Poor Invasion of Trophoblastic Cells but Normal Plaque Formation in Fibroblastic Cells despite *actA* Deletion in a Group of *Listeria monocytogenes* Strains Persisting in Some Food Processing Environments[▽]

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Received 27 November 2009/Accepted 14 March 2010

We determined mammalian cell invasion and virulence gene (*inlA*, *inlB*, and *actA*) sequences of *Listeria monocytogenes* strains belonging to a molecular subtype (RAPD 9) that often persists in Danish fish-processing plants. These strains invaded human placental trophoblasts less efficiently than other *L. monocytogenes* strains, including clinical strains, and they carry a premature stop codon in *inlA*. Eight of 15 strains, including the RAPD 9 and maternofetal strains, had a 105-nucleotide deletion in *actA* that did not affect cell-to-cell spread in mouse fibroblasts. The RAPD 9 strains may still be regarded as of low virulence with respect to human listeriosis.

Listeria monocytogenes is a Gram-positive pathogenic bacterium that can cause foodborne listeriosis, which affects immunocompromised individuals, causing septicemia and meningitis, and pregnant women, causing preterm delivery, miscarriage, or stillbirth. It is a ubiquitous environmental bacterium, and it is therefore continuously introduced to food-processing plants, where some molecular subtypes are able to persist despite thorough cleaning and disinfection procedures (1, 27, 32, 42). Such persistent strains are likely to contaminate the food products and may be the cause of foodborne infections (30).

We have shown that specific molecular subtypes of *L. monocytogenes* can persist for years in the seafood-processing environment (45), and strains representing a particularly prevalent, persistent molecular subtype, RAPD type 9 ([RAPD 9] random amplified polymorphic DNA), had a lower virulence potential than clinical strains in simple eukaryotic models (12). However, in a more complex biological model using oral dosing of pregnant guinea pigs, the tested RAPD 9 strain (strain La111) surprisingly infected the placentas and fetuses just as efficiently as a clinical strain (13). We therefore hypothesized that this specific subtype may have an altered (enhanced) ability to invade placental cells (e.g., trophoblasts) or an enhanced ability to spread intracellularly.

Several virulence factors are important for *L. monocytogenes*, and mutations in key virulence genes lead to less-virulent *L. monocytogenes* strains (8). The surface proteins InlA and InlB interact with their respective receptors, E-cadherin

(23) and Met, gC1qR, or proteoglycans (4, 15, 34) and mediate internalization of *L. monocytogenes* into nonphagocytic cells. *L. monocytogenes* strains with mutations in *inlA* (premature stop codons [PMSCs]) have been found in France (29), the United States (25), and Japan (9), and these mutations lead to attenuation in the invasion of intestinal epithelial cells (25, 28, 33), but it is not known if invasion into trophoblasts is affected. ActA is important for cell-to-cell spread (5) and is involved in invasion of epithelial cells (39), and ActA-mediated cell-to-cell spread plays a major role in crossing the fetoplacental barrier in both a guinea pig and a mouse model (3, 22).

The purpose of this study was to determine if the high level of prevalence of a RAPD 9 strain in guinea pig fetuses after oral dosing could be explained by increased invasion into and spread between trophoblastic and fibroblastic cells, respectively. Subsequently, we sequenced selected virulence genes to determine if strain variations in cell invasion and spread could be explained by differences in sequences.

Strains, culture conditions, and characterization. Fifteen *L. monocytogenes* strains representing different origins, RAPD types, serotypes, and lineages were used (Table 1). Four strains (N53-1, La111, H13-1, and M103-1) represent a unique, persistent subtype (RAPD 9) of *L. monocytogenes*. The bacteria were grown in brain heart infusion (BHI) broth (catalog no. CM0225; Oxoid) and enumerated on BHI agar. For all cell assays, bacteria were grown at 37°C with aeration for 20 h. Serogrouping was done using *Listeria* O antisera types 1 and 4 (catalog nos. 223001 and 223011; Becton Dickinson) according to the manufacturer's instructions, and lineage separation was done as in Wiedmann et al. (44). RAPD typing was done according to Vogel et al. (43). Caco-2 cell invasion was done according to Jensen et al. (12).

Invasion, intracellular growth, and cell-to-cell spread. Invasion and intracellular growth were studied in the human cho-

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▽ Published ahead of print on 26 March 2010.

TABLE 1. Characteristics of the *Listeria monocytogenes* strains used in this study

Strain	Origin ^a	Serotype	Lineage	RAPD type ^b	Mutation in indicated gene ^c :			Invasion level in indicated cell line ^d :			No. of plaque formations/ 10 ² bacteria invading L929 cells	Source or reference
					<i>inlA</i>	<i>inlB</i>	<i>actA</i>	Caco-2	L929	JAR		
N53-1	Smoke house equipment	1/2a	2	9	PMSC	SPM ^{1,2}	Del	10 ^{2e}	5 × 10 ¹ -1 × 10 ²	10 ⁴	72	45
La111	Smoke house equipment	1/2a	2	9	PMSC	SPM ^{1,2}	Del	10 ^{2e}	5 × 10 ¹ -1 × 10 ²	10 ⁴	75	43
H13-1	Smoke house equipment	1/2a	2	9	PMSC	SPM ^{1,2}	Del	10 ^{2e}	5 × 10 ¹ -1 × 10 ²	10 ⁴	81	45
M103-1	Slaughter house equipment	1/2a	2	9	PMSC	SPM ^{1,2}	Del	10 ^{2e}	5 × 10 ¹ -1 × 10 ²	10 ⁴	140	45
La22	Cold-smoked salmon	1/2a	2	12	FL	SPM ^{1,2}	FL	10 ^{3e}	5 × 10 ¹ -1 × 10 ²	10 ⁵ -10 ⁶	104	42
7418	Spreadable sausage	1/2b	1	14	FL	SPM ²	FL	10 ⁵ -10 ^{6e}	5 × 10 ¹ -1 × 10 ²	10 ⁶	54	18
3849-97	Maternofetal fluid (35 + 6, L)	4	1	NT	FL	SPM ²	Del	10 ⁵ -10 ⁶	5 × 10 ¹ -1 × 10 ²	10 ⁵	85	35
3272-03	Maternofetal fluid (35 + 2, L)	1	2	NT	FL	SPM ^{1,2}	FL	10 ⁵ -10 ⁶	5 × 10 ¹ -1 × 10 ²	10 ⁵ -10 ⁶	292	35
3495-04	Maternofetal fluid (31 + 4, L)	1	1	NT	FL	NM	Del	10 ⁵ -10 ⁶	5 × 10 ¹ -1 × 10 ²	10 ⁵ -10 ⁶	101	35
4810-98	Maternofetal fluid (38 + 4, D)	4	1	NT	Del	SPM ²	FL	10 ⁵ -10 ⁶	5 × 10 ¹ -1 × 10 ²	10 ⁶	63	35
12443	Monkey maternofetal fluid	1/2a	2	73	FL	SPM ^{1,2}	Del	10 ⁵ -10 ^{6e}	5 × 10 ¹ -1 × 10 ²	10 ⁶	133	37
Scott A	Human clinical isolate (blood)	4b	1	72	FL	SPM ²	FL	10 ^{6e}	5 × 10 ¹ -1 × 10 ²	10 ⁶	234	Campden ^f
4446	Human clinical isolate (blood)	4b	1	71	FL	SPM ²	Del	10 ⁵ -10 ^{6e}	5 × 10 ¹ -1 × 10 ²	10 ⁵ -10 ⁶	51	18
LO28	Human feces	1/2c	2	69	PMSC	NM	FL	10 ^{3e}	5 × 10 ¹ -1 × 10 ²	10 ⁴ -10 ⁵	84	41
EGD	Rabbit isolate from 1926	1/2a	2	68	FL	NM	FL	10 ^{4e}	5 × 10 ¹ -1 × 10 ²	10 ⁴ -10 ⁵	87	W. Goebel ^g

^a The maternofetal strains are described according to week and day of gestation and the outcome of the fetus (L, live; D, dead).

^b NT denotes a bacterium that did not belong to any of the RAPD types represented by the other strains and to which a specific RAPD type was not assigned.

^c The presence or absence of mutations in the virulence genes *inlA*, *inlB*, and *actA* is indicated by the following: PMSC, premature stop codon; FL, full-length sequence; Del, deletion; SPM¹, single-point mutation (alanine to threonine); SPM², single-point mutation (valine to isoleucine); NM, no mutation.

^d Invasion levels are presented as CFU/ml and plaque formation as the number of plaques formed per 10² invading bacterial cells.

^e Unpublished results and results previously published by Jensen et al. (11, 13).

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FIG. 1. Invasion of *L. monocytogenes* strains into human trophoblastic JAR cells. Strains were grown in BHI broth at 37°C for 20 h and adjusted to 1.0×10^6 CFU/ml before the JAR monolayer was infected. Invasion is expressed as the number of intracellular CFU/ml relative to the number of CFU/ml added to the well. Strains were sorted according to origin. Dark columns represent lineage I strains and light columns represent lineage II strains. The columns show the averages of the results of one trial carried out in duplicate, and the error bars show standard deviations. The results are representative of those of three independent experiments. Mann-Whitney analysis showed that the *L. monocytogenes* RAPD 9 strains as a group differed significantly from the maternofetal strains with respect to invasion ability ($P = 0.0195$). Comparison of lineage I and lineage II strains showed that lineage I strains invaded to a significantly higher level than lineage II strains ($P = 0.0343$).

riocarcinoma cell line JAR (LGC Standards no. HTB-144), and invasion and plaque formation were studied in the mouse fibroblast cell line L929 (European Collection of Animal Cell Cultures [ECACC] no. 85011425). All cell assay experiments were carried out in duplicate in three independent trials. The JAR cells were propagated in F12 (catalog no. BE12-615; Lonza) and the L929 cell line in Dulbecco's modified Eagle's medium (DMEM) (catalog no. BE12-604F; Lonza), both supplemented with 10% fetal bovine serum (FBS) (catalog no. DE14-830; Lonza) and 25 μ g/ml gentamicin (catalog no. 15750-037; Gibco). Cells were incubated at 37°C with 5% CO₂. Invasion and intracellular growth were studied with a gentamicin protection assay as previously described (12). JAR cells were adjusted to 5×10^4 cells/ml and L929 cells to 3×10^5 cells/ml; 1 ml was added to 24-well tissue culture plates (catalog no. 92024; TPP) and incubated for 24 h. The monolayers of JAR and L929 cells were infected with 1×10^6 CFU/ml or 5×10^4 CFU/ml of bacteria, respectively. The cells were washed in saline water and overlaid with medium containing 50 μ g/ml gentamicin. The cells were washed after 1 h with saline water and either lysed immediately with 0.1% Triton X-100 or overlaid with medium containing 25 μ g/ml gentamicin and incubated for 2 h, 3.5 h, or 5 h before being lysed. The number of intracellular bacteria was determined by plating appropriate dilutions on BHI agar plates. For the plaque formation assay, 4 ml adjusted L929 cells was added to 6-well tissue culture plates (catalog no. 92006; TPP), incubated for 24 h to reach a monolayer, and then washed twice in saline water before 3 ml of bacteria culture (5×10^4 CFU/ml in cell culture medium) was added. The plates were centrifuged for 5 min at $150 \times g$.

FIG. 2. Plaque formation by *L. monocytogenes* in mouse fibroblastic L929 cells. Strains were grown in BHI broth at 37°C for 20 h and adjusted to 5×10^4 CFU/ml before infection of the L929 monolayer. Data are expressed as the number of plaques for 10^2 invading bacteria. Strains have been sorted according to their origins. Dark columns represent lineage I strains, and light columns represent lineage II strains. Check-patterned columns represent strains with a 105-nucleotide deletion in *actA*. Columns show averages of the results from one trial carried out in duplicate, and error bars show standard deviations. The results are representative of those of three independent experiments. Kruskal-Wallis analysis showed no significant difference in the number of plaques formed when strains were grouped according to origin ($P = 0.8326$), and Mann-Whitney analysis showed no significant difference when strains were grouped according to lineage ($P = 0.2721$) or the presence of the *actA* deletion ($P = 0.6126$).

After 1 h, the L929 cells were washed in saline water, followed by the addition of a 2-ml overlay consisting of DMEM, 0.5% agarose, and 10 μ g/ml gentamicin. After 4 days of incubation, the plaques were visualized by the addition of 2 ml DMEM containing 0.5% agarose and 0.01% neutral red (catalog no. N2889; Sigma). *L. monocytogenes* strains 10403S and 10403S with *actA* deleted (kindly provided by D. Portnoy, University of California) were included as positive and negative controls, respectively, for plaque formation (data not shown). The plaque areas were measured, using ImageJ software, and plaque size was normalized to the size of the well. The measurement included 10 plaques per strain from one experiment. The Mann-Whitney test was used to compare groups of *L. monocytogenes* strains with full-length sequences and strains with an *actA* deletion with respect to either number of plaques or plaque size. The Kruskal-Wallis test was used to test for associations between invasive ability, number of plaques, and plaque size among groups of strains of different origins. All analyses were performed using GraphPad Prism statistical software. P values of <0.05 were considered significant.

The group of persistent RAPD 9 strains invaded the trophoblasts at a significantly lower level than the maternofetal strains did ($P = 0.0195$), but EGD and LO28 also displayed a low level of invasion in JAR cells (Fig. 1). Since *L. monocytogenes* invades placental trophoblasts in an InlA-E-cadherin-dependent manner (21), the observed lower level of invasion of the RAPD 9 strains and LO28 in JAR cells is supported by the presence of a premature stop codon in *inlA* in these strains (see below). Lineage I strains invaded trophoblastic cells at a significantly higher level than lineage II strains ($P = 0.0343$). The two strains derived from food samples, 7418 and La22, also invaded

TABLE 2. Primers used for sequencing of *Listeria monocytogenes* virulence genes

Primer	Sequence (5'–3')	Annealing temp (°C)	Reference
1F 1R	GGA AAA ATG TGC TGG AAC CGG GTC TAT ATC CGT TAT CTG A	45	This study This study
1.5F 1.5R	ATC GAT GGA GTG GAA TAC TT GTG CCT ATA TCT TTT AAC TGG TTA C	48	This study This study
2F 2R	CCG CTA GCT AAT TTG ACG A GCA AGT GAG CTT ACG TCA	41.7	This study This study
4F 4R	CTT GGA GCT AAC CAA ATA AGT AAC A TTT TAC GGG CTT AGC TGG TT	48	This study This study
4.5F 4.5R	GTG AAA AAT GTG ACT GGC GC TCC GTC GCA AAA TCC CAT TT	58	This study This study
InlA seq F InlA R	GTG GAC GGC AAA GAA ACA AC ATA TAG TCC GAA AAC CAC ATC T	58	25 25
actA F actA R	AGT CAG TTG CGG ATG CTT CTG TCT GTT GTC TCG CTG TTT TCG	58	This study This study
InlB1-2F InlB1-2R	GAT GTG GTT TTC GGA CTA TAT GCT AAT GCT CTT AAA TCG CT	47	This study This study
InlB2F InlB2R	TTC TTT GGA GCA TAA TGG TA CGC TTT AGT CCA GCC AAT AT	48	This study This study
InlB3F InlB3R	TAC TGA AAA AAA TGG TGG GCA T TTG ACC TTC GAT GGT TGC TT	47	This study This study

the trophoblasts at a very high level, comparable to that of the maternofetal strains. The high level of invasion of the maternofetal and human clinical strains was also seen in Caco-2 cells (Table 1). There was no difference in intracellular growth in JAR cells between the RAPD 9 strains and the other strains (results not shown).

All strains invaded the L929 cells at a similar level (Table 1), and all strains were able to form plaques in L929 cells (Table 1 and Fig. 2). One maternofetal strain (3272-03) formed a very high number of plaques, almost double the numbers of the other strains. We did not find differences between the number of plaques formed when strains were grouped according to origin ($P = 0.8326$), lineage ($P = 0.2721$), or presence of the *actA* deletion ($P = 0.6126$) (see below); however, lineage I strains formed significantly larger plaques than lineage II strains ($P = 0.0160$).

Sequencing of virulence genes. The *inlA*, *inlB*, and *actA* genes were amplified by PCR from total isolated DNA and sequenced in both directions by DNA Technology (Århus, Denmark). The *inlA* and *inlB* genes were fully sequenced, and the *actA* gene was partly sequenced. The primers (DNA Technology, Århus, Denmark) used for PCR amplification and sequencing are shown in Table 2. The *actA* region was chosen, as previous studies proposed a link between deletions of repeats in this region of proline-rich repeats and reduced *in vitro* motility and plaque formation (36, 38).

We have hypothesized that single-point mutations in *inlA* could cause a lower level of invasion by RAPD 9 strains in Caco-2 cells (11, 12), and here we identified a premature stop codon in all four RAPD 9 strains. A nucleotide substitution from cytosine to thymidine at position 1474 results in a stop

codon at position 492 (Fig. 3A), reported to lead to export of InlA instead of its incorporation into the cell membrane (16, 28). LO28 was the only other strain containing PMSC, which was already known. The maternofetal strain 4810-98 had a small deletion of 9 nucleotides starting at position 2214 (data not shown). This deletion did not influence the ability of this strain to invade trophoblasts. The deletion was present at the membrane-anchoring region of InlA and therefore might not influence the activity of InlA.

Three of 15 strains contained a complete *inlB* sequence, but 12 strains had two nucleotide substitutions, one from guanine to adenine at position 350, resulting in a substitution from alanine to threonine (position 117), and one from guanine to adenine (position 395), leading to a substitution from valine to isoleucine (position 132) (Fig. 3B). The first mutation was seen in the four RAPD 9 strains, two maternofetal strains (12443 and 3272), and one food strain (La22). The other type of mutation was seen in all of the strains except EGD, LO28, and the maternofetal strain 3495-04. None of the strains had any nonsense mutations in *inlB*.

Eight of 15 strains contained a deletion of 105 nucleotides (35 amino acids) in *actA* (Fig. 3C). The deletion encodes amino acid 305 to amino acid 340, which is the central region of proline-rich repeats required for binding to the vasodilator-stimulated phosphoprotein (VASP) (6, 19). The deletion was seen not only in the four RAPD 9 strains, but also in four of the clinical strains, of which two have caused fetal infection. This suggests that the deletion in the *actA* repeat region does not influence the ability to cause fetal infection. Also, this deletion did not influence the number of plaques formed ($P = 0.6126$) (Table 1).

Discussion. *L. monocytogenes* subtypes that persist in the food industry have caused listeriosis outbreaks, in the United States, for example (30, 31), and although the subtype RAPD 9, which is common in Danish fish-processing plants, has not been linked to outbreaks (Birgitte Smith, unpublished data), the apparent ability of a strain (La111) of this subtype to invade guinea pig fetuses (13) led us to study invasion of this subtype in trophoblastic cells and the sequences of its virulence genes.

The InlA receptor E-cadherin is present on the cell wall of trophoblastic cells and is responsible for InlA-E-cadherin-dependent entry of *L. monocytogenes* into trophoblasts (21). RAPD 9 strains invade Caco-2 cells, which are of another InlA-E-cadherin-dependent cell line, at a lower level than strains of other origins and RAPD types (11), and we found in the present study that they were poor invaders of trophoblastic cells as well (Fig. 1). This was likely caused by a premature stop codon in *inlA* in the four RAPD 9 strains. A strain lacking *inlA* is attenuated in its ability to invade cells from the placental barrier (8, 21), and our study is the first to demonstrate that strains with PMSCs in *inlA* are affected in their ability to invade placental cells. Strains with truncated InlA cannot be regarded as nonvirulent *per se*, as cases of maternofetal listeriosis or bacteremia have been caused by strains expressing truncated InlA (8, 10).

InlB is important for the interaction with several cell line receptors; however, full expression of InlB may not be a prerequisite for virulence, as strain F2365, isolated during the 1985 listeriosis epidemic in California, contains a stop codon in *inlB* at position 100 (26). We expected that InlB was important for invasion of *L. monocytogenes* into L929 fibroblast cells, as it influences invasion into Vero cells (40). We did find the same single-point mutations (SPMs) at amino acid positions 117 and 132, but these did not affect fibroblast invasion. The SPMs are located in the leucine-rich region of InlB, which is important for the interaction with the Met receptor (34). The contradiction between our results and the results seen by Temoin et al. (40) could be explained by the use of two different cell lines (Vero cells and L929 cells) or could be due to the presence of other mutations in other genes.

ActA is responsible for the formation of the actin tail, and strains lacking ActA are less virulent and unable to form plaques (5). ActA is also required for crossing of the fetoplacental barrier in guinea pigs and mice (3, 22). The *actA* deletion found in several strains in our study has been described for other *L. monocytogenes* strains (14, 24, 38, 44). Jiang et al. (14) found that a strain containing the deletion is unable to form plaques in the mouse fibroblastic cell line L929, but the control strain and the deletion strain in that study were not isogenic, and therefore other genetic differences could be involved. Neither Sokolovic et al. (38), Chen et al. (7), nor Moriishi et al. (24) found any correlation between plaque formation and *actA* deletion in either the rat epithelial cell line L2 or in L929.

All four tested RAPD 9 strains had exactly the same sequence (and mutations) for all three sequenced virulence genes, indicating that these strains are genetically highly similar. They were isolated from four different processing environments over a period of 8 years (45). By amplified fragment length polymorphism (AFLP) subtyping, we found that N53-1 (isolated in 2002), H13-1 (isolated in 2003), and La111 (iso-

lated in 1996) were identical, whereas M103-1 (isolated in 2003) was marginally different.

The paradox that a strain (La111) with an otherwise low level of virulence that harbors a mutation in *inlA* is able to infect guinea pig fetuses indicates that InlA does not play a crucial role in placental infection of guinea pigs. This finding is also supported by a study with pregnant guinea pigs (2). In contrast, full-length *inlA* is important for human placental infection (2, 8, 10, 21), which indicates that InlA and InlB are species specific. The discrepancy between *in vivo* (guinea pig) and *in vitro* (cell lines) results could therefore be explained by the species specificity of InlA and InlB. InlA interacts with the human and guinea pig E-cadherin but not with the mouse E-cadherin (20), whereas InlB interacts with the human and mouse Met receptor but not with the guinea pig Met receptor (17). Like humans, the gerbil is permissive to both the InlA-E-cadherin and the InlB-Met pathways, and it has recently been established that InlA and InlB have interdependent roles in fetoplacental invasion in this species. Consequently, *L. monocytogenes* targets only the placenta *in vivo* if both InlA and InlB pathways are functional (8). Hence, we believe that the RAPD 9 subtype may be regarded as a group of *L. monocytogenes* with a low level of virulence.

Nucleotide sequence accession numbers. The *inlA*, *inlB*, and *actA* gene sequences have been deposited in GenBank under accession numbers GU735663 to GU735675, GU079614 to GU079626, and GU060665 to GU060678, respectively.

We thank Vi Nguyen for excellent handling of the cell lines.

A.H. was supported by Microbial Opportunistic Pathogens (grant no. 2052-03-0013) and by the Danish Research Council for Technology and Production Sciences (project 274-08-042). C.T.G. was funded by a Ph.D. grant from the Technical University of Denmark.

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