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Truncated Internalin A and Asymptomatic *Listeria monocytogenes* Carriage: In Vivo Investigation by Allelic Exchange

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Allelic exchange of the region coding for the C terminus of InlA between one epidemic (with an 80-kDa InlA) and one asymptomatic (with a 47-kDa InlA) carriage *Listeria monocytogenes* strain confirmed the need for this region for internalin entry in vitro. Interestingly, restoration of internalin A functionality did not result in full virulence in chicken embryo assays.

Listeria monocytogenes is a gram-positive bacterium responsible for food-borne infections, ranging from asymptomatic fecal carriage to severe gastroenteritis to life-threatening infections, such as septicemia, meningitis, and mother-to-child infections (1, 3, 24). This facultative intracellular pathogen has a unique ability to cross three barriers during infection: the intestinal barrier (16), the blood-brain barrier (9), and/or the placental barrier (14, 24). Its pathogenicity is due to its ability to enter, reside in, and multiply in not only phagocytic but also nonphagocytic cells. E-cadherin mediates internalization in nonphagocytic cells (i.e., epithelial cells) by interacting with the cell wall-associated protein, internalin A (InlA) (7, 18). The interaction of internalin A with E-cadherin on enterocytes is an early critical step for the onset of listeriosis in vivo (16).

Understanding how bacteria cross the intestinal barrier is a key issue in the study of food-borne disease, but some aspects of this intestinal phase have not been elucidated yet. For example, between 1 and 6% of the general population carry *L. monocytogenes* without manifestation of any symptoms (5, 10, 22). Recently, the pathogenic potentials of sporadic and epidemic-associated *L. monocytogenes* carriage isolates were compared. Out of 14 human carriage isolates, 5 were both virulence attenuated toward 14-day-old chick embryos and impaired in their internalization process into Caco-2 cells (19, 20). These five strains produced truncated forms of internalin A, from 47 to 60 kDa, instead of the commonly encountered 80-kDa internalin A, because of point mutations in *inlA*. Internalin A analysis from 10 additional food isolates revealed that the expression of truncated internalin A may not be rare or specific to human carriage, since all 10 food isolates that were studied produced truncated forms of internalin A and were less invasive (21).

Considering the relationship observed in vivo between the structure of these isolates' internalin A and their invasiveness toward chick embryos, it can be postulated that pathogenic potential is directly conditioned by the efficiency of internalin-mediated entry into chick cells.

In order to verify this hypothesis, chromosomal *inlA* replacements and exchange have been realized between one epidemic isolate (Scott A) and one isolate carried asymptomatically by humans and known to produce a truncated form of internalin A (47 kDa) (Fig. 1) (19). Genetic manipulation of these two wild-type bacteria might lead to a better understanding of the contribution of this internalin A-truncated phenotype to the complex interplay that results in disease.

Phenotypic and genotypic analyses of allelic exchange mutants. In previous studies, low invasiveness of isolates from various origins, such as strain H1, was linked to the production of truncated forms of internalin A (20, 21). In order to clarify this apparent correlation, we sought to correct this defect. Among the genetic tools used for these functional analyses of genes, allelic exchange of the *inlA* region found in the H1 strain with the corresponding *inlA* region found in the epidemic-associated strain Scott A was performed. This strategy was preferred to the introduction of replicative expression vector that, first, requires one step of chromosomal gene deletion, and second, can induce differences in initial in vivo transcription of the gene of interest. We used the thermosensitive derivative host delivery vector, pVE6007, described by Maguin et al. (17) combined with an Ori^+ derivative vector, pORI19, described by Law et al. (11) for higher efficiency in the double-crossover events within *inlA* homologous genes. Identification of recombinants of interest was done by Western immunoblotting as previously described (20). Sodium dodecyl sulfate (SDS) extract protein profiles performed prior to the hybridization step of the Western blot analysis confirmed that PP1 was a derivative of strain H1 (Fig. 2A). An 80-kDa internalin A was detected in clone PP1 (Fig. 2B). We did not find any difference between the mutant and the wild-type strain H1 with respect to colony aspect, growth in brain heart infusion (BHI) broth at 37°C, hemolysis on blood agar plates, and development of zones of opacity on COMPASS *Listeria* selective agar due to production of P_i-phospholipase C (data not shown).

Similarly, comparison of SDS extract protein profiles confirmed that strain C3 was a derivative of the parental strain Scott A (Fig. 2A); this clone produced the 47-kDa internalin A (Fig. 2B). We did not find any difference between the mutant

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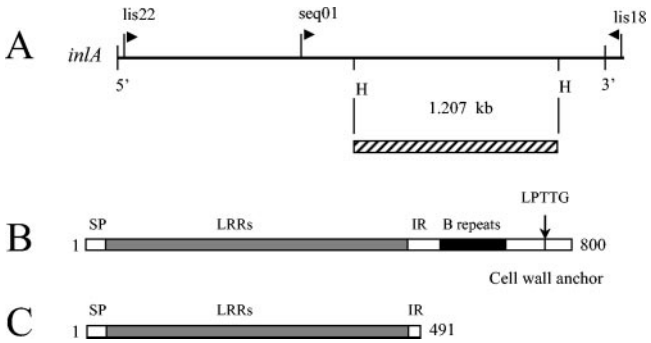

FIG. 1. Schematic map of *inlA*. (A) Primers seq01 (5′-AATCTAG CACCACGTTCGGG-3′) and lis18 (5′-TCTCCTTGATTCTAG-3′) were used for specific *inlA* PCR amplification. The amplified DNA fragment digested with HindIII restriction enzyme (H) is represented by a hatched rectangle and cloned into the pORI19 plasmid. Structural organization of internalin A for isolates Scott A (B) and H1 (C) is shown. SP, signal peptide.

and the wild-type strain Scott A with respect to colony aspect, growth in BHI broth at 37°C, hemolysis on blood agar plates, and development of zones of opacity on COMPASS *Listeria* selective agar due to production of P_i-phospholipase C.

To complete analysis, *inlA* coding sequences from H1, Scott A, C3, and PP1 were sequenced, and these nucleotide sequences were compared (data not shown). As expected, one single point mutation was detected at position 1414 in the coding sequence of *inlA* from C3 and H1. This mutation created a nonsense codon (TAG), resulting in the production of a protein with a theoretical molecular mass of 47 kDa lacking the B repeat region and the LPXTG motif. Sequence analysis showed that this mutation was absent in the coding sequence from PP1. The putative protein produced is an 800-amino-acid protein whose characteristics include common structural features shared by other proteins of the internalin multigene family, i.e., the leucine-rich repeat (LRR) region, the highly conserved interrepeat (IR) region, the B-repeat region, and the LPXTG motif which mediates anchoring of the protein to peptidoglycan (Fig. 1B).

The truncated form of internalin A confers altered invasiveness of *L. monocytogenes* in vitro. As previously described, in vitro assays with Caco-2 cell lines showed that adhesion and invasion rates of parental strain Scott A (4.5 and 23.5%) were higher than those observed with parental strain H1 (0.3 and 0.5%) (19, 20).

In order to verify that the sole factor responsible for the low entry ability of strain H1 was the expression of truncated internalin A, we tested the ability of the two mutants, C3 and PP1, expressing each internalin A variant, to enter into cells permissive for internalin-mediated entry. Infection of Caco-2 cells with the C3 mutant strain showed a phenotype close to the strain H1 phenotype (Fig. 2C). Conversely, restoration of the production of a full-length form of internalin A in strain H1 restored the phenotype observed for parental strain Scott A (Fig. 2C), indicating that functional restoration was complete in vitro. The strategy used, by limiting final genetic modifications in the two strains, allowed us to conclude that a nonsense mutation in *inlA*, and hence an altered structure of internalin A, is solely responsible of the reduced ability of *L. monocytogenes* strain H1 to enter Caco-2 cells.

We subsequently tested whether or not these constructions had any interference in the efficiency of the following steps of infection. The plaque-forming assay allowed us to verify that the cell-to-cell spread process was not affected in strains PP1 and C3. Indeed, the two mutants tested were able to continue the infectious cycle and to form plaque of dissemination in the Caco-2 monolayers as rapidly as the wild-type strains Scott A and H1 (Fig. 2D). The number of plaques that appeared 24 h after the initial infection time of 2 h was in accordance with the respective rates of entry evaluated previously (Fig. 2C).

The strategy used, by limiting final genetic modifications in the two strains, allowed us to conclude that the truncated form of internalin A produced by strains such as H1 clearly abolished listerial internalization into human Caco-2 cells. Following mutagenesis, its functional restoration, on the other hand, was complete in vitro. Collectively, the data reinforced the pivotal role played by internalin A in vitro in the internalization process (7, 15). The consequences of the nonsense point mutation observed in H1 and C3 are consistent with the internalin functionality model, in which the presence of IR and LRR regions stabilize LRR structures and show efficient internalin–E-cadherin interactions (15), while the C-terminal region is critical for efficient anchoring of internalin A on the bacterial surface (Fig. 1) (12). Considering the *inlA* sequence of wild isolate H1, we can hypothesize from the deduced structure that, first, the IR region is partially truncated and probably results in impaired folding of the LRR region and, second, the absence of a C-terminal part probably results in the release of internalin A from the bacterial surface. Whether this soluble form of internalin A is able to interact with E-cadherin is not known, but it would be of great interest to evaluate the possible protection offered by this bound truncated internalin A to epithelial cells against a subsequent infection caused by the *L. monocytogenes* strains that produce complete internalin A.

Evaluation of the role of truncated internalin A in virulence toward chick embryos. Based on these in vitro results, we hypothesized that the production of the truncated internalin A was responsible for the previously observed low in vivo virulence of strain H1 (19). However, survival of 14-day-old chick embryos inoculated with mutants PP1 and C3 did not allow us to clearly confirm this hypothesis (Fig. 3).

As previously described (19), infection of chick embryos with parental strain Scott A resulted in 100% mortality within 3 days, whereas infection with parental strain H1 resulted in 20% mortality after 6 days. Similar embryo mortality rates were observed with eggs contaminated with internalin A-mutated C3 and PP1 strains. Indeed, at the third day after inoculation, 20% mortality was observed. After 6 days of infection, 100% mortality was reached, as observed for wild-type Scott A.

The choice of the model to address internalin A function is critical, because *L. monocytogenes* exhibits a stringent host tropism due to the specific ligand-receptor interaction. Indeed, internalin A can bind to human E-cadherin (hEcad), chicken E-cadherin, or guinea pig E-cadherin but not to mouse and rat E-cadherins (13, 18). This host specificity relies on the nature of the 16th amino acid, a proline in hEcad and chicken E-cadherin and a glutamic acid in mouse and rat E-cadherins (13). Consequently, mouse or rat models are not appropriate

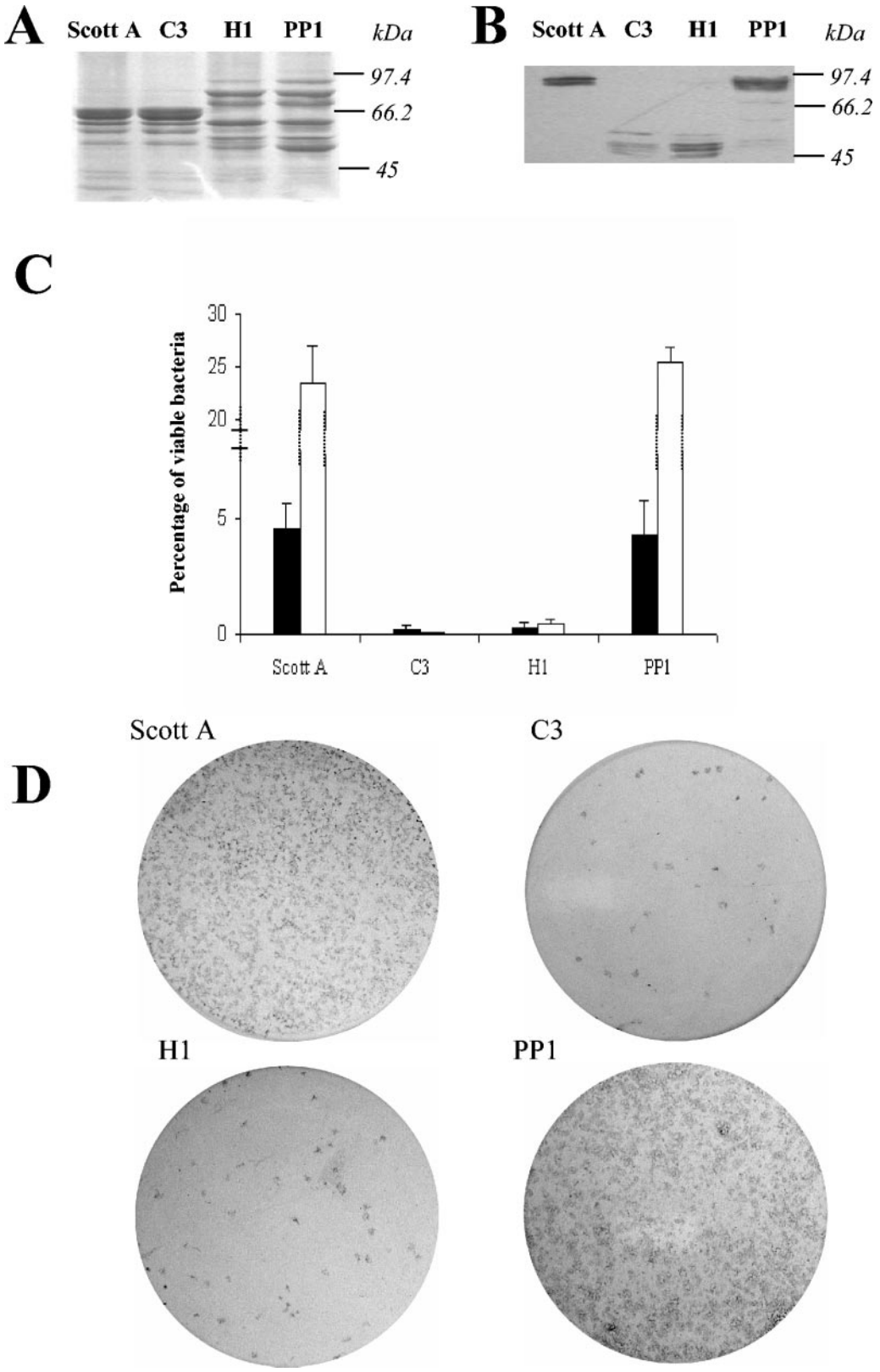

FIG. 2. Molecular and functional properties of the recombinant *L. monocytogenes* strains (C3 and PP1) in comparison with their respective parental strains (Scott A and H1). (A) SDS-polyacrylamide gel electrophoresis of the SDS extract fraction from BHI-grown *L. monocytogenes* cells stained with Coomassie blue R-250. Molecular mass markers are indicated to the right of the panel. (B) Immunoblot analysis of internalin A from the same SDS extract fractions after labeling with monoclonal antibody L7.7. The order of the samples and molecular mass markers are the same as in panel A. (C) Listerial adhesion to (black columns) and entry into (white columns) Caco-2 cells. The results are expressed as the mean percentage (of initial inoculum) of viable recovered bacteria per well from two independent experiments analyzed in triplicate. Vertical bars depict the standard deviation. (D) Plaque formation by *L. monocytogenes* in Caco-2 cells. The initial inoculum ranged from 1.65×10^4 to 7.3×10^4 bacteria per well.

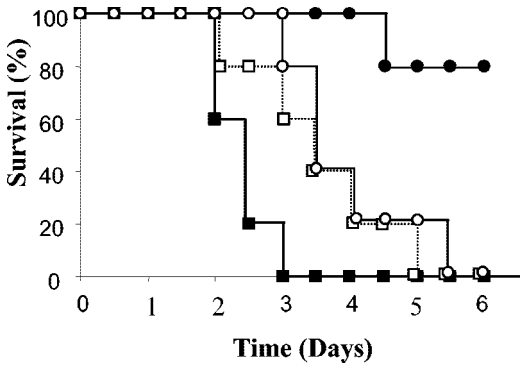

FIG. 3. Survival curve of chick embryos inoculated at the 14-day-old stage with *L. monocytogenes*. Wild strains Scott A (■) and H1 (●) and derivative strains C3 (□) and PP1 (○) were inoculated at doses ranging from 0.5×10^2 to 1×10^2 CFU of bacteria per egg via the chorioallantoic membrane. The survival of embryos was monitored daily for 6 days.

for addressing oral human infection and disease and particularly for addressing internalin A function in vivo. More recently, transgenic mice expressing hEcad solely in enterocytes have been used to test the in vivo relevance of results acquired by use of more reductionist in vitro approaches (16).

Strikingly, first of all, the restoration of functional internalin A in mutant PP1 was not enough to reach Scott A infectiousness levels toward chick embryos, even though the final mortality rate was similar to that caused by the epidemic strain. Secondly, the truncation of internalin A in Scott A resulted in a longer time to observe 100% mortality after infection but did not result in the attenuation of virulence observed with strain H1. Given these findings, it is unlikely that the virulence differences observed between parental strains H1 and Scott A could be explained solely by differences in internalin structure. Among the other virulence factors currently identified (2, 23), factors implicated in entry, intracellular multiplication, and cell-to-cell spread within human epithelial cells have to be excluded, as judged by adhesion, entry, and plaque formation rate obtained in Caco-2 cells.

Factors implicated in high virulence have yet to be identified. Interestingly, Scott A, as all major food-borne listeriosis epidemic strains, is a serovar 4b strain, while H1 belongs to the serovar 1/2a. These serovar 4b strains belong to a unique genetic group (25, 26) and have been characterized by a specific combination of genes (4, 8). Some of these genomic regions, moreover, have been shown to be flanked on one side by the virulence-associated gene encoding internalin A (6). The virulence differences observed between H1 and Scott A may be explained by this set of genes that could confer a particular ability to cause disease.

Taken together, these results provide evidence of the necessary but not sufficient role of internalin A to induce in vivo infection. Indeed, other factors, perhaps serovar dependent, may explain the high virulence observed with the epidemic strain Scott A.

Nucleotide sequence accession numbers. DNA sequences of the *inlA* allele from strains Scott A, C3, and PP1 were deposited in the EMBL, GenBank, and/or DDBJ databases under accession numbers AJ784775, AJ784776, and AJ784777, respectively.

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