Differential *inlA* and *inlB* Expression and Interaction with Human Intestinal and Liver Cells by *Listeria monocytogenes* Strains of Different Origins

Hadewig Werbrouck,^{1*} Koen Grijspeerdt,¹ Nadine Botteldoorn,¹ Els Van Pamel,¹ Nancy Rijpens,¹ Jo Van Damme,² Mieke Uyttendaele,³ Lieve Herman,¹ and Els Van Coillie¹

Ministry of the Flemish Community, Institute for Agricultural and Fisheries Research, Unit Technology and Food—Product Quality and Food Safety, Brusselsesteenweg 370, 9090 Melle, Belgium¹; Laboratory of Molecular Immunology, Rega Institute for Medical Research, Minderbroedersstraat 10, 3000 Leuven, Belgium²; and Department of Food Safety and Food Quality, Laboratory of Food Microbiology and Preservation, Faculty of Bioscience Engineering, Ghent University, Ghent, Belgium³

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

Listeria monocytogenes is a gram-positive, facultatively intracellular, food-borne pathogen that has the capacity to cause severe infections, such as gastroenteritis, septicemia, abortion, and meningitis in humans and animals (15). Immunocompromised individuals, elderly people, pregnant women, fetuses, and neonates are most susceptible to listeriosis, which is characterized by a low infection rate but a high mortality rate. *L. monocytogenes* is widely distributed in nature and is generally transferred to humans by contaminated food (15, 65). Despite the fact that many food products are contaminated with this pathogen (59, 60), the incidence of listeriosis is relatively low. This contradiction may be explained by differences in the contamination levels of the food products. However, another explanation may be the existence of differences in the virulence potentials of individual strains (4, 13, 24, 26, 41, 44, 51, 63, 66). Furthermore, the fact that listeriosis also occurs in immunocompetent persons indicates that, besides host immunity, other factors, such as the virulence potential of the *L. monocytogenes* strain, could play a role in susceptibility to listeriosis (14, 32, 41, 53).

While many pathogens are taken up only by professional phagocytes, *L. monocytogenes* can also invade cells that are normally nonphagocytic, such as epithelial cells (17), hepatocytes (10, 18, 67), and endothelial cells (23). Consequently, *L. monocytogenes* is able to cross three significant barriers, namely, the intestinal epithelial cell barrier, the blood-brain endothelial cell barrier, and the fetoplacental endothelial cell barrier. There is evidence that invasion of the intestinal barrier is the first step in the infection process. After translocation of this barrier, the bacteria infect the lymph nodes and subsequently reach the spleen and the liver via transport through lymph and blood. Several studies have clearly shown that intracellular bacterial multiplication occurs mainly in hepatocytes and that invasion of hepatocytes is probably a key virulence mechanism in the development of a systemic host infection (5, 18, 19, 49). *L. monocytogenes*-infected epithelial, monocytic, and endothelial cells have been shown to release chemoattractants, such as interleukin-8 (IL-8), which leads to an influx of neutrophils to the infectious foci (12, 20, 28, 30, 48, 56). This early defense mechanism serves to keep the infection at a level that can be resolved by the later T-cell-mediated response. The level of chemokine production by infected cells may be a critical factor for clearance of *L. monocytogenes*.

Several virulence genes involved in the *L. monocytogenes* infection process have been identified, and their roles have

* Corresponding author. Mailing address: Institute for Agricultural and Fisheries Research, Unit Technology and Food—Product Quality and Food Safety, Brusselsesteenweg 370, 9090 Melle, Belgium. Phone: 32 (0)9 272 30 80. Fax: 32 (0)9 272 30 01. E-mail: Hadewig.Werbrouck@ilvo.vlaanderen.be.

been well established (6, 7, 64). At least two surface-associated proteins mediate the entry into mammalian cells, the internalins InlA (16) and InlB (10), which are colocalized at the bacterial surface. These two genes are organized in an operon, and transcription of the genes can occur both individually or together, by PrfA-independent and PrfA-dependent mechanisms, respectively (31, 36). InlA, which binds to the host cell receptor E-cadherin, promotes invasion of enterocytes and crossing of the intestinal barrier (34, 35, 38, 45). Recently, truncated, nonfunctional forms of InlA in a number of *L. monocytogenes* strains belonging to serotypes 1/2a and 1/2c (24), leading to a reduced ability to invade enterocytes (42, 50), have been described. Clinical strains were shown to express full-length InlA far more frequently than were food strains, supporting the critical role of full-length InlA in human listeriosis (24). InlB, which interacts with three distinct host cell receptors (ligands)—Met (57), gC1q-R (3), and glycosaminoglycans (27)—mediates entry into a wide variety of cell types, such as hepatocytes, epithelial cells, fibroblasts, and endothelial cells, which is critical for the development of systemic infection (10, 18, 36, 43). Ami, a bacterial cell-surface autolysin with an amidase activity, has been shown to play a role in adhesion to eukaryotic cells (39).

The objective of the present study was to investigate phenotypic differences between *L. monocytogenes* strains of different origins (clinical and nonclinical strains). Therefore, the in vitro invasion capacity for specific human cell types (i.e., monocytic THP-1 cells, enterocytic Caco-2 cells, and hepatocytic HepG2 cells) and the capacity to induce production of the proinflammatory cytokine IL-8 were compared. The significant differences between clinical and nonclinical strains in intestinal and liver cell invasion led us to study, by real-time reverse transcription (RT)-PCR, an extended group of *L. monocytogenes* strains for the mRNA expression levels of *inlA*, *inlB*, and *ami*, all important for host cell infection. Also, a selection of *L. monocytogenes* strains used in this study was subjected to partial DNA sequence analysis of the *inlA* gene to determine the functionality of the InlA protein.

MATERIALS AND METHODS

***L. monocytogenes* strains, growth conditions, and serotype identification by PCR.** Two groups of *L. monocytogenes* strains (a total of 64) of different origins were studied. One group included 26 human sporadic clinical strains, generously provided by Marc Yde (Belgian Listeria Reference Centre, Institute of Public Health, Brussels, Belgium), and the animal epidemic clinical laboratory strain EGD (2, 10), which was obtained from Pascale Cossart (Institute Pasteur, Paris, France) (Table 1). The second group included 23 food, 3 feed, and 11 farm-environmental strains, referred to as nonclinical strains (Table 1).

The *L. monocytogenes* strains were grown (overnight) at 37°C with shaking (150 rpm) in brain heart infusion broth (Oxoid Ltd., London, United Kingdom). Numbers of CFU were determined by plating the appropriate serial dilutions on tryptone soya agar (TSA) (Oxoid Ltd.) plates.

A multistep PCR-based serotype identification method was carried out, permitting classification of *L. monocytogenes* into five serotype groups: 1/2a (3a), 1/2b (3b), 1/2c (3c), 4a/c, and 4b (4d,4e) (1).

Culture of cell lines. All cell lines were cultured at 37°C and 5% CO₂. The human monocytic cell line THP-1 (ATCC TIB-202) was cultured in RPMI 1640 (Cambrex Bio Science Verviers, Verviers, Belgium) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Invitrogen Ltd., Paisley, United Kingdom). The human enterocytic cell line Caco-2 (ECACC 86010202) was cultured in Eagle's minimum essential medium with Earle's salts (EMEM) (Cambrex Bio Science Verviers) supplemented with 10% heat-inactivated FCS and 2 mM glutamine (Cambrex Bio Science Verviers). The human hepatocytic carcinoma cell line HepG2 (ATCC HB-8065) was cultured in EMEM supplemented with

10% heat-inactivated FCS, 2 mM glutamine, and 0.02% human recombinant insulin (100 U/ml) (Novo Nordisk A/S, Bagsvaerd, Denmark).

Cell invasion assays. To study invasion of the different cell lines by *L. monocytogenes*, the gentamicin protection assay was performed. Because of the difficulty of harvesting the different strains at the same moment in the late logarithmic growth phase to start the invasion assay, bacterial stocks were made and frozen in several portions until use. For preparation of these stocks, bacteria were collected at an optical density at 600 nm (OD₆₀₀) of 0.85 to 0.95 (late logarithmic growth phase) (UVIKON 860 spectrophotometer; Serlabo, Drogenbos, Belgium), washed twice in Dulbecco's phosphate-buffered saline with Ca²⁺ and Mg²⁺ (DPBS) (Cambrex Bio Science Verviers), resuspended in PBS with 15% glycerol, and stored at -80°C. Numbers of CFU were determined after an aliquot was thawed and were always compared with the live bacterial counts measured before freezing. No remarkable differences in live bacterial counts were observed after one freezing cycle. Suspension THP-1 cells were seeded in 96-well tissue culture plates at 2 × 10⁵ cells per well in culture medium supplemented with 16 nM phorbol myristate acetate (Sigma-Aldrich, St. Louis, Mo.) and incubated for 24 h to induce adhesion of the cells to the plate. Caco-2 and HepG2 cells were seeded in 96-well tissue culture plates at 2 × 10⁴ and 1 × 10⁵ cells per well, respectively, and grown at 37°C for 8 (Caco-2) or 3 (HepG2) days to obtain confluent monolayers with a density of about 2 × 10⁵ cells per well. Before infection, attached cells (THP-1) or cell monolayers (Caco-2 and HepG2) were washed once with serum-free cell culture medium. For infection of THP-1 cells, 4 × 10⁵ bacteria (multiplicity of infection [MOI] = 2) in 200 µl of RPMI 1640 plus 1% FCS were used per well. Caco-2 and HepG2 cells were infected with 200 µl of bacterial cell suspension containing 4 × 10⁶ bacteria (MOI = 20) in EMEM plus 1% FCS. After addition of the bacteria, plates were centrifuged for 10 min at 1,000 × g (37°C) to bring the bacteria into contact with the cell monolayers. Then, plates were incubated for 1 h at 37°C (5% CO₂) to allow the bacteria to invade the cells. Subsequently, cells were washed three times with serum-free medium to remove uninvaded cells. Next, 200 µl, for THP-1 cells, or 100 µl, for Caco-2 and HepG2 cells, of the appropriate culture medium containing 0% FCS (THP-1) or 1% FCS (Caco-2 and HepG2) and 50 µg/ml gentamicin (Invitrogen Ltd.), to kill extracellular bacteria, was added and plates were incubated (1 or 24 h) at 37°C (5% CO₂). After 1 h, cells were washed three times with PBS, and 200 µl of 1% Triton X-100 (MP Biomedicals Europe, Asse-Relegem, Belgium) in distilled water was added to each well. Plates were incubated for 10 min at 37°C (150 rpm) to lyse the eukaryotic cell culture. The numbers of intracellular bacteria were determined by plating the appropriate serial dilutions on TSA. The detection limit of the assay was 33.33 CFU/ml. After 24 h, the cell culture supernatant was harvested, filtered (0.22 µm), and stored at -20°C until the IL-8 concentration was determined. In each assay, a negative control (uninfected cells) was included. All invasion assays were performed in triplicate and repeated three times.

Detection of IL-8 in cell culture supernatant. The concentration of IL-8 in the culture supernatant of the infected cells was determined by enzyme-linked immunosorbent assay (ELISA). For IL-8 detection, a polyclonal goat anti-human IL-8 antibody for coating (61) and a monoclonal mouse anti-human IL-8 antibody (R&D Systems, Abingdon, United Kingdom) were used as described previously (68). As a positive control for IL-8 production, cells were stimulated for 24 h with 5 µg/µl lipopolysaccharide (LPS) (*Escherichia coli* O111:B4) (Difco Laboratories, Detroit, Mich.). Uninfected cells were included as a negative control. The detection limit of this assay was 0.075 ng/ml.

DNA sequencing and sequence analyses. From the *L. monocytogenes* strains, partial DNA sequences of the following genes were determined: *inlA*, *inlB*, *ami*, *rpoD*, *uflA*, and the 16S rRNA gene. DNA fragments of the different genes were generated by PCR with the appropriate primers (with annealing at 53°C) (Table 2). The PCR products were purified with the High Pure PCR Product Purification kit (Roche Diagnostics, Penzberg, Germany) and subsequently sequenced with the respective forward primer, using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, Calif.), and analyzed with the ABI Prism 310 Genetic Analyzer System (Applied Biosystems). To sequence the complete PCR fragment of the *inlA* gene, several internal primers were used (Table 2). Nucleic acid sequences were aligned with Kodon Software (Applied Maths Inc., Austin, Tex.).

Sample collection, cell lysis, and RNA isolation. *L. monocytogenes* strains were grown at 37°C until an OD₆₀₀ of 0.85 to 0.95 was reached [(±1 to 3) × 10⁹/ml]. One milliliter of the culture was harvested, and total RNA was extracted with the RNeasy Bacteria Reagent and RNeasy Mini kit (QIAGEN Inc., Valencia, Calif.) as described by the manufacturer with some minor modifications: the bacterial pellet was resuspended in 100 µl of Tris-EDTA buffer containing 5 mg/ml of lysozyme (Roche Diagnostics) and 13 mg/ml of proteinase K (Promega Inc., Madison, Wis.) and incubated for 10 min at 37°C for bacterial cell lysis.

TABLE 1. Tested *L. monocytogenes* strains and their characteristics

Strain ^a	Origin	Serotype as determined by ^b :		Group
		Agglutination	PCR	
EGD†		1/2a	1/2a (3a)	Animal clinical laboratory reference, epidemic
MB 676*†	CSF	4b	4b (4d,4e)	Human clinical, sporadic
MB 677*†	CSF	4b	4b (4d,4e)	Human clinical, sporadic
MB 687	CSF	1/2b	1/2b (3b)	Human clinical, sporadic
MB 688	Unknown	1/2b	1/2b (3b)	Human clinical, sporadic
MB 689†	Unknown	4b	4b (4d,4e)	Human clinical, sporadic
MB 691*†‡§	Unknown	1/2a	1/2a (3a)	Human clinical, sporadic
MB 2314*†	Amniotic fluid	4b	4b (4d,4e)	Human clinical, sporadic
MB 2315*†§	CSF	1/2a	1/2a (3a)	Human clinical, sporadic
MB 2316†	Blood	4b	4b (4d,4e)	Human clinical, sporadic
MB 2317†	Blood	1/2a	1/2a (3a)	Human clinical, sporadic
MB 2318	Blood	4b	4b (4d,4e)	Human clinical, sporadic
MB 2319*†	Blood	1/2a	1/2a (3a)	Human clinical, sporadic
MB 2320*†	CSF	4b	4b (4d,4e)	Human clinical, sporadic
MB 2321*†‡	Blood	1/2c	1/2c (3c)	Human clinical, sporadic
MB 2322*†	Blood	1/2b	1/2b (3b)	Human clinical, sporadic
MB 2323*†	Blood	1/2b	1/2b (3b)	Human clinical, sporadic
MB 2640	Blood	ND	4b (4d,4e)	Human clinical, sporadic
MB 2641†§	Blood	3a	1/2a (3a)	Human clinical, sporadic
MB 2642	Blood	1/2b	1/2b (3b)	Human clinical, sporadic
MB 2643	Blood	4b	4b (4d,4e)	Human clinical, sporadic
MB 2644†	Blood	1/2a	1/2a (3a)	Human clinical, sporadic
MB 2645	Blood	4b	4b (4d,4e)	Human clinical, sporadic
MB 2646	CSF	4b	4b (4d,4e)	Human clinical, sporadic
MB 2647†	Blood	1/2a	1/2a (3a)	Human clinical, sporadic
MB 2648	CSF + blood	1/2b	1/2b (3b)	Human clinical, sporadic
MB 2649†	Blood	1/2a	1/2a (3a)	Human clinical, sporadic
MB 700*†	Meat salad	4b	4b (4d,4e)	Food
MB 701†‡	Meat salad	1/2c	1/2c (3c)	Food
MB 704*†‡§	Vegetable/egg salad	1/2a	1/2a (3a)	Food
MB 707	Cheese	1/2b	1/2b (3b)	Food
MB 711*†	Meat	4b	4b (4d,4e)	Food
MB 712*†	Vegetable/egg salad	3b	1/2b (3b)	Food
MB 715†‡	Vegetable/egg salad	1/2c	1/2c (3c)	Food
MB 716	Egg salad	3b	1/2b (3b)	Food
MB 720*†	Minced meat	4b	4b (4d,4e)	Food
MB 1828†	Fish salad	ND	1/2a (3a)	Food
MB 1840	Fish salad	ND	1/2b (3b)	Food
MB 1899†‡	Meat	ND	1/2c (3c)	Food
MB 1909*†	Fish salad	ND	1/2b (3b)	Food
MB 1911	Fish salad	ND	1/2b (3b)	Food
MB 1956†	Fish salad	ND	1/2a (3a)	Food
MB 1975†	Smoked fish	ND	1/2a (3a)	Food
MB 1984†	Minced meat	ND	1/2a (3a)	Food
MB 1992†	Cheese	ND	1/2a (3a)	Food
MB 2003†	Cheese	ND	1/2a (3a)	Food
MB 2010	Fish salad	ND	1/2b (3b)	Food
MB 2022*†	Cheese	ND	4b (4d,4e)	Food
MB 2094†	Smoked fish	ND	1/2a (3a)	Food
MB 2403†	Udder	1/2a	1/2a (3a)	Farm-environmental
MB 2404*†	Udder	4b	4b (4d,4e)	Farm-environmental
MB 2408†	Water	4b	4b (4d,4e)	Feed
MB 2409	Raw milk	1/2b	1/2b (3b)	Farm-environmental
MB 2414	Udder	3b	1/2b (3b)	Farm-environmental
MB 2415†	Silage	1/2a	1/2a (3a)	Feed
MB 2416	Water	1/2b	1/2b (3b)	Feed
MB 2421*†	Feces	4b	4b (4d,4e)	Farm-environmental
MB 2422	Feces	4b	4b (4d,4e)	Farm-environmental
MB 2423†	Air	1/2a	1/2a (3a)	Farm-environmental
MB 2429†	Udder	1/2a	1/2a (3a)	Farm-environmental
MB 2430	Feces	1/2b	1/2b (3b)	Farm-environmental
MB 2432*†§	Udder	1/2a	1/2a (3a)	Farm-environmental
MB 2434	Feces	1/2b	1/2b (3b)	Farm-environmental
MB 2459*†	Fish	ND	1/2a (3a)	Food

^a MB, bacterial collection of the Institute for Agricultural and Fisheries Research, Unit Technology and Food—Product Quality and Food Safety, Melle, Belgium. Symbols: *, tested for in vitro invasion and IL-8 production in human cell lines; †, analyzed for the partial DNA sequence of the 3' end of the *inlA* gene; ‡, contains a truncated, nonfunctional InlA protein; §, underestimated relative *inlA* expression because of the presence of additional polymorphisms in the HW RT-82 primer-binding site.

^b For the conventional agglutination serotyping method, see reference 55; for the PCR-based serotyping method, see reference 1. ND, not determined.

TABLE 2. Primers used for sequencing and real-time PCR^a

Gene ^b	Primer sequence (5' to 3')		Primer set ^c
	Forward	Reverse	
<i>inlA</i>	AATCTAGCACCCTGTCTGGG TTTCTATAATAACAAGGTAAGT GACGTAAGC ATGACGGTGTAAACAACATCT CCAACAACCTGGAGGGAACACI	TTCTGCAAAAGCATCATCTG	Seq01† (50) and HW-59 HW RT-3†
<i>inlA</i> *	GAACCAGCTAAGCCIGTAAAAAG	CGCCIGTTTGGGCATCA	HW-76† HW RT-83†
<i>inlB</i>	AGTGATGATGGCGATTATG	CCAATGCTAAATTGATACCAGTGTAGTAAT	HW RT-81† and HW RT-82
<i>inlB</i> *	GGAAAAGCAAAAGCAIGATTTC	TCCATCAACATCATAACTTACTGTGTAAA	HW-1 and HW RT-6
<i>ami</i>	TTATTGGCACCTCCGCTA	TTTCAGTAGGATGGATTGGGA	HW RT-46 and HW RT-9
<i>ami</i> *	GGACTGCAACGGCAAGAACATG	CTGTGTGTGCGCGAAGCA	HW-27 and HW-28
<i>rpoD</i>	CTGACGATGCAGATGAIGAG	GTAATTCGTATGCTGCGTGA	HW RT-13 and HW RT-14
<i>rpoD</i> *	TGGATTCTGCAAGCGATAACC	GCACCGGAATACGGATIGTT	HW-37 and HW-38
<i>tufA</i>	TCATGATGCCAGTTGAGGATGT	TGTAACAATACCGATTACGTACGTAGTACG	HW RT-49 and HW RT-50
<i>tufA</i> *	GCTGAAGCTGGCGACAACA	CTTGACACAGTTGGATATCTTCAC	HW-31 and HW-32
16S rRNA	AGTAACACGTGGGYAACCTG	CAGCAGCCGCGGTAATAC	HW RT-59 and HW RT-60
16S rRNA*	CTTCGCAATGGACGAAAGT	ACGATCCGAAAACCTTCTTCATAC	HW-41 and HW-42 HW RT-34 and HW RT-35

^a All primers were designed based on the complete *L. monocytogenes* genomes of strain EGD-e (serotype 1/2a) (GenBank accession number NC_003210) and strain F2365 (serotype 4b) (GenBank accession number NC_002973).

^b *, used for real-time PCR.

^c †, used to sequence the complete PCR fragment of the *inlA* gene.

Total RNA was eluted in 50 µl of diethyl pyrocarbonate (DEPC)-treated deionized water. To remove the residual DNA, a DNase I treatment was performed for 2 h at 37°C in a final volume of 200 µl containing 100 U of RNase-free DNase I (Roche Diagnostics), 40 U of RNase inhibitor (Applied Biosystems), 4 mM Tris-HCl (pH 7.5), and 0.6 mM MgCl₂. Subsequently, DNase I was inactivated and the total RNA was further purified by standard phenolization and precipitation techniques (52). After precipitation, the RNA pellet was dissolved in 50 µl of DEPC-treated water and stored at -20°C until use.

cDNA synthesis. The RT reaction was performed in a total volume of 20 µl containing 1 µl of RNA template, 5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1 mM (each) deoxynucleoside triphosphate (Amersham Biosciences AB, Uppsala, Sweden), 2.5 µM random hexamers (Applied Biosystems), 20 U of RNase inhibitor (Applied Biosystems), and 25 U of Multiscribe reverse transcriptase enzyme (Applied Biosystems). To check for residual DNA, each RNA sample was also subjected to a cDNA synthesis reaction without addition of Multiscribe reverse transcriptase enzyme (NoRT). The reaction mixtures were successively incubated for 10 min at room temperature, 15 min at 42°C, 5 min at 99°C, and 5 min at 5°C.

Real-time quantitative PCR. Real-time quantitative PCR was carried out with the ABI Prism 7000 Sequence Detection System using SDS 1.0 application software (Applied Biosystems). Optimization of real-time RT-PCR is essential for accurate quantification. Based on known DNA sequences of different strains, downloaded from the EMBL/GenBank database, three or four primer sets were designed for each gene with ABI Prism Primer Express 2.0 software. When a single-nucleotide polymorphism was detected between different strains (serotypes) in the primer-binding site, the nucleotide in the corresponding primer was replaced by an inosine (Table 2). This was performed because a polymorphism might lead to a higher cycle threshold (*C_T*) value due to a decrease in amplification efficiency during PCR, resulting in a misinterpretation of the result. The primers were purchased from Invitrogen and tested. Taking into account the *C_T* (as low as possible), the slope of the standard curve (near -3.3), primer dimers (absent), and the magnitude of the signal generated by the given set of PCR conditions (as high as possible), the best primer set was selected for each gene (Table 2). To check for possible additional polymorphisms in the primer-binding sites of the selected primers, partial DNA sequences of *inlA*, *inlB*, *ami*, *rpoD*, *tufA*, and the 16S rRNA gene were determined for a random selection of at least 25 *L. monocytogenes* strains used in the expression study.

Real-time quantitative PCR was performed in a 25-µl reaction volume containing 5 µl of the appropriate cDNA, 12.5 µl of SYBR Green PCR master mix (Applied Biosystems), and a 600 nM concentration of the appropriate gene-specific primers. The following cycle profile was used: one cycle at 95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 1 min. The data were collected during each elongation step. A melting-curve analysis between 65°C and 95°C was performed after each PCR to check the specificity of the amplification

product. To check for residual DNA, the NoRT cDNA reaction was also tested. When a ΔC_T of >4 between the sample and its respective NoRT was obtained, the DNA contamination level was considered negligible. In each run, a negative control (without cDNA) was included. For each gene, a threshold was determined. The *C_T* was defined as the PCR cycle at which the fluorescence generated within a reaction exceeds the threshold. The *C_T* values obtained by real-time PCR were quantified by using a relative standard curve. For this purpose, a stock solution of cDNA was prepared, from which a twofold serial dilution was made.

Data analysis of the relative expression levels of *ami*, *inlA*, and *inlB*. To analyze the variability of the used housekeeping genes, the software program geNorm 3.3 was applied (62). This program calculates, firstly, the gene stability measure *M* (which must be lower than 1.5) for each used housekeeping gene. The highest *M* value reflects the gene with the least stable expression level. In a second step, this program calculates for the used housekeeping genes a normalization factor, which can be applied for data processing.

The relative *inlB* and *ami* expression levels were normalized to the relative quantities of three different housekeeping genes by using geNorm (relative expression level of gene of interest/normalization factor): two genes encoding mRNA (*rpoD* and *tufA*) and one gene encoding rRNA (the 16S rRNA gene). The gene stability measure *M* was 0.723 for *rpoD*, 0.708 for *tufA*, and 0.658 for 16S rRNA. The low *M* values and the small differences between these values indicated that each of these housekeeping genes is constitutively expressed under the experimental conditions used here and that each of the three used housekeeping genes is suitable as internal control. Consequently, *inlA* expression was normalized only to *tufA*, to reduce the real-time PCR assay number. *tufA* was selected because it encodes mRNA and the *M* value of *tufA* is lower than that of *rpoD*.

Statistics. To detect significant differences in the in vitro invasion and IL-8 induction data, an analysis of variance (ANOVA) procedure was used. When there were repetitions available, the average value weighted by the inverse of the variance was used for the analysis. *P* values smaller than 0.05 were considered to be significant. It was verified that the basic assumptions of the ANOVA (e.g., homogeneity of variances and homoscedasticity) were fulfilled. The statistical power of the ANOVA was assessed with a power analysis. In principle, power values below 80% do not permit statistical judgments that are accurate and reliable.

For the real-time RT-PCR data, the results were analyzed with the nonparametric Kruskal-Wallis ANOVA, because assumptions for the standard ANOVA were not met.

Standard Pearson correlation coefficients (*R*²) and the associated significance levels were also calculated.

All statistical analyses were done with Statistica 7 (StatSoft, Tulsa, Okla.).

Nucleotide sequence accession numbers. DNA sequences have been deposited in the GenBank database under accession numbers AM076675 to AM076716.

RESULTS

Serotype identification by PCR. Because a number of the *L. monocytogenes* strains included in this study had not yet been serotyped, a PCR-based serotype identification method was carried out for all strains and compared with the available results of the conventional agglutination method (55). For all strains for which the serotype was known, the serotype group determined by the PCR-based method (Table 1) corresponded to the serotype determined previously by the conventional method (Table 1). For strains with an unknown serotype, the serotype identification obtained by the PCR-based method was considered in this study.

In vitro invasion of human THP-1, Caco-2, and HepG2 cells. To study the invasion potential of clinical and nonclinical strains in specific human cell types, the gentamicin protection assay was performed. Three human cell lines, representing different targets in the infection process, were studied: monocytic THP-1 cells, enterocytic Caco-2 cells, and hepatocytic HepG2 cells. For the THP-1 cells, a total of 12 strains were tested; for the Caco-2 and HepG2 cells, a total of 21 strains were analyzed. The characteristics of these strains are reported in Table 1. All in vitro invasion assays were performed in triplicate in three independent experiments.

While this study was being conducted, the existence of non-functional forms of InlA in a number of *L. monocytogenes* strains, leading to reduced invasion capacity in the enterocytic cell line Caco-2, was reported (24, 42). This led us to analyze the strains used in our in vitro invasion assay for the presence of functional InlA by sequencing the 3' end of the *inlA* gene (see below). The *inlA* gene of 18 of the 21 tested strains encoded full-length InlA (80 kDa). However, in two clinical strains (MB 691 [serotype 1/2a] and MB 2321 [serotype 1/2c]) and one nonclinical strain (MB 704 [serotype 1/2a]) (Table 1), the presence of a nonsense mutation in the *inlA* gene was detected, indicating a truncated nonfunctional InlA protein with a theoretical molecular mass of 47 kDa (MB 691 and MB 704) or 68 kDa (MB 2321). These strains were excluded from further statistical analysis of the Caco-2 cell invasion data.

After study of the invasion into the different cell lines, a power analysis was performed to check whether the sample size was adequate to obtain an accurate statistical analysis. In the case of the data obtained after 1 h of infection, the power value was 99.99% for the Caco-2 and HepG2 cells; for the THP-1 cells, however, the power value was only 18.5%.

After 1 h of infection of THP-1 cells, no clear difference in the invasion capacity of *L. monocytogenes* between the tested clinical and nonclinical strains was observed (Fig. 1, upper panel). Although a low power value was calculated for the THP-1 cell invasion data (see above), this result gives a strong indication that invasion of THP-1 is not dependent on the origin of the strains. In Caco-2 cells, clear differences in invasion capacity were observed between the tested strains (Fig. 1, middle panel). In spite of the expression of a nonfunctional InlA, the strains MB 691, MB 2321, and MB 704 were able to invade with low capacity ($\pm 10^4$ CFU/ml) into Caco-2 cells. Seven of the eight clinical strains with full-size InlA showed a lower invasion capacity ($\pm 10^5$ CFU/ml) than did the nonclinical strains ($\pm 10^6$ CFU/ml) ($P < 0.001$). In the HepG2 cells, 9

FIG. 1. Assays of invasion by *L. monocytogenes* strains of different origins (clinical and nonclinical strains) into three human cell lines, monocytic THP-1 cells (upper panel), enterocytic Caco-2 cells (middle panel), and hepatocytic HepG2 cells (lower panel). Results are expressed as mean log₁₀ CFU/ml (plus standard deviations) for three independent experiments analyzed in triplicate. Numbers of intracellular bacteria were determined after 1 h. ●, strains with a truncated, nonfunctional InlA protein; C, clinical strains; NC, nonclinical strains.

of the 10 tested clinical strains and only 1 of the 11 tested nonclinical strains showed a lower invasion capacity than the other strains (Fig. 1, lower panel). When all 10 clinical and 11 nonclinical strains were considered, a significant difference was noticed ($P < 0.001$).

IL-8 production by human cells after *L. monocytogenes* infection. In THP-1 and in HepG2 cells, induction of the proinflammatory cytokine IL-8 after *L. monocytogenes* infection was examined by analysis of IL-8 immunoreactivity in the supernatant of infected cells. The results are shown in Fig. 2.

For the monocytic THP-1 cells, differences in IL-8 production between the *L. monocytogenes* strains were observed. The power value (20%) was too low to calculate significances; however, no correlation with the origins of the strains was noticed (Fig. 2, upper panel). The high level of IL-8 production after induction of monocytic cells with *L. monocytogenes* is on the order of the IL-8 induction level after LPS treatment of THP-1 cells.

FIG. 2. IL-8 production after infection of human monocytic THP-1 cells (upper panel) and human hepatocytic HepG2 cells (lower panel) with *L. monocytogenes* strains of different origins (clinical and non-clinical strains). IL-8 was detected by ELISA. Mean values (plus standard deviations) for three independent experiments carried out in triplicate are shown. C, clinical strains; NC, nonclinical strains; —, negative control; LPS, lipopolysaccharide (positive control).

For the HepG2 cells, the power value (95%) was sufficient for calculating significances. Although the overall IL-8 production levels were low, it can clearly be observed that 9 of the 10 clinical strains induced lower IL-8 levels than the majority of the nonclinical strains ($P < 0.001$) (Fig. 2, lower panel). Also, the positive control stimulator LPS yielded low IL-8 production in this cell line. These cytokine production levels are in accordance with the invasion capacities of the strains into HepG2 cells (Fig. 1, lower panel), with a low invasion capacity corresponding to low IL-8 production and vice versa.

Analysis of the partial *inlA* gene sequence of *L. monocytogenes* strains. Based on the results of Jacquet et al. (24), which indicated that the functionality of the InlA protein in *L. monocytogenes* is dependent on the serotype, the partial DNA sequence of the 3' end of the *inlA* gene was determined from all used *L. monocytogenes* strains belonging to serotypes 1/2a, 1/2c, and 3a and from some strains belonging to serotypes 1/2b, 3b, and 4b. In total, 44 strains were analyzed: 22 from 1/2a, 3 from 1/2b, 4 from 1/2c, 1 from 3a, 1 from 3b, and 13 from 4b (Table 1).

No nonsense mutations were detected in the tested *L. monocytogenes* strains belonging to serotype 1/2b, 3b, or 4b, indicating that these strains express full-length InlA. In contrast, in the four tested strains belonging to serotype 1/2c a point mutation or deletion, creating a nonsense mutation, was detected in the *inlA* gene, indicating the expression of nonfunctional, truncated InlA proteins (Table 1). The only tested serotype 3a strain also expressed full-length InlA. The majority of the serotype 1/2a strains expressed full-length InlA (20 of 22). Only

2 of the 22 serotype 1/2a strains expressed truncated InlA, namely, the clinical strain MB 691 and the nonclinical strain MB 704 (Table 1). All five strains isolated from cerebrospinal fluid (CSF) or amniotic fluid expressed full-size InlA.

Optimization of real-time quantitative RT-PCR. The PCR efficiencies for all genes (*inlA*, *inlB*, *ami*, *rpoD*, *tufA*, and the 16S rRNA gene), assessed by determination of the slope of the standard curve, were $>90\%$ [$E = (10^{-1/\text{slope}} - 1) \cdot 100$] and were optimal for all assays for each gene (data not shown). The correlation coefficients were >0.99 (data not shown). The reproducibility and reliability of the assay were assessed by repeating the cDNA synthesis and real-time PCR three times under identical conditions. The interassay coefficients of variation for cDNA synthesis and for real-time PCR were 13.78% and 4.33%, respectively.

To check the DNA sequence of the primer-binding sites of the studied genes, a random selection of at least 25 strains was analyzed. In the cases of *inlB*, *ami*, *rpoD*, *tufA*, and the 16S rRNA gene, no unexpected polymorphisms were detected. Unfortunately, two additional polymorphisms were detected in the primer-binding site of the reverse primer of the *inlA* gene in some strains belonging to serotype 1/2a. Therefore, the primer-binding sites of *inlA* were analyzed for all strains of serotypes 1/2a, 1/2c, and 3a belonging to genetic serotype lineage II predicted cluster (40). Five of the 22 strains showed additional polymorphisms at the primer-binding site (MB 691 [1/2a], MB 704 [1/2a], MB 2315 [1/2a], MB 2432 [1/2a], and MB 2641 [1/2a]). Because the presence of a polymorphism in the primer-binding site might lead to an underestimation of the relative *inlA* expression level, these strains were excluded from further statistical analysis of *inlA* expression (Fig. 3).

To check for the presence of chromosomal DNA, a NoRT was performed for each gene and for each sample. The DNA contamination level for all samples was negligible.

Relative expression of the virulence genes *inlA*, *inlB*, and *ami*. The unexpected observation that the majority of the tested clinical strains are less invasive than nonclinical strains in enterocytic Caco-2 and hepatocytic HepG2 cells led us to study the relative quantitative expression levels of *inlA* and *inlB* in an extended group of clinical and nonclinical *L. monocytogenes* strains. These genes are both important for invasion in the respective cell lines. Additionally, *ami*, involved in adhesion to eukaryotic cells, was analyzed. This extended group of *L. monocytogenes* strains comprised 27 clinical and 37 nonclinical strains. (Table 1).

In Fig. 3, the results of the expression study are shown. Significant statistical differences were observed in *inlA* ($P < 0.001$) and *inlB* ($P < 0.001$) expression levels between the clinical and nonclinical *L. monocytogenes* strains, with the clinical strains showing lower *inlA* and *inlB* expression levels than the nonclinical strains. When the *inlA* and *inlB* expression levels were compared with each other, a correlation coefficient (R^2) of 0.82 was found. The calculated correlation coefficients between the mRNA expression data (*inlA* and *inlB*), the invasion (1 h) capacity into Caco-2 and HepG2 cells, and the IL-8 production 24 h after *L. monocytogenes* infection of HepG2 cells are illustrated in Table 3. These data clearly show that the results of the different tests are strongly correlated to each other.

FIG. 3. *inlA*, *inlB*, and *ami* mRNA expression levels in the late logarithmic growth phase of clinical and nonclinical *L. monocytogenes* strains. The bars represent *inlA*, *inlB*, and *ami* mRNA expression levels (plus standard deviations) relative to *tufA* (for *inlA*) or to three housekeeping genes (*rpoD*, *tufA*, and the 16S rRNA gene) (for *inlB* and *ami*). *n*, number of experiments; ▲, strains that are underestimated in relative *inlA* expression level because of the presence of additional polymorphisms in the primer-binding site of the reverse primer.

For *ami* expression, no significant difference was noticed between the clinical and nonclinical strains (Fig. 3). No correlation was found between *ami* expression and *inlA* or *inlB* expression either.

DISCUSSION

At present, any *L. monocytogenes* strain is considered potentially pathogenic for humans, because little information is

TABLE 3. Calculated correlation coefficients (R^2) between mRNA expression data (*inlA* and *inlB*), invasion capacities into Caco-2 and HepG2 cells, and IL-8 production at 24 h after *L. monocytogenes* infection of HepG2 cells

Parameter	Variable	R^2				IL-8 production by HepG2 cells
		mRNA expression level		Invasion (1 h)		
		<i>inlA</i>	<i>inlB</i>	Caco-2 cells	HepG2 cells	
mRNA expression level	<i>inlA</i>	1.00	0.82	0.78	ND ^a	ND
	<i>inlB</i>	0.82	1.00	ND	0.81	0.58
Invasion (1 h)	Caco-2 cells	0.78	ND	1.00	ND	ND
	HepG2 cells	ND	0.81	ND	1.00	0.85
IL-8 production	HepG2 cells	ND	0.58	ND	0.85	1.00

^a ND, not determined.

available about the correlations between the virulence potential and specific characteristics of the strains. However, many studies suggest differences in virulence potential between *L. monocytogenes* strains (4, 9, 13, 24–26, 41, 51, 63, 66). The objective of this study was to track phenotypic differences in virulence between *L. monocytogenes* strains of different origins by comparing clinical and nonclinical strains at different levels.

For the monocytic THP-1 cells, no differences in invasion capacity or in IL-8 production of *L. monocytogenes* strains of different origins were observed. Phagocytosis of *L. monocytogenes* by THP-1 cells could be one of the reasons why differences in mRNA expression levels of *inlA* and *inlB*, observed in this study, do not influence the invasion capacity of *L. monocytogenes* in these cells. This is in contrast to the invasion of the enterocytic Caco-2 and the hepatocytic HepG2 cells, where a significant difference between clinical and nonclinical strains was observed, with the majority of the clinical strains showing a lower invasion capacity than the nonclinical strains. Other studies also showed variations in invasion efficiencies into Caco-2 cells between different *L. monocytogenes* strains, but no clear correlation with the origin of the strains was found (26, 63). However, in these studies the growth phase of the tested bacteria is not clearly mentioned; this is possibly an important aspect. Our own experience indicates that the expression of virulence genes is upregulated in the late logarithmic growth phase (data not shown). Other studies have also shown that a stress condition, such as entry into the stationary phase, can trigger the expression of virulence genes by transcriptional control (11, 58). Therefore, in this study all *L. monocytogenes* strains were tested in the late logarithmic phase. The unexpected observation that the majority of the clinical strains show a lower invasion capacity into Caco-2 and HepG2 cells than do the nonclinical strains is inconsistent with several reports in which a low entry level is correlated with low virulence (46, 47). It is possible that the use of another cell line and/or another in vitro assay (plaque-forming assay instead of invasion assay) in these studies is an explanation for the contradictory results. On the other hand, the group of nonclinical *L. monocytogenes* strains used in the current study contains a wider variety of serotypes than did the avirulent group studied by Roche et al. (46), which mainly contained *L. monocytogenes* strains belonging to serotype 1/2a. In accordance with the results of Roche et al. (46), our nonclinical 1/2a *L. monocytogenes* strains ex-

pressed on average lower *inlA* and *inlB* mRNA levels than did strains of the other serotypes, resulting in lower invasion rates. Furthermore, the recently described truncated, nonfunctional forms of InlA in some *L. monocytogenes* strains were also mainly observed in strains belonging to serotype 1/2a or 1/2c; this may also explain the low invasion capacity observed in the study of Roche et al. (46). In our study, the strains expressing nonfunctional, truncated InlA were still able to invade Caco-2 cells with low capacity. This means that additional bacterial factors probably also mediate invasion into enterocytic cells (8).

Because of the differences in invasion capacity into Caco-2 and HepG2 cells, the mRNA levels of *ami*, *inlA*, and *inlB*, all important for adhesion and invasion of eukaryotic cells, were studied in an extended group of *L. monocytogenes* strains. The *inlA* and *inlB* expression levels were shown to be significantly lower in the clinical strains than in the nonclinical strains, in contrast with *ami* expression, for which no difference was noticed. These results are in accordance with the observed invasion capacities in enterocytic and hepatocytic cells. The high correlation between the *inlA* and *inlB* mRNA expression levels suggests that these genes are probably transcribed together. Cotranscription, as well as coregulation, of *inlA* and *inlB* has already been described by others (31, 36). To our knowledge, this is the first report to describe differences in *inlA* and *inlB* expression levels between clinical and nonclinical strains by real-time PCR. Different expression levels determined by real-time RT-PCR have also been described for the hemolysin gene (*hly*) of *L. monocytogenes* strains, although no clear difference was found between human clinical and nonclinical isolates (51). Nevertheless, it is also important to note that some nonclinical strains also express low *inlA* and *inlB* levels and vice versa. These results only reinforce the fact that the virulence and infection process of *L. monocytogenes* is a complex phenomenon. It is also known that the infection dose, host factors, and particularly the immune status of the host play a key role in the infection process. In addition, a recent epidemiological study wherein the expression of full-length or truncated InlA in a large group of *L. monocytogenes* strains of different origin was determined showed that 96% of the clinical strains express full-size InlA, in contrast to 65% of the nonclinical strains (24). This higher frequency of functional InlA in the clinical strains indicates that InlA plays an important role in human listeriosis, although some strains with a nonfunctional InlA also seem to be able to cause listeriosis. Our results, however, show that clinical strains express on average a lower level of *inlA* and *inlB* than do nonclinical strains. The fact that no difference in *ami* expression levels between both groups of strains was noticed indicates that the observed differential expression of specific virulence genes is not a general phenomenon. A possible explanation for our finding that clinical strains express lower levels of *inlA* and *inlB* transcripts, resulting in a lower capacity to invade host cells, is that a lower degree of cell invasion would be used by virulent *L. monocytogenes* strains in vivo as an immune evasion strategy, to prevent the nonspecific immune system from clearing the infection in an early phase. The lower IL-8 production in liver cells by clinical *L. monocytogenes* strains supports this hypothesis, as IL-8 attracts neutrophils to the inflammatory foci, leading to the control of infection (12). This means possibly that intracellular growth may not be a

feature of the initial phase of listeriosis and that intracellularity only becomes critical with the onset of adaptive immunity. This notion is also supported by the observation that the attenuated phenotype of *hly* mutants becomes apparent only at the stage of liver and spleen infection (21). Immune evasion by low invasion rate has also been hypothesized for infection of poultry by different *Salmonella enterica* serotypes. Entry of the avian *Salmonella enterica* serovar Gallinarum (specific pathogenic for poultry) in avian cells did not induce a strong inflammatory response, suggesting that this pathogen might not be limited by the immune system, in contrast to the other *Salmonella* serotypes that are less specifically pathogenic for poultry and that induced strong inflammatory responses (29). Whether the differences in *inlA* and *inlB* expression observed in vitro in this study play a role in vivo has still to be evaluated in animal models. No clear differences in *inlA* and *inlB* expression levels between clinical strains isolated from CSF or amniotic fluid and those isolated from blood were observed. This result is in accordance with another study, in which it was suggested that InlB is not an important virulence factor for central nervous system infection (22, 25, 54). However, it has recently been shown that the interaction of InlA–E-cadherin could also play a role in crossing of the placental barrier (33).

From our experiments, it cannot be deduced whether or not the observed correlation between invasion capacity into HepG2 cells and IL-8 production is direct. In endothelial cells, it has been shown that LLO (listeriolysin O), and not InlB, induces IL-8 production (30, 48). However, in macrophages it has been noticed that InlB itself can activate the NF- κ B pathway, which is important for IL-8 production (37). Complementatation of *L. monocytogenes* with a plasmid expressing *inlB* or analysis of the effect of a mutant deficient in LLO production should give more information about the relation between *inlB* expression level and IL-8 production in HepG2 cells after *L. monocytogenes* infection.

Our analysis of the functionality of InlA indicates that in none of the *L. monocytogenes* strains belonging to serotype 1/2b, 3b, 4b, or 3a was a nonsense mutation detected in the *inlA* sequence, indicating that these strains all express full-length InlA. Serotype 1/2a expressed either full-size (91%) or truncated (9%) InlA. All five strains isolated from CSF or amniotic fluid expressed full-size InlA. These results are in agreement with the results of Jacquet et al. (24).

In conclusion, we have shown that the average *inlA* and *inlB* mRNA expression levels differ between *L. monocytogenes* strains of different origins and that clinical strains show a statistically significantly lower expression level than nonclinical strains. Furthermore, these results have been confirmed by biological assays, and a strong correlation has been found between *inlA* and *inlB* expression levels and the capacity to invade enterocytic Caco-2 and hepatocytic HepG2 cells, respectively, and to induce the proinflammatory cytokine IL-8 after *L. monocytogenes* infection of HepG2 cells. Although the physiological relevance of this observation must be confirmed by in vivo studies, our results indicate that fine tuning of the transcription of virulence genes, resulting in differences in expression levels, may play a role in the virulence of *L. monocytogenes* and may differentiate *L. monocytogenes* strains.

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