarizes the key steps of the pathophysiology of *Listeria* infection.
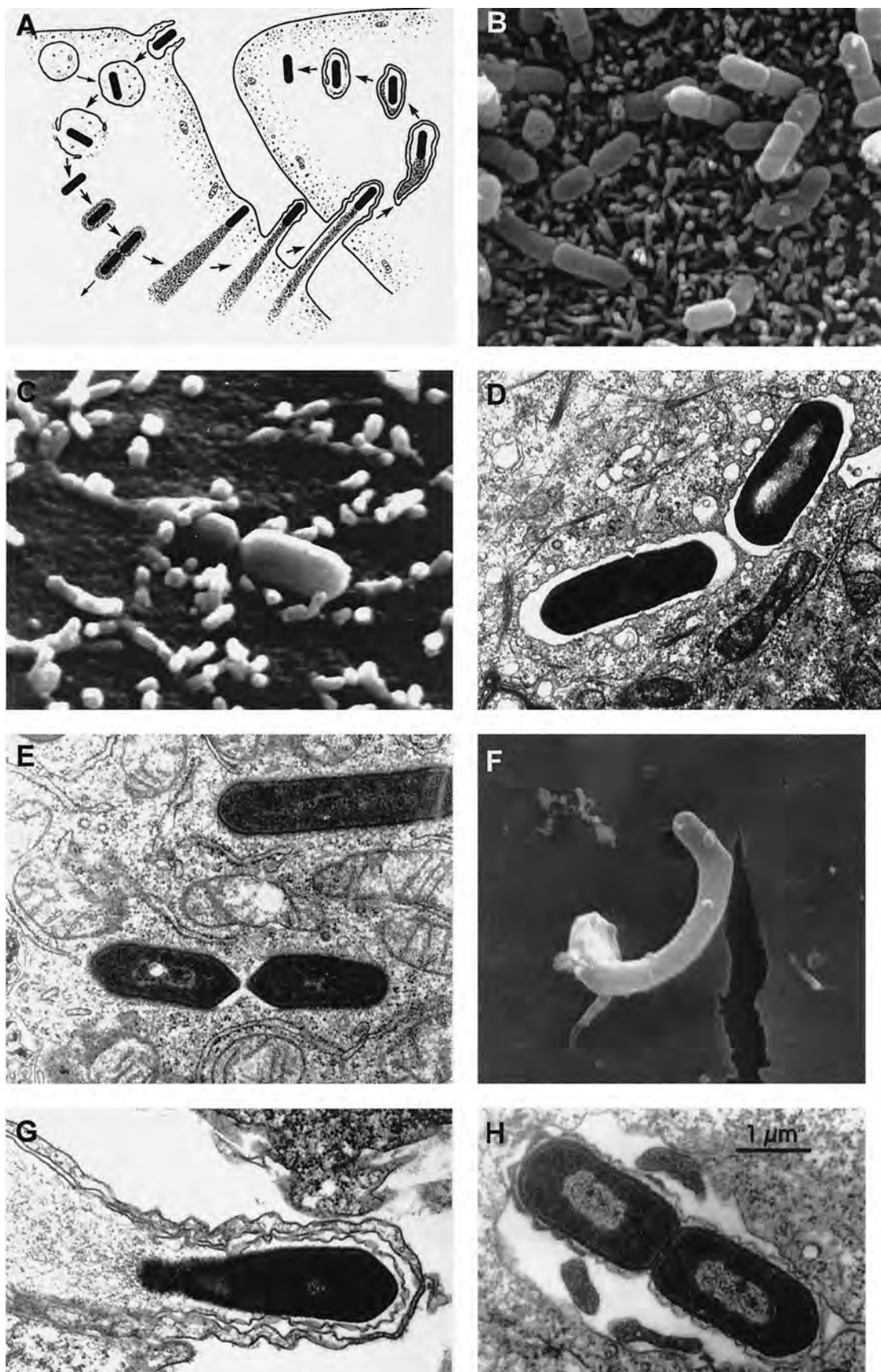
INTRACELLULAR INFECTIOUS CYCLE

In addition to multiplying within macrophages, *Listeria* organisms are invasive pathogens that can induce their own internalization in various types of cell that are not normally phagocytic. These include epithelial cells (194, 435, 518), fibroblasts (359, 641), hepatocytes (149, 242, 701), endothelial cells (157, 245, 498), and various types of nerve cell, including neurons (151). *Listeria* organisms have been also shown to be taken up by and survive within dentritic cells (253, 345). In the interior of all cells that *L. monocytogenes* is able to penetrate, whether macrophages or nonprofessional phagocytes, it develops an intracellular life cycle with common characteristics (Fig. 3).

Internalization

The cycle begins with adhesion to the surface of the eukaryote cell and subsequent penetration of the bacterium into the host cell. The invasion of nonphagocytic cells involves a zipper-type mechanism, in that the bacterium gradually sinks into diplike structures of the host cell surface until it is finally engulfed. During this process, the target cell membrane closely surrounds the bacterial cell and does not form the spectacular local processes or membrane ruffles characteristic of invasion by *Salmonella* and *Shigella* spp. (147, 322, 358, 435, 642) (Fig. 3B and C). The structures, mechanisms, and signal transduction cascades involved in the interaction between the bacterium and the cell during phagocytosis are only beginning to be elucidated.

The multisystemic nature of listerial infection indicates that *L. monocytogenes* probably recognizes a number of different eukaryotic receptors. The C3bi and C1q complement receptors have been reported to be involved in *L. monocytogenes* uptake by phagocytic cells (10, 115, 155, 156). *L. monocytogenes* is also efficiently internalized in the absence of serum, indicating that nonopsonic receptor-ligand interactions are also involved in host cell recognition by *Listeria* spp. (509). The eukaryote cell receptors used by *Listeria* spp. include the transmembrane glycoprotein E-cadherin (435), the C1q complement fraction receptor (61), the Met receptor for hepatocyte growth factor (HGF) (615a), and components of the extracellular matrix (ECM) such as heparan sulfate proteoglycans (HSPG) (12) and fibronectin (217). The macrophage scavenger receptor was shown to bind *L. monocytogenes* lipoteichoic acids and hence may also be involved in *Listeria*-macrophage interactions (160, 240). The bacterial ligands identified to date are all surface proteins, such as the internalins InlA and InlB, the actin-polymerizing protein ActA, and p60 (see below). Recently, several putative adhesion factors of *L. monocytogenes* have been identified by screening transposon mutants for defective attachment to eukaryotic cells. Among these putative adhesins are the surface protein Ami, an autolysin with a C-terminal cell wall-anchoring domain similar to InlB (60, 429, 445) (see below), and Lap, a 104-kDa surface protein involved in attach-

ment to Caco-2 cells (581a). Also recently, a 24.6-kDa fibronectin-binding protein has been identified in *L. monocytogenes* by screening a genomic library in *Escherichia coli* for fibronectin binding (215). Bacterium-host cell interactions involving lectins (482) may also be involved in *L. monocytogenes* adhesion to eukaryotic cells (174). Indirect evidence has been obtained for an *L. monocytogenes* adhesin containing α-D-galactose, involved in uptake by mouse CB1 dendritic cells (253) and human HepG22 hepatocarcinoma cells (114) upon recognition of a carbohydrate receptor at the eukaryotic cell surface. The presence in *L. monocytogenes* of lectin-like ligands mediating binding to D-glucosamine, L-fucosylamine, and *p*-aminophenyl-α-D-mannopyranoside moieties has also been indirectly demonstrated (110).

Intracellular Proliferation and Intercellular Spread

During invasion, *Listeria* bacteria become engulfed within a phagocytic vacuole (194) (Fig. 3D). Little is known about the characteristics of the *Listeria*-containing vacuolar compartment, but the vacuoles become acidified soon after uptake (25). There is evidence that *L. monocytogenes* ensures its intravacuolar viability by preventing phagosome maturation to the phagolysosomal stage (11). Thirty minutes after entry, the bacteria begin to disrupt the phagosome membrane (194), and within 2 h, about 50% of the intracellular bacterial population is free in the cytoplasm (655) (Fig. 3E). This membrane disruption step is essential for listerial intracellular survival and proliferation (220) and is mediated by the hemolysin in combination with phospholipases (see below).

Once in the cytosol, bacteria multiply (Fig. 3E), with a doubling time of approximately 1 h (194, 518), i.e., approximately three times slower than in rich medium. In *L. monocytogenes*, intracellular growth does not involve the upregulation of known stress proteins (261) (see below), and auxotrophic mutants requiring aromatic amino acids or purines for growth in minimal medium grow inside cells at rates similar to those of the wild-type parent strain (413). This indicates that the cytoplasmic compartment is permissive for *Listeria* proliferation. However, three metabolic genes (*purH*, *purD*, and *pyrE*, involved in purine and pyrimidine biosynthesis) and an arginine ABC transporter (*arpJ*) have been shown to be induced within cells (336). Mutation of these genes had no major effect on intracellular proliferation and virulence (336), which can mean that certain nutrients are present in the host cell cytoplasm at concentrations that, although not limiting, are sufficiently low that *L. monocytogenes* has to upregulate certain metabolic

genes to grow efficiently within cells. Recent experimental evidence indicates that pathogenic *Listeria* spp. may exploit hexose phosphates from the host cell cytoplasm for efficient intracellular growth (543) (see below). Evidence has also been recently presented that antimicrobial peptides from the host cell cytosol, such as ubiquicidin found in macrophages (283a), may play a role in restraining intracellular proliferation of *L. monocytogenes*. Nothing is known about the listerial mechanisms counteracting these inhibitory peptides.

Intracytoplasmic bacteria are immediately surrounded by a cloud of a fine, fuzzy, fibrillar material composed of actin filaments, which later (around 2 h postinfection) rearranges to form an actin tail of up to 40 μm at one of the poles of the bacterium (see Fig. 6B). This actin tail consists of two populations of cross-linked actin filaments, one formed by relatively long, axially arranged actin bundles and the other by shorter, randomly arranged filaments. The polar assembly of actin filaments propels the bacterial cell in the cytoplasm with a mean speed of 0.3 μm/s, in a fashion reminiscent of the "action-reaction" principle that governs rocket movement. Moving *Listeria* cells leave an evanescent trail of F-actin undergoing depolymerization at the distal end, the steady-state length of which is proportional to the speed of bacterial movement. This movement is random, so some bacteria eventually reach the cell periphery, come into contact with the membrane, and push it, leading to the formation of finger-like protrusions with a bacterium at the tip (Fig. 3F and G). These pseudopods penetrate uninfected neighboring cells and are in turn engulfed by phagocytosis, resulting in the formation of a secondary phagosome delimited by a double membrane (Fig. 3H), with the inner membrane originating from the donor cell (Fig. 3G). Bacteria escape rapidly (within 5 min) from the newly formed vacuole by dissolving its double membrane, reach the cytoplasm, and initiate a new round of intracellular proliferation and direct intercellular spread (117, 453, 549, 601, 652, 654–656) (Fig. 3A). In tissue culture, this infectious cycle is reflected in the formation of plaques in the cell monolayer (641, 672). Actin-based intracytoplasmic movement and cell-to-cell spread are mediated by the listerial surface protein ActA (see below).

VIRULENCE FACTORS

Hemolysin

The hemolysin gene, *hly*, was the first virulence determinant to be identified and sequenced in *Listeria* spp. Subsequent

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FIG. 3. Stages of listerial intracellular parasitism. (A) Scheme of the intracellular life cycle of pathogenic *Listeria* spp. (reproduced from reference 655 with permission of the Rockefeller University Press). In anticlockwise order: entry into the host cell by induced phagocytosis, transient residence within a phagocytic vacuole, escape from the phagosome into the cytoplasm, cytosolic replication and recruitment of host cell actin onto the bacterial surface, actin-based motility, formation of pseudopods, phagocytosis of the pseudopods by neighboring cells, formation of a double-membrane phagosome, escape from this secondary phagosome, and reinitiation of the cycle. (B to H) Scanning and transmission electron micrographs of cell monolayers infected with *L. monocytogenes* (B) Numerous bacteria adhering to the microvilli of a Caco-2 cell (30 min after infection). (C) Two bacteria in the process of invasion (Caco-2 cell, 30 min postinfection). (D) Two intracellular bacteria soon after phagocytosis, still surrounded by the membranes of the phagocytic vacuole (Caco-2 cell, 1 h postinfection). (E) Intracellular *Listeria* cells free in the host cell cytoplasm after escape from the phagosome (Caco-2 cell, 2 h postinfection). (F) Pseudopod-like membrane protrusion induced by moving *Listeria* cells, with the bacterium being evident at the tip (brain microvascular endothelial cell, 4 h postinfection; taken from reference 245 with permission). (G) Section of a pseudopod-like structure in which a thin cytoplasmic extension of an infected cell is protruding into a neighboring noninfected cell (notice that the protrusion is covered by two membranes) (Caco-2 cell, 4 h postinfection). (H) Bacteria in a double-membrane vacuole formed during cell-to-cell spread (Caco-2 cell, 4 h postinfection).

characterization of the *hly* locus led to discovery of the chromosomal virulence gene cluster in which most of the genetic determinants required for the intracellular life cycle of pathogenic *Listeria* spp. reside (see below). The *hly* product, Hly, was also the first virulence factor for which a precise role in the pathogenesis of *Listeria* infection was demonstrated. Hly is a key virulence factor essential for pathogenicity, having a vital role not only in intracellular parasitism but also in several other functions in the interaction of listeriae with their vertebrate host.

Characterization and role in escape from phagosome. In 1941, Harvey and Faber (269) demonstrated for the first time the production of a soluble hemolysin by *L. monocytogenes*. During the 1960s and 1970s, various researchers tried to purify this hemolysin and characterize its biochemical and toxic properties (218, 303, 305, 475, 620, 683). Jenkins et al. (303) were the first to provide evidence that the *Listeria* hemolysin is similar in function and antigenicity to streptolysin O (SLO) from *Streptococcus pyogenes*. Kingdon and Sword (334) showed that the hemolysin was inhibited by cholesterol, that its optimum pH was below 7, and that it had cytotoxic properties in phagocytic cells. They were also the first to suggest that the hemolysin might be involved in the disruption of phagosomal membranes (15, 334). In 1985, Vicente et al. (676) reported the molecular cloning of a chromosomal fragment from *L. monocytogenes* that conferred hemolytic activity on *E. coli*. These authors were the first to use molecular genetics in the study of listerial virulence factors. Finally, in 1987, Geoffroy et al. (207) provided the first unambiguous evidence that the hemolysin of *L. monocytogenes* is an SLO-related cytolysin belonging to the family of cholesterol-dependent, pore-forming toxins (CDTX). This toxin was given the name listeriolysin O (LLO), and one of its key characteristics was determined, its low optimum pH (5.5) and the narrow pH range at which it is active (4.5 to 6.5) (207). Later, the 58-kDa CDTX hemolysin of *L. ivanovii*, ivanolysin O (ILO), was purified and characterized (669), and it was also shown that the weakly hemolytic but nonpathogenic species *L. seeligeri* produces, albeit in small amounts, an LLO-related CDTX (208, 376).

The strong correlation between hemolytic activity and pathogenicity in the genus *Listeria* (the pathogenic species *L. monocytogenes* and *L. ivanovii* are hemolytic [see Fig. 5], whereas the nonpathogenic species are not; the exceptional case of *L. seeligeri* will be discussed below) (250, 605, 626) and the observation that the spontaneous loss of hemolysin production results in avirulence (288) led Gaillard et al. (195) and various other groups to generate isogenic hemolysin mutants of *L. monocytogenes* by transposon mutagenesis (108, 323, 518). These mutants were much less virulent in mice (increase of >4 log units in the LD_{50}). Spontaneous revertants from which the transposon was lost recovered both hemolytic activity and pathogenicity, and reintroduction of the *hly* gene in *trans* into the nonhemolytic mutants restored virulence to wild-type levels (108, 195, 323, 518). Genetic analysis of the point of insertion for one of these mutants led, in 1987, to the identification and characterization of the hemolysin gene (432, 436). Cell culture studies of the effects of *hly* inactivation showed that hemolysin is required for the survival and proliferation of *L. monocytogenes* within macrophages and nonprofessional phagocytes (194, 359, 518). Electron microscopy of *L. mono-*
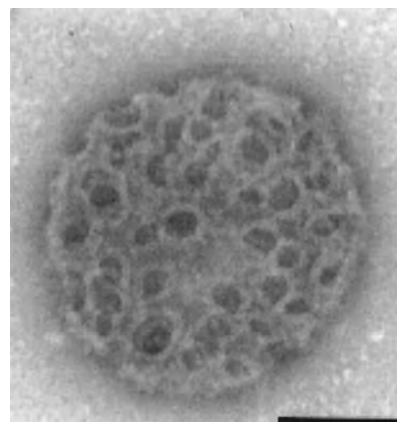

FIG. 4. Pore-forming activity of LLO. Sheep erythrocyte ghost after treatment with purified LLO, showing the ring-shaped oligomeric structures of the toxin attached to the membrane. (Bar, 100 nm.) (Reproduced from reference 300 with permission of the publisher).

cytogenes-infected cultured epithelial cells revealed that *hly* mutants remained within the vacuolar compartment, whereas hemolytic bacteria were free in the cytoplasm (194). The heterologous expression of *hly* in *Bacillus subtilis* conferred on this nonpathogenic bacterium the ability to escape from the phagocytic vacuole and to proliferate within cells (although it did not become pathogenic, indicating that virulence factors other than LLO were required for infection) (45). These studies demonstrated the key role played by LLO in the intracellular infectious cycle of pathogenic *Listeria* spp., as a mediator of phagosome membrane disruption. LLO not only mediates lysis of the primary phagosomes formed after the uptake of extracellular bacteria but is also required for the efficient escape of *L. monocytogenes* from the double-membrane vacuole that forms upon cell-to-cell spread (202) (Fig. 3D and H). The pores or membrane lesions caused by LLO (Fig. 4) probably facilitate the access of *Listeria* phospholipases to their substrates (see following section), leading to total dissolution of the physical barrier that delimits the phagosomal compartment.

Sequencing of the genes for LLO (436), ILO, and the *L. seeligeri* hemolysin (seeligerilysin [LSO]) (257) revealed a high degree of similarity at both the protein (86 to 91%) and nucleotide (76 to 78%) levels and confirmed that the deduced proteins are members of the CDTX family of cytolysins. This family of toxins has 23 recognized members from different gram-positive genera, all of which are very similar in structure (40 to 70% amino acid sequence similarity) and show antigenic cross-reactivity (7, 8, 633). Portnoy et al. (519) demonstrated that, despite their structural and functional similarity, not all LLO-related toxins can mediate escape from phagosomes. These authors expressed the genes encoding SLO and perfringolysin O (PFO, the CDTX from *Clostridium perfringens*) in *B. subtilis* and observed that only PFO facilitated intracellular growth. However, unlike LLO, PFO damaged host cells (519). The *pfo* gene was also unable to complement an *hly* mutation in *L. monocytogenes* for virulence in mice. The resulting strain was hemolytic and able to escape from phagosomes, but PFO again damaged the host cell, preventing the intracellular proliferation of the bacteria (310). This indicates that LLO has

evolved structural features to prevent cytoxicity which are not present in PFO from an extracellular pathogen. By selecting for *pfo*-expressing *L. monocytogenes* mutants capable of normal intracellular growth, the same research group identified PFO mutations capable of mediating lysis of the vacuole but devoid of toxic effects on the infected host cell (311). One type of mutation decreased the cytosolic half-life of PFO (see below). Another type of mutation, very interestingly, modified the pH optimum of PFO, making it similar to that of LLO, with low levels of activity at pH 7.4 (a pH similar to that of the eukaryotic cell cytoplasm). This low optimal pH, similar to that within phagosomes, is thought to provide the basis for compartmentalized activity of LLO in an acidified vacuole and for protection against the potential deleterious effect on cell membranes of leakage of the toxin into the cytoplasm. This is probably a key element in the adaptation of *L. monocytogenes* to intracellular parasitism. Direct evidence for a link between the low optimum pH and compartment-specific pore-forming activity of LLO in macrophage phagosomes has been provided by a recent study using the membrane-nonpermeating fluorophore 8-hydroxypyrene-1,3,6-trisulfonic acid (25). It was shown that *L. monocytogenes*-containing phagosomes acidify rapidly after bacterial uptake. This was followed by an increase in pH and dye release from the vacuole. Loss of the fluorophore was prevented by lysosomotropic agents, such as ammonium chloride and bafilomycin A1 (25). Bafilomycin A1 was also shown to block *L. monocytogenes* escape from the primary vacuole in epithelial cells (102). These experiments indicate that a lowering in pH results in activation of LLO present in the phagosome lumen, leading to membrane destabilization, pH equilibration, and consequent deactivation of LLO.

Recent experimental evidence has shown that a PEST-like sequence present at the N terminus of LLO and absent in PFO is responsible for the rapid degradation of the listerial toxin in the host cell cytosol (127a). PEST-like sequences are thought to target eukaryotic proteins for phosphorylation and degradation, and deletion of this sequence in *L. monocytogenes* LLO led to increased cytotoxicity and lower virulence. When the sequence was introduced in PFO and the chimeric toxin was expressed in *L. monocytogenes*, bacteria were less cytotoxic than those expressing wild-type PFO and were able to replicate intracellularly. These data suggest that adoption of a PEST motif in LLO is another strategy used by *L. monocytogenes*, together with the low pH optimum, to restrict the activity of the toxin to the host cell vacuole, thereby preserving the intracellular niche for bacterial proliferation (127a). A recent report has also shown that *L. monocytogenes* mutants expressing a PEST-deleted *hly* allele, although fully hemolytic, are strongly impaired in the ability to escape from the phagocytic vacuole. This suggests that the PEST motif in LLO may also play a specific role in the disruption of the phagosomal membrane, e.g., by serving as a ligand for targeting to that membrane other factors required for its efficient disruption (381a).

Structure-function. CDTXs typically have a conserved undecapeptide, ECTGLAWEWWR, in the C-terminal region (7, 8). Single-amino-acid substitutions in this undecapeptide in LLO produce *L. monocytogenes* strains with various degrees of attenuation, proportional to the loss of cytolytic activity of the respective hemolysin variants (440). Mutational analyses of the undecapeptide in several other CDTXs have also demon-

strated the importance of this motif for activity (511, 583, 608). LSO, the *L. seeligeri* hemolysin, has an Ala-to-Phe substitution in this conserved motif (257), and this may be one of the reasons for the lower hemolytic activity of this species. The undecapeptide contains the Cys residue that confers on CDTXs the characteristic after which this group of toxins was formerly designated (i.e., "thiol activated or oxygen labile"), that is, inhibition by oxidation or thiol-reacting compounds and reactivation by thiol-reducing agents (8, 633). It was formerly assumed that activation by thiol-reducing agents was due to the breakage of an intramolecular bond. However, once the sequences of many of these toxins became available, it was clear that only one Cys residue was present in most of the toxins of the family. It was also thought that this residue was essential for activity, presumably serving as the ligand in the interaction with membrane cholesterol (see below) (8, 633). However, replacement of this residue with Ala in LLO (440) and in other toxins of the family (511, 583) did not significantly affect activity, although it did render the molecule insensitive to inactivation by oxidation or thiol-alkylating agents. Replacement with Gly or bulkier amino acids, however, abolished most of the activity, indicating that the mechanism underlying Cys-mediated reversible inhibition of cytolytic function probably involves steric hindrance due to formation of toxin dimers or heterodimers with other proteins via disulfide bridges. Indeed, ILO copurifies with a major 27-kDa protein from the culture supernatant of *L. ivanovii* (349, 669) (the small, secreted internalin i-InlE [169]), and this heterodimer is resolved by treatment with thiol-reducing agents (669). Its nonessential role for cytolytic activity in vitro raises the question of why there is such strong selection pressure for conservation of the Cys residue among CDTXs. This residue may have a major role in vivo, for example, as a modulator of toxin activity (activation in low pH, reducing environments) or as a facilitator of host membrane targeting for other virulence-associated proteins (for example, i-InlE) that may associate with the CDTX in a heterodimer.

Cholesterol irreversibly inhibits the cytolytic activity of CDTXs, and these toxins are active only against cholesterol-containing membranes, reflecting that this membrane compound is a key toxin target (8, 633). The observation that soluble CDTXs form highly stable amphiphilic complexes with cholesterol led to the development of a simple one-step method to purify these toxins from the culture supernatant by selective precipitation with cholesterol (669, 670). With this technique, it was shown that a cytolytically inactive, truncated form of LLO lacking the C-terminal region including the undecapeptide was nonetheless able to form cholesterol-toxin complexes. The conclusion was that LLO possesses functional domains for cholesterol binding located outside the C-terminal region and independent from those involved in cytolysis. It also appeared clear that the undecapeptide containing the Cys residue was not essential for interaction of the toxin with cholesterol (670). Unfortunately, the ability of these truncated LLO molecules to bind to membranes was not tested. Such an experiment would have indicated whether cholesterol acts as the primary binding receptor for LLO. If this were so, then prior treatment of the toxin with cholesterol should block the cholesterol-binding sites on the molecule and prevent the interaction of the toxin with the target membrane. However, recent experiments have demonstrated that preincubation with cho-

lesterol does not impede the binding of LLO-cholesterol complexes to target membranes (300). This is consistent with experimental data showing that PFO mutations in critical residues of the undecapeptide resulting in impaired binding to membranes (see below) did not affect binding specificity for cholesterol (608). The mechanism by which CDTXs disrupt membranes involves the transition from monomeric, water-soluble toxin to noncovalently bound oligomeric, insoluble arc- and ring-shaped toxin structures that insert into the membrane, forming large pores about 20 to 30 nm in diameter (8, 41, 42, 452, 609, 633) (Fig. 4). An interesting observation was that although cholesterol-complexed LLO binds to target membranes, the toxin does not oligomerize to form pores (300). All these findings suggest that cholesterol is involved not in the initial binding of soluble toxin monomers to the membrane but at a subsequent stage of membrane-toxin interaction that leads to polymerization and membrane intercalation. Indeed, early observations of CDTX activity, showing that at 4°C cytolysis does not occur although binding is unaffected (8, 633), did already indicate that membrane binding and disruption are independent events in CDTX function. This is consistent with experimental data showing that most of the anti-LLO monoclonal antibodies that neutralize cytolytic activity do not affect binding to target membranes (123, 464). The ability of noncytolytic LLO-cholesterol complexes to bind cell membranes is also consistent with such complexes being able to induce cytokine expression (474), IL-1 release (707), and the induction of lipid second messengers (618), host cell responses that require prior interaction of the toxin with membrane receptors. This observation raises the interesting possibility that LLO released extracellularly and rendered noncytolytic by the formation of complexes with free cholesterol present in body fluids may be targeted to the cell membranes and mediate signaling events upon binding.

Membrane binding was not affected in LLO mutants with impaired cytolytic activity due to amino acid substitutions in the ECTGLAWEWWR undecapeptide, including Trp-to-Ala substitutions, which almost totally abolish toxin function (440). These data suggested that the motif itself is not primarily involved in the initial interaction of the toxin monomer with the target membrane. This contrasts with other experimental data showing that weakly hemolytic PFO mutants with Phe replacements in the three Trp residues of the Cys-containing region (Trp436, Trp438, or Trp439) had significantly lower erythrocyte membrane-binding activities (608). Circular dichroism spectral analyses of the membrane-associated form of these PFO mutant toxins suggested that both Trp438 and Trp439 are involved in a conformational change that occurs during oligomerization and pore formation (462). Thus, the impaired interaction of PFO mutant toxins with the membrane may reflect either that the motif is directly involved in the interaction with the membrane or that it is required for correct toxin folding during oligomerization and membrane insertion during pore formation. The latter interpretation is consistent with the observed inhibition of pneumolysin (PLY; the CDTX from *Streptococcus pneumoniae*) self-interaction by derivatization of the single Cys residue with the thiol-active agent dithio(bis)nitrobenzoic acid (213), suggesting that the domain carrying the undecapeptide is involved in toxin autoaggregation. The precise role of the conserved undecapeptide in

CDTX function is still not understood and further research on this aspect is clearly needed.

A picture of the possible mechanism underlying membrane disruption and cytolysis by CDTXs has begun to emerge in the last few years from crystallographic studies, functional modeling, and detailed structural analyses performed with SLO, PLY, and, in particular, PFO (214, 461, 493, 494, 566, 611). Given the high degree of sequence similarity between members of this toxin family it can be assumed with a reasonable degree of certainty that most of the predicted functional features are extrapolable to LLO. CDTXs are composed of four domains of which the first three would be involved in toxin oligomerization and membrane disruption and the fourth primarily in binding to the membrane. This is consistent with experimental data showing that the membrane-binding activity is associated with the C-terminal half of the toxin molecule (491) and that neutralizing antibodies that map to domain 4 inhibit binding to cellular membranes (130), whereas neutralizing epitopes that do not inhibit binding to membranes map to domain 1 (123). Membrane contact would facilitate the interaction of toxin molecules with cholesterol, which is buried in the membrane. Binding to cholesterol would then trigger a conformational change in the protein, increasing its hydrophobicity and membrane affinity, favoring a more intimate interaction between toxin monomers and the lipid layer. The resulting hydrophobic monomers would become partially embedded in the membrane, diffuse laterally, and collide with other monomers to form polymeric aggregates. This would explain why initial binding of CDTXs to the membrane is reversible and temperature independent whereas the subsequent steps are irreversible and are blocked at 4°C. At this low temperature, membrane dynamics are presumably restricted, resulting in kinetic energy requirements incompatible with membrane penetration, lateral diffusion, and oligomerization. A region that interacts with the membrane has recently been identified in domain 3. This region contains two transmembrane domains that undergo an α-helix to β-strand transition, creating a pair of hairpin structures that penetrate the membrane. There is some debate over the extent of membrane penetration; some studies claim that the pore is not a hole in the bilayer but instead an unusual phospholipid phase structure (214), whereas others favor the view that the the two amphipathic β-hairpins completely span the bilayer, creating a true pore (611).

Other roles in infection. Evidence is accumulating that LLO is a multifunctional virulence factor with many important roles in the host-parasite interaction other than phagosomal membrane disruption. Exogenous and endogenous exposure to LLO may induce a number of host cell responses, such as cell proliferation and focus formation in transfected fibroblasts (131), activation of the Raf–Mek–mitogen-activated protein (MAP) kinase pathway in epithelial cells (647, 647a, 685), mucus exocytosis induction in intestinal cells (97), modulation of internalization via calcium signaling (680) and cytokine expression in macrophages (355, 474), degranulation and leukotriene formation in neutrophils (619), apoptosis in dendritic cells (252), phosphoinositide metabolism (618), lipid mediator generation (618), NF-κB activation (330), and expression of cell adhesion molecules in infected endothelial cells (153, 330, 351). More details of these aspects of LLO can be found below.

LLO also plays a critical role in the protective immune response to *L. monocytogenes* infection in two ways. First, the LLO-mediated release of bacteria into the cytosol and subsequent intracellular growth are essential for major histocompatibility complex (MHC) class I-restricted listerial antigen presentation and the induction of specific protective cytotoxic CD8$^+$ T cells (23, 33, 71, 284, 285). Second, LLO is itself a major protective antigen recognized by *Listeria*-specific CD8$^+$ CTLs during listerial infection (32, 59, 281, 574, 625). LLO has been shown to be processed very efficiently into peptides that are presented by MHC class I molecules (677). One LLO peptide, nonamer LLO 91–99, is an immunodominant epitope that induces CD8$^+$ CTLs, which protect in vivo against *L. monocytogenes* infection and confer significant anti-*Listeria* immunity on naive mice upon passive transfer (265, 495). Pore formation by exogenous LLO has also been shown to mediate the delivery of soluble antigens to the TAP-dependent cytosolic MHC class I antigen presentation pathway (121, 122). This may be an additional mechanism involved in the generation of CD8$^+$ CTLs against antigens secreted by extracellular *L. monocytogenes* bacteria. LLO also elicits a potent humoral response, and several studies have shown that the detection of LLO-specific antibodies can be used in the serodiagnosis of *L. monocytogenes* infection in humans and animals (34, 211, 212, 248, 382, 394, 396). An old paradigm of cell-mediated immunity, that antibodies are not involved in protection against intracellular parasites (328), has recently been challenged by Edelson et al. (163), who showed that a murine anti-LLO neutralizing monoclonal antibody increases resistance to *Listeria* infection in mice. It is therefore possible that the humoral arm of the immune system is also involved in protection against *L. monocytogenes* infection and that LLO plays a key role here also.

Phospholipases

Pathogenic *Listeria* spp. produce three different enzymes with phospholipase C (PLC) activity that are involved in virulence. Two, PlcA and PlcB, are present in *L. monocytogenes* and *L. ivanovii*; the third, SmcL, is specific to *L. ivanovii*.

Characterization. Production of phospholipase activity by *L. monocytogenes* was first described in 1962 by Fuzi and Pillis (191), who reported opacity reactions in egg yolk agar that correlated in intensity with the hemolytic activity of the strains tested (see Fig. 12). This activity was detected in hemolytic fractions of *L. monocytogenes* culture supernatants and was at first wrongly identified as being due to the hemolysin itself (303, 304, 332). However, the application of careful fractionation procedures led to separation of the hemolytic and phospholipase activities (305, 620). The *L. monocytogenes* phospholipase was characterized as a secreted phosphatidylcholine (PC) cholinephosphohydrolase (PC-PLC;EC 3.1.4.3) similar to that responsible for the lecithinase activities of *Bacillus cereus* and *C. perfringens* (375). The purified PC-PLC/lecithinase is a zinc-dependent enzyme with an apparent molecular mass of 29 kDa, active over a wide pH range (5.5 to 8.0), and with a broad substrate spectrum (206, 223). On lipid micellar dispersions, this PLC rapidly hydrolyzes PC, phosphatidylethanolamine (PE), and phosphatidylserine (PS). It also, to a lesser extent, hydrolyzes sphingomyelin (SM) but does not or only very

weakly hydrolyzes phosphatidylinositol (PI) (206, 223). However, PI is rapidly hydrolyzed in erythrocyte ghosts and in PI-PC-cholesterol liposomes, leading to the formation of inositol-l-phosphate (223). The purified enzyme is atoxic for mice; it is weakly hemolytic in guinea pig, horse, and human erythrocytes but not in sheep erythrocytes (206).

Lecithinase activity, like hemolytic activity, is an easily recognizable phenotypic marker closely associated with pathogenicity in the genus *Listeria* (528) (see Fig. 12). As for *hly*, transposon mutagenesis was used to identify the lecithinase gene. Two types of lecithinase-deficient, attenuated-virulence mutants were isolated. In the first type, the transposon was inserted into *mpl* (532), which encodes a zinc-metalloprotease (140), and the first gene of a 5.7-kb operon located immediately downstream from *hly* (434) (see Fig. 8). The second type of lecithinase-deficient mutant resulted from transposon insertion into the *actA* gene, located downstream from *mpl* in the operon and encoding the actin nucleator ActA (672) (see following section). Cloning of the DNA region targeted by the transposons in this mutant led to identification of the structural gene encoding the nonspecific listerial PLC, *plcB*. This gene occupies the third position in the 5.7-kb operon, called the lecithinase operon (see Fig. 8). The sequence of the *plcB* product, PlcB, is very similar to those of the PC-PLC precursor of *B. cereus* (38.7% identity over 253 amino acids) and the α-toxin precursor from *C. perfringens* (22.4% identity over 223 amino acids) (672). Western immunoblotting of PlcB in the *L. monocytogenes* culture supernatant detects two antigenically related bands of 33 and 29 to 30 kDa (206, 470, 532). This indicates that PlcB, like the *B. cereus* and *C. perfringens* PC-PLCs (309, 657), is secreted as an inactive proenzyme that is processed in the extracellular medium by proteolytic cleavage. The secreted PlcB proenzyme contains 264 amino acids, including a 26-residue N-terminal propeptide of 2.9 kDa, which is cleaved to give the faster-migrating mature, active enzyme. PlcB must be secreted in an inactive form to prevent bacterial membrane damage due to degradation of its phospholipids. The lecithinase-deficient *mpl* insertion mutant described above produced only the larger PlcB band corresponding to the inactive proenzyme (532), and complementation of this mutant with *mpl* restored the lecithinase phenotype and production of the active 29- to 30-kDa PlcB form (520). Thus, Mpl, which is encoded by the first gene of the lecithinase operon and, like PlcB, is also a zinc-dependent metalloenzyme, is involved in processing of the PC-PLC/lecithinase into its mature and active form in the *L. monocytogenes* culture supernatant.

The *plcA* gene, which encodes a PI-specific PLC, was identified in *L. monocytogenes* earlier than *plcB* by chromosome walking in the region upstream from *hly* (82, 377, 431) (see Fig. 8). Its secreted product, PlcA, is a 33-kDa polypeptide similar to the PI-PLCs from *Bacillus thuringiensis, B. cereus*, and *Staphylococcus aureus* (around 30% primary sequence identity) and eukaryotic PI-PLCs, such as that produced by *Trypanosoma brucei* (431). The purified PlcA enzyme is highly specific for PI, with a pH optimum between 5.5 and 6.5 in Triton X-100-mixed micelles, suggesting that, like LLO, it would be active in acidified phagocytic vesicles. No hydrolyzing activity was observed with PC, PS, PE, PI 4-phosphate, or PI 4,5-bisphosphate (222). The enzyme is able to hydrolyze glycosyl PI (GPI)-anchored eukaryotic membrane proteins (222, 431), although much less
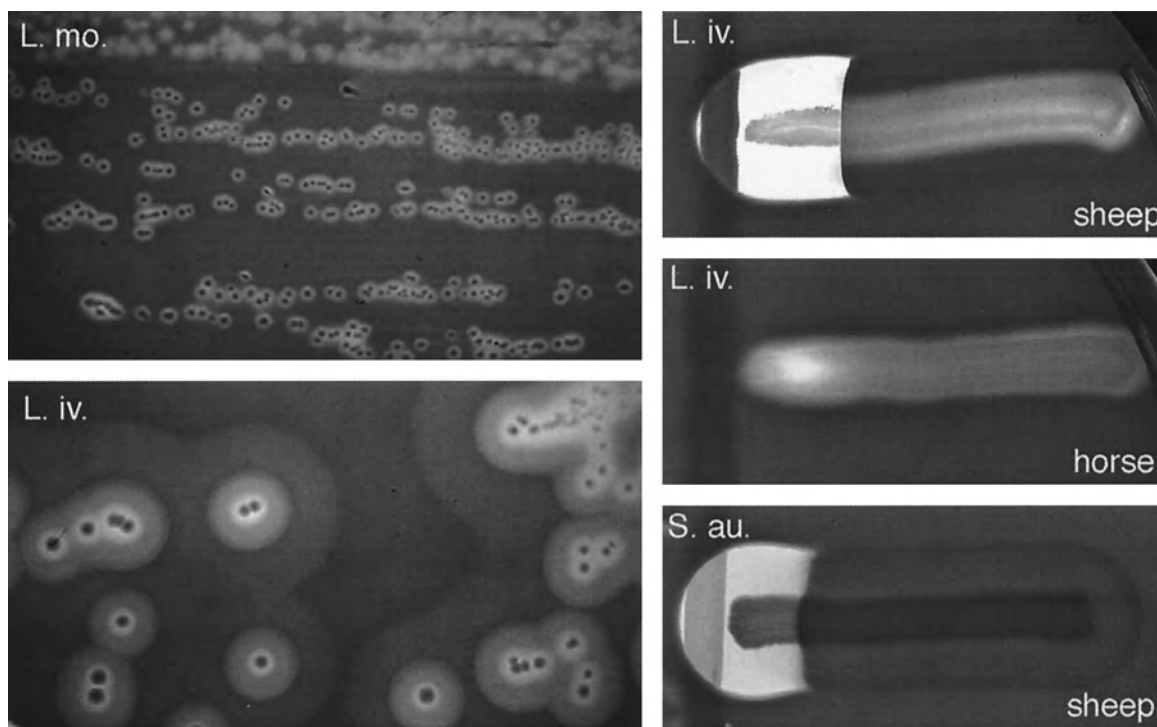
FIG. 5. Hemolytic activities of pathogenic *Listeria* spp. Left panels, spontaneous hemolysis of *L. monocytogenes* (L. mo.) and *L. ivanovii* (L. iv.) on sheep blood agar; compare the narrow ring of β-hemolysis produced by *L. monocytogenes* (here exceptionally evident) with the striking multizonal lytic activity of *L. ivanovii*, which is due to the production of an additional cytolytic factor (the sphingomyelinase SmcL). Bacteria were cultured at 37°C for 36 h and then left at 4°C for 48 h. Right panels, synergistic (CAMP-like) hemolysis of *L. ivanovii* and β-toxin (sphingomyelinase)-producing *S. aureus* (S. au.) with *R. equi* (vertical streak); *L. ivanovii* and *S. aureus* elicit identical shovel-shaped patches of synergistic hemolysis, reflecting functional relatedness of the sphingomyelinases C produced by these two bacteria (see text and reference 226 for details). The CAMP-like effect results from the total lysis of the halo of sphingomyelinase-damaged erythrocytes exposed to a cholesterol oxidase from *R. equi* (467). As shown in the two upper right panels, the *L. ivanovii* sphingomyelinase is active towards sheep erythrocytes (which contain 51% sphingomyelin in the membrane) but not towards horse erythrocytes (13.5% sphingomyelin only). Note that the inner zone of spontaneous hemolysis that surrounds *L. ivanovii* colonies, due to the activity of the hemolysin (ivanolysin O), is not influenced by the source of erythrocytes. Bacteria were cultured at 37°C for 24 h.

efficiently than the orthologous enzyme from *B. thuringiensis* (200).

The animal pathogen *L. ivanovii* produces an additional PLC that is specific for SM. This SMase was purified from *L. ivanovii* culture supernatant after selective removal of ILO by sequestration with cholesterol and shown to contribute to the hemolytic activity of this species (669). In contrast to *L. monocytogenes*, which is weakly hemolytic, *L. ivanovii* produces a strong bizonal lytic effect on sheep blood agar, with an inner halo of complete hemolysis surrounded by a ring of incomplete hemolysis similar to that for β-toxin-producing *S. aureus* and *B. cereus* (546, 607, 669) (Fig. 5). The outer ring of incomplete hemolysis is completely lysed if erythrocytes are exposed to a soluble product released by the actinomycete *Rhodococcus equi* (546, 626, 667, 668) (a cholesterol oxidase [467]), giving rise to a characteristic shovel-shaped patch of synergistic hemolysis (Fig. 5). This CAMP-like hemolytic reaction is routinely used for the identification of *L. ivanovii* (605, 606, 668). The gene encoding the *L. ivanovii* SMase, *smcL*, has recently been identified and sequenced. Its predicted protein product, SmcL, is highly similar (around 50% identity) to the known bacterial SMases from *S. aureus* (β-toxin), *B. cereus*, and *Leptospira interrogans* (226). Gene disruption and complementa-

tion studies have demonstrated that *smcL* is the determinant responsible for the differential hemolytic properties of *L. ivanovii* (225, 226). SmcL is functionally very similar to the *S. aureus* β-toxin in terms of membrane-damaging activity, as revealed by the similar ability of the two molecules to elicit shovel-shaped CAMP-like reactions with *R. equi* and the lytic selectivity for SM-rich membranes (both enzymes lyse sheep erythrocytes, in which SM accounts for 51% of total membrane phospholipids, but not horse erythrocytes, in which the SM content is only 13.5%) (Fig. 5). However, SmcL does not cause the hot-cold lysis typical of β-toxin (unpublished data), indicating that, despite their structural and functional similarities, there are differences in the mechanisms underlying membrane damage between these two homologous bacterial SMases.

Role in virulence. The observation that Hly⁻ mutants of *L. monocytogenes* are able to escape to the cytosol and grow within certain human epithelial cells (Henle 407 and HeLa cells) suggested that listerial membrane-active products other than Hly may be involved in phagosome disruption (518). Such a role for a listerial phospholipase was first shown for PlcB. Sequential insertional mutagenesis of the lecithinase operon of *L. monocytogenes* revealed that a *plcB* mutant formed smaller plaques in infected J774 macrophage monolayers and accumu-

lated within double-membrane vacuoles (672). This indicated that the PlcB phospholipase is required for efficient lysis of the secondary phagosomes formed after listerial cell-to-cell spread (Fig. 3H). Using in-frame deletion mutants, Hly was shown to be dispensable for efficient lysis of the primary vacuole of Henle 407 cells as long as PlcB was produced, suggesting that PlcB also mediates escape from primary phagosomes in these cells (414). Immunofluorescence studies with infected cells showed that PlcB accumulates in *L. monocytogenes*-containing vacuoles, colocalizing with the endosomal-lysosomal marker Lamp1 (415). In the absence of Hly, a Δmpl mutation caused defects in escape from primary phagosomes and cell-to-cell spread similar to those in a $\Delta plcB$ mutant or a double Δmpl $\Delta plcB$ mutant, consistent with Mpl's being involved in the activation of pro-PlcB (414, 415). Using the Δmpl mutant, it was shown that intracellular activation of proPlcB is mediated not only by Mpl, but also via an Mpl-independent pathway presumably mediated by a lysosomal cysteine protease. PlcB activation by both pathways is sensitive to bafilomycin A1, an inhibitor of the vacuolar proton pump ATPase, indicating that this activation is, as for LLO, low-pH dependent and hence vacuolar compartment specific (415). A recent study has shown that intracellularly multiplying bacteria contain pools of preformed pro-PlcB. These pools are suddenly secreted when pH decreases below 7.0 by an unknown mechanism involving the Mpl-mediated release of active enzyme (416). The amount of the processed form of PlcB, but not that of proPlcB, is significantly lower in cells infected with an ActA⁻ mutant, indicating that proPlcB proteolytic activation occurs predominantly in secondary vacuoles (415). This is consistent with PlcB's playing a key role in cell-to-cell spread. PlcB has recently been shown to be required for intercellular spread from macrophages to different types of mammalian cells, including brain microvascular endothelial cells in vitro (246), and for spread in murine brain tissue in vivo (589). This provides experimental evidence for a major role of PlcB-facilitated cell-to-cell spread in the in vivo physiopathogenesis of *Listeria* infection.

plcA-deficient strains were initially isolated during a search for molecular determinants involved in cell-to-cell spread based on the screening of a library of transposon-induced mutants for small-plaque formation (82, 641). These *plcA* insertion mutants were 1,000-fold less virulent in mice, failed to grow in mouse organs, and were defective in intracellular proliferation in macrophages (82). However, this severe virulence deficiency was due to a polar effect on the downstream gene *prfA*, which encodes the transcriptional activator of *Listeria* virulence genes with which *plcA* is cotranscribed (see below). Subsequent studies with an in-frame *plcA* deletion mutant revealed only a slight reduction in virulence and in the ability to escape from the primary phagosomes of murine bone marrow macrophages (83) (see below). Another study showed that the expression of *plcA* in the nonpathogenic species *L. innocua* promotes transient intraphagolysosomal multiplication of this otherwise nonintracellularly replicating bacterium (597). The relevance of this observation to the role of *plcA* in pathogenesis is, however, unknown. Insight into the contribution of PlcA to virulence could only be obtained by systematic comparison of the behavior of single and double *plcA* and *plcB* deletion mutants in mice and cell culture infection assays (629). As expected, the absence of PlcA led only to a minor

defect in in vivo virulence (threefold increase in mouse LD_{50}) and in escape from primary vacuoles. Cell-to-cell spread was unaffected. The lack of PlcB, on the other hand, resulted in more significant attenuation (20-fold increase in LD_{50}) and, as noted earlier, defective cell-to-cell spread, but escape from primary phagosomes of bone marrow macrophages was not impaired. However, a double $\Delta plcA$ $\Delta plcB$ mutant was found to be severely impaired in virulence (500-fold increase in LD_{50}) and in its ability to escape from the primary phagosome and to spread from cell to cell (414). In a Δhly background, PlcB was found to be essential, but PlcA made only a minor contribution to escape from primary vacuoles (414). Overall, these data indicated that PlcA has only a minor individual role in virulence but acts synergistically with PlcB and Mpl (which themselves also have an accessory role in virulence) to achieve, in conjunction with LLO, optimal levels of escape from primary and secondary phagosomes.

Recent experimental evidence has indicated that the *L. ivanovii* SMase also plays an accessory role in pathogenesis similar to that of PlcA and PlcB. A lack of SMase production in *L. ivanovii* results in a moderate decrease in virulence (fourfold increase in mouse LD_{50}) and in intracellular growth rate in bovine epithelial cells. Complementation with the *smcL* gene facilitated intracellular survival of the nonpathogenic species *L. innocua* in macrophages and conferred on an *L. monocytogenes* mutant devoid of any known membrane-damaging virulence determinant (*hly*, *plcA*, and *mpl*/*plcB*) as well as of the *actA* gene the ability to proliferate within epithelial cells. Electron microscopy of these cells showed that SmcL mediates the disruption of primary vacuoles and the release of bacteria into the cytosol (225, 226).

There are several possible reasons to explain the presence in pathogenic *Listeria* spp. of such a panoply of phospholipases with redundant functions in phagocytic vacuole destabilization. First, the relative amounts of membrane phospholipids differ between cells (5). Second, in a given cell type, membrane phospholipids are asymmetrically distributed in the lipid bilayer, with some mostly present in the outer (SM) or inner (PE, PS, and PI) lipid leaflets and others equally represented in the two lipid layers (PC) (5). Third, membrane asymmetry is inverted during phagocytosis, and in double-membrane phagosomes, there is presumably a mixed situation, with bacteria bound by membranes with normal and inverted lipid asymmetries. Last, transmembrane asymmetry is also modified in a number of physiological and pathological conditions, such as during apoptosis or during cellular activation associated with increased levels of intracellular Ca^{2+} (39, 360), so it is likely that variations in phagosome membrane lipid composition occur during the host cell adaptive response to infection. Consequently, (i) the contribution of each phospholipase to phagosome disruption may differ according to cell type and (ii), in the same cell type, the sequential or combined action of several phospholipases with different substrate specificities and mechanisms of action may be important for optimal bacterial escape from phagosomes with variable membrane lipid bilayer topology and composition. In this scenario, the PlcB phospholipase, which is able to degrade most membrane phospholipids, is likely to be a key player in phagosome disruption whatever the composition and distribution of lipids in the membrane, leading to complete dissolution of the physical barrier that sepa-

rates bacteria from their cytoplasmic niche. The role of PlcA, in contrast, is more difficult to understand if we consider that PlcB has been reported to efficiently hydrolyze PI in biological membranes and liposomes in vitro (see above). PI is moreover a minor lipid in eukaryotic membranes. However, it is clear that PlcA potentiates the membrane-damaging activity of PlcB, which may not depend exclusively on enzymatic activity itself but on particular structural features of the enzyme molecule affecting its membrane insertion and the accessibility to its specific substrate in natural conditions. The role in pathogenesis played by the cleavage of GPI-anchored surface proteins by PlcA is unknown. The production by *L. ivanovii* of an additional phospholipase specific for SM is particularly intriguing if we take into account that *plcA* and *plcB* are sufficient for pathogenesis in *L. monocytogenes*. It is possible that the hemolysin and the PlcA/PlcB phospholipase tandem of *L. ivanovii* are less efficient in disrupting the phagocytic vacuole and that SmcL is required to facilitate disruption of the SM-rich luminal leaflet of phagosomes. Another possibility, suggested by the selective membrane-damaging activity of the enzyme for sheep erythrocytes, is that SmcL is involved in the pathogenic tropism of *L. ivanovii* for ruminants (226). An attractive hypothesis is that SmcL has specifically evolved in *L. ivanovii* because some of the ruminant cell types or vacuolar compartments important in pathogenesis have, like ruminant erythrocytes, membranes containing large amounts of SM. Consistent with a role for SmcL in host adaptation, expression of *smcL* was shown to promote intracellular growth of the *L. monocytogenes* mutant devoid of any membrane-damaging factor in bovine MDBK cells but not in canine MDCK cells, a similar kidney-derived epithelial cell line from a mammalian species that is not naturally susceptible to *L. ivanovii* (225).

Besides their mechanical function in escape from the phagosome, listerial PLCs may also play an important role in pathogenesis by subverting the host signaling pathways mediated by phospholipid hydrolysis products such as diacylglycerol (DAG), ceramide (CER), and inositol phosphates. These lipid metabolites regulate key cell processes, including cell growth and differentiation, apoptosis, and the synthesis of cytokines and chemokines (346, 596), and hence their generation during *Listeria* infection may play an important role in adapting host cell and tissue inflammatory responses to the needs for intracellular life and host tissue colonization. Higher levels of lipid turnover with accumulation of DAG and CER have been shown to occur 3 to 4 h after infection of murine J774 macrophages by *L. monocytogenes*. An involvement of bacterial PLCs in this DAG and CER generation activity was suggested by the fact that cells infected with a ΔplcA ΔplcB mutant showed a smaller increase in the levels of these mediators (629). However, this double mutant still induced the generation of DAG and CER (629), suggesting the involvement of endogenous phospholipases in this host cell response. Pathogenic *Listeria* spp. also induce phosphoinositide metabolism and DAG accumulation in infected human umbilical vein endothelial cells (HUVECs), and this response was shown, using mutant bacteria and exogenously added purified proteins, to be dependent on the synergistic interaction between LLO and PlcA (617). Since prior treatment with LLO was a prerequisite for the observation of such a response upon external stimulation of the cells, it was suggested that LLO-induced pores provide

PlcA with access to PI in the inner membrane leaflet (617). Similar LLO-PlcA cooperation-dependent induction of phosphoinositide hydrolysis and concomitant cell activation has been observed in neutrophils (619). The listerial phospholipases PlcA and PlcB have also been shown to be required for persistent NF-κB activation in P388D1 macrophages (272) and for full induction of CER generation associated with NF-κB activation and enhanced E-selectin expression and neutrophil rolling and adhesion in HUVECs (600). An association between PlcA and PlcB expression, intracellular Ca^{2+} signaling, and listerial internalization has been reported in J774 macrophages during early time points of infection (680), but the underlying mechanism and true relevance of these observations are unknown. Recently, using *L. ivanovii* isogenic mutants and heterologous expression in an *L. monocytogenes* double *plcA plcB* mutant, it has been shown that the SmcL SMase mediates CER generation in infected host cells. This required the previous release of bacteria into cytosol and was associated with increased apoptosis of infected MDBK cells (González-Zorn et al., unpublished data).

ActA

A great deal of attention has been paid in recent years to the study of the actin-based intracellular motility of pathogenic *Listeria* spp. (27, 87, 105, 105a, 147, 366a, 401, 402, 515, 581, 630, 636). No classical motor proteins such as myosin have been found to be involved in bacterial intracellular motility, and the actin tail of moving *Listeria* organisms is functionally similar to the poorly understood actin polymerization process observed in lamellipodia, responsible for the locomotion of eukaryotic cells. Therefore, interest in listerial actin-based motility has transcended microbial pathogenicity, as it provides cell biologists with a relatively simple model for dissecting the molecular mechanisms underlying actin cytoskeleton assembly and function. This model has the enormous advantage that a single bacterial protein is sufficient to promote the actin recruitment and polymerization events responsible for intracellular movement. This protein is ActA (142, 339), the product of the *actA* gene (672).

The central role of ActA in listerial intracellular motility and virulence was initially revealed by the unusual phenotype of an isogenic *actA* mutant of *L. monocytogenes* in infected tissue culture cells (339). After entry, these bacteria were capable of escaping into the host cytoplasm, but could not move intracellularly and accumulated as microcolonies in the perinuclear area of the cell (Fig. 6). The mutant was unable to recruit actin, as shown by staining with fluorescein isothiocyanate-labeled phalloidin, a fungal toxin that binds to F-actin and paralyzes the actin cytoskeleton. In addition, the *actA* mutant was highly attenuated in the mouse infection model (142, 339).

The *actA* gene encodes a secreted 639-amino-acid protein with a 610-residue mature form that is attached to the bacterial cell wall via its C-terminal region (Fig. 6). Several lines of experimental evidence have demonstrated the essential role of ActA in actin-based bacterial motility. Transfection experiments in epithelial cells showed that ActA is targeted to the mitochondrial surface and that these organelles recruit actin filaments in a way similar to *actA*-expressing bacteria (513). Microfilament-associated proteins such as α-actinin and fim-
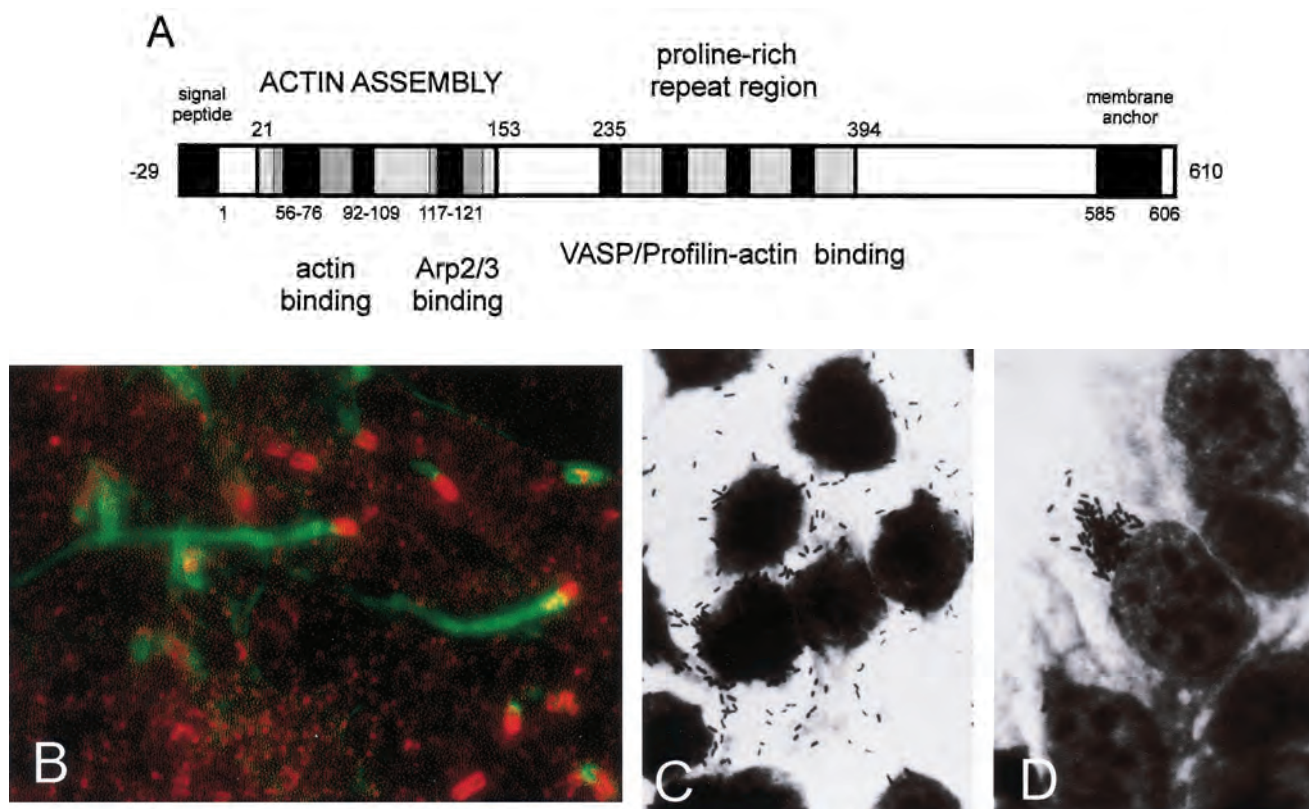
FIG. 6. Actin-based intracellular motility. (A) Schematic structure and functional motifs of ActA (see text for details; amino acids numbered according to references 339 and 672). (B) Cos-1 cells infected with *L. monocytogenes* and labeled with FITC-phalloidin (actin stain, green) and rhodamine-conjugated anti-*Listeria* antibody (red) at 4 h postinfection; moving bacteria with actin tails are visible. (C and D) Giemsa stain of Caco-2 cells infected with *L. monocytogenes* wild-type (C) and Δ*actA* mutant (D) at 4 h postinfection; Δ*actA* mutant bacteria do not move intracellularly and do not spread to neighboring cells, growing in the cytoplasm as a microcolony close to the nucleus.

brin were also recruited to the ActA-decorated mitochondria in transfected cells. Thus, ActA, whether anchored to the bacterial or mitochondrial membrane, can induce the formation of an actin cytoskeleton. Although transfection experiments conclusively demonstrated that ActA alone was essential for actin filament recruitment, they did not show how this protein generates intracellular bacterial motility. This was achieved by heterologous expression of the *actA* gene in *L. innocua* and *S. pneumoniae*, bacteria that are otherwise incapable of actin-based motility. This demonstrated that ActA itself mediates actin polymerization-driven bacterial movement (340, 631).

Mature ActA from *L. monocytogenes* can be arbitrarily divided into three distinct domains: (i) N-terminal domain (amino acids 1 to 234), which is rich in cationic residues; (ii) central region of proline-rich repeats (amino acids 235 to 394); and (iii) C-terminal domain (amino acids 395 to 610), with a highly hydrophobic stretch spanning residues 585 to 606 that anchors the protein to the bacterial surface (Fig. 6). Early structure-function studies with *actA* deletion constructs expressed in transfected cells (189, 513) or in a *Listeria* background and tested in *Xenopus* egg extracts (365, 409) permitted an initial attribution of specific functions to these domains of the ActA protein. The N-terminal region plays an essential role, as it contains all the information necessary to initiate F-actin assembly and bacterial movement (Fig. 6). A domain rich in positively charged residues (amino acids 129 to 153) in this region was identified as being strictly required for actin assembly. Amino acids 117 to 121 were shown to be critical for filament elongation, and deletion of amino acids 21 to 97 led to discontinuous actin tail formation, suggesting a role in maintenance of the dynamics of actin-based motility for this domain. Interestingly, a synthetic peptide corresponding to part of this domain (residues 33 to 74) was shown to interact with F-actin in vitro (365). In the central region, a domain of proline-rich repeats (amino acids 265 to 396) is required for binding of the focal contact proteins vasodilator-stimulated phosphoprotein (VASP) and Mena (see below). *Listeria* mutants lacking this region of ActA are still able to recruit actin to the bacterial surface and to move intracellularly, albeit at significantly lower speeds. This indicates that while the VASP and Mena proteins are not essential for the initiation of actin assembly on the bacterial surface, they are important for the efficiency of the process (366, 472, 632). There is significant structural and functional similarity between the C-terminal half of ActA (including the proline-rich repeat region) and zyxin (224), a eukaryotic protein associated with focal adhesions and actin stress fibers that may be considered a eukaryotic homolog of the listerial surface protein.

The mechanism by which ActA induces actin polymerization and bacterial motility is now beginning to be understood. ActA

is not detected in the actin tail (341, 471), so clearly the listerial surface protein is not necessary for stabilization of the actin meshwork left behind by moving *Listeria* cells. In the proximal region of this tail, F-actin filaments tend to be unidirectionally polarized, with barbed ends oriented towards the bacterial cell surface, indicating that actin nucleation is occurring at this point (655, 656). This suggested that ActA could act as an actin nucleator. However, globular (G)-actin monomer-binding activity could not be unequivocally demonstrated in ActA until very recently (96, 708), so the question arose whether ActA must be modified in vivo in the mammalian cell to have adequate actin-binding function (e.g., by phosphorylation [70]), or whether another host molecule interacts with ActA and cooperates with it to trigger actin nucleation. Systematic searches for such a host factor among known actin cytoskeleton-binding, cross-linking, and regulatory proteins revealed that many, including α-actinin, tropomyosin, talin, vinculin, fimbrin (plastin), filamin, villin, gelsolin, ezrin/radixin, cofilin, profilin, coronin, Rac, CapZ, Arp3, and VASP, colocalize with *Listeria*-associated actin filaments (86, 105, 117, 126, 139, 338, 361, 409, 514, 564, 650, 653).

Profilin and VASP were found exclusively at the point of actin assembly, in close association with the bacterial cell (88, 653), suggesting that they are actively involved in initiation of the *Listeria*-induced actin polymerization process. Profilin is a G-actin-binding protein, and its function in actin assembly is to bring ATP-actin to the barbed end of the filament in a complex that lowers the critical concentration for polymerization. Profilin interacts with polyproline (503), and poly-L-proline beads have been used to deplete profilin from cell extracts, resulting in the abolition of actin tail formation and bacterial motility (653). As profilin colocalizes with ActA at the site of actin polymerization and ActA has four polyproline motifs, it was thought that profilin was involved in actin nucleation by recruiting G-actin to the site of ActA expression on the bacterial surface. However, profilin was shown to associate only with bacteria in cell extracts or within infected cells, and not with ActA-expressing bacteria grown in culture medium, indicating that it requires an additional host factor to interact with ActA (653). VASP, in contrast, was shown to interact directly with ActA in vitro (88). VASP and its homolog Mena, which is also present between the bacterial surface and the growing actin tail (210), normally colocalize with actin filaments in focal contacts and in dynamic membrane processes (515, 538). VASP and Mena belong to the *Drosophila* Enabled (Ena)/VASP family of proteins, known to be involved in neural development. They are organized into three domains, an EVH1 (Ena-VASP homology 1) domain, a proline-rich central domain, and an EVH2 C-terminal domain. VASP has been shown to act as a ligand for profilin, vinculin, and zyxin (88, 210, 319, 537). Thus, by binding to ActA and profilin, VASP and Mena may establish a direct connection between intracellular *Listeria* cells and the cytoskeletal components of the host cell (210, 367). The EVH1 domain of the Ena/VASP-like proteins has been shown to bind directly to the proline-rich motif FPPPP, repeated three to four times in ActA, depending on the *L. monocytogenes* strain. This proline-rich ActA sequence is a novel polyproline motif that mimics the natural interaction between Ena/VASP proteins with their endogenous ligands in the focal adhesion proteins zyxin and vinculin. VASP has also been

shown to interact with filamentous (F)-actin via its C-terminal EVH2 domain (85, 367, 472). These data strongly suggest that VASP may act as a bridge between polymerization-competent profilin/actin complexes and ActA and between ActA and the actin tail itself, thereby favoring assembly of the actin network that is used for bacterial propulsion across the cytosol. However, profilin is not essential for listerial actin-based motility (409), and the same is true for Ena/VASP-like proteins because, as indicated above, deletion of their ligand motif on ActA does not prevent (although it does impair) listerial actin-based motility.

A major clue to the mechanism by which ActA induces actin polymerization came from recent experiments showing that the N-terminal 264 amino acids of the protein interact with the Arp2/3 complex, resulting in a dramatic increase in actin nucelation activity (690, 691). First identified in *Acanthamoeba*, the Arp2/3 complex, which includes two actin-related proteins, Arp2 and Arp3, caps pointed ends, cross-links actin filaments, and has weak actin nucleation activity (403, 456). This complex was shown to initiate actin assembly on the surface of *Listeria* cells in a reconstituted cell-free system (689). The Arp2/3 complex is activated by proteins of the Wiskott-Aldrich syndrome protein (WASP)/Scar family, which connect the cytoskeleton with the signaling pathways involved in the dynamic remodeling of the actin framework (404, 562, 700, 704). It has been demonstrated that the p21-Arc subunit of the Arp2/3 complex binds to the C-terminal domain of Scar1 and the related WASP. Strikingly, the N-terminal 264 amino acids of ActA, the C-terminal p21-Arc-binding fragment of Scar1, and the C-terminal fragment of WASP are similar in sequence, and all increase the nucleation activity of the purified Arp2/3 complex (404). Indeed, C-terminal fragments of Scar1 that bind Arp2/3 completely block actin tail formation and *Listeria* motility in cell extracts and in cells overexpressing the corresponding Scar1 constructs, although *Listeria* organisms are able to initiate actin cloud formation. Motility is restored by adding purified Arp2/3 complex (419). Thus, Arp2/3 is clearly an essential host cell factor for actin-based motility, and the major role of ActA is probably to regulate the activity of this multiprotein complex. Recent studies have demonstrated that the N-terminal region of ActA plays a critical role in binding and stimulating the nucleation activity of the Arp2/3 complex (514a, 626a, 708). Two domains in this ActA region (residues 56 to 76 and 92 to 109), with sequence similarity to WASP homology 2 domains, bind to actin monomers (708) (Fig. 6). A third N-terminal domain (residues 115 to 150, in particular the positively charged motif 117-KKRRK-121), with sequence similarity to the Arp2/3 homology region of WASP family proteins, binds Arp2/3 and competes for this binding with the the WASP family proteins N-WASP and Scar1 (514a, 708) (Fig. 6). It thus appears that ActA uses a mechanism similar to that of the endogenous WASP family proteins to promote Arp2/3-dependent actin nucleation.

However, using pure proteins in solution with assembled F-actin in the presence of ATP (the hydrolysis of which releases free energy for actin assembly), Loisel et al. (389) demonstrated recently that Arp2/3 in combination with VASP or WASP is not sufficient for actin-based motility. Wild-type movement was only reconstituted upon addition of capping protein and actin depolymerizing factor (ADF or cofilin), pre-

viously shown to be required for the depolymerization of actin tails (564) and to increase the rate of propulsion of *L. monocytogenes* in highly diluted, ADF-limited cell extract (86). In accordance with these observations, a cofilin homology sequence similar to that in WASP family proteins has been identified in the N-terminal fragment of ActA and shown to be critical for stimulating actin nucleation with the Arp2/3 complex in vitro and for actin-based motility in cells (626a). These data indicate that adequate turnover and the maintenance of high levels of ATP-G-actin and profilin-actin are essential for elongation of the actin tail and motility (389).

Gelsolin, a protein that caps barbed ends and severs actin filaments and which concentrates behind motile bacteria, was also shown to increase the length of actin tails and to support higher intracellular velocities. However, unlike ADF, gelsolin is not essential for *Listeria* motility (361). Loisel et al. (389) also confirmed that neither profilin nor VASP is essential for actin-based motility, although their absence causes the rate of bacterial movement to decrease 3- to 10-fold. Similarly, the actin filament cross-linking protein α-actinin, which is uniformly distributed over the actin tail (117), is not required for movement, but in its absence there is an apparent lack of rigidity in the actin tail and a greater tendency for bacteria to drift in solution (389). These findings emphasize that while relatively few proteins are sufficient to build the core of the actin motor of *L. monocytogenes*, many other host cell proteins, some with as-yet-unknown functions, may be involved in the actin cytoskeletal processes that lead to ActA-mediated listerial intracellular motility. Among these proteins is LaXp180 (506), recently identified using the yeast two-hybrid system aproach and shown to colocalize with intracellular *Listeria* cells. LaXp180 is a putative binding partner of the phosphoprotein stathmin, involved in the regulation of microtubule dynamics. By immunofluorescence it was demonstrated that stathmin also colocalizes with intracellular ActA-expressing *L. monocytogenes* (506). The role in infection of these newly identified proteins that are recruited to the listerial cell surface remains to be elucidated.

The motile force for intracellular bacterial movement is thought to be provided by the continuous deposition of actin monomers at the interface between the bacterial cell surface and the growing tail of tangled actin filaments immobilized in the cytosol that serves as a support structure for propulsion. To be effective, this motion system requires that actin deposition occur at only one pole of the bacterial cell, pushing it forward. This is indeed the case, as shown by the fact that the actin tail extends only from one end of the bacterium. This unidirectional actin polymerization appears to be related to the asymmetrical distribution of ActA on the bacterial cell. ActA accumulates at one of the bacterial poles and is totally absent from the other (the newly formed pole after bacterial division), which corresponds to the leading edge of movement opposite the place where actin tail formation occurs (341, 656). The basis of this polar ActA expression is not fully understood, but it clearly depends on the bacterial cell cycle and septum formation. Recent experiments with *S. pneumoniae* cells and polystyrene beads hemispherically decorated with purified ActA have provided convincing evidence that asymmetric distribution of ActA is indeed sufficient for directional actin assembly and movement (81, 631).

Because actin-based intracellular motility and intercellular spread are crucial for *Listeria* virulence, it is not surprising that the second pathogenic species of the genus, *L. ivanovii*, also possesses these features (320). The *actA* gene from *L. ivanovii*, i-*actA*, encodes a protein larger than ActA from *L. monocytogenes*. The similarity between the two proteins is also limited (34% identity) (230, 348). However, ActA and i-ActA have a similar overall structure (charged N-terminal region, central region of polyproline motifs, and C-terminal membrane anchor region), and the functional domains required for actin recruitment and VASP binding are also conserved (209). The two proteins do indeed have the same function, and i-*actA* restores actin tail formation in an *actA* mutant of *L. monocytogenes* (230). Remarkably, the ActA proteins are the only polypeptides encoded by the central virulence gene cluster that have the same function but a high degree of sequence divergence between *L. monocytogenes* and *L. ivanovii* (see below). Differences in the interactions of the two species with the eukaryote host may have resulted in different selection pressures for conservation of *actA* genes in *L. monocytogenes* and *L. ivanovii*. Alternatively, actin-based motility may only require a set of conserved functional modules correctly exposed in space, the core protein carrying these modules tolerating significant deviations and even the insertion of other species-specific functional motifs. *L. seeligeri* has also been shown to contain *actA*-homologous sequences (232). However, this species cannot induce actin polymerization in mammalian cells even if it is manipulated so that it can escape from the phagosomes of infected cells (321). The avirulence of this strain may have led to the mutational corruption of a protein with a function that is no longer required.

Two other intracellular bacteria, *Shigella flexneri* and *Rickettsia* spp., and vaccinia virus have similar mechanisms of intracellular movement and cell-to-cell spread by continuous polar actin assembly (38, 190, 231, 278, 389). Bacterially induced actin polymerization has also been studied in great detail in *S. flexneri*, and interestingly, the bacterial protein involved in this process, IcsA, has no sequence similarity to ActA. IcsA also has a polar distribution on the bacterial surface and induces polar actin deposition but uses a different mechanism of actin nucleation, involving activation of the Cdc42 effector WASP to form a ternary IcsA-WASP-Arp2/3 complex (105a, 164). Thus, it appears that ActA and IcsA have evolved convergently, with both subverting the signaling pathway that controls actin cytoskeletal dynamics but by different strategies, accounting for their structural differences.

Recently, evidence has been presented that ActA may also be involved in the entry of *L. monocytogenes* into eukaryotic cells, probably by recognition of an HSPG receptor (12). Indeed, putative heparan sulfate (HS)-binding motifs, typically consisting of clusters of positively charged amino acids with interdispersed hydrophobic residues, have been identified in the N-terminal region of ActA. One such motif is very similar to the HS ligand of the *Plasmodium* circumsporozoite protein, and treatment of target cells with a synthetic peptide containing this malarial HS-binding motif significantly inhibited listerial attachment. In addition, by using CHO cells deficient in HS synthesis, it was shown that the absence of ActA reduced the binding and uptake of *L. monocytogenes* only in cells containing this glycosaminoglycan. A family of HSPGs, the syndecans, are bound to cell membranes via a membrane-spanning

core protein, colocalize with actin bundles at the eukaryotic cell surface, and are thought to act as bridges between the ECM and the cortical cytoskeleton (530, 710). It therefore seems plausible that the interaction of ActA with an HSPG receptor induces transmembrane signaling events leading to cytoskeletal rearrangements and phagocytosis. Definitive confirmation that ActA plays a major role in *L. monocytogenes* internalization by host cells has recently been obtained by using an in-frame Δ*actA* mutant. Loss of ActA production in this mutant resulted in a decrease in invasiveness of up to a factor of 60 to 70 in epithelial cells, which was fully recovered upon complementation with the *actA* gene (Suárez et al., submitted for publication).

Although it is a major surface-exposed virulence factor, ActA is not presented by either MHC class I or class II molecules. This is only achieved if ActA is manipulated so that it is delivered in soluble form to the cytosolic MHC class I-processing compartment. However, the CD8$^+$ CTLs so elicited against the listerial surface protein are not protective in vivo (120).

Internalins

Internalins are the protein products of a family of virulence-associated genes found in pathogenic *Listeria* spp. The first members of this family to be characterized, InlA and InlB, encoded by the *inlAB* operon, were identified in *L. monocytogenes* by screening a bank of transposon-induced mutants for impaired invasiveness in Caco-2 cell monolayers. InlA was shown to function as an invasin, mediating bacterial internalization by these normally nonphagocytic epithelial cells, and was therefore named internalin (193). A large number of internalin homologs have since been identified in both *L. monocytogenes* and *L. ivanovii* (150, 168, 169, 527) (Fig. 7). An element common to all internalins is a leucine-rich repeat (LRR) domain consisting of a tandem repeat arrangement of an amino acid sequence with leucine residues in fixed positions (317). The typical LRR unit of internalins consists of 22 amino acids, with leucine or isoleucine residues at positions 3, 6, 9, 11, 16, 19, and 22 (— —L— —L— —L—L— —N—I— —I/L— —L). This sequence forms a novel, right-handed helix designated parallel β-helix, with a turn after every LRR unit, first identified in the pectate lyase of *Erwinia chrysanthemi* (277, 705). The LRR domain is thought to be involved in specific protein-protein interactions. LRR domain proteins are numerous among eukaryotes, in which they are involved in a variety of functions, such as cell adhesion, as is the case for decorin, fibromodulin, and the glycoprotein Ibg, or receptor-ligand interactions and signal transduction pathways, as is the case for Trk, CD14, and the adenylate cyclase of *Saccharomyces cerevisiae* (337). LRR proteins are less frequent among prokaryotes and most are virulence factors, like YopM of *Yersinia pestis* and *Yersinia pseudotuberculosis*, IpaH of *Shigella flexneri*, and the filamentous hemagglutinin of *Bordetella pertussis* (55, 264, 407). The occurrence in pathogenic *Listeria* spp. of a multigene family of LRR-containing proteins is unusual among prokaryotes.

The internalins best characterized in terms of structure and function are InlA and InlB. InlA comprises 800 amino acids, which can be subdivided into two functional regions. The N-terminal half of InlA contains, in addition to a signal peptide (a

FIG. 7. Structure of the members of the internalin multigene family from *L. monocytogenes* and *L. ivanovii* (reproduced with modifications from reference 358 with permission of the publisher with data from Dominguez-Bernal et al. [unpublished data]). S, signal peptide; B, B-repeats; C, Csa domain repeats C-repeat; D, D-repeats. See text for details.

feature common to all internalins), 15 LRR units (A repeats), whereas the C-terminal half contains three longer repeat sequences (B repeats) and a cell wall anchor comprising the sorting motif LPXTG followed by a hydrophobic membrane-spanning region of approximately 20 amino acids and a few positively charged amino acids (Fig. 7). This distal LPTXG motif, like similar motifs in other surface proteins covalently linked to the peptidoglycan in gram-positive bacteria, has been shown to be responsible for the attachment of InlA to the bacterial cell envelope in a process mediated by the enzyme sortase (105b, 135, 179, 371, 465, 466, 590). InlB comprises 630 amino acids with seven LRR units in the N-terminal part (149). The LPXTG motif and the hydrophobic tail are absent from the C-terminal part of InlB. Instead, InlB has a 232-amino-acid region consisting of tandemly arranged repeats about 80 amino acids long, each starting with the sequence GW. This domain,

known as the cell surface anchor (Csa) domain, is responsible for the attachment of InlB to the bacterial surface (60). The Csa domain mediates a novel mechanism of protein-bacterial surface association in which InlB is partially buried in the cell wall, interacts with the cytoplasmic membrane, and uses lipoteichoic acid as a ligand (313). This type of association appears to be relatively weak, as significant amounts of InlB are found in the supernatant of *L. monocytogenes* cultures and InlB is released from the bacterial surface upon incubation in buffers with high Tris-HCl concentrations (60, 455). Another surface protein of *L. monocytogenes*, the putative autolysin/adhesin Ami, also associates with the bacterial cell wall via a Csa domain (313).

inlAB mutants are severely impaired in host cell invasion, and both InlA and InlB have been shown to be required for entry of *L. monocytogenes* into normally susceptible nonphagocytic cells in vitro (143, 149, 193, 196, 197, 242, 246, 385). The invasion processes mediated by InlA and InlB are apparently similar from a mechanistic point of view (of the zipper type [417, 642]), but the two proteins follow different signaling pathways to achieve entry (see below). InlA and InlB are sufficient to trigger internalization by appropriate host cells, as shown by their ability to confer invasiveness on normally noninvasive bacteria, such as *L. innocua* and *Streptococcus faecalis*, and to induce the internalization of latex beads coated with the purified proteins (62, 63, 373, 455).

Functional analysis of InlA has shown that the N-terminal region encompassing the LRR domain and the interrepeat region is necessary and sufficient for bacterial entry into permissive cells (373). Monoclonal antibodies directed against the LRR domain block InlA-mediated entry, indicating that this structure is essential in the entry process (435). The host cell receptor for InlA is E-cadherin, a calcium-dependent intercellular adhesion glycoprotein composed of five extracellular domains and a cytoplasmic tail (205, 703). This receptor was identified by affinity chromatography on a column with covalently coupled InlA onto which a Caco-2 cell extract was loaded. Definitive evidence that E-cadherin is a principal receptor for InlA was obtained by transfection experiments using fibroblasts nonpermissive for invasion by *L. monocytogenes*; expression of the gene of the chicken homolog of E-cadherin, L-CAM, made these fibroblasts fully susceptible to invasion by recombinant *L. innocua* expressing the *inlA* gene but not to invasion by wild-type *L. innocua* or by a ΔinlA mutant of *L. monocytogenes* (435). E-cadherin accumulates at the gap junctions between intestinal epithelial cells but is also present over the entire basolateral face of these cells, consistent with experimental data showing that *L. monocytogenes* preferentially penetrates polarized intestinal Caco-2 cells via their basolateral surface (196, 650). E-cadherin is also present on the surface of hepatocytes, dendritic cells, brain microvascular endothelial cells, and the epithelial cells lining the choroid plexus and placental chorionic villi, all of which are potential targets during *Listeria* infection. The cadherin specific to the nervous system, N-cadherin, does not promote InlA-mediated entry (435), which correlates with the observation that direct invasion of neurons by *L. monocytogenes* is not efficient (151). However, certain sensory neurons seem to be permissive for InlA-mediated internalization of *L. monocytogenes* because they may express E-cadherin (146). InlA presumably interacts,

via its LRR region, with the first extracellular domain of E-cadherin (372). This interaction leads to bacterial adherence, but entry is mediated by the intracytoplasmic domain of E-cadherin, which presumably leads to actin cytoskeleton rearrangement via α- and β-catenins (372a).

Upon contact with susceptible eukaryotic cells, InlB induces membrane ruffling and tyrosine phosphorylation of several proteins, including Gabl. Cbl, and Shc, implicated in membrane localization and activation of the phosphatidylinositol 3-kinase (PI3K) p85/p110. This PI3K, known to be involved in the control of the actin cytoskeleton, is stimulated by InlB, resulting in activation of PLC-γ1 and in increased levels of phosphoinositide second messengers. Inhibition studies have shown that p85/p110 PI3K does not participate in InlA-mediated uptake but is indispensable for InlB-mediated phagocytosis (45a, 296, 297). Met, a receptor tyrosine kinase which physiologically serves as ligand for hepatocyte growth factor (HGF), has been recently identified as a signaling receptor for InlB. Interaction with InlB tyrosine phosphorylates the 145-kDa subunit of Met, a binding partner of PI3K. Met-deficient cell lines are unable to mediate InlB-dependent entry, suggesting a major role for this receptor in *L. monocytogenes* invasion (615a). Using affinity chromatography, a second receptor for InlB has been identified in gC1q-R, the cellular ligand of the globular part of the C1q complement fraction. Pretreatment of target cells with soluble C1q or anti-gC1q-R antibodies impaired InlB-mediated entry, and transfection of the gC1q-R gene in nonpermissive cells promoted internalization of InlB-coated beads (61). However, gC1q-R lacks a transmembrane domain, suggesting that it may function as a coreceptor rather than a signaling receptor. As for InlA, the LRR region of InlB is sufficient for entry into mammalian cells (62, 297). The X-ray crystal structure of the InlB LRR domain has been recently determined: it consists of an elongated structure resembling a bowed tube with an extensive solvent-exposed surface area. Three regions were identified as potential candidates for mediating protein-protein interaction: two were in the concave face of the tube, consisting, respectively, of clusters of hydrophobic and negatively charged amino acids; the other was at the tip and contained two calcium ions, which may serve as bridging molecules in the interaction with host cell receptors (412a).

Evidence indicates that InlA and InlB have different cell specificities and may therefore play an important role in cell and host tropism. Dramsi et al. (149) analyzed in detail the effect of in-frame *inlA* and *inlB* deletions and corresponding gene complementations in an internalin double mutant on *L. monocytogenes* entry into various cell types. They showed that InlA but not InlB mediates entry into human Caco-2 intestinal cells and that InlB is necessary for invasion of murine TIB73 hepatocytes, whereas InlA is not. However, in the human hepatocyte cell line HepG-2, both InlA and InlB were required for internalization. More recently, the entry of *L. monocytogenes* into HUVECs and brain microvascular endothelial cells has been shown to be dependent on InlB but not on InlA (157, 246, 247, 498). The available data indicate that the role of InlA in invasion is restricted to E-cadherin-expressing cells, whereas InlB mediates entry into a wider range of cell types from various animal species, including certain epithelial and fibroblast cell lines, such as Vero, HEp-2, HeLa, CHO, L2, and

S180 cells, as well as hepatocytes and endothelial cells (150, 246, 435, 455, 498). InlA has also been shown to contribute to at least 50% of serum-independent phagocytosis by macrophages (584), indicating that at least one macrophage receptor recognizes this listerial surface protein.

A major breakthrough in our understanding of InlA function has been the recent discovery by Lecuit et al. (372) that a Pro residue at position 16 in the first extracellular domain of E-cadherin is critical for this interaction with InlA. The presence of a Pro residue at position 16 of E-cadherin correlated with permissivity to invasion of the host cell. Thus, cells expressing mouse and rat E-cadherin, both of which have a Glu residue at this position, do not allow InlA-dependent entry, whereas cells expressing human E-cadherin, L-CAM, and guinea pig E-cadherin, with Pro16, are totally permissive to invasion by InlA-expressing bacteria. A Pro16→Glu substitution in human E-cadherin abolished interaction with InlA, whereas replacement of the Glu16 residue with a Pro in mouse E-cadherin resulted in a gain of function (372). These findings are important in that they are the first to identify a possible molecular basis for host specificity in a bacterial pathogen. They also make necessary reevaluation of the available experimental information on the role of InlA and InlB in virulence, in particular, data obtained from in vivo studies in mice and rats, in which no clear pathophysiological role of the *inlAB* determinant could be identified despite its proven involvement in cell invasion in vitro. In such in vivo studies, for example, the *inlAB* locus has been shown to affect only very slightly the persistence of *L. monocytogenes* in mouse liver and not to be essential for the invasion of hepatocytes in vivo (14, 149, 150, 197, 242, 385). Similarly, no role for the *inlAB*-encoded internalins was detected in intestinal invasion and translocation in mouse and rat ligated ileal loop models (119, 523) or in the development of murine cerebral listeriosis (589). Despite the different cell specificities of InlA and InlB, a double Δ*inlAB* mutant was 10 times less invasive than a single *inlA* mutant in Caco-2 cells, indicating that the two internalins synergize to optimize invasiveness. Such synergistic activity of InlA and InlB is also evident in human hepatocytes, with Δ*inlA,* Δ*inlB,* and Δ*inlAB* mutations reducing the rate of entry by factors of 2 to 5, 10, and 100, respectively (149). However, the heterologous expression of *inlB* did not confer on *L. innocua* the ability to invade murine hepatocytes (149). It is therefore possible that the absence of InlA recognition by E-cadherin in mouse tissues impairs the targeting of *L. monocytogenes* to certain critical cell types in vivo, thereby preventing the appropriate interaction of InlB with host cell surface receptors.

The study of Lecuit et al. (372) raises important questions about the usefulness of the widely used mouse model, at least for those aspects in which InlA and InlB are potentially involved. Indeed, as described above, oral infection with *L. monocytogenes* leads to invasion of the intestinal epithelium in guinea pigs, which have the permissive type of E-cadherin, but not in mice and rats, which express a *Listeria*-nonpermissive isoform of the intercellular adhesion molecule.

The observation that InlB alone is not sufficient to mediate entry of *L. innocua* into murine hepatocytes whereas complementation with the inlB gene restores full invasiveness to a Δ*inlAB* mutant (149) clearly shows that other *L. monocytogenes*-specific products are involved in the interaction with host

cells. This is also indicated by the significant level of residual invasiveness of *inlAB* mutants both in vitro (143, 150, 157, 196, 197, 247, 253, 385, 435) and, especially, in vivo (14, 149, 196, 242, 385, 589). It seems very likely that other members of the Inl repertoire play a role similar to that of InlA and InlB in the interaction with the eukaryotic cell surface. In addition to the *inlAB* operon, six other genes encoding putative surface-associated internalins (*inlC2, inlD, inlE, inlF, inlG,* and *inlH*) have been identified in *L. monocytogenes*. These internalin genes are distributed in three loci, two of which contain three internalin genes each and correspond to a single internalin islet that has presumably evolved differently in two isolates of *L. monocytogenes* serovar 1/2a (150, 527) (see Fig. 7 and below). All these newly identified internalins have signal peptides and LRR domains with various numbers of repeat units in their N-terminal halves and, like InlA, an LPXTG motif at their C-terminal ends, suggesting that they are covalently bound to the bacterial cell wall. Deletion of the *inlGHE* gene cluster did not affect entry into a number of mammalian cell lines, suggesting that these internalins, unlike InlA and InlB, are not involved in invasion. However, counts of the Δ*inlGHE* mutant in the liver and spleen were 3 and 2.5 log_{10} units below those of the wild-type parental strain, indicating that the missing internalins are somehow important for host tissue colonization in vivo (527). Deletion of the *inlGHE*-homologous *inlC2DE* gene cluster and of the additional *inlF* gene identified in the other strain also had no effect on entry into various cell types; however, here the absence of these internalin loci did not affect the bacterial counts in mouse organs (150). This, together with evidence indicating that internalin gene homologs are also present in the nonpathogenic species *L. innocua* (193), suggests that members of this multigene family may have biological functions not exclusively related to virulence.

All the above-mentioned internalins are large, cell wall-associated proteins which, until very recently (see below), had been found exclusively in *L. monocytogenes*. Their genes are preferentially expressed extracellularly and, with the exception of the *inlAB* operon, are independent of the listerial virulence regulator PrfA (75, 527). A new group of LRR proteins has recently been identified in both *L. monocytogenes* and *L. ivanovii*. Structurally, they are very similar to the large internalins but lack the C-terminal (B) repeat region, the LPXTG motif, and the membrane anchor (Fig. 7). The internalin proteins of this class are therefore generally smaller (around 30 kDa versus the 80 and 71 kDa of InlA and InlB, respectively) and are released in soluble form into the culture supernatant. InlC (167), also called IrpA (143), is the prototype of this group of small, secreted internalins (167) and is the only secreted internalin detected to date in *L. monocytogenes*. Unlike the genes encoding the large, cell wall-associated internalins, all the genes encoding these secreted internalins are PrfA dependent. Indeed, this property was fundamental to the discovery of this subfamily of internalins: InlC was identified in the culture supernatant as a major 30-kDa protein that was overproduced in *L. monocytogenes* after complementation with the *prfA* gene in multicopy, which results in the artificial upregulation of PrfA-dependent proteins (143, 167, 384). In *L. ivanovii*, five PrfA-dependent secreted internalins (i-InlC, i-InlD, i-InlE, i-InlF, and i-InlG) have been reported (168, 169, 350), three of which (i-InlE, i-InlF, and i-InlG) are encoded in a large viru-

lence gene cluster containing additional genes encoding secreted internalins (225; Dominguez-Bernal et al., unpublished; see also below). Internalization and intracellular growth were found to be similar for an *L. monocytogenes inlC* deletion mutant and the parental wild-type strain when tested in several cell types in vitro. The *inlC* mutation, however, resulted in significantly lower virulence in mice (decrease in 1 $\log_{10}$ in the bacterial load in organs) after infection by the intravenous or oral route (143, 167). A significant loss of virulence in mice has also been observed for the Δ*i-inlE* and Δ*i-inlF* mutants of *L. ivanovii* (169). Like most PrfA-dependent genes (see below), *inlC* is preferentially expressed in the cytoplasm, especially at late stages of infection, when bacteria are in the process of active intercellular spread (75, 167). This suggests that InlC may be involved in the dissemination of infection. However, cell-to-cell spread was found not to be affected in an *inlC* mutant (143, 167, 246). No intracellular target has yet been identified for InlC, and the role in virulence of the small, secreted internalins remains unknown.

Other Virulence Factors

The listerial proteins reviewed so far are true virulence factors because they primarily accomplish tasks specifically required for parasitization of the vertebrate host and have evolved exclusively in pathogenic *Listeria* species. In addition to these virulence factors, other listerial products have been identified that also contribute to survival within the host. Although some of them have a major influence on the outcome of the host-parasite interaction, their participation in pathogenesis is more indirect, as they are probably involved in general housekeeping functions also necessary for saprophytic life. This does not exclude the possibility that some of these bacterial products may have evolved specific characteristics and differ in certain aspects from their counterparts in nonpathogenic *Listeria* spp. as a result of adaption to the lifestyle of a facultative intracellular pathogen.

p60 (*iap*). On agar plates, *L. monocytogenes* gives rise spontaneously to colonies with an altered, stable, rough phenotype. These bacteria form long filamentous structures composed of chains of individual cells (354). Although these rough mutants produce wild-type levels of Hly, their virulence is attenuated (288). This lack of virulence correlates with impaired invasiveness, particularly in fibroblasts (354). This invasion deficit results from a defect in the production of a major 60-kDa extracellular protein, p60, that is found both in the culture supernatant (354) and associated with the cell wall (570) and which, when added exogenously, disaggregates the bacterial chains and restores invasiveness (354). The p60 protein is encoded by the *iap* (for invasion-associated protein) gene, which in *L. monocytogenes* encodes a 484-residue polypeptide containing a central repeat region of Thr-Asn units (343). The expression of *iap* is independent of PrfA (72, 75) and is controlled at the posttranscriptional level (342). p60-homologous proteins are synthesized by all *Listeria* spp., but in each species there are specific differences in amino acid sequence that can be used for identification purposes in PCR-based and immunological assays (74, 77). Only the *L. monocytogenes* protein is able to restore invasiveness to p60-defective rough mutants (73). There is some experimental evidence supporting a role of

p60 in intestinal invasion and in vivo survival (279, 282). A recent study using recombinant p60 from *L. monocytogenes* has shown that the protein binds specifically to Caco-2 intestinal cells (499). The p60 protein has a murein hydrolase activity required for normal septum formation and essential for cell viability (702), which makes it difficult to determine the precise role of p60 in virulence because *iap* mutations are lethal. It has recently been shown that p60 is a major antigen in the protective response against *L. monocytogenes* (203, 204, 267).

Antioxidant factors. Macrophages become listericidal after their immunological activation by cytokines such as IFN-γ or by engagement of complement receptor 3 during C3b-facilitated opsonic phagocytosis of *L. monocytogenes* (84, 115, 146, 181). Although it is unclear how macrophages kill intracellular parasites such as *L. monocytogenes*, it is thought that the generation during the oxidative burst of reactive oxygen intermediates (ROI) and, via interaction of the superoxide anion with nitric oxide (NO), reactive nitrogen intermediates (RNI) plays a key role. In vitro experimental data indeed suggest that ROI and RNI contribute significantly to the macrophage-mediated killing of *L. monocytogenes* (28, 37, 57, 294, 483, 484). Some studies suggest a major involvement of ROI (294, 454, 484), whereas others indicate that both ROI and RNI are required and may even act in synergy (3, 490). Iron is required for the catalytic reactions leading to the generation of ROI and RNI, and the correct intracellular homeostasis of this element has been reported to be essential for adequate macrophage listericidal activity (181). In vivo, inducible NO synthase (NOS) has been shown to be expressed during listerial encephalitis by macrophages in microabscesses at the sites of active bacterial multiplication (315). Inhibition of NO synthesis by a specific NOS inhibitor was also found to exacerbate listerial infection in mice (28, 56).

In spite of this evidence, catalase and superoxide dismutase (SOD), enzymes that are produced by bacteria primarily to detoxify the endogenous free oxygen radicals generated during prokaryotic oxidative metabolism but which also play a key role in counteracting the oxygen-dependent microbicidal mechanism of the host during infection (175, 185, 256, 258, 445, 658), appear to have only a minor involvement, if any, in *Listeria* virulence. Thus, loss of catalase activity in an *L. monocytogenes* transposon-induced mutant had no measurable effect on proliferation in mouse organs (369). Studies of a *cat* deletion mutant confirmed that catalase is not involved in *L. monocytogenes* virulence in the mouse model. This lack of a significant role for catalase in pathogenesis is consistent with the reported isolation of spontaneous catalase-negative *L. monocytogenes* mutants from listeriosis patients (76, 165, 643). Similarly, loss of SOD activity by deletion of the *sod* gene (65) led to at most a slight decrease in the proliferation capacity of *L. monocytogenes* in vitro in mouse bone marrow-derived macrophages (which exhibit a clearly detectable oxidative burst in response to this pathogen) and in the organs of experimentally infected mice. These effects were slightly more pronounced with a *cat sod* double mutant (Brehm et al., unpublished data), indicating that catalase and SOD may together provide some levels of resistance to bacterial killing.

The insignificant protective role of catalase and SOD in virulence is consistent with the kinetics of *L. monocytogenes* populations within macrophages, with most bacteria being

killed very rapidly (within 3 min) in the vacuolar compartment after uptake (127, 533). It is also consistent with the particular characteristics of *L. monocytogenes* intracellular parasitism, in which bacteria multiply in the cytoplasm, a comparment where toxic concentrations of ROI and RNI are unlikely to be present, thereby rendering catalase and SOD activities unnecessary for bacterial survival. Indeed, in listerial intracellular parasitism, only a small percentage of bacteria need to survive destruction in the phagosome and reach the cytoplasm to initiate a productive infection (128). For this "minimal" survival, *Listeria* cells may exploit other more specialized resources to cope with oxidative stress, such as stress response mechanisms (see below). Microbicidal mechanisms other than the production of ROI or RNI, such as lysosomal enzymes (270), defensins (374, 469), and cytosolic antimicrobial peptides (283a), may play a critical role in the host defense against *Listeria* infection (262, 616), but nothing is known about the relevance to pathogenesis of such oxygen-independent killing mechanisms and the putative listerial factors that may counteract them.

Metal ion uptake. Iron is a key element for all living cells, including prokaryotic cells, serving as cofactor for a large number of enzymes and essential proteins involved in electron transport processes. In animal host tissues, iron is not freely available because it is sequestered by ferric transferrin in serum and by ferritin and heme compounds within cells. Thus, bacterial pathogens have evolved specialized mechanisms to capture iron for growth in host tissues, and these mechanisms play an important role in virulence (502a). In *L. monocytogenes*, iron not only stimulates growth in synthetic medium but, if administered to infected mice in salt form, also increases bacterial proliferation rates in the liver and spleen and decreases the LD_{50} (639, 644). In humans, hypersideremia after massive blood transfusions has been identified as an important risk factor for listeriosis (594). Unlike many other bacteria, *Listeria* does not seem to secrete extracellular iron scavengers (siderophores).

Three different iron uptake systems have been described in *L. monocytogenes*. One involves the direct transport of ferric citrate to the bacterial cell by a citrate-inducible system (2). Another system involves an extracellular ferric iron reductase, which uses as a substrate naturally occurring iron-loaded catecholamines and siderophores (2, 21, 111, 112, 133). The third system may involve a bacterial cell surface-located transferrin-binding protein (263), although the existence of such a mechanism has been questioned (43). No experimental evidence is currently available on the contribution of these iron uptake systems to the pathogenesis of *L. monocytogenes* infection.

In addition to its role as an essential nutrient, iron is also used by pathogenic bacteria as a signal molecule for the regulation of virulence gene expression. This sensory system is based on the marked differences in free iron concentrations between the environment and intestinal lumen (high) and host tissues (low) (387). Expression of *hly* has been shown to be induced in conditions of iron limitation, indicating that this metal ion plays a role in the regulation of virulence gene expression in *L. monocytogenes* (see below).

Recently, a P-type ATPase involved in copper transport, CtpA, has been identified in *L. monocytogenes*. A *ctpA* knockout mutant was impaired in in vivo survival in tissues of infected mice but had normal intracellular growth in HeLa and J774 cell monolayers (182). Copper is a cofactor in a variety of redox enzymes, such as SOD, that are involved in the disposal by bacteria of toxic ROI generated during the oxidative burst of macrophages (2a). However, the marginal implication of SOD in *L. monocytogenes* virulence (see above) indicates that the involvement of CtpA in pathogenesis may be more indirect and related to the regulation of metal ion homeostasis.

Stress response mediators. As indicated above, a small proportion of *L. monocytogenes* cells survive the stressful, hostile conditions of the phagocytic vacuole and reach the cytoplasm, where they proliferate, leading to the spread of the infection (128). Survival under stress involves an adaptive response mediated by a set of conserved proteins that are upregulated in vitro upon exposure to heat shock, low pH, oxidative agents, toxic chemical compounds, starvation, and, in general, any situation in which bacterial growth is arrested. These proteins may be chaperones that assist in the proper refolding or assembly of stress-damaged proteins or proteases that degrade damaged proteins, ensuring that essential physiological pathways function correctly in stressed cells (229).

Analysis of the stress response of *L. monocytogenes*, including that to heat and cold shock conditions, has revealed similarities to that of other prokaryotes (24, 261, 507, 508). In facultative intracellular pathogenic bacteria, for example, *Salmonella*, general stress proteins have been shown to be induced upon infection of macrophages (78, 95). In *L. monocytogenes*, in contrast, stress proteins appear not to be required for intracellular proliferation. This has been shown by Hanawa et al. (261), who found that none of the 32 listerial proteins selectively induced in J774 macrophages infected for 5 h were the same as any of the stress proteins produced when bacteria were subjected to various environemental stresses. It has also been shown that the alternative sigma factor σ^B is not involved in *L. monocytogenes* virulence, although its expression is activated in stress conditions and is required for osmotolerance (26, 695). This may be explained by the particular characteristics of *L. monocytogenes* intracellular parasitism, which develops mainly in a nonaggressive environment, the host cell cytoplasm, much less hostile than that of the phagolysosome in which *Salmonella* bacteria proliferate. However, adaptation to sublethal environmental stresses has been shown to protect *L. monocytogenes* against the lethal action of toxic compounds (392), indicating that in this bacterium there is a "stress-hardening" adaptive response that may be important for survival in hostile conditions. There is evidence that the adaptive response of *L. monocytogenes* to acidic conditions, such as those encountered in the stomach and in certain foods, may be relevant to pathogenesis. Thus, development of acid tolerance results in increased internalization by Caco-2 cells and higher survival rates in lipopolysaccharide-activated macrophages (101a). Acid adaptation also results in higher loads of *L. monocytogenes* bacteria in the intestine and the mesenteric lymph nodes after oral infection in mice (576a). Stressful conditions are also likely to be encountered during the transient residence in the phagolysosome. Consistent with this, the stress response of *L. monocytogenes* involves the induction of *hly*, which encodes the principal factor involved in escape from the phagocytic vacuole (634, 635) (see below).

Recently, a group of virulence-associated stress mediators

involved in intravacuolar survival and subsequent escape to the cytoplasm have been identified in *L. monocytogenes*. All these stress proteins have highly conserved counterparts in *B. subtilis*, in which they have stress tolerance-related functions and are involved in the signaling cascade of sporulation. One of these stress proteins is ClpC, an ATPase belonging to the heat shock protein-100 (HSP-100)/Clp family, a class of highly conserved proteins involved in stress tolerance in many prokaryotic and eukaryotic organisms (585, 638, 684). The locus encoding this protein, *clpC*, was identified by Rouquette et al. (569) by transposon mutagenesis and selection for iron-dependent growth in synthetic medium. The original intention was to identify genes involved in iron uptake (567). However, the phenotype of the mutants isolated, the mutations of which all mapped to the *clpC* locus, had nothing to do with iron metabolism but was instead related to a defect in resistance to the nutritional stress caused by iron deprivation (569). *clpC* is upregulated in heat shock conditions (42°C) and preferentially expressed during stationary phase, a pattern consistent with this gene's being induced by general stress conditions. The *clpC* mutants were found to be highly susceptible to several stresses, including high temperature and high osmolarity in addition to iron limitation. Their growth in bone marrow macrophages and in vivo survival in the organs of infected mice were also severely impaired, resulting in a substantial loss of virulence (decrease of 3 $\log_{10}$ units in bacterial counts) (568, 569). Electron microscopy showed that *clpC* mutants remained confined to the phagocytic vacuole, indicating either that ClpC is involved in lysis of the phagosomal membrane (568) or, more probably, that the loss of ClpC reduces bacterial fitness to the extent that *L. monocytogenes* is no longer capable of surviving within the phagosome or producing sufficient quantities of active membrane-damaging virulence factors.

Expression of *clpC* was found to be almost abolished in an *L. monocytogenes prfA** mutant, in which the PrfA regulon is constitutively overexpressed (see below), and in culture conditions that trigger the activation of PrfA. This suggests that the listerial virulence gene transcriptional activator protein negatively controls ClpC. This repressor effect is apparently released as *L. monocytogenes* enters the stationary phase, indicating that the stress-induced *clpC*-activating regulatory pathways prevail over PrfA (548). No canonical PrfA boxes (the binding site for PrfA in PrfA-regulated promoters) were identified in the promoter region controlling the expression of *clpC*, suggesting that expressional crosstalk between PrfA and ClpC is mediated by an intermediary negative regulator activated by PrfA. Expressional interplay between a true virulence determinant and a stress mediator has been described in other facultative intracellular pathogens, such as *Salmonella* spp. In these bacteria, the stress response mediator RpoS induces the synthesis of the positive transcriptional regulator SpvR, which controls *spv* plasmid virulence genes important for intracellular survival and the development of systemic infection (95, 251). In *L. monocytogenes* the situation appears to be the converse, because activation of the central virulence regulator PrfA triggers the downregulation of a general stress response effector. As discussed above, these differences probably stem from the differences in intracellular behavior of the two bacteria. Thus, *Salmonella* organisms proliferate within a vacuole, presumably

a hostile environment in which stress proteins and virulence factors involved in intraphagolysosomal survival would be simultaneously required. In contrast, *Listeria* cells rapidly escape from the phagosome and actively multiply in the more permissive environment of the host cell cytoplasm. The repression of *clpC* by PrfA is entirely consistent with the observed activation of the PrfA system when bacteria enter the cytoplasm (see below) and with the nonrequirement for stress proteins for the proliferation of *L. monocytogenes* in this cellular compartment (see above). The coupling of PrfA activation with ClpC downregulation in *L. monocytogenes* is a good example of the clever strategies that facultative intracellular pathogens have evolved to coordinate gene expression and to ensure that genes encoding factors superfluous at a given step of the infectious cycle are appropriately turned off. It has been recently shown that ClpC is required for adhesion and invasion of *L. monocytogenes*, possibly by modulating the expression of InlA, InlB, and ActA (460a).

ClpE is, like ClpC, a member of the Clp family of HSP-100 stress proteins and is also required for prolonged survival at 42°C and involved in the virulence of *L. monocytogenes* (460). A double *clpC clpE* mutant was found to be totally avirulent in the mouse model and was readily eliminated from liver tissue. *clpE* expression was found to be upregulated in a *clpC* mutant, suggesting that ClpC and ClpE have redundant roles in stress tolerance and that the absence of one is compensated for by upregulation of the other (460).

ClpP is a 22-kDa protein belonging to a family of stress proteases highly conserved in prokaryotes and eukaryotes (198). Deletion of *clpP* showed that ClpP is required for growth under stress conditions and for survival in macrophages in vitro and in mouse tissues in vivo. In the *clpP* mutant, LLO production was low under stress conditions, and virulence and hemolytic activity were totally restored by complementation with *clpP*. This suggests that the ClpP protease is important for the observed stress-induced upregulation of Hly (see above).

ClpC, ClpE, and ClpP are regulated by CtsR, the first gene of the operon including *clpC* (459). *L. monocytogenes* CtsR is a homolog of the *B. subtilis* CtsR repressor of stress response genes. A *ctsR* deletion mutant of *L. monocytogenes* displayed enhanced survival under stress conditions and normal virulence in mice, whereas a wild-type strain engineered to overexpress *ctsR* had significantly attenuated virulence, presumably due to a restricted stress response (459). This is consistent with CtsR being a negative regulator of Clp proteins. It would be interesting to investigate whether repression of *clpC* is due to PrfA-mediated activation of *ctsR*, resulting in silencing of the CtsR-controlled stress response regulon.

Another locus that may be involved in stress tolerance and virulence in *L. monocytogenes* has recently been identified. This locus, *lisRK*, encodes a two-component system and was identified in an acid-tolerant transposon-induced mutant of *L. monocytogenes*. Deletion of the gene encoding the histidine kinase component of this LisRK system, *lisK*, resulted in greater resistance to acid and high ethanol concentrations in the stationary phase and, apparently, also in lower virulence (109). The mechanism underlying the phenotype of the mutant is unclear.
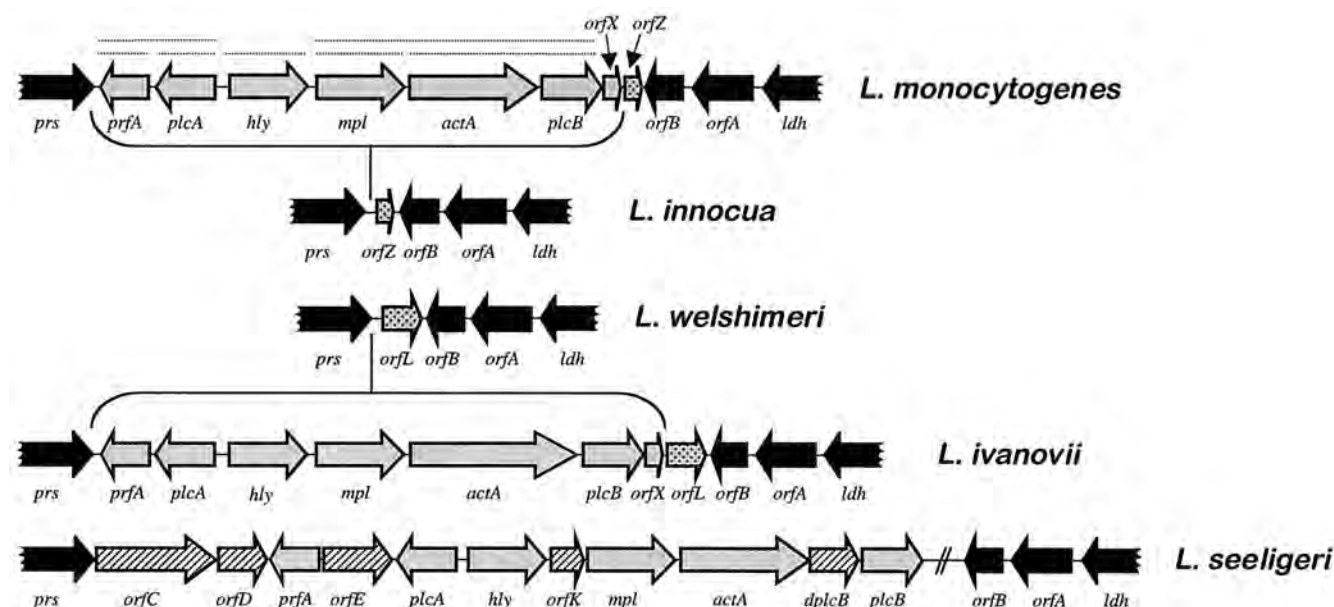
FIG. 8. Physical and transcriptional organization of the central virulence gene cluster (LIPI-1) of *L. monocytogenes* and structure of the locus in other *Listeria* spp. Genes belonging to LIPI-1 are in grey, and the flanking loci are in black. Dotted lines above *L. monocytogenes* LIPI-1 genes indicate known transcripts. LIPI-1 is inserted in a chromosomal region delimited by the *prs* and *ldh* genes, encoding the housekeeping enzymes phosphoribosyl-pyrophosphate synthase and lactate dehydrogenase, respectively. In the *plcB-ldh* intergenic region, two ORFs, *orfA* and *orfB*, are found in all *Listeria* spp., indicating that the insertion point of LIPI-1 is between the *prs* and *orfB* loci. In the *plcB-orfB* intergenic region of *L. monocytogenes*, there are two small ORFs, *orfX* and *orfZ* (stippled), which delimit the putative excision point of LIPI-1 in *L. innocua*. In the *plcB-orfB* intergenic region of *L. ivanovii*, two small ORFs, *orfX* (encoding a homolog of the *orfX* product from *L. monocytogenes*) and *orfL* (stippled), are also present, which, like those in *L. monocytogenes*, delimit the deletion point of LIPI-1 in the nonpathogenic species *L. welshimeri*. *orfZ* and *orfL* encode polypeptides with significant similarity to bacteriophage proteins (respectively Orf6 of the A511 *Listeria* phage and a serine/threonine protein phosphatase from λ [88a, 670a]), suggesting that LIPI-1 was originally acquired by phage transduction (see text for details). The additional ORFs found in the *L. seeligeri* virulence gene cluster are hatched (the *orfK* product shows 39% similarity with the phosphatidylinositol phospholipase PlcA and *dplcB* is a truncated duplicate of the *plcB* gene). The figure has been constructed using data from references 88a, 232, 350, and 672 and unpublished results from J.K. (*L. seeligeri*) and B.G.-Z. and J.-A.V.-B. (*L. ivanovii*). (Adapted from reference 670a with permission of the publisher.)

GENETIC ORGANIZATION AND EVOLUTION OF VIRULENCE DETERMINANTS

Although plasmids have been reported in *Listeria* spp. (370, 504, 521), all the listerial virulence determinants identified to date are chromosomally encoded. The virulence genes of *Listeria* spp., as in other pathogenic bacteria, are mostly organized into discrete genetic units known as pathogenicity islands (PAIs). PAIs are presumably acquired by bacteria via horizontal gene transfer, sometimes as part of mobile genetic elements, and consequently play a key role in the evolution of bacterial virulence (49, 249, 259, 260).

Central Virulence Gene Cluster

Six of the virulence factors responsible for key steps of *L. monocytogenes* intracellular parasitism (*prfA, plcA, hly, mpl, actA*, and *plcB*) are physically linked in a 9-kb chromosomal island formerly known as the *hly* or PrfA-dependent virulence gene cluster (88a, 350) and now referred to as LIPI-1 (for *Listeria* pathogenicity island 1) (670a). Figure 8 shows the physical and transcriptional organization of this "intracellular life" gene cassette. The *hly* monocistron, encoding LLO, occupies the central position in this locus. Downstream from *hly* and transcribed in the same orientation is the lecithinase operon, comprising the *mpl, actA*, and *plcB* genes (434, 672).

These genes are transcribed either as a single 5.7-kb mRNA under control of the *mpl* promoter or as shorter transcripts, one specific for *mpl* and the other starting at a promoter immediately upstream from *actA* and covering the *actA* and *plcB* genes and three additional small open reading frames (ORFs). The *plcA-prfA* operon is located upstream from *hly* and is transcribed in the opposite orientation, either as a bicistronic mRNA of 2.1 kb or in monocistronic transcripts of 1.2 and 0.8 to 0.9 kb, respectively (187, 188, 433).

Phylogenetic analyses based on the sequence of the 16S and 23S rRNA have revealed that the genus *Listeria* comprises two evolutionary branches, one relatively distant, corresponding to *L. grayi*, and the other containing the remaining species. The latter are in turn divided into two sublines of descent, one comprising *L. monocytogenes* and the closely related nonpathogenic species *L. innocua*, and the other containing *L. ivanovii* and the nonpathogenic species *L. seeligeri* and *L. welshimeri* (98, 579) (Fig. 9). *L. ivanovii*, which has an intracellular life cycle similar to that of *L. monocytogenes* (320), carries a copy of LIPI-1 (232, 350, 670a) (Fig. 8). The genetic structures of LIPI-1 from *L. monocytogenes* and *L. ivanovii* are identical, but the corresponding DNA sequences exhibit only 73 to 78% similarity (350, 670a), a degree of divergence compatible with the genetic distance that separates the two species. One gene of LIPI-1, *actA*, is specially dissimilar, reflecting a divergent
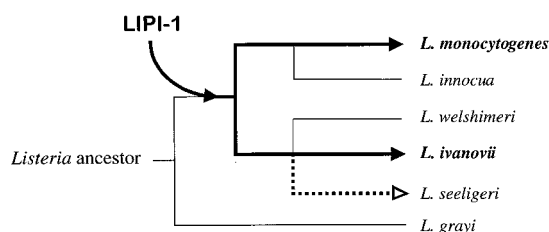
FIG. 9. Evolution of the central virulence gene cluster (LIPI-1) in the genus *Listeria*. The presence of LIPI-1 at the same chromosomal position in various species belonging to distinct evolutionary branches of the genus suggests that the virulence gene cluster was acquired before speciation (see text for details). Thick lines indicate the evolutionary pathway followed by LIPI-1; solid arrows indicate conservation of a functional LIPI-1 (in *L. monocytogenes* and *L. ivanovii*), whereas the dotted lines with an open arrowhead indicate functional corruption of the virulence gene cluster (in *L. seeligeri*). *L. innocua* and *L. welshimeri* presumably arose by excision of LIPI-1 in the corresponding evolutionary branches. The dendrogram is a schematic reconstruction of the phylogeny of *Listeria* according to references 98 and 579. (Adapted from reference 670 with permission of the publisher.)

evolution in these two *Listeria* spp. This lack of similarity is also reflected at the amino acid level (34% identity) even though the corresponding ActA proteins have similar functions in actin-based motility (230, 348). LIPI-1 is absent from the genomes of the nonpathogenic *Listeria* spp. with one exception, *L. seeligeri* (88a, 350, 670a). Although in this species LIPI-1 genes are present in a structurally intact form (232, 257), they are not expressed correctly due to the insertion of a divergently transcribed ORF (*orfE*) between *plcA* and *prfA*, disrupting the positive autoregulatory loop crucial for adequate PrfA-dependent virulence gene activation (321, 350) (Fig. 8) (see below). The *L. seeligeri* LIPI-1 contains additional ORFs (*orfCD*) and duplications of the *plcA* and *plcB* genes (350, 670a). In *L. monocytogenes, L. ivanovii*, and *L. seeligeri*, LIPI-1 is stably inserted at the same chromosomal position (Fig. 8), has a DNA composition similar to that of the core genome, lacks any obvious traces of mobility factors (integrases, transposases, or insertion sequences [IS]), and its insertion point is devoid of typical integration signals (direct repeats or tRNA genes) (88a, 350, 670a). These data are consistent with LIPI-1's having been acquired a long time ago by a common listerial ancestor (350, 670a).

It is clear that LIPI-1 provides the host bacterium with access to a new niche, animal host tissues, which imposes selection pressures totally different from those encountered in the environment. The evolutionary history of the genus *Listeria* can thus be interpreted such that the acquisition of LIPI-1 by a clone of the primordial listerial ancestor was a crucial event that led to separation of the genus into two different phylogenetic branches, one corresponding to bacteria that remained saprophytic, resulting in the current species *L. grayi*, and the other to bacteria which, by virtue of the intracellular life cassette, became parasitic (350, 670a) (Fig. 9). It is reasonable to assume that the outcome of LIPI-1 in each of the two lines of descent of the parasitic branch also played a key role in the further diversification of the genus, as follows: (i) stabilization, in *L. monocytogenes* and *L. ivanovii*; (ii) early deletion (possibly when LIPI-1 still had its original mobility functions), in *L.*

innocua and *L. welshimeri*; and (iii) functional inactivation, in *L. seeligeri* (Fig. 9). The persistence of the LIPI-1 genes in intact form in *L. seeligeri* is intriguing. Because these genes no longer confer any virulence properties on the host bacterium, one would expect them to be eliminated or corrupted during evolution. One explanation for their conservation is that the *prfA*-inactivating insertion was a relatively recent event in the evolution of *L. seeligeri*, the additional ORFs present in LIPI-1 of this species representing subsequent gene insertions that were tolerated by the afunctional PAI. Alternatively, the gene cluster present in *L. seeligeri* may represent an ancestral form of LIPI-1 which is still evolving and which, in its present form, provides an advantage in a specific natural habitat other than the mammalian host but which also requires phagosome escape functions for survival (e.g., in protists that take up bacteria from soil) (350).

Two virulence determinants present in LIPI-1, *hly* and *plcB*, have homologs which are exclusively present in low-G+C gram-positive bacteria closely related to *Listeria*, such as *Bacillus* and *Clostridium*. Homologs of two other LIPI-1 genes, *plcA* and the thermolysin-related zinc-metalloprotease gene *mpl*, are also common in this branch of gram-positive bacteria. This clearly suggests that the source of LIPI-1 genes is within the same phylogenetic division to which the genus *Listeria* belongs. PrfA, a member of the Crp/Fnr superfamily of transcription factors, is widespread in both gram-negative and gram-positive bacteria (221). ActA is the only LIPI-1-encoded protein for which no obvious structural homolog has been identified yet in prokaryotes. ActA carries functional domains present in eukaryotic proteins involved in the assembly of the actin cytoskeleton, such as vinculin, zyxin, and the WASP family of proteins (see above). It remains to be determined whether this reflects convergent evolution or indicates horizontal gene transfer between *Listeria* and eukaryotic cells. Whether LIPI-1 was acquired in a single recombinational event or whether it specifically evolved in the *Listeria* ancestor by a stepwise assembly process also remains unclear. Recent sequence analyses of the *prs-ldh* intergenic region of *L. innocua* and *L. welshimeri* have provided evidence that LIPI-1 was indeed once present in the chromosome of these species and that it became entirely excised, possibly in a single event, from the chromosome of these bacteria. Consistent with this, two small ORFs at the right end of LIPI-1 have been shown to exhibit significant similarity to bacteriophage or viral proteins, suggesting that the gene cluster (or at least part of it) was originally mobilizable by phage transduction (88a, 670a) (Fig. 8).

Internalin Islands

Internalins belong to a multigene family exclusive to *Listeria*. Except for the *inlC* and *inlF* genes of *L. monocytogenes*, the members of this family are always found in clusters of two to several genes, all oriented in the same direction (Fig. 10). The largest of the known internalin islands is LIPI-2, a chromosomal locus specific to *L. ivanovii* which contains 10 *inl* genes (see below). All *inl* genes have very similar sequences and encode highly homologous proteins with variable numbers of LRR repeats and, in the case of the large, surface-associated internalins, B and Csa (or GW) repeats. These repetitive DNA sequences are naturally prone to recombination, suggesting
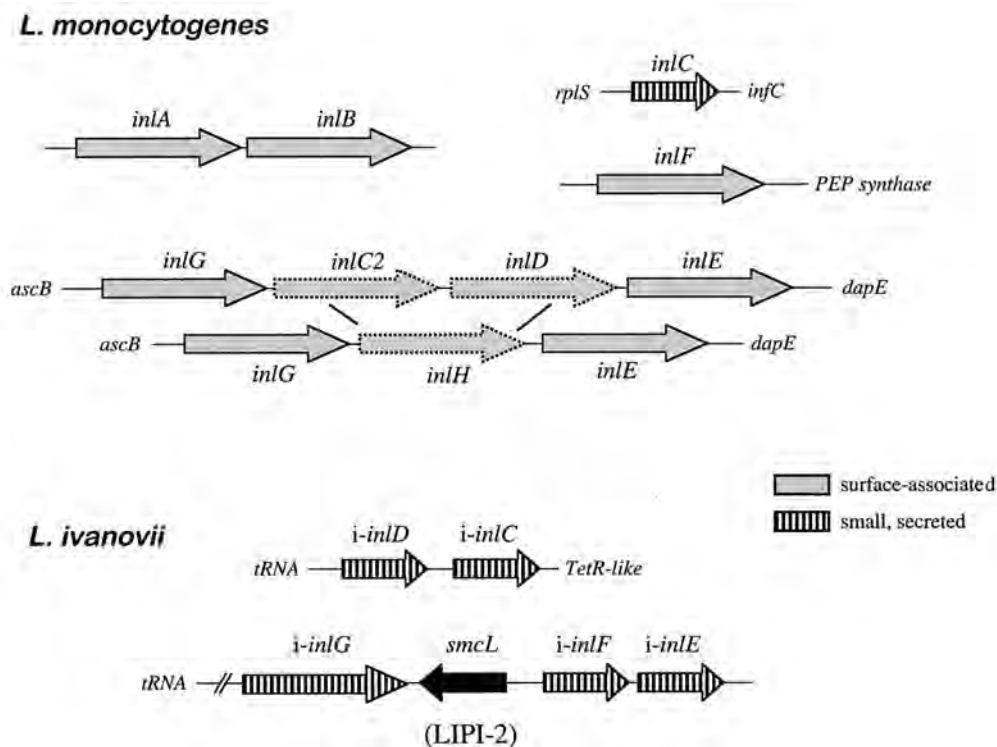
FIG. 10. Internalin islands and islets of *L. monocytogenes* and *L. ivanovii*. The scheme shows the different gene arrangements found for the same internalin locus in two isolates of *L. monocytogenes*, possibly resulting from rearrangements between *inl* genes (*inlH* presumably arose by recombination of the 5′-terminal part of *inlC2* and the 3′-terminal part of *inlD* [350, 527]). See text for details.

that the diversity of *inl* genes on the listerial chromosome is the result of gene duplications and intra- and intergenic rearrangements. There is evidence for such recombination events in *inl* genes. Comparison of the same internalin locus in two different *L. monocytogenes* strains revealed a different gene organization. The two strains shared only two structurally identical *inl* genes (*inlG and E*), and two internalin genes present in one isolate (*inlC2* and *inlD*) (150) presumably recombined to generate a new internalin gene in the other (*inlH*) (527) (Fig. 10). In *L. ivanovii*, a tandem fusion of two small, secreted internalin genes in LIPI-2 has led to the generation of a hybrid gene encoding a larger, secreted internalin, *i-inlG* (Dominguez-Bernal et al., unpublished). (Fig. 7).

The origin of the *inl* genes is intriguing. LRR domains are widespread in eukaryotic proteins but rare in bacterial proteins, suggesting that they are a relatively recent acquisition by prokaryotes. *inl* genes are also found in the nonpathogenic species *L. innocua* (193; European *Listeria* Genome Consortium, unpublished). The presence of members of the internalin multigene family in *Listeria* spp. representing distinct phylogenetic branches of the genus suggests that *inl* genes were already present in the common listerial ancestor and, consequently, that evolutionarily they are probably as old as LIPI-1. However, *inl* loci have a different distribution, gene organization, and location in the different *Listeria* spp. The *inlG(C2D/H)H* internalin islet of *L. monocytogenes* is present between two housekeeping genes (*ascB* and *dapE*) that are contiguous in *L. ivanovii* and other *Listeria* spp. (350). Also, *inlC* of *L. monocytogenes* is inserted alone, whereas its counterpart in *L. ivano-*

vii, i-inlC, is adjacent to another *inl* gene (*i-inlD*), and both are inserted at a different place of the chromosome (168). Another example is the large internalin island LIPI-2 of *L. ivanovii*, which is inserted between two housekeeping genes that are otherwise contiguous in *L. monocytogenes*. The analysis of the insertion points of *i-inlCD* and LIPI-2 revealed that these two loci are associated with tRNA genes (168, 225). tRNA loci are commonly used as a target for integration of lysogenic phages and other mobile elements (539) and are frequently found at the insertion sites of PAIs in gram-negative bacteria (259, 260). Consequently, *inl* genes have probably been not only transmitted vertically during evolution but also exchanged by horizontal transfer within the genus *Listeria*, possibly carried by phages, which in these bacteria are abundant, have interspecies infectivity, and are capable of DNA transduction (287, 338).

The conservation of such a repertoire of *inl* homologs in the genome of pathogenic *Listeria* spp. must necessarily reflect that each of the individual internalins plays a role in the biology of these bacteria. It has been suggested that variability in the exposed face of internalin LRR motifs is important to create a variety of specific protein-protein interactions (412b). Consistent with this notion, a direct involvement in receptor-facilitated invasion of permissive host cells has been demonstrated for the LRR domains of InlA and InlB (62, 373) and a role in cell and host tropism has been demonstrated for these internalins (149, 372) (see above). In *L. monocytogenes*, genes encoding large, surface-anchored internalins are abundant (150, 193, 527), whereas there seem to be very few genes encoding small, secreted internalins (only one, *inlC*, has been identified to

date) (167). In *L. ivanovii*, the reverse seems to be true, with many genes encoding small, secreted internalins and no genes encoding large, surface-associated internalins (168, 169, 226; Dominguez-Bernal et al., unpublished). It is therefore possible that the different gene complements in these two *Listeria* spp. and the structural diversity of the internalins they encode facilitate the recognition of a variety of host receptors and are thus at least partly responsible for their different pathogenic characteristics.

In line with this assumption is the recent discovery in *L. ivanovii* of LIPI-2, a large (22 kb) gene cluster composed of many small, secreted internalin genes and the *smcL* gene, encoding the *L. ivanovii* SMase (225). LIPI-2 is specifically present in *L. ivanovii* within the genus *Listeria* and may play a significant role in the pathogenic tropism of this species for ruminants. At least for the *smcL* product, there are indications for such a role in host tropism, as it selectively lyses sphingomyelin-rich membranes (e.g., those from sheep erythrocytes) (226) and promotes intracellular proliferation in bovine MDBK cells but not in canine MDCK cells (225). Most of the LIPI-2 *inl* genes are PrfA dependent, whereas *smcL* is absolutely independent of PrfA and is transcribed in the opposite orientation (226). This suggests that LIPI-2 was formed by the insertion of *smcL* into a preexisting core of *inl* genes. The *smcL*-encoded enzyme is homologous to the *S. aureus* β-toxin and the *B. cereus* SMase (226), suggesting that the gene was acquired by *L. ivanovii* via horizontal transfer from either of these two low-G+C gram-positive bacteria. LIPI-2 has a DNA composition significantly different from that of the genome of *L. ivanovii* and is unstable, leading to a 17-kb deletion that affects the right part of the PAI. These characteristics, together with the expressional subordination of most of its genes to the LIPI-1-encoded PrfA protein, indicate that LIPI-2 is a more recent development that LIPI-1 in the evolutionary history of the genus *Listeria*. Spontaneous LIPI-2 deletion mutants are significantly impaired in virulence for mice and sheep and exhibit altered organ tropism in the latter animals (Dominguez-Bernal et al., unpublished).

REGULATION OF VIRULENCE GENE EXPRESSION

Evidence for coordinate regulation of virulence genes in *L. monocytogenes* was first provided by the analysis of spontaneous and transposon-induced mutants that were noninvasive and had a combined deficiency in hemolysin (Hly) and lecithinase (PlcB) production (325). Genetic analysis of these mutants revealed that they were affected in the expression of the *prfA* gene, located immediately downstream from *plcA* in LIPI-1 (Fig. 8) (228, 379, 433). *Trans*-complementation experiments in these mutants and in a heterologous host, *B. subtilis*, confirmed that the *prfA* gene product, a 27-kDa protein called PrfA (for positive regulatory factor A), was required for the expression of *hly* and the other genes of LIPI-1 (89, 188, 380, 433). Accordingly, PrfA$^-$ mutants had a markedly higher LD$_{50}$ in mice (around 3 log units) and were unable to grow in host tissues (89, 433, 613). Spontaneous *prfA* deletion mutants arise frequently in *L. monocytogenes* strains kept in laboratory conditions. Indeed, the current type strain of the species, SLCC 53^T (also called CIP 82110^T and ATCC 15313), one of the

original strains isolated by Murray et al. in 1926 (458) (see Introduction), is one such mutant (324, 380, 433).

PrfA, Master Regulator of Virulence

PrfA is the only regulator identified to date in *Listeria* spp. which is directly involved in the control of virulence gene expression. This protein is the main switch of a regulon including the majority of the known listerial virulence genes. Some of the members of the regulon, including LIPI-1 genes (with *prfA* itself; see below) and several genes of the subfamily of secreted internalins (e.g., *inlC* of *L. monocytogenes* and i-*inlE* of *L. ivanovii*), are tighly regulated by PrfA; others, such as the *inlAB* operon, are only partially regulated by PrfA (148, 167, 350, 385, 433, 544). However, the expression of some listerial virulence genes is totally independent of PrfA, as is the case for the SMase gene of *L. ivanovii, smcL* (226), and the *inlGHE* internalin locus of *L. monocytogenes* (527). There is evidence that PrfA also negatively regulates some *L. monocytogenes* genes, such as the stress response mediator gene *clpC* (548) and the motility-associated genes *motA* (439) and *flaA* (544). This suggests that PrfA has a more global regulatory role in *L. monocytogenes*.

The deduced amino acid sequence of the PrfA protein shows similarities (20% overall identity) to that of the cyclic AMP (cAMP) receptor protein of *E. coli* (Crp, also known as CAP), on the basis of which the listerial regulator has been included in the Crp/Fnr family of bacterial transcription factors (347, 364). Crp/Fnr-like proteins have a role in virulence gene regulation in other mammalian and plant pathogens (221, 347, 694). Although most members of the Crp family of transcription factors are from gram-negative bacteria, Crp/Fnr-related transcriptional regulators have been also described in gram-positive bacteria (298). The PrfA homologs encoded by the *prfA* genes of *L. ivanovii* and *L. seeligeri* have a primary structure 80% identical to that of the *L. monocytogenes* virulence regulator protein. In *L. ivanovii*, PrfA also plays a central role in the coordinate regulation of virulence gene expression. In *L. seeligeri*, virulence genes are not expressed, but complementation with the *plcA-prfA* bicistron from *L. monocytogenes* activates transcription of the seeligerilysin O (*slo*) gene, leading to bacterial escape from the phagosome and intracellular bacterial multiplication (321). This suggests that the avirulence of this *Listeria* species is at least partly due to the insertion of the divergently transcribed *orfE* between the *plcA* and *prfA* genes (Fig. 8), resulting in a PrfA$^-$ phenotype even if *prfA* is intact and the corresponding PrfA protein is potentially active (see preceding section). Whether the complemented *L. seeligeri* strain is virulent has not yet been tested.

Two structural features that are important for Crp function are also present at the corresponding positions in PrfA (Fig. 11). One is a helix-turn-helix (HTH) motif in the C-terminal region (amino acids 171 to 191) which, as in Crp, mediates the interaction of the protein with specific DNA sequences in target promoters (612). These target sequences, called PrfA-boxes, are 14-bp palindromes centered on position −41.5 relative to the transcription start site in PrfA-dependent promoters (187, 437, 672) (Fig. 11). DNA footprinting experiments have shown that PrfA binds in vitro to the PrfA-box (136). The second structural feature of Crp that is shared by PrfA is a
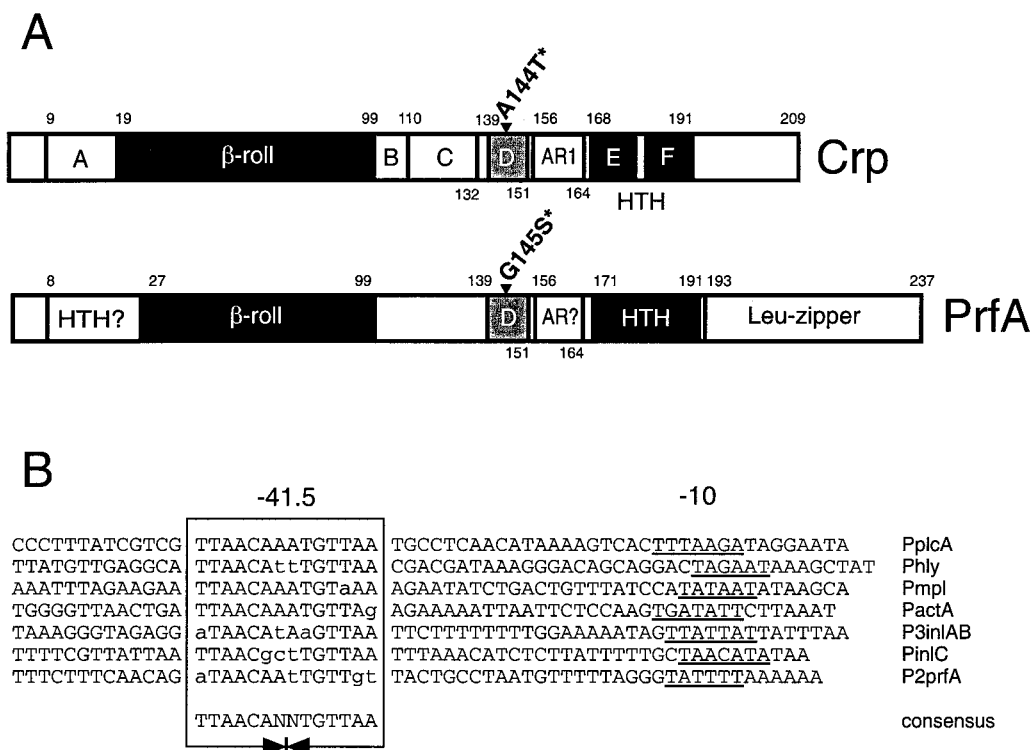
FIG. 11. Structure of PrfA (A) and its target DNA sequences in PrfA-regulated promoters (B). See text for details. In PrfA, amino acid coordinates correspond to the peptide sequence deduced from the *prfA* gene (position 1 corresponds to the Met residue of the first triplet), whereas in Crp they correspond to the actual amino acid position in the primary structure of the native protein, which lacks the residue encoded by the first triplet. Therefore, the amino acid numbering of Crp is shifted one position with respect to that of PrfA, and hence, position 144 of Crp, where the Crp* mutation Ala→Thr lies, aligns exactly with position 145 of PrfA, where the Gly→Ser substitution leading to the PrfA* mutant phenotype is located. In Crp, A to F designate the α-helical stretches of the protein and AR1 is activating region 1 (two other activating regions [AR2 and AR3] are embedded within the β-roll structure). In PrfA, structures specific for this protein are in light gray. (B) N indicates any nucleotide. (Adapted from references 66 and 221.)

series of short antiparallel β-strands delimited by Gly residues, which form a β-roll structure involving most of the N-terminal half of the protein (residues 19 to 99) (Fig. 11). In Crp, this structure forms the binding site for the activating cofactor, cAMP (344). However, cAMP is not detected and is not known to function as an intracellular messenger in gram-positive bacteria (291). Accordingly, most of the Crp residues involved in cAMP binding are not conserved in PrfA, and the addition of exogenous cAMP to *L. monocytogenes* does not lead to PrfA-mediated transcriptional activation (673). The role of this putative β-roll structure in PrfA is currently unknown. Two other functionally important regions of Crp, the D α-helix (amino acids 138 to 155), involved in transmission of the allosteric effect from the cAMP-binding N-terminal domain to the DNA-binding C-terminal domain, and activation region 1 (residues 156 to 164), involved in Crp-RNA polymerase interaction with class II promoters, are also particularly well conserved in PrfA (Fig. 11). PrfA does have some features that are unique among the members of the Crp-related family of transcriptional regulators, an additional HTH motif in the N-terminal region of unknown function, and a 25-amino-acid extension at its C terminus, which may form a leucine zipper (Fig. 11). Mutant forms of PrfA lacking this putative leucine zipper motif were found to be inactive (221).

Environmental Control of Virulence Gene Expression

Virulence factors are primarily required to colonize animal tissues and to overcome the host defense mechanisms brought into play during infection. Therefore, their synthesis outside the host may represent a burden compromising the ability of *L. monocytogenes* to survive in the natural environment and limiting its potential for transmission. Evidence is accumulating that *L. monocytogenes* has evolved signal-sensing and signal-transducing mechanisms to exploit the diverse physicochemical environments it may encounter to adapt virulence gene expression to the particular needs of saprophytic versus parasitic lifestyles. Temperature is one such environmental variables. Thus, PrfA-dependent transcription is weak below 30°C (environmental temperature) but becomes induced at 37°C (body temperature of warm-blooded animals) (124, 378, 544). The mechanism underlying this thermoregulation is unknown.

An increase in temperature is, however, not sufficient for maximal levels of transcriptional activation of *prfA* and the genes of the PrfA-dependent regulon to be reached in *L. monocytogenes*. Wild-type strains express PrfA-regulated genes very weakly in rich media (e.g., brain heart infusion [BHI]) at 37°C, but transcription of these genes is strongly activated if the bacteria are cultured in BHI supplemented with activated

charcoal (543–545, 548). The presence of this absorbent does not affect bacterial growth, indicating that the effect on virulence gene transcription involves a signal-sensing regulatory mechanism rather than a nutritional stress response due to sequestration of essential nutrients (545). One possible explanation for the "charcoal effect" is that activated charcoal absorbs a substance that is present in BHI but not in infected tissues and that allows *L. monocytogenes* to detect that it is growing outside an adequate host compartment. One such substance might be iron, because low free iron availability defines the environment of host tissues (387) and there is evidence that *hly* expression is activated in conditions of iron limitation (101, 113, 207). The specific binding of PrfA to target sequences in the *hly* and *actA* promoters is also strongly inhibited under high-iron conditions (51). However, charcoal-treated BHI does not retain its virulence-activating properties after removal of the charcoal by filtration (Novella et al., unpublished data), suggesting that the mechanism responsible is not related to the sequestration of a component of the culture medium.

Although the mechanism of charcoal-mediated activation of virulence genes remains unknown, the charcoal effect clearly demonstrates that signals from the growth medium play a critical role in regulating the expression of the PrfA regulon. It also shows that *L. monocytogenes* needs to sense a suitable combination of environmental signals of physical and, possibly, chemical nature to upregulate the PrfA regulon. This may represent a fail-safe mechanism by which *Listeria* bacteria prevent the expression of virulence genes in situations in which they are not required even if the temperature rises above the critical activation threshold. An induction of *prfA* and PrfA-dependent virulence genes similar to that observed with charcoal takes place when *L. monocytogenes* bacteria growing in BHI are transferred to minimal essential medium (MEM) (54, 635). MEM does not support normal growth of *L. monocytogenes* (635), so it is unclear whether the virulence gene-activating effect is due to a sensory mechanism involving chemical signals, a stress response to starvation, or both.

L. monocytogenes has been shown to respond to various stresses, such as exposure to high temperature (42°C) and oxidants or entry into stationary phase, by increasing, via induction of *prfA*, the expression of a set of genes including *hly* (634, 635). The upstream mediator involved in the activation of *prfA* under stress conditions is unknown. Detailed transcriptional analysis of the genes of LIPI-1 at 42°C revealed selective induction of *hly* and *plcA*, transcription of the genes of the lecithinase operon (*mpl, actA,* and *plcB*) remaining at basal levels (Ripio et al., unpublished). This expression pattern is identical to that observed if bacteria are confined within a phagocytic vacuole (75). It is therefore possible that a transient stress response involving the transcriptional activation of some genes of the PrfA regulon (those requiring lysis of the phagocytic vacuole) takes place when *Listeria* cells are still confined within the acidified phagosome, during the early stages of cell infection after invasion (see below). A simple decrease in pH to between 5.0 and 6.0 (i.e., the pH of acidified phagosomes) reduces *hly* expression in vitro (30, 124), indicating that other growth-arresting stress conditions found within phagosomes may be required for the induction of *hly* and the synergistic phagosome-disrupting virulence determinant *plcA*. It is also possible that de novo synthesis of *hly* plays a lesser role in phagosome disruption than acid-induced release of preformed intracellular stocks of the toxin, as postulated for PlcB (416).

Although wild-type *L. monocytogenes* cells produce low to undetectable levels of virulence factors in BHI, they are as virulent in mice as *prfA** strains (545), which overexpress virulence genes constitutively in vitro (see below). As described above, wild-type strains can strongly induce PrfA-dependent virulence genes, up to expression levels similar to those in constitutive *prfA** mutants, if grown with charcoal or in MEM. These observations led to the suggestion that the high levels of virulence gene induction reached by wild-type strains in vitro upon exposure to charcoal or MEM may correspond to the levels of expression required in vivo by *L. monocytogenes* to survive and proliferate in infected host tissues (545). A recent study has provided support to this hypothesis by showing that, despite their differences in spontaneous levels of expression in vitro, there is no significant difference in intracellular virulence gene activation between a wild-type strain and a *prfA** mutant (75). Indeed, several PrfA-dependent virulence genes, such as *plcA* (336), *actA* (54, 137, 451), and *inlC* (167), and PrfA synthesis itself (540), have been shown to be highly induced intracellularly. Investigation of *L. monocytogenes* intracellular gene expression using the green fluorescent protein as a reporter revealed that the *actA* promoter is activated within 30 min to 1 h of infection. This activation depended upon the ability of *L. monocytogenes* to reach the host cytosol, as it was not observed in a Δ*hly* mutant unable to escape from the phagocytic vacuole (185). All these findings indicate that the expression of *prfA* and PrfA-dependent genes is upregulated if *L. monocytogenes* senses activatory signals specific to the host cytosolic compartment.

Carbon Source Regulation of Virulence Genes

Fermentable carbohydrates cause a strong repression of virulence genes in *L. monocytogenes* (30, 67, 124, 444, 543). This downregulation occurs only if the carbohydrate is added in amounts sufficient to promote bacterial growth, suggesting that the underlying regulatory mechanism is related to catabolite repression (CR) (444). However, an *L. monocytogenes* mutant lacking CcpA, a central mediator of CR in gram-positive bacteria, did not show any alteration in carbon source regulation of virulence genes (29). Sugar-mediated repression affects not only the divergently transcribed virulence genes, *plcA* and *hly*, which have overlapping promoters, but also *plcB*, belonging to another transcriptional unit with no immediate physical link to *plcA* and *hly* and the promoter of which (P*actA*) is strictly dependent on PrfA (50, 67, 544) (Fig. 8). This suggests that repression is mediated by a *trans*-acting pleiotropic factor and somehow involves PrfA. Fermentable sugars such as cellobiose (336, 444, 541) and glucose (unpublished data) reduce PrfA-dependent virulence gene expression without affecting the levels of PrfA protein, indicating that this regulation is mediated by a repressor mechanism that either reduces the activity of the PrfA protein or blocks the binding of PrfA to its target promoters. The repressor mechanism appears to be situated hierarchically over the positive control pathway that activates the PrfA virulence regulon (see below), because fermentable carbohydrates annulate the induction of virulence genes by char-

coal treatment (67, 543). Control of the synthesis of virulence-associated products by carbon source has also been reported for other pathogenic bacteria, such as *E. coli* (STb heat stable enterotoxin) (80), *Actinobacillus actinomycetemcomitans* (leukotoxin) (449), and *Pseudomonas aeruginosa* (PlcH hemolytic phospholipase) (575). Such a CR-like regulatory mechanism therefore seems to be widespread among pathogenic bacteria, suggesting that it may be important for the coordinate control of bacterial virulence. The actual relevance of this regulatory mechanism to the modulation of virulence expression in vivo is presently unknown.

An interesting observation is that in strain NCTC 7973 of *L. monocytogenes*, virulence gene expression was downregulated by the β-glucosides cellobiose and arbutin but not by common sugars, such as glucose, mannose, or fructose (67, 500, 501). Although the behavior of NCTC 7973 is anomalous and there is evidence that this strain has accumulated several regulatory mutations (30, 67, 543), it suggests that sugars may use two independent mechanisms to bring about virulence gene repression in *L. monocytogenes*, one responding to any carbon source and another specific for β-glucosides. This is also suggested by the recent characterization of a mutant derived from the wild-type strain EGD, in which a clear uncoupling between glucose- and β-glucoside-induced PrfA-dependent virulence factor expression was observed (67). In addition, PlcB production is inhibited in NCTC 7973 by cellobiose and arbutin, but not by salicin (67), indicating that repression by β-glucosides is a complex phenomenon in which various sugar-sensing mechanisms, with different substrate specificities, may be involved. Recently, a 4-kb operon specific to *L. monocytogenes, bvrABC*, encoding a putative β-glucoside-specific phosphoenolpyruvate-sugar phosphotransferase system similar to that of the *bgl* and *arb* operons of *E. coli* and *Erwinia chrysanthemi* and the *bgl* regulon of *B. subtilis*, has been reported to be involved in virulence gene repression by cellobiose and salicin (67). Transcription of the enzyme II permease gene, *bvrB*, was found to be induced by cellobiose and salicin but not arbutin, and a *bvrAB* knockout mutation abolished the repression exerted by cellobiose and salicin. β-Glucosides are unique to the plant kingdom and are presumably abundant in decaying vegetation. Thus, Bvr-mediated sensing of β-glucosides may allow *L. monocytogenes* to recognize its presence in a soil environment and consequently switch off virulence genes. It is presently unknown how the the *bvr* locus triggers repression of PrfA-dependent genes. Another locus possibly involved in regulation of *hly* expression by cellobiose has also recently been identified by transposon mutagenesis (292).

In contrast to glucose and other common fermentable carbohydrates, glucose-1-phosphate (G1P) (in general, any hexose phosphate) efficiently stimulates the growth of *L. monocytogenes* without causing virulence gene repression (543). Sugar phosphate utilization is not constitutive in *L. monocytogenes*; it is a strictly PrfA-dependent phenotype that is coexpressed with other virulence factors (e.g., Hly, ActA, and PlcB), suggesting a role in pathogenesis. Consistent with this, the capacity to grow on hexose phosphate as a sole carbon source is also present in the pathogenic species *L. ivanovi* (as a PrfA-dependent phenotype as well) but absent from nonpathogenic *Listeria* spp. As previously mentioned, PrfA-dependent genes become selectively activated in the cytoplasm of the host cell. In this compartment, the sugar phosphates G1P and glucose 6-phosphate are available as exogenous carbon sources for bacterial growth as a result of glycogen mentabolism. A role of hexose phosphate uptake in *Listeria* intracellular proliferation has recently been confirmed with the identification and mutagenesis of the PrfA-dependent gene *hpt*, encoding a sugar phosphate transporter (Chico-Calero et al., submitted for publication). The lack of repression of virulence genes observed with hexose phosphates indicates that uptake of these sugars bypasses the regulatory circuit responsible for triggering the CR response.

Regulatory Mechanism of PrfA

The clearest evidence that PrfA is a functional homolog of Crp was provided by the characterization of *prfA** mutants of *L. monocytogenes* (544). These mutants were first identified by Ripio et al. (545) when testing a panel of *L. monocytogenes* strains for Hly and PlcB production as markers of PrfA-dependent virulence gene expression. Three different phenotypes were observed (Fig. 12). Phenotype 1 was exhibited by most of the strains tested and was characterized by low to undetectable levels of virulence factor production in BHI and the capacity to substantially (by 10- to 25-fold) increase this production upon culture medium supplementation with charcoal. Phenotype 2 was exhibited by only a few strains and was characterized by spontaneously high levels of virulence factor production and by unresponsiveness to charcoal, the levels of virulence factor production already being maximal. The third phenotype was exhibited by only one strain (LO28) and was characterized by intermediate levels of spontaneous Hly and PlcB production on BHI and responsiveness to charcoal. It was concluded that phenotype 1 corresponded to the wild type of *L. monocytogenes* and that phenotypes 2 and 3 represented mutant or variant phenotypes (545) (Fig. 12).

All phenotype 2 variants were strains that had been kept under laboratory conditions for a long time. Some were well-known collection strains widely used in research on listerial virulence, such as NCTC 7973 (Fig. 12) and the EGD Mackaness strain used in the laboratory of one of us (P.B.), originally obtained from R. J. North at the Trudeau Institute and thereafter renamed EGD-A to prevent confusion with the wild-type EGD strain currently used as a reference strain by the European *Listeria* Genome Consortium (545). Phenotype 2 variants were probably introduced inadvertently into research as model strains because their conspicuous hyperhemolytic phenotype facilitated the selection of Hly⁻ mutants during early studies on the role of hemolysin in *Listeria* virulence. Phenotype 2 variants arise spontaneously at a very low frequency from wild-type strains during subculture in the laboratory and may be induced by chemical mutagenesis (171, 326, 545). The *prfA* genes in all phenotype 2 strains tested to date have a point mutation at codon 145, leading to an amino acid substitution (Gly→Ser) in the PrfA protein. Complementation studies have shown that this mutation is responsible for the constitutive overexpression of virulence genes exhibited by phenotype 2 strains (544).
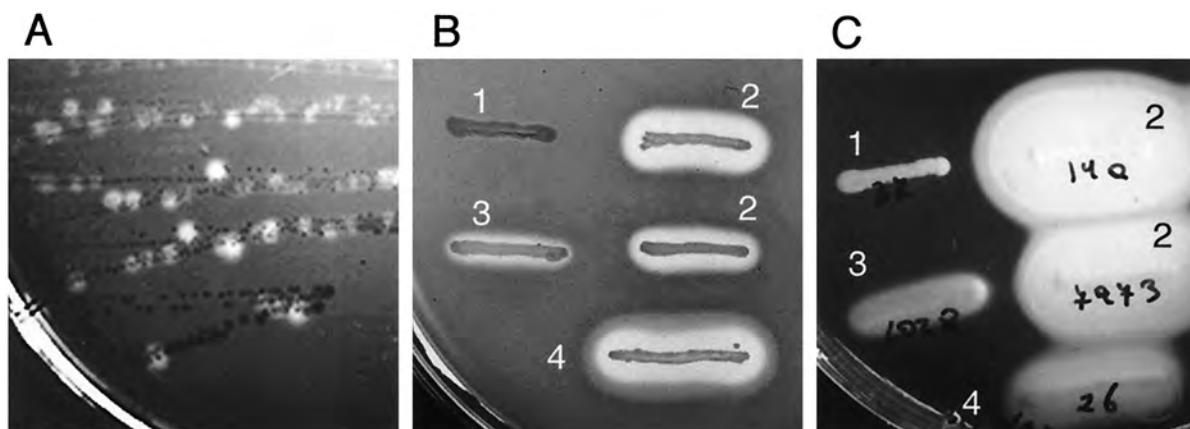
FIG. 12. Hemolysin (Hly) and lecithinase (PlcB) phenotypes of *L. monocytogenes*. (A and B) Sheep blood agar; (C) egg yolk agar. (A) Original plate from which *prfA** mutants of *L. monocytogenes* were first isolated and identified; the culture shows a mixture of colonies of the wild-type isolate P14 (weakly hemolytic) and its spontaneous *prfA** derivative, P14-A (strongly hemolytic) (see text and references 544 and 545 for details). (B and C) Hly and PlcB phenotypes of *L. monocytogenes* (streaks 1, 2, and 3) compared with those of *L. ivanovii* (streak 4). 1, weak to undetectable hemolytic and lecithinase activities typical of the *L. monocytogenes* wild type (clinical strain P37); 2, strong hemolytic and lecithinase activities of *prfA** mutant (strains P14-A and NCTC 7973); 3, intermediate variant phenotype found in strain L028, of unknown molecular basis (L028 has a wild-type *prfA*); 4, strong hemolytic and lecithinase activities by *L. ivanovii* (clinical isolate P26); all the strains of this species characteristically overexpress PrfA-dependent virulence genes to levels similar to those of *prfA** mutants of *L. monocytogenes*.

A PrfA protein with the Gly145Ser substitution renders the virulence regulon insensitive to a variety of environmental repressor signals, such as growth in rich medium, low temperature, and the presence of readily fermentable carbon sources (30, 543, 544). Gly-145 of PrfA aligns with Ala-144 of Crp (544, 673), a position at which any bulkier substitution (e.g., Ala144Thr) results in a mutant Crp* protein that no longer requires the cofactor cAMP to become transcriptionally active (344) (Fig. 11). Like Crp* mutant forms, the Gly145Ser PrfA protein has greater binding affinity for the specific target DNA sequence (673). The Gly145Ser mutation also increases the capacity of the PrfA-RNA polymerase complex to initiate transcription in vitro (Vega et al., unpublished data). From these observations, it has been suggested that PrfA functions via a cofactor-mediated allosteric transition mechanism similar to that of Crp and that the Gly145Ser mutation represents a cofactor-independent PrfA* form that is "frozen" in an active conformation (544, 673).

The regulatory model currently proposed for PrfA is depicted in Fig. 13. This model is based on two premises. The first is that PrfA has two functional states, inactive and active, and shifts from one to the other upon binding of a low-molecular-weight cofactor, not yet identified, that transduces the stimulatory environmental signals to the PrfA transcriptional machinery (544, 673). Evidence for the existence of this PrfA-activating cofactor has been obtained by the fractionation of bacterial extracts on sucrose gradients, in which a low-molecular-weight component seems to stimulate PrfA-target DNA complex formation (221). The second premise is that PrfA is always present, at least in minimal amounts, in the bacterial cytoplasm, which is also the case. *prfA* is expressed in two ways: (i) under the control of growth-regulated promoters in the *plcA-prfA* intergenic region, generating a monocistronic transcript from which PrfA is synthesized in low but detectable amounts even if bacteria are exposed to PrfA regulon-nonactivating conditions (186, 187, 433, 540, 541, 673); and (ii) under the

control of a PrfA-dependent promoter in front of the *plcA* gene, which generates a *plcA-prfA* bicistronic transcript (83, 186, 187, 433) (Fig. 13; see also Fig. 8), creating an autoregulatory loop which is critical for the adequate activation of *prfA* (378, 433, 541, 543, 544, 673). In the model proposed, the levels of virulence gene expression depend primarily on the functional status of the PrfA protein. This in turn controls the levels of *prfA* expression via positive feedback, resulting in strong PrfA-dependent transcriptional activation due to a rapid increase in PrfA in the active conformation, provided that the intracellular levels of the environmentally regulated stimulatory cofactor remain high (see the legend to Fig. 13). This positive-control model is highly versatile and makes possible a fine-tuned adaptive response to rapidly changing environmental conditions, such as would be encountered by *L. monocytogenes* during its transition from free to parasitic life and during passage across the various physiological barriers, tissues, and compartments of the infected host. The model provides a coherent explanation of all the available experimental data on PrfA regulation and is also compatible with the possible existence of additional regulatory elements modulating the activity of PrfA or its interaction with the target DNA. These elements include the so-called PrfA-activating factor (Paf), a proteinaceous substance that may be a fraction of the RNA polymerase and that facilitates the formation of the PrfA/RNA polymerase/target DNA complex (50, 51, 136, 221), and the putative mediators of carbon source regulation of virulence gene expression (see above).

Fine Regulation of Virulence Determinants

A second level of regulation is provided by the differential response of PrfA-dependent promoters according to the structure of their PrfA-boxes (Fig. 11). Binding affinity to PrfA-boxes is affected by the number of nucleotide mismatches they carry, becoming weaker as the sequence diverges from the
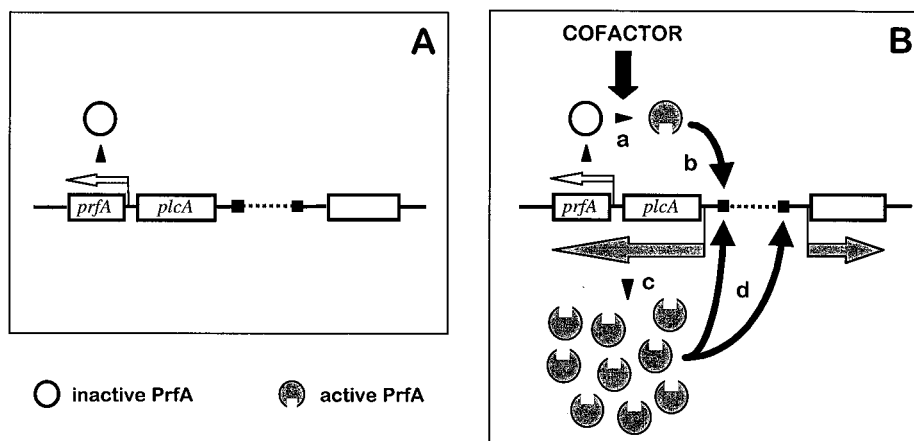
FIG. 13. Model for the mechanism of PrfA-mediated regulation. This model predicts that PrfA can undergo an allosteric transition between inactive (light gray) and active (dark gray) forms upon interaction with a low-molecular-weight hypothetical cofactor (see text for details). (A) Resting PrfA system. There is no cofactor, and the PrfA protein is synthesized at low, basal levels from the monocistronic transcripts generated from growth phase-regulated promoters in front of the *prfA* gene (small light gray arrow). (B) Activated PrfA system. If *L. monocytogenes* senses a suitable combination of environmental signals (i.e., 37°C and cytoplasmic environment), the intracellular concentration of the cofactor increases, leading to activation of the PrfA protein (a), which binds with increased affinity to the PrfA-boxes (black squares) in PrfA-regulated promoters (b); the transcriptionally active PrfA form causes the synthesis of more PrfA (in active conformation) via the positive autoregulatory loop generated by the PrfA-dependent bicistronic *plcA-prfA* transcript (c), thereby inducing the transcription of all PrfA-dependent genes (d) (dark gray arrows represent PrfA-dependent transcripts; the empty rectangle on the right represents any PrfA-dependent gene). The PrfA regulon remains switched on as long as the cofactor is present in the bacterial cytoplasm, but is rapidly switched off if the activating environmental signals cease and the concentration of the cofactor drops. (Reproduced from reference 673 with permission of the publisher.)

perfect inverted repeat. Promoters with perfectly symmetrical palindromes (e.g., P*hly* and P*plcA*) are more sensitive and respond more efficiently to activation by PrfA than those with nucleotide mismatches (e.g., P*mpl*, P*actA*/*plcB*, and P*inlA*) (Fig. 11), which require larger amounts of the regulatory protein for full activation (53, 72, 186, 187, 544, 613, 698). This complementary *cis*-acting regulatory mechanism appears to be critical for the temporal and spatial expression of virulence genes and consequently for the correct development of the intracellular infectious cycle of *L. monocytogenes*. Early work by Freitag et al. (186, 187) and Camilli et al. (83) using different combinations of mutants affected in the monocistronic or bicistronic expression of *prfA* demonstrated that transcription of *prfA* from its own promoters in the *plcA-prfA* intergenic region was sufficient to mediate escape from the phagocytic vacuole and subsequent intracellular proliferation, but not to mediate cell-to-cell spread, which required a functional autoregulatory loop. These observations suggested that small amounts of PrfA synthesized during the early stages of bacterium-cell interaction are sufficient to activate rapidly and fully the high-affinity promoters of the *hly* and *plcA* genes, the products of which are involved in phagosome disruption. In contrast, activation of the low-affinity *actA* promoter, which directs the expression of the *actA* and *plcB* determinants involved in cell-to-cell spread, requires higher levels of PrfA, as provided by induction of the bicistronic transcript in the cytoplasm. Consistent with this, a recent study comparing wild-type bacteria with *hly* and *actA* mutants (which are confined in the phagosome and host cell cytoplasm, respectively) has shown that the PrfA-dependent membrane-damaging determinants *hly* and *plcA* are predominantly expressed in the phagosomal

compartment, whereas the *actA* and *mpl* genes are selectively induced in the cytoplasm of the infected host cell (75).

As described above, experimental evidence has recently been obtained that PrfA synthesis is indeed activated in the host cell cytoplasm, correlating with higher activity of the PrfA-dependent *plcA* promoter that directs the expression of the *plcA-prfA* bicistron (540). However, *prfA** mutants, in which PrfA-dependent expression is constitutively activated, are highly cytotoxic for the host cell (unpublished data). This may result from excess, unrestricted production of toxic virulence factors such as Hly and the phospholipases. In other words, activation of the PrfA system in the cytoplasm may be deleterious and incompatible with intracellular parasitism unless modulated by additional regulatory mechanisms (especially when *L. monocytogenes* reaches high intracellular population densities after multiplication). *prfA* transcript levels are surprisingly low within host cells (75), suggesting that a high turnover of *prfA* mRNA may be a mechanism that helps limit PrfA synthesis within host cells. The reported short half-life of Hly (127a, 311, 677) and PlcB (415) in the host cell cytoplasm may also play an important role in posttranslationally regulating the intracellular levels of cytotoxic virulence factors. This is clearly suggested by the observation that, whereas the levels of *actA* transcription in the host cell cytoplasm are only three times higher than those of *hly*, the amounts of the corresponding proteins are very different, with 70 times more ActA than Hly (451). Additional negative control mechanisms acting at the transcriptional level may also contribute to modulate *prfA* expression within the host cell. For example, uptake of glucose from the host cell cytoplasm may counteract, by carbon source-mediated repression (see above), the stimulatory effect of the

intracellular environment on PrfA function. Freitag et al. (186, 187) reported that in the absence of PrfA, transcription from the two promoters immediately upstream from *prfA* is increased, and deletion of the −35 region of one of these two promoters (P2) (see Fig. 11 and 13), with a structure reminiscent of a PrfA-box, leads to higher levels of *prfA* transcription. This suggests that, by binding to the *plcA-prfA* intergenic region, PrfA may short-circuit the autoamplification loop, thereby downregulating its own expression. One study did not confirm the existence of such a PrfA-mediated negative autoregulatory mechanism (613), but a more recent study has shown that the transcription of a reporter gene under the control of the P2*prfA* promoter is significantly repressed by PrfA (72).

Some *L. monocytogenes* virulence genes, although part of the PrfA regulon, can also be expressed independently of PrfA. This may provide additional expressional characteristics important for the specific role of individual virulence factors in pathogenesis. A clear example is the internalin operon, *inlAB*, which is expressed from three promoters, two of which are PrfA independent. The third promoter is PrfA dependent, but its PrfA-box has two substitutions, resulting in a low binding affinity and inefficient induction by the listerial virulence regulator (53, 136, 148, 613). This promoter configuration is obviously responsible for the peculiar expression characteristics of the *inlAB* operon, which, in contrast to other PrfA-regulated loci, is significantly expressed during extracellular growth in broth medium at 37°C and positively regulated by elevated iron concentrations (i.e., PrfA-downregulating conditions) but poorly expressed within the cell cytoplasm or in MEM (i.e., PrfA-activating conditions) (53, 75, 101, 167). This is consistent with the role of the products of this operon, the internalins InlA and InlB, as invasins mediating internalization from the extracellular space and with no known involvement in intracellular proliferation.

The *hly* gene, which is primarily PrfA dependent, has an alternative means of expression via a weak PrfA-independent promoter (141). This explains why *L. monocytogenes prfA* deletion mutants have weak hemolytic activity (141, 544) and low-level expression of *hly* is detectable in *L. monocytogenes* in conditions of PrfA downregulation, such as extracellular growth in broth culture. As discussed earlier (617, 619), low concentrations of exogenous Hly released by extracellular bacteria may contribute to pathogenesis by triggering cell responses, and the PrfA-independent promoter of the *hly* gene may be important in this respect.

HOST CELL RESPONSES TO INFECTION

Signal Transduction Pathways Associated with Epithelial Cell Invasion

Little is known about the signaling cascades triggered by *Listeria* organisms during entry into cells that are normally nonphagocytic. InlB has been shown to be an agonist of the lipid kinase p85/p110 (PI 3-kinase [PI3K], and the InlB-mediated uptake of *L. monocytogenes* by Vero cells has been shown to be associated with activation of the PI3K (296, 297). In addition to InlB, this activation also requires tyrosine phosphorylation in the host cell, because uptake is inhibited by

genistein treatment, which blocks tyrosine-specific protein kinases. The mechanism by which the PI-3 kinase mediates uptake is unknown. However, the products of the reaction catalyzed by PI3K, phosphoinositide-3,4-bisphosphate and phosphoinositide-3,4,5-triphosphate, may directly interfere with the actin cytoskeleton by uncapping the barbed ends of actin filaments, thereby affecting cytoskeleton dynamics. In addition to phosphoinositide-phosphates, leukotrienes are also thought to be involved in *L. monocytogenes* signaling during the invasion of epithelial cells. Nordihydroguaretic acid, an inhibitor of 5-lipooxygenase activity, which generates leukotrienes from arachidonic acid, also inhibits the invasion by *L. monocytogenes* of Hep-2 cells (430). However, it is not yet known at which step of the putative signaling cascade leukotrienes are involved, or whether the leukotriene signal is initiated by InlA- or InlB-receptor interactions.

The treatment of various types of cell with the protein kinase C (PKC) inhibitor genistein (or staurosporine) inhibits invasion by *L. monocytogenes*, suggesting that PKC activity is critical for regulating the cytoskeletal changes necessary for listerial uptake (253, 353, 648, 675). It has recently been reported (648) that the invasion of HeLa cells by *L. monocytogenes* activates not only ERK-1 and ERK-2, but also two other MAP kinases (p38 and JNK) and the MAP kinase kinase MEK-1. ERK-2 is also phosphorylated upon invasion by LLO-negative mutants and is a downstream target of MEK-1. These data suggest that MEK-1/ERK-2 activation is one step in the signaling cascade leading to *L. monocytogenes* uptake by host epithelial cells. ERK-2 activation is not sensitive to wortmannin treatment, showing that it is not a downstream event of PI3K activation. Obviously, the PI3K and MEK-1/ERK-2 pathways may form two components of converging or independent signal transduction systems required for *L. monocytogenes* invasion. Another protein tyrosine kinase, pp60^{c-src}, is clearly activated during the entry of *L. monocytogenes* into epithelial cells because specific inhibition of pp60^{c-src} by herbimycin blocks *inlAB*-dependent entry (664). Treatment of host cells with TcdB toxins led to a dramatic breakdown of the normal actin cytoskeleton but did not abrogate *L. monocytogenes* invasion (or actin-based motility), indicating that the small GTP-binding proteins of the Rho and Ras subfamilies are not involved in the entry process (162).

The *L. monocytogenes* invasion process has been found to be sensitive to treatment with cytochalasin D for all cell types tested, demonstrating the importance of microfilaments for internalization (194, 359). Several recent reports have demonstrated that microtubules also play some role in invasion. The uptake of *L. monocytogenes* by mouse dendritic cells, mouse bone marrow-derived macrophages, and human brain endothelial cells is sensitive to nocodazole treatment, which disrupts microtubules (246, 253, 352).

Activation of NF-κB and Modulation of Host Gene Expression in Macrophages

Differential gene expression in eukaryotic cells depends largely on modification of the transactivating activity of inducible transcription factors. NF-κB, which is involved in regulation of the expression of many immunologically important genes (622), is the prototype of a family of transcription fac-

tors. NF-κB DNA-binding activity in response to *L. monocytogenes* infection has been studied in the macrophage-like cell line P388D$_1$ (272, 273), endothelial cells (330, 600), and epithelial cells (271). A rapid invasion-independent increase of the NF-κB RelA/p50 DNA-binding activity is observed in macrophages within 10 to 20 min of addition of the bacteria. Studies with in-frame deletion mutants have shown that NF-κB is induced with biphasic kinetics upon *L. monocytogenes* infection. The first transient induction of NF-κB requires only the adhesion of *L. monocytogenes* to the P388D$_1$ cells. This induction also occurs upon infection with mutants of *L. monocytogenes* that have lost one or more of the known virulence genes and involves lipoteichoic acid, a cell surface component of *L. monocytogenes*. A second, permanent induction of NF-κB is detectable after release of the *Listeria* bacteria into the cytoplasm of the host cell. This occurs exclusively with virulent *L. monocytogenes* strains and requires the synthesis of the bacterial phospholipases PlcA and PlcB in the infected host cell. It has been speculated that DAG and CER, likely products of the two enzymes (see above), act as second messengers in the induction of persistent NF-κB activation.

The expression of cytokines upon treatment of mammalian cells with *L. monocytogenes* or *L. monocytogenes*-derived products has been analyzed in many different cell systems. Infections were performed with defined cell populations, such as mouse bone marrow-derived macrophages (BMM), macrophage-like cell lines, and complex mixtures of cells, like spleen cell preparations (356, 357). We will briefly review some of the studies and focus on those in which defined cell types were used as hosts for *L. monocytogenes*.

Early studies by E. A. Havell (274–276) showed that murine fibroblasts infected with *L. monocytogenes* produce significant amounts of alpha/beta interferon (IFN-α/β) and IL-6 and that mouse alveolar macrophages respond to *L. monocytogenes* infection by producing IL-6 and TNF-α. Differences in the induction of cytokine expression were assessed for macrophages infected with live and killed *L. monocytogenes*-infected macrophages. Live and killed bacteria differ in IL-1, IL-6, and TNF-α production (447, 709), whereas IL-12 is induced in vitro by live and killed *Listeria* cells (94).

Using various well-defined mutants of *L. monocytogenes*, it has been shown that viable bacteria rapidly induce IL-1α, IL-1β, IL-6, and TNF-α mRNAs in the mouse macrophage-like cell line P388D$_1$, whereas killed bacteria induce only IL-1β mRNA (355). Hly$^-$ mutants are unable to induce IL-1α, IL-6, and TNF-α but do induce IL-1β mRNA. The infection of BMM with *L. monocytogenes* also induces the proinflammatory cytokines (132). However, an Hly$^-$ *L. monocytogenes* mutant was found to induce the same types and amount of cytokines as the wild-type strain, indicating that intracellular growth is not necessary for the transcriptional induction of these cytokines in BMM.

Not all the bacterial products mediating the induction of cytokines upon *L. monocytogenes* infection have been identified. The bacterial cell wall polymer lipoteichoic acid has been shown to induce the expression of the proinflammatory cytokines IL-1α/β, IL-6, and TNF-α in BMM (353) and in human monocytes (40). Nishibori et al. (474) analyzed cytokine gene expression upon treatment of spleen cells with purified LLO. Total spleen cell preparations containing macrophages and NK cells responded rapidly with increases in the synthesis of IL-1α, IL-6, IL-10, IL-12, TNF-α, and IFN-γ mRNAs. By depleting the cell preparations of either NK cells or macrophages, the contribution and interaction of the two cell types to cytokine production were assessed. It was found that IL-1α, IL-12, and TNF-α were produced mainly by macrophages, whereas IFN-γ was clearly produced by NK cells stimulated by macrophages, probably via TNF-α and IL-12.

The synthesis of cytokine receptor mRNAs in BMM infected with *L. monocytogenes* seems to be regulated inversely to the corresponding cytokines (132). TNF receptor type I and IFN-γ receptor are transcriptionally downregulated following the infection of BMM with *L. monocytogenes*, whereas IL-1 receptor type II mRNA is unaffected. In contrast, TNF receptor type II expression is upregulated. The bacterial requirements for this downregulation of cytokine receptor expression are unknown, but the downregulation is specific for infection with *L. monocytogenes* and was not found upon infection with nonpathogenic *L. innocua* strains.

Phagocytosis of *L. monocytogenes* is required for transcriptional induction of the heat shock protein 70 (hsp70) gene in macrophages. The synthesis of LLO seems to accentuate the hsp70 response (598). In contrast, expression of the gene encoding MHC class II antigen I-Ab is downregulated upon phagocytosis, at a time when the bacteria are still located mainly in the phagosomal compartment (595). Again, LLO seems to be important for this host response. Another important host cell gene that is downregulated on infection of macrophages by *L. monocytogenes* encodes antigen H-2K, a major component of the class I MHC molecule. Unlike downregulation of the gene encoding antigen I-Ab from the class II MHC, this event requires not only LLO, but also the expression of one or more genes of the listerial lecithinase operon (595).

Host Responses during Infection of Endothelial Cells

Host cell responses elicited in endothelial cells during infection or by treatment with listerial exotoxins include lipid second-messenger generation (617, 618), NF-κB activation (330, 600), stimulation of cytokines and chemokines, and induction of cell adhesion molecule expression (152, 153, 247, 330, 351, 699). HUVECs were shown to respond to *L. monocytogenes* infection or treatment with purified LLO by increasing phosphoinositide metabolism and inducing the production of the vasoactive inflammatory mediators platelet-activating factor and prostaglandin I$_2$. This LLO-triggered second-messenger induction is further stimulated by the phospholipase PlcA, which acts in synergy with LLO (see above). It has been speculated that the pore-forming activity of LLO might facilitate the uptake of PlcB by the HUVECs, resulting in DAG and inositol phosphate generation (617). Listerial phospholipase-mediated increased levels of CER were also found in HUVECs during *L. monocytogenes* infection. This increase is followed by an elevation in the activity of the transcription factor NF-κB (152, 600). The cell adhesion molecules for which expression was analyzed in *L. monocytogenes*-infected HUVECs and BMECs include E-selectin, P-selectin, VCAM-1, and ICAM-1 (152, 153, 351, 699). P-selectin expression peaked 30 to 60 min after infection, whereas the induction of E-selectin, ICAM-1, and VCAM-1 started 4 to 8 h postinfection.
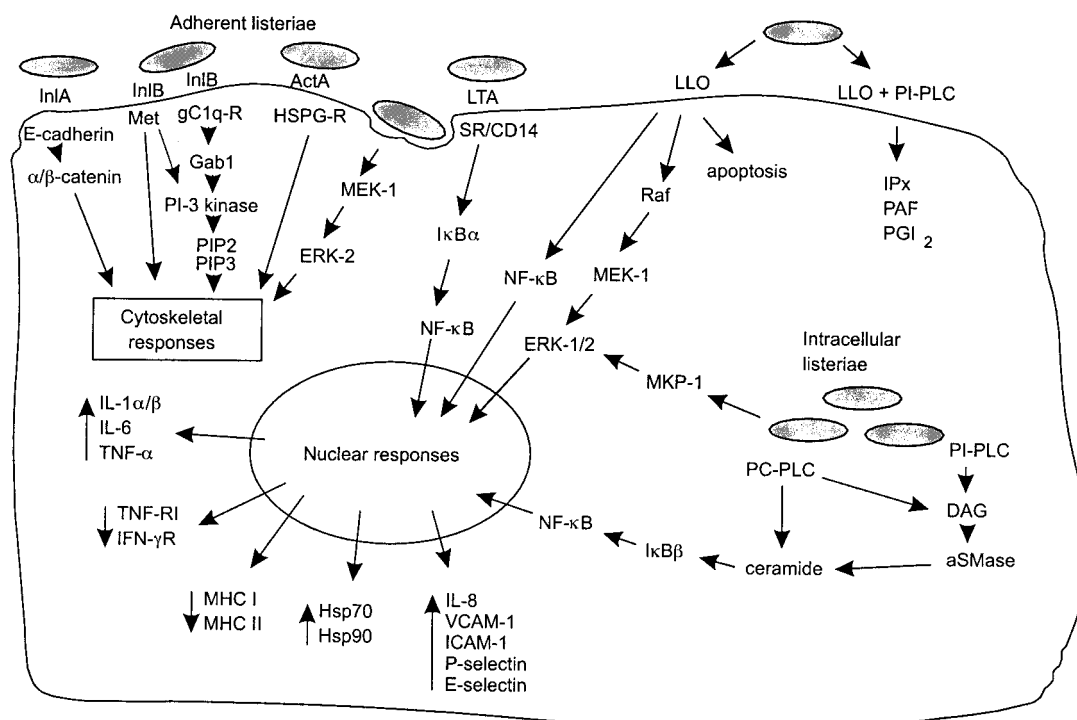
FIG. 14. Summary of modulated signal transduction pathways and host cell responses identified during *L. monocytogenes* infection of murine bone marrow-derived macrophages, murine P388D₁ and J774 cell line macrophages, human Caco-2 and HeLa epithelial cells, mouse dendritic cells, and human umbilical vein endothelial cells. Abbreviations: aSMase, acidic sphingomyelinase; gC1q-R, complement C1q receptor; Hsp70, heat shock protein 70; Hsp90, heat shock protein 90; HSPG-R, HSPG receptor; ICAM-1, intercellular adhesion molecule 1; IFN-γR, IFN-γ receptor; IPx, inositolphosphates; LTA, lipoteichoic acid; MEK-1, mitogen-activated protein kinase kinase 1; Met, receptor tyrosine kinase for HGF; MKP-1, mitogen-activated protein kinase phosphatase 1; PAF, platelet-activating factor; PGI₂, prostaglandin I₂; PIP2, phosphatidylinositol-(3,4)-bisphosphate; PIP3, phosphatidylinositol-(3,4,5)-trisphosphate; PI-PLC, phosphatidylinositol-specific phospholipase C; SR, scavenger receptor; TNF-RI, TNF receptor type 1; VCAM-1, vascular cell adhesion molecule-1. Reproduced with modifications from reference 357 with permission of Elsevier Science.

Cell adhesion molecule expression is partly dependent on LLO expression (351). E-selectin expression and the concomitant induced adhesion of polymorphonuclear leukocytes to HUVECs is particularly dependent on PlcA and PlcB synthesis by *L. monocytogenes*. These data show that the phospholipases are important virulence factors in the *L. monocytogenes*-endothelial cell interaction, interfering with signal transduction pathways mediated by lipid second messengers and causing changes in gene expression (600). The expression of IL-6 and IL-8 is also induced during the infection of HUVECs, but no data are available concerning the bacterial requirements for the induction of cytokine and chemokine expression upon infection (247).

Apoptosis

L. monocytogenes has recently been shown to induce apoptosis in hepatocytes (560), Caco-2 epithelial cells (662), and dendritic cells, in which it was also demonstrated that LLO is the bacterial component triggering this event (252). *L. monocytogenes*-infected hepatocytes undergo apoptosis in vitro and in the infected mouse. It has been suggested that hepatocyte apoptosis, which is linked to neutrophil recruitment, eliminates infected cells rapidly from the tissue. Thus, apoptosis would inhibit the spread of *L. monocytogenes* to the neighboring cells and simultaneously would make the intracellular *Listeria* cells

accessible to effector cells. In dendritic cells, which are important antigen-presenting cells, LLO-induced apoptosis should result in lower levels of antigen presentation to immune cells, thereby downregulating the immune response.

Figure 14 summarizes the information available on the signaling cascades involved in the host cell response to *Listeria* infection.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In the last 15 years, listeriosis has undergone a transformation from an infectious disease of limited importance to one of the most topical food-borne infections and a source of major concern for health authorities and the food industry alike. In the same period of time, the work of various groups in Europe and the United States has made the causal agent of this disease, *L. monocytogenes*, one of the best-characterized intracellular parasites at both the molecular and cellular levels. One particular aspect of the interaction between *L. monocytogenes* and the eukaryotic host cell, the actin-based motility mediated by the surface protein ActA, is today a true paradigm of cell biology. Despite the major progress made towards understanding the mechanisms of virulence of *Listeria* spp., our current insight into the process of pathogenesis is still very partial and fragmentary. We have unraveled fundamental details of the

intracellular parasitism practiced by these bacteria and the molecular determinants responsible for key stages in their life cycle within the cells of the host. However, we know very little about the mechanisms used by pathogenic *Listeria* spp. to penetrate the organism, to cross the host's physiological barriers, and to interact with the target cells in which they proliferate, causing disease. The same holds true for the strategies used by *Listeria* spp. to resist and counteract the defense mechanisms of the host for long enough after intestinal translocation to produce invasive disease in the target organs. Only about 15 listerial genetic determinants have been found to date to be associated with pathogenesis, including true virulence genes and housekeeping genes required for survival in the host. This number is very small compared with the nearly 3,000 genes predicted for the 2.9-Mbp genome of *L. monocytogenes* (438, 679; European *Listeria* Genome Consortium, unpublished). It is therefore clear that there must be other, as yet unknown loci in the *Listeria* chromosome that are required for the development of infection.

To date, studies of the molecular determinants of *Listeria* virulence were mainly based on the analysis of mutants affected in conspicuous phenotypes displayed under in vitro conditions. The clearest illustration of this is the central virulence gene cluster, which was discovered by genetic analysis of transposon mutants affected in hemolytic and lecithinase activities, two phenotypes easily detectable on agar plates. With hindsight, it was a clear stroke of luck that the mutated *hly* and *plcB* genes were located in the same chromosomal locus together with other essential virulence genes such as *prfA* and *actA*. In other cases, the in vitro phenotype used to identify potential virulence genes during mutagenesis was a priori more directly related to functions relevant to *Listeria* pathogenesis, such as loss of invasiveness in eukaryotic cells. The extensively studied *inlAB* operon was identified in this way. However, although the *inlAB* products have been clearly shown to have an invasin function in cell monolayers in vitro, no clear role for this locus in pathogenesis has been demonstrated yet in infection models in vivo. This illustrates the limitations inherent to the approaches used to date in the molecular analysis of *Listeria* virulence, particularly when combined with in vitro models of infection that do not necessarily reproduce reliably the natural conditions encountered by *L. monocytogenes* in the tissues of the host.

This scenario is likely to change radically in the immediate future with the availability of the complete sequence of the genome of *L. monocytogenes*. This will make it possible to shift from an investigation largely based on reductionist analysis of the role in virulence of individual microbial products in an isolated form, to a holistic approach to *Listeria* pathogenesis. In conjunction with appropriate in vivo expression technologies, which are beginning to be applied to *Listeria* spp. with promising results (158, 192), the genomic approach will make it possible to identify systematically the entire set of genes required by *L. monocytogenes* for its survival and proliferation in the various tissues and compartments of the host. The comparative genomic investigation of pathogenic versus nonpathogenic *Listeria* species, and of environmental versus epidemic strains of *L. monocytogenes*, will lead to the discovery of new pathogenicity determinants, providing us at the same time with insight into the evolution of virulence in this bacterial genus. Progress in human genomics will also make it possible to define the host components and processes engaged during *Liste-*

ria infection, as shown by a recent study in which cellular responses to *L. monocytogenes* were monitored using oligonucleotide microarrays (97a). Characterization of the expression profiles of both the host cell and the pathogen at various stages of the infection process will undoubtedly represent a quantum leap in our understanding of host-microparasite interaction.

The shift towards genomic technology as the primary approach for analyzing the molecular determinants of *Listeria* pathogenesis will not make reductionist strategies of investigation less important. Indeed, functional analysis of the genome of *L. monocytogenes* will bring to light a growing number of potential virulence factors, and the intimate mechanisms of interaction of these factors with the molecules of the host will still require investigation by the classical techniques of molecular and cell biology. The importance of this reductionist approach is illustrated by the recent study by Lecuit et al. (372), suggesting that the host tropism of *L. monocytogenes* may depend on a single residue of E-cadherin, the cell receptor for InlA. It has required the continuous efforts of various research groups over several years to make the first steps towards elucidating the molecular mechanisms of actin-based motility, which involves only one *Listeria* protein. This indicates the enormity of the task ahead of us if we are to understand in precise molecular terms the way in which *Listeria* interacts with its various vertebrate hosts to produce infection.

Fundamental research on *Listeria* molecular biology and pathogenesis can be exploited in the rational design of novel biotechnological tools to fight disase. The characterization of the listerial intracellular life cycle and of the role that hemolysin plays in it, together with the knowledge accumulated concerning the immunobiology of experimental listeriosis in the mouse, has made *L. monocytogenes* a most promising candidate as antigen delivery system for the development of novel recombinant oral live vaccines (254). By its phagosome-disrupting activity, Hly permits the antigens secreted by the producer bacteria to be released into the cytoplasm and thus to be presented by MHC class I molecules, inducing a cytotoxic response useful for combating intracellular parasite infections or tumoral disease (327). Indeed, successful vaccination of mice using *L. monocytogenes*-based recombinant live vaccines has been reported for several viral and tumor model systems (183, 227, 293, 307, 418, 492, 496, 497, 614, 688). Recently, a plasmid containing model antigen genes under the control of a eukaryotic promoter and an endolysin gene from a *Listeria* phage under the control of the strictly PrfA-dependent promoter of the *actA* gene (which is selectively activated in the host cell cytoplasm) were introduced into an *L. monocytogenes* strain attenuated by a deletion in the lecithinase operon. Bacteria lysed upon entry into the cytosol and generated an MHC class I-restricted immune response against the model antigens expressed from the plasmid (137). These and similar experiments (104) show that *L. monocytogenes* is also a good potential candidate for a vector to transfer DNA into eukaryotic cells, of potential value for DNA vaccines or for gene therapy (637). Increasing our knowledge of the molecular and cellular mechanisms of listerial pathogenesis by genomic research will make it possible to improve these *Listeria*-based biotechnological tools by endowing them with optimal biosafety and target cell specificity characteristics. Progress in *Listeria* genomics

and comprehensive understanding of the complete physiological pathways of *L. monocytogenes* may also enable us to identify targets for the development of novel antimicrobial agents to treat listeriosis and other bacterial infections, or of inhibitory substances for selectively controlling the survival and growth of *L. monocytogenes* in foods.

We are still very far from completely understanding at the molecular level how pathogenic *Listeria* spp. interact with the host to cause infection. Many questions remain unanswered. For example, what is the true role of the large internalin multigene family in *Listeria* biology and pathogenesis? Why does *L. monocytogenes* preferentially infect the CNS and the placenta? Why is it that only certain serovars of *L. monocytogenes* are associated with listeriosis and certain clones of serovar 4b are associated with epidemic episodes of invasive disease? Why do pregnant women develop neither meningoencephalitis nor septicemic disease while suffering miscarriage due to *Listeria* infection? What mechanisms are involved in the direct invasion of neighboring cells during cell-to-cell spread? What pathways are involved in transduction of the signals involved in the induction of phagocytosis during cell invasion? In the coming years of the new era of postgenomic *Listeria* research, we will certainly witness spectacular progress in understanding of the complex molecular mechanisms used by these versatile bacteria to survive as parasites in the tissues and cells of the vertebrate host, and we will no doubt find answers to these and many other questions.

ACKNOWLEDGMENTS

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