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The *inlA* Gene of *Listeria monocytogenes* LO28 Harbors a Nonsense Mutation Resulting in Release of Internalin

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Internalin is a surface protein that mediates entry of *Listeria monocytogenes* EGD into epithelial cells expressing the cell adhesion molecule human E-cadherin or its chicken homolog, L-CAM, which act as receptors for internalin. After observing that entry of *L. monocytogenes* LO28 into S180 fibroblasts, in contrast to that of EGD, did not increase after transfection with L-CAM, we examined both the expression and the structure of internalin in strain LO28. We discovered a nonsense mutation in *inlA* which results in a truncated protein released in the culture medium. Mutations leading to release of internalin were also detected in clinical and food isolates. These results question the role of internalin as a virulence factor in murine listeriosis.

Listeria monocytogenes is a gram-positive bacterium responsible for severe food-borne infections (7). It is able to induce its own uptake into nonphagocytic cells, such as intestinal epithelial cells, and to spread from cell to cell using an actin-based motility process (for a review, see reference 8). Several *L. monocytogenes* strains have been used to analyze these processes and their regulation. In our laboratory, *L. monocytogenes* LO28 (serovar 1/2c), originally isolated from the feces of a healthy pregnant woman, has been used to study actin-based motility and regulation of virulence factors and to establish a chromosomal map (15). Invasion genes were mainly studied for strain EGD (serovar 1/2a), which was the first *L. monocytogenes* strain to be isolated during an epidemic among laboratory animals (13). EGD and LO28 are fully virulent in the mouse model (1, 6).

EGD entry into mammalian cells is mediated by the bacterial surface proteins InlA (internalin) (5) and InlB (2; for a recent review, see reference 8), which are both necessary and sufficient for entry into various cultured cell lines, each having its own specificity. InlA is an 800-amino-acid protein required for entry into the epithelial cell line Caco-2 and other cell lines expressing the cell adhesion molecule E-cadherin, which acts as a receptor for internalin (5, 10, 12). InlB mediates entry in many other cell lines; its receptor is unknown. Internalin contains two repeat regions, a leucine-rich repeat region (LRR) and a B-repeat region. The C-terminal part of internalin contains an LPXTG motif which mediates anchoring of the protein to the peptidoglycan. While internalin can be detected in culture supernatants, it is mainly associated with the bacterial surface, and our group has shown recently that the associated form of internalin promotes entry into cells (9).

In the course of a comparative study, the invasiveness of strain LO28 was compared to that of strain EGD in the mouse fibroblast line S180 and in an S180 derivative transfected with L-CAM, the chicken homolog of E-cadherin. In agreement with previous results (12), entry of EGD into fibroblasts expressing L-CAM was approximately 60-fold higher than entry into S180 cells. This was not the case for strain LO28, which entered both S180 and S180(L-CAM) cells poorly. This result

prompted us to examine both the expression and the structure of internalin in strain LO28.

The presence of internalin in LO28 was analyzed in total cell extracts and in culture supernatants by using two internalin-specific monoclonal antibodies (MAbs), i.e., L7.7, which is specific for the LRR, and J14.1, which is specific for the C-terminal part of internalin (Fig. 1) (11). The amounts of internalin detected in total extracts with MAb L7.7 were similar for both strains, strongly suggesting that expression of the *inlA* gene in LO28 was similar to that in EGD. However, the apparent molecular weight of the protein was clearly lower than that of EGD internalin, raising the possibility that LO28 internalin was either degraded or truncated. Moreover, a larger quantity of internalin was detected in LO28 culture supernatants than in EGD supernatants. Taken together, these results suggested that LO28 InlA could be released in the supernatant due to the absence of a cell wall anchoring motif. This hypothesis was supported by two observations: (i) in Western blot analysis of total extracts, LO28 internalin could not be detected with MAb J14.1 (Fig. 1), and (ii) LO28 internalin could not be detected on the bacterial surface by immunofluorescence staining with
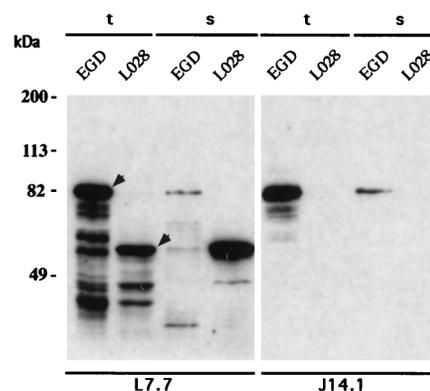

FIG. 1. Western blot analysis of internalin in strains LO28 and EGD. The presence of internalin in sonicated total protein extracts (t) and culture supernatants (s) was analyzed, using an LRR-specific MAb, L7.7, and a C-terminus-specific MAb, J14.1 (11). The amount of material loaded corresponds to 10 μg of protein from bacteria harvested in exponential phase ($A_{600} = 0.5$). The estimated molecular masses for the LO28 and EGD internalins (indicated by arrowheads) are 60 and 80 kDa, respectively.

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Phase contrast   Immunofluorescence
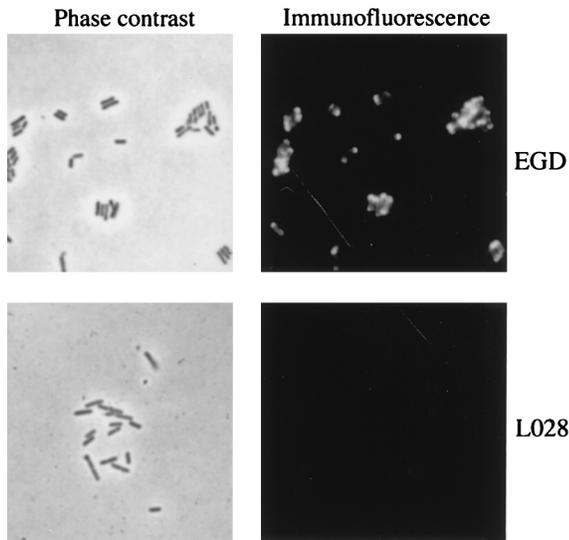

EGD

L028

FIG. 2. Detection of internalin on the bacterial surface by immunofluorescence. Cells of strains LO28 and EGD were harvested at the end of the log phase ($A_{600} = 0.8$) and labelled with the LRR-specific MAb L7.7 (11). Internalin was revealed by a Texas red-labelled goat anti-mouse immunoglobulin antibody (Biosys). Bacteria were visualized by phase-contrast microscopy and immunofluorescence staining.

MAb L7.7 under conditions where EGD internalin could be detected (Fig. 2). The structure of the *inlA* gene was thus examined.

On the basis of Southern blots and PCR experiments, the *inlA* genes had been considered identical in length in strains EGD and LO28 (3, 5, 14). To confirm these results, a specific PCR amplification of LO28 and EGD chromosomal DNAs was performed with primers OML18 and OML22, which flank *inlA* in EGD (Fig. 3). Amplification of both DNAs resulted in the production of a 2.4-kb fragment corresponding to the expected size for *inlA*. *Hin*dIII digestion of these PCR products yielded the same three fragments of 1.2, 1.0, and 0.2 kb, suggesting that LO28 and EGD *inlA* genes are identical in size. This result did not exclude, however, the possibility of a nonsense mutation in LO28 *inlA*. To address this issue, we decided to clone the LO28 *inlA* gene and examine its nucleotide sequence. An 881-bp PCR fragment corresponding to the central part of *inlA* in EGD was used as a probe to clone, by colony hybridization under high-stringency conditions, the LO28 *inlA*
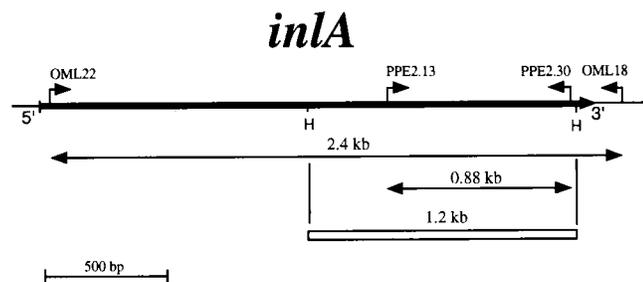
inlA

FIG. 3. The *inlA* gene. Primers OML18 (5′-TCTCCTTGATTCTAG-3′), OML22 (5′-AAAAAACGATATGTATG-3′), PPE2.13 (5′-CTTACCTAGTTA TACAAA-3′), and PPE2.30 (5′-TTCATTGTACTTGTTGTG-3′) were used for specific *inlA* PCR amplification. The two *Hin*dIII restriction sites of *inlA* (H) are indicated. The amplified DNA fragments are shown. The sequenced DNA fragment (1.2 kb) is represented by an open rectangle.

TABLE 1. *L. monocytogenes* strains used in this study

Strain[a]	Serovar	Source and specific property
EGD	1/2a	Mackaness (1962)
LO28 (Bof 343)	1/2c	Vicente et al. (1985)
CLIP 42398 (BUG 1573)	4b	French epidemic, 1993
CLIP 63713 BUG 1559	4b	French epidemic, 1995
CLIP 61634 (BUG 1574)	1/2a	Clinical isolate, 1995; septicemia
CLIP 62416 (BUG 1575)	4b	Clinical isolate, 1995; septicemia
CLIP 63869 (BUG 1576)	4b	Clinical isolate, 1995; septicemia
CLIP 64419 (BUG 1577)	1/2b	Clinical isolate, 1995; meningitis
CLIP 66572 BUG 1560	4b	Clinical isolate, 1995; meningitis
CLIP 67325 (BUG 1578)	1/2b	Clinical isolate, 1995; perinatal form
CLIP 61657 (BUG 1579)	1/2a	Clinical isolate, 1995
CLIP 62624 (BUG 1580)	1/2a	Clinical isolate, 1995; meningitis
CLIP 63976 (BUG 1581)	4b	Clinical isolate, 1995; perinatal form
CLIP 64966 (BUG 1582)	4b	Clinical isolate, 1995; perinatal form
CLIP 67086 (BUG 1561)	1/2c	Clinical isolate, 1995; septicemia
CLIP 67989 (BUG 1583)	4b	Clinical isolate, 1995
CLIP 61523 (BUG 1584)	ND[b]	Food isolate, 1995
CLIP 62177 (BUG 1585)	ND	Food isolate, 1995
CLIP 64846 (BUG 1652)	1/2a	Food isolate, 1995; meat
CLIP 66462 (BUG 1586)	ND	Food isolate, 1995
CLIP 68314 (BUG 1587)	ND	Food isolate, 1995
CLIP 68942 (BUG 1588)	ND	Food isolate, 1995
CLIP 61842 (BUG 1589)	ND	Food isolate, 1995
CLIP 64467 (BUG 1590)	ND	Food isolate, 1995
CLIP 64920 (BUG 1563)	1/2c	Food isolate, 1995; dairy product
CLIP 68091 (BUG 1591)	ND	Food isolate, 1995
CLIP 68454 (BUG 1592)	ND	Food isolate, 1995
CLIP 69373 (BUG 1564)	3a	Food isolate, 1995; fish

[a] CLIP refers to the Pasteur Institute collection, and BUG and Bof are the Cossart laboratory collection names. Strains indicated in bold letters secrete internalin (this work).
[b] ND, not determined.

gene from a *Hin*dIII chromosomal fragment library constructed in pUC18. Four clones harboring the same 1.2-kb fragment were obtained. The inserts of two of them were totally sequenced. The four silent mutations previously identified during a survey study of *inlA* polymorphism in several *L. monocytogenes* strains were detected (14). Moreover, we found, compared to EGD *inlA* (5), 10 additional single point mutations in LO28 *inlA* and a deletion of an adenine at position 1637. This frameshift mutation leads to the creation of a nonsense codon, TAA, for position 1729, generating an open reading frame encoding a 63-kDa protein lacking a cell wall
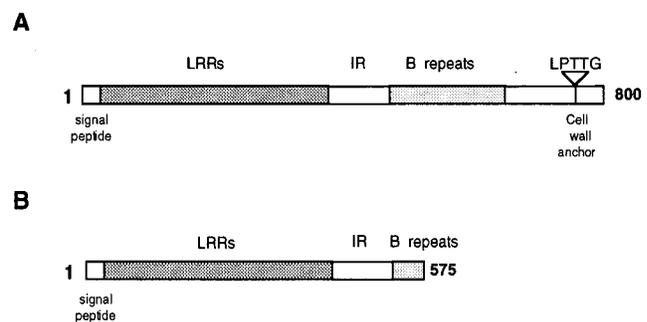

FIG. 4. Structural organization of InlA in strains EGD (A) and LO28 (B). LRRs, leucine-rich repeats; IR, interrepeats.
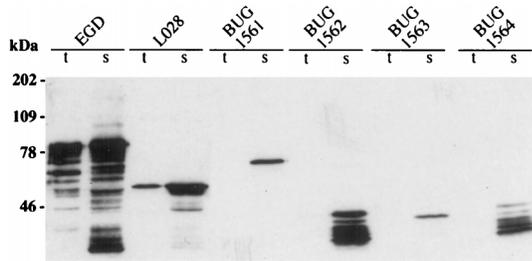
FIG. 5. Western blot analysis of internalin in food and clinical isolates. The presence of internalin in total cell extracts (t) and culture supernatants (s) from the indicated strains was analyzed with the LRR-specific MAb L7.7 (11). The amount of material loaded corresponds to 200 μl of cultures containing bacteria in stationary phase (A_{600} = 1.4).

anchor (Fig. 4). This result is in complete agreement with the apparent molecular weight of the protein detected by Western blotting and mainly present in the culture supernatant (Fig. 1).

The unexpected structure of internalin in strain LO28 led us to analyze the structure of internalin in 26 other *L. monocytogenes* strains, including 14 clinical strains and 12 food isolates (Table 1). Four strains expressed truncated forms of internalin, with apparent molecular masses of 40, 45, 47, and 75 kDa (Fig. 5). Three of them (BUG 1562, BUG 1563, and BUG 1564) were food isolates, and one (BUG 1561) was a clinical isolate responsible for a case of septicemia. For all four, the protein was detected only in the culture supernatants and could not be detected on the bacterial surface by immunofluorescence (data not shown). From this preliminary epidemiological study, it appears that truncation of internalin is not a rare event. This result was unexpected since the *inlA* gene regions encoding the LRR and B-repeat regions had been shown to be genetically conserved (14). However, we had noticed several years ago that the capacity of LO28 to invade Caco-2 cells was much lower than that of EGD (4). Furthermore, like LO28, the four strains that secreted internalin failed to show enhanced invasion in L-CAM-transfected fibroblasts compared to that in nontransfected fibroblasts (Table 2). Taken together these data suggest that the low level of entry of LO28 into Caco-2 cells and L-CAM-transfected fibroblasts is due to the absence of surface-associated internalin. Whether the low level of entry is due to the soluble form of internalin, to an InlB-mediated mode of entry, or to another factor, such as another member of the internalin family, is not known.

The role of internalin in listeriosis has not been completely

TABLE 2. Entry of EGD and of bacterial strains secreting internalin into L-CAM-transfected fibroblasts

Strain	S180(L-CAM)/S180 ratio[a]
EGD	60 ± 18
LO28	3.3 ± 1.7
BUG 1561	1.6 ± 0.7
BUG 1562	1.0 ± 0.2
BUG 1563	1.1 ± 0.6
BUG 1564	2.0 ± 1.5

[a] Ratio of the percentage of bacterial entry into S180(L-CAM) fibroblasts to that into nontransfected S180 fibroblasts. Invasion assays were performed by a slight modification of the method of Mengaud et al. (12) with a multiplicity of infection of approximately 15 bacteria per cell.

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elucidated. Since LO28 is fully virulent in the mouse model (1), the data presented here suggest that internalin is not absolutely critical for virulence in mice and/or that its function may be redundant with those of other proteins. One may note that most in vivo experiments have been performed with the mouse model. In this model, some aspects of human listeriosis, such as meningitis or meningoencephalitis, cannot be easily reproduced. Whether internalin plays a role in these clinical forms or whether it is one of the many factors participating in listeriosis remains to be thoroughly investigated.

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