Multiple point mutations in virulence genes explain the low virulence of *Listeria monocytogenes* field strains

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In order to understand the causes of the low virulence of *Listeria monocytogenes* field strains, five low-virulence strains were analysed. These five strains showed changes in relation to invasion, phosphatidyl-inositol phospholipase C (PI-PLC) activity, plaque formation and *in vivo* virulence. Molecular analyses revealed the same mutations in the *plcA*, *inlA* and *inlB* genes in all five strains. The Thr262Ala substitution in the PI-PLC protein was responsible for the absence of PI-PLC activity. This residue, conserved in certain *L. monocytogenes* species, is located at the outer rim of the active site pocket and could impair the cleavage activity of the enzyme. The low invasion rate of these strains was due to a nonsense codon leading to a lack of InlA protein synthesis, and to an Ala117Thr substitution in the leucine-rich repeat of InlB, which altered the interaction with the Met receptor. Single *trans* complementation with the *inlA*_{EGDe}, *inlB*_{EGDe} or *plcA*_{EGDe} genes restored the capacity of low-virulence strains either to enter epithelial and fibroblastic cells or to express PI-PLC activity. Complementation by allelic exchange of the *plcA*_{EGDe} gene on the chromosome and *trans* complementation with either the *inlA*_{EGDe} or the *inlB*_{EGDe} gene restored the ability to form plaques, but only partly restored the *in vivo* virulence, suggesting that there were other gene mutation(s) with consequences that could mainly be observed *in vivo*. These results indicate that the low virulence of *L. monocytogenes* strains can be explained by point mutations in a number of virulence genes; these could therefore be important for detecting low-virulence strains. Moreover, the fact that all the strains had the same substitutions suggests that they have a common evolutionary pathway.

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INTRODUCTION

Listeria monocytogenes is a Gram-positive facultative intracellular pathogen responsible for severe food-borne infections of both humans and animals. *L. monocytogenes* is able to invade, survive and multiply inside phagocytic and non-phagocytic cells. The most important virulence genes are clustered in a 10 kb locus on the chromosome and are

Abbreviations: i.v., intravenous; LLO, listeriolysin-O; LRR, leucine-rich repeat; PC-PLC, phosphatidylcholine phospholipase C; PI-PLC, phosphatidyl-inositol phospholipase C; PFA, plaque-forming assay; s.c., subcutaneous.

The GenBank accession numbers for the *plcA*, *inlA* and *inlB* nucleotide sequences were: AY367414, EF990797 and EF990802 for strain BO43; AY367410, EF990793 and EF990798 for strain CNL895807; AY367411, EF990796 and EF990801 for strain CNL895795; AY367412, EF990794 and EF990799 for strain 416; and AY367413, EF990795 and EF990800 for strain 417.

Three supplementary figures, showing immunofluorescence labelling of actin tails, nucleotide and amino acid substitutions observed in *plcA*, *inlA* and *inlB* genes of the Group III strains, and a schematic representation of the crystal structure of PI-PLC, and also a supplementary table, listing the PCR primers used, are available with the online version of this paper.

positively regulated by the transcriptional activator positive regulatory factor A (PrfA). Invasion of mammalian cells mainly requires the expression of two internalin-family proteins, InlA and InlB, encoded by the *inlA* and *inlB* genes, respectively (Gaillard *et al.*, 1991; Dramsi *et al.*, 1995). Both proteins are necessary and sufficient for the bacterium to invade various cell lines (Lecuit *et al.*, 1997; Braun *et al.*, 1999). InlA interacts with host cell E-cadherin, in a species-specific manner, to mediate the invasion of epithelial cells. InlB promotes the invasion of a wide variety of mammalian cells, such as hepatocytes and fibroblast cells, through interaction with three receptors: Met, gC1qR and glycosaminoglycans (Shen *et al.*, 2000; Braun *et al.*, 2000; Jonquieres *et al.*, 2001).

After invading mammalian cells, *L. monocytogenes* escapes from the phagocytic vacuole by using a pore-forming bacterial toxin, listeriolysin-O (LLO), encoded by the *hly* gene, and two phospholipases, phosphatidyl-inositol phospholipase C (PI-PLC) and phosphatidylcholine phospholipase C (PC-PLC), encoded respectively by the *plcA* and *plcB* genes. Free in the cytosol, *L. monocytogenes* grows rapidly and employs ActA to induce polymerization of host

actin filaments, which the bacteria use for moving intracellularly. Such movements cause protrusions which may contact neighbouring cells, resulting in the formation of double-membrane vacuoles, which are lysed by PC-PLC and LLO (Gaillard *et al.*, 1986; Vazquez-Boland *et al.*, 1992). This cell-to-cell spread allows plaque formation in cell monolayers, which can be used to estimate the virulence of *L. monocytogenes* strains (Roche *et al.*, 2001).

L. monocytogenes is a pathogenic species, but some strains have been shown to have low virulence (Tabouret *et al.*, 1991; Norrung & Andersen, 2000; Roche *et al.*, 2001). In a previous study, 26 low-virulence *L. monocytogenes* field strains were identified using a method combining a plaque-forming assay (PFA) with subcutaneous (s.c.) injection into the left hind footpad of mice (Roche *et al.*, 2001). The low virulence of these *L. monocytogenes* strains was found to be a stable trait. They also exhibited a low lethality in mice compared to virulent strains (Roche *et al.*, 2003). These low-virulence strains, unrelated in origin and isolation date, were assigned to one of four groups based on their phenotypic characteristics, using cluster analysis (Roche *et al.*, 2005). The cause of the low virulence of the 11 strains belonging to Group I has been identified. In fact, all these strains exhibit a mutated PrfA.

The present study was designed to identify virulence factors or cell-invasion mechanisms that were impaired in five out of six low-virulence Group III strains, which exhibited exactly the same phenotypic characteristics. These five strains are referred to as 'Group III strains' for the remainder of this article.

METHODS

Strains, plasmids and culture conditions. The virulent *L. monocytogenes* strain EGDe and the isogenic mutants EGDe Δ inlA, EGDe Δ inlB and EGDe Δ inlA Δ inlB originated from P. Cossart's laboratory (Institut Pasteur, Paris, France) as did the *Listeria innocua* BUG499 strain. The EGDe Δ actA2 strain originated from E. Domann's laboratory (Institute of Medical Microbiology, Hospital of the Justus-Liebig University Giessen, Giessen, Germany). *Escherichia coli* TG1 was used for plasmid construction. The characteristics of the five low-virulence Group III strains are described in Table 1. Strains harbouring plasmid pHT1618 (Lereclus & Arantes, 1992) were grown in a culture medium containing 100 μ g ampicillin ml⁻¹ for *E. coli*, and 10 μ g tetracycline ml⁻¹ for *Listeria*. Strains harbouring plasmid pP1 (Dramsi *et al.*, 1995) were grown with 150 μ g erythromycin ml⁻¹ for *E. coli*, and 8 μ g erythromycin ml⁻¹ for *Listeria* in liquid medium, and 1 mg erythromycin ml⁻¹ for both *E. coli* and *Listeria* in agar medium.

Cell lines and culture conditions. A human adenocarcinoma HT-29 cell line (ECACC no. 85061109), a human epithelial Caco-2 cell line (ECACC no. 86010202) and a green monkey fibroblastic Vero cell line (ECACC no. 84113001) were grown in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g glucose l⁻¹ (Invitrogen) supplemented with 2 mM L-glutamine (Invitrogen) and 10% fetal calf serum (Life Technologies) for HT-29 and Caco-2 cells, or 5% fetal calf serum for Vero cells. For Caco-2 cells, non-essential amino acids were added (M7145, Sigma). Antibiotics (100 IU penicillin ml⁻¹ and 100 μ g streptomycin ml⁻¹) were routinely added to the culture medium, except for the virulence assays. J774-A1 macrophages (ECACC no. 85011428) were grown in DMEM supplemented with 10% fetal calf serum, 4 mM L-glutamine and 100 mM sodium pyruvate.

Cell culture assays. Cell invasion assays were performed with Caco-2 or Vero cells, as described previously (Velge *et al.*, 1997). The results

Table 1. Characteristics of the strains used in this study (Roche *et al.*, 2001)

Invasion and plaque-forming assays were carried out in duplicate and repeated twice for each strain. <DT, Below the detection threshold.

Strain	Source	Serovar	Invasion rate on HT-29 cells (mean $\pm$ SD) (%)	PFA (mean $\pm$ SD)*	s.c. test (splens)		LD ₅₀ or maximum dose injected§	Enzyme activities		
					log(number) (mean $\pm$ SD)†	Ratio‡		LLO	PC-PLC	PI-PLC
<i>L. innocua</i> (BUG499)		6a	<0.001	0	<DT	0:5	>0.76 $\times 10^9$	0	0	0
EGDe (BUG1600)	Clinical animal case	1/2a	2.02 $\pm$ 0.98	6.35 $\pm$ 0.12	5.54 $\pm$ 0.46	5:5	4.62	16	6.37	8.30
CNL895807	Milk	1/2a	0.15 $\pm$ 0.08	0	<DT	0:5	>1.24 $\times 10^9$	32	4.52	0.92
CNL895795	Dairy product	1/2a	0.05 $\pm$ 0.02	0	<DT	0:5	>1.03 $\times 10^9$	32	5.82	0.36
416	Dairy product	1/2a	0.07 $\pm$ 0.46	0	<DT	0:5	9.25	32	4.36	0.39
417	Dairy product	1/2a	0.05 $\pm$ 0.02	0	2.81 $\pm$ 1.47	2:5	>1.19 $\times 10^9$	8	5.42	0.56
BO43	Milk	1/2a	0.45 $\pm$ 0.17	0	2.53	1:5	9.05	64	5.35	0.67

*log(number of plaques per 10⁷ c.f.u. deposited). Values are from two independent experiments performed in duplicate.

†log of the number of *Listeria* recovered from the spleens of immunocompetent Swiss mice 3 days after s.c. injection of 10⁴ c.f.u. in 50 μ l into their left hind footpads. Values are from five infected mice.

‡Ratio of infected mice to inoculated mice.

§LD₅₀ after s.c. inoculation or maximal dose injected.

||Enzyme activities in haemolytic units for LLO, enzyme units ml⁻¹ for PC-PLC, and pmol min⁻¹ ml⁻¹ for PI-PLC.

were expressed as the log of the number of invading bacteria recovered after 3.5 h. Experiments were carried out in duplicate and repeated twice for each strain.

PFA were performed as described previously (Van Langendonck *et al.*, 1998). The number of plaques was counted and the diameters of 20 plaques were measured with an Anastigmat Loupe $\times 4$ including a micrometer device (Peak).

Double fluorescence labelling of actin and bacteria. J774-A1 macrophages were grown on eight-chamber tissue-culture glass slides (Falcon) with 5×10^5 cells per well. Cells were activated with *Salmonella enterica* serovar Typhimurium LPS ($0.1 \mu\text{g ml}^{-1}$) the night before the assay. Cells were infected for 1 h with 1×10^6 c.f.u. and then incubated in culture medium containing $100 \mu\text{g}$ gentamicin ml^{-1} for 5 h to kill extracellular bacteria. Cells were washed and fixed overnight with cold 80 % acetone, saturated with $200 \mu\text{l}$ 10 % rabbit serum, and then incubated for 1 h with $100 \mu\text{l}$ of a 1:50 diluted anti-*Listeria* polyclonal antibody conjugated with fluorescein isothiocyanate (5688-1995, Biogenesis). Cellular F-actin was stained for 20 min in darkness at room temperature with $100 \mu\text{l}$ 1 % rhodamine-labelled phalloidin. Preparations were examined under a fluorescence microscope.

Nucleotide sequencing and cloning of virulence genes. The *inlA* and *inlB* genes were amplified by PCR from total isolated DNA with AccuPrime Taq high-fidelity DNA polymerase (Invitrogen). An *inlA*–*inlB* fragment was amplified using primers *inlA*-Fwd and O24 for Group III strains and *inlA*-Fwd and *inlB*-Rev5 for the EGDe strain (see Supplementary Table S1). Nucleotide sequencing was carried out by Genome Express. Nucleotide sequences were aligned using Vector NTI (Informax).

The *plcA*_{EGDe}, *inlA*_{EGDe} and *inlB*_{EGDe} genes were amplified with Phusion enzyme (Ozyme) using O37/O39, *inlA*-Fwd2/*inlA*-rev and *inlB*-Fwd/*inlB*-rev5 primers, respectively (Supplementary Table S1). The purified and digested *plcA* fragment was then introduced into digested pHT1618 vector, and the purified and digested *inlA* and *inlB* fragments were introduced into digested pP1 vector. DNA plasmids from *E. coli* transformants were isolated using the QIAprep plasmid kit (Qiagen). After verification by endonuclease restriction and sequencing analysis, recombinant plasmids were introduced into Group III strains by electroporation, as described elsewhere (Park & Stewart, 1990).

Complementation by allelic exchange mutagenesis. The thermosensitive pMAD vector was used for allelic replacement of the *plcA* gene on the chromosome (Arnaud *et al.*, 2004). A DNA fragment of the EGDe strain was amplified with *plcAF3*/*plcAR3* primers using the Phusion enzyme (Supplementary Table S1). The fragment was purified and digested, and cloned into the digested pMAD vector. The recombinant vector was extracted from *E. coli*, and a three-step procedure was used for allele replacement in *L. monocytogenes*. In brief, the pMAD-*plcA*_{EGDe} plasmid was introduced into the 416 strain by electroporation. The following steps were carried out as described elsewhere (Arnaud *et al.*, 2004), except that the sensitive temperature used was 42°C and the concentration of X-Gal was $200 \mu\text{g ml}^{-1}$. Several colonies were checked for erythromycin sensitivity and loss of the plasmid was analysed using PCR (Supplementary Table S1).

PI-PLC activity assays. Detection of PI-PLC activity was checked on PI-TY agar, as described elsewhere (Notermans *et al.*, 1991), and was analysed in the culture supernatant with tritium-labelled L- α -phosphatidyl-inositol, as previously described (Roche *et al.*, 2005). Experiments were performed in triplicate for each strain.

Western blot analysis. Secreted proteins were obtained as described elsewhere (Bannam & Goldfine, 1999). Protein pellets were separated

in 12 % SDS–polyacrylamide gels. Western blots were probed with anti-PI-PLC antibody (provided by H. Goldfine's laboratory, University of Pennsylvania School of Medicine) (Bannam & Goldfine, 1999) and bound antibodies were detected using chemiluminescence (ECL+, GE Healthcare). Total bacterial extracts were separated in 10 % SDS–polyacrylamide gels. Western blots were probed with rabbit anti-InlB antibody (provided by P. Cossart's laboratory, Institut Pasteur, Paris, France) (Braun *et al.*, 1999).

Mouse virulence assays. The virulence of the strains was assessed after s.c. injection into the left hind footpad or after intravenous (i.v.) injection, as described previously (Roche *et al.*, 2001). The results were expressed as the mean of the log number of c.f.u. per positive organ.

Statistical analysis. The spleen colonization of i.v.-inoculated mice was analysed using a non-parametric Mann–Whitney test. Invasion assays were analysed with an analysis of variance followed by a Tukey–Kramer test. These statistical tests were performed with InStat software 2.03 (GraphPad).

RESULTS

Characteristics of the low-virulence strains

Previously published phenotypic characterization of the low-virulence strains (Roche *et al.*, 2001) showed that Group III strains (strains CNL895807, 416, 417, CNL895795 and BO43) had similar LLO and PC-PLC activities to those of the virulent EGDe strain but no PI-PLC activity. Moreover, these strains did not form plaques in HT-29 cell monolayers (Table 1), exhibited no virulence in mouse infection assays (Table 1) and had an $\text{LD}_{50} > 1 \times 10^9$ c.f.u., similar to the results obtained with *L. innocua*. The *prfA* gene and the intergenic regions containing PrfA boxes were not mutated, suggesting that the transcription of virulence genes could occur. The low virulence of these strains could be related to multi-causal effects: low invasion capability, incapacity to form plaques and/or lack of PI-PLC activity.

Fluorescence labelling of ActA

As the incapacity of the Group III strains to form plaques could also be related to a non-functional ActA protein, the presence of actin tails was investigated using fluorescence microscopy with J774-A1 macrophages, which overcome the low invasion capability of bacteria. However, all Group III strains exhibited the same number of actin tails with a similar appearance to those observed with the virulent EGDe strain, unlike the EGDe Δ *actA2* strain, which did not form actin tails (see Supplementary Fig. S1). These results provide evidence that the ActA protein of Group III strains is functional and that the absence of plaques on cell monolayers is not related to ActA.

Expression of virulence proteins

To determine whether the lack of invasion and PI-PLC activity was related to an absence of InlA, InlB or PI-PLC

proteins, Western-blot analysis was carried out to investigate the presence of these proteins. A protein was detected by the anti-PI-PLC serum in the precipitated culture supernatant of all the Group III strains and the EGDe strain, but not in the supernatant of the *L. innocua* strain. This result indicates that the lack of PI-PLC activity was not due to an absence of protein expression (Fig. 1a). Similarly, analysis of InlB expression detected a band at 63 kDa for all the Group III strains and the EGDe strain. However, the 417 strain expressed a lower quantity of InlB than the EGDe strain (Fig. 1b). Western-blot analysis carried out by the CNR (Centre National de Référence des *Listeria*, Institut Pasteur) detected the InlA protein for the EGDe strain, but not for any of the Group III strains (data not shown). The lack of InlA synthesis could thus explain the defective invasion of these strains into epithelial cells.

Nucleotide sequencing of the virulence genes

To understand why InlA was not synthesized and to determine whether non-functional PI-PLC and InlB proteins were expressed, the *plcA*, *inlA* and *inlB* genes were sequenced. Nucleotide sequencing of the *inlA* gene identified the insertion of an adenine at position 13 in all the Group III strains, creating a nonsense codon in the coding sequence after the twenty-fifth amino acid (see Supplementary Fig. S2a), thus explaining the absence of InlA. The *inlB* sequencing revealed nine nucleotide substitutions in all Group III strains (Supplementary Fig.

S2a), leading to two mutations in InlB: Ala117Thr and Val132Ile (Supplementary Fig. S2b). These mutations were located in the leucine-rich repeat (LRR) region, which is involved in the interaction of InlB with Met, its cellular receptor. Only the first substitution led to a change in the hydrophobic nature of the amino acid.

As previously shown, all the Group III strains, none of which exhibited PI-PLC activity, showed the same 12 substitutions in the *plcA* gene (Supplementary Fig. S2a) that led to three mutations in PI-PLC (Supplementary Fig. S2b) (Roche *et al.*, 2005). Analysis of the mutations showed that the isoleucine–valine substitution at position 17 (Ile17Val) was located in the signal sequence of the protein. The second substitution was located at position 119, between β -sheet II and the D helix, and led to the substitution of a phenylalanine by a tyrosine (Phe119Tyr). The last substitution was located between β -sheet VII and the G helix and changed the threonine in position 262 into an alanine (Thr262Ala). Only the Phe119Tyr and Thr262Ala mutations led to a change in the physico-chemical properties of the amino acids.

Trans complementation with a plasmid encoding *plcA*

In order to demonstrate that the substitutions in the *plcA* gene were responsible for the lack of PI-PLC activity, the pHT1618 plasmid carrying the *plcA*_{EGDe} gene and its intergenic region was inserted into all Group III strains. Interestingly, *trans*-complementation restored the PI-PLC activity for the five strains (Fig. 2a). The *trans*-complemented CNL895795, 416 and BO43 strains exhibited a very high enzymic activity compared to the EGDe strain, in contrast to the *trans*-complemented strains CNL895807 and 417, which exhibited a lower activity. However, all *trans*-complemented strains exhibited PI-PLC activity on PI-TY agar, unlike their parental strains (data not shown). These results indicate that mutations in the *plcA* gene account for the lack of PI-PLC activity in the Group III strains.

Trans complementation with a plasmid encoding *inlA*

The nonsense codon at the 5' end of the *inlA* gene explains why the Group III strains did not express InlA and invaded the Caco-2 cells on average 55-fold less than the EGDe strain, since the invasion of Caco-2 cells is known to be InlA-dependent (Lecuit *et al.*, 1997) (Fig. 2b). *Trans* complementation of the Group III strains with plasmid pP1 carrying the *inlA*_{EGDe} gene allowed invasion of Caco-2 cells at the same rate as the EGDe strain. In fact, a statistically significant ($P < 0.01$) 100-fold higher invasion rate was observed for these *trans*-complemented strains than for their parental strains, or their parental strains transformed with the empty plasmid (Fig. 2b). These results indicate that *inlA* mutation explains the inability of the Group III strains to invade epithelial cells.

Fig. 1. Western blot analysis. (a) PI-PLC proteins from culture supernatants were precipitated with TCA and separated by 12% SDS-PAGE. (b, c) InlB proteins from total extract were separated by 10% SDS-PAGE. Western-blot analysis was carried out on parental strains (b) and complemented strains (c). Proteins were transferred to a nitrocellulose membrane and probed with antibodies raised against either PI-PLC or InlB protein. PI-PLC and InlB proteins were revealed using peroxidase-labelled goat anti-rabbit antibody and anti-mouse antibody, respectively, and the Uptilight enhanced chemiluminescence substrate.

Fig. 2. Phenotypic characterization of *trans*-complemented strains. (a) PI-PLC activity (pmol ml⁻¹ min⁻¹) measured using [³H]- α -phosphatidyl-inositol. Activity was measured three times for each strain. This figure shows representative results of one experiment because of the variability of this method, which did not allow us to calculate the means but gave the same trend. (b) Invasion assay in human intestinal epithelial Caco-2 cells. (c) Invasion assay in fibroblastic Vero cells. Ø, non-complemented (hatched bars); pP1, complemented with empty plasmid (black bars); *inA/inB*, complemented with pP1 plasmid harbouring *inA*_{EGDe} or *inB*_{EGDe} genes (spotted bars). Invasion assays were carried out in duplicate and repeated twice for each strain. The bars represent the mean invasion rates and the error bars indicate SD. ***P*<0.01; ****P*<0.001.

Trans complementation with a plasmid encoding *inlB*

To analyse the role of the *inlB* mutations, invasion assays were performed in fibroblastic Vero cells, whose invasion is *InlB*-dependent (Dramsi *et al.*, 1995). This was confirmed by the similar invasion rate of the EGDe Δ *inlA* strain to that of the EGDe strain (Fig. 2c). The Group III strains invaded Vero cells 100-fold less than the EGDe strain and at the same rate as EGDe Δ *inlB* and EGDe Δ *inlA* Δ *inlB* strains. The Group III strains *trans*-complemented with the pP1 plasmid carrying the *inlB*_{EGDe} gene showed a statistically significant ($P < 0.001$) 100-fold higher invasion rate in fibroblastic cells than their parental strains, or parental strains complemented with the empty plasmid (Fig. 2c). Their invasion rates were similar to those of the EGDe Δ *inlB*+*inlB*_{EGDe} strain and three logs superior to those of the EGDe Δ *inlB* or EGDe Δ *inlB*+pP1 strains. Western blots showed that *trans*-complemented strains had a large quantity of *InlB* (Fig. 1c).

Complementation with the *plcA* gene by allelic exchange on the chromosome

Since double or triple complementations were technically impossible due to plasmid incompatibility and defective growth, the strains were complemented with the *plcA*_{EGDe} gene on the chromosome by allelic exchange, a method shown to be useful for restoring the virulence of *L. monocytogenes* strains (Olier *et al.*, 2005). The *plcA*_{EGDe} gene and its intergenic region were inserted into strain 416 with the pMAD vector (Arnaud *et al.*, 2004). The integrants obtained by double crossover were checked for PI-PLC activity on PI-TY agar and confirmed using a radioactivity-based assay. Homologous recombination on the chromosome allowed different alleles of the *plcA* gene to be obtained. The resulting alleles represented a combination of the three different amino acids (at positions 17, 119 and 262) of the *plcA*_{EGDe} and *plcA*₄₁₆ genes. Sequencing showed that strain 59, which possesses the three amino acids of the EGDe strain in positions 17, 119 and 262 (profile 17_{EGDe}-119_{EGDe}-262_{EGDe}), exhibited PI-PLC activity (Table 2). This activity was also restored in strain 114 (profile 17₄₁₆-119₄₁₆-262_{EGDe}) and in strain 2 (profile 17₄₁₆-119_{EGDe}-262_{EGDe}). In contrast, strains 112 (profile 17_{EGDe}-119_{EGDe}-262₄₁₆), 6 (profile 17₄₁₆-119_{EGDe}-262₄₁₆) and 57 (profile 17_{EGDe}-119₄₁₆-262₄₁₆) did not exhibit enzymic activity. These results suggest that only the Thr262Ala mutation was responsible for the lack of PI-PLC activity in the Group III strains.

Restoration of plaque formation with Caco-2 cells

To restore plaque formation, strains complemented with the *plcA* gene by allelic exchange were *trans*-complemented with the pP1-*inlA*_{EGDe} vector. For the remaining experiments, only strains 112 (which exhibits no PI-PLC activity) and 114 (which does exhibit PI-PLC activity) were used. As

Table 2. Different strains obtained by allelic exchange of *plcA*_{EGDe} and *plcA*₄₁₆

Strain	Phenotype*			Halo†	Activity (pmol ml ⁻¹ min ⁻¹)‡
	aa 17	aa 119	aa 262		
<i>L. innocua</i>				No	0.31
EGDe	EGDe	EGDe	EGDe	Yes	11.57
416	416	416	416	No	0
59	EGDe	EGDe	EGDe	Yes	4.07
2	416	EGDe	EGDe	Yes	14.17
114	416	416	EGDe	Yes	11.85
6	416	EGDe	416	No	0.25
57	EGDe	416	416	No	0.21
112	EGDe	EGDe	416	No	0

*Amino acid at position 17, 119 or 262 for the different strains obtained by allelic exchange of *plcA*_{EGDe} and *plcA*₄₁₆.

†Ability to form a turbid halo on PI-PLC agar according to the method of Notermans *et al.* (1991).

‡PI-PLC enzymic activity measured with [³H]- α -phosphatidyl-inositol (Roche *et al.*, 2005). Activity was measured three times for each strain with similar results. The table shows representative results of one experiment.

expected, the *trans*-complemented strains 114 and 112 acquired the capacity to invade Caco-2 cells, with a statistically significant ($P < 0.001$) approximately 1000-fold higher invasion rate than those of parental strains or strains complemented with the empty plasmid (Fig. 3a). No plaques were found for the parental 416 strain or the strain complemented with the *inlA*_{EGDe} gene (Table 3). Interestingly, when strain 114, which exhibited PI-PLC activity, was complemented with the *inlA*_{EGDe} gene, its plaque-forming capacity was restored, unlike the parental strain or the strain transformed with the empty plasmid. The 114+*inlA*_{EGDe} strain formed smaller but more numerous plaques than the EGDe strain (Table 3). This result is specific to the T262A substitution, as the 112+*inlA*_{EGDe} strain was unable to form plaques in Caco-2 cell monolayers.

Restoration of plaque formation with Vero cells

As both the *plcA* and *inlA* genes were necessary to restore *in vitro* virulence in Caco-2 cells, we decided to check the role of the double *plcA*_{EGDe}-*inlB*_{EGDe} complementation in Vero cells. The complementation of strains 112 and 114 with the pP1-*inlB*_{EGDe} plasmid showed a statistically significant ($P < 0.001$) 100-fold higher invasion rate than those of the parental strains (Fig. 3b), whereas the invasion rates of the parental 112 and 114 strains were similar to those of strains 416 and EGDe Δ *inlB*. Only strain 114+*inlB*_{EGDe} was able to form a similar number of plaques to the EGDe strain, but the elongated shape of the plaques made it impossible to measure their diameter (Table 3). As observed with *plcA*_{EGDe}-*inlA*_{EGDe} complementation, double complementation with *plcA*_{EGDe}

Fig. 3. Cell invasion assays on epithelial Caco-2 cells (a) and fibroblastic Vero cells (b) for parental strains and *trans*-complemented strains. Ø, non-complemented (hatched bars); pP1, complemented with empty plasmid (black bars); *inlA/inlB*, complemented with pP1 plasmid harbouring *inlA*_{EGDe} or *inlB*_{EGDe} genes (spotted bars). Experiments were carried out in duplicate and repeated twice for each strain. The bars represent the mean invasion rates and the error bars indicate SD. *** $P < 0.001$.

and *inlB*_{EGDe} genes was necessary to restore plaque formation.

In vivo assay

Since plaque-forming capacity was restored for strains complemented with either *plcA/inlA* or *plcA/inlB* genes, the virulence level of these strains was investigated in a mouse model. As murine E-cadherin is not recognized by InlA (Lecuit *et al.*, 1999), only the strains complemented with both *inlB* and *plcA* genes were tested. None of the complemented strains showed spleen colonization after s.c. inoculation, indicating that the double complementation did not restore virulence by this route of infection. After i.v. inoculation, the 114 + *inlB*_{EGDe} strain showed a statistically significant ($P < 0.005$) sixfold higher spleen colonization [median log(c.f.u. per organ) = 3.72] than the 112 + *inlB*_{EGDe} (2.92) and 416 + *inlB*_{EGDe} strains (2.76). This result suggests that the virulence was restored to a lesser extent than that of the EGDe strain (5.47). The same level of colonization observed in strains 416 (3.91) and

114 + *inlB*_{EGDe} could be explained by the negative effect induced by the plasmid and already observed in the invasion assays (Figs 2b, c and 3a, b).

DISCUSSION

We had previously identified 26 low-virulence field strains which were assigned to four groups (Roche *et al.*, 2005). For five out of six strains in group III, the same mutations in three genes, *plcA*, *inlA* and *inlB*, were responsible for their inability to form plaques; this ability was restored by complementation with the corresponding genes of the virulent EGDe strain. However, although complementation with *plcA*_{EGDe} and *inlB*_{EGDe} partly restored the virulence of these strains in mice after i.v. inoculation, it was not sufficient to restore the *in vivo* virulence after s.c. inoculation. As InlA is unable to interact with murine E-cadherin, the absence of *in vivo* virulence of the Group III strains could not be due to the InlA mutation. These results suggest that other genes are altered in Group III strains, for

Table 3. Virulence of double-complemented low-virulence strains assessed by PFA

ND, Not determined.

Strains	PFA with Caco-2 cells		PFA with Vero cells (no. of plaques) [†]
	Diameter of plaques (mm)*	Number of plaques [†]	
EGDe	0.81 ± 0.14	4.4	5.59
416	—	0	0
416 + pP1	—	0	0
416 + <i>inlA</i> _{EGDe}	—	0	ND
416 + <i>inlB</i> _{EGDe}	ND	ND	0
112	—	0	0
112 + pP1	—	0	0
112 + <i>inlA</i> _{EGDe}	—	0	ND
112 + <i>inlB</i> _{EGDe}	ND	ND	0
114	—	0	0
114 + pP1	—	0	0
114 + <i>inlA</i> _{EGDe}	0.66 ± 0.07	6.02	ND
114 + <i>inlB</i> _{EGDe}	ND	ND	5.71

*Mean ± SD of the diameter of 20 plaques.

[†]Number of plaques was expressed as the log(number of plaques per 10⁷ c.f.u. deposited).

example the *agrA* and *inlJ* genes, which are only expressed *in vivo*, or genes involved in adaptation to mouse immune defences (Autret *et al.*, 2003; Sabet *et al.*, 2005).

Restoration of PI-PLC activity by allelic exchange highlighted that the Thr262Ala mutation was likely responsible for the absence of PI-PLC activity of the Group III strains, whereas the two other mutations were silent mutations. Threonine at position 262 is conserved in the different *L. monocytogenes* strains sequenced (strains EGDe, 10403S and F6845 of serovar 1/2a, and strains CLIP80459, F2365 and H7858 of serovar 4b), despite large genotypic differences between the different serovars (Doumith *et al.*, 2004). These results demonstrate that Thr262 plays a key role in PI-PLC activity. The crystallographic structure of PI-PLC shows that *myo*-inositol, the polar head-group of the PI substrate, interacts with side-chains of amino acids, including Asp204, which is spatially close to Thr262 (Moser *et al.*, 1997). Thr262, which is located on the outer rim of the active site pocket, could interact with the glycerolipid part of PI. The Thr262Ala substitution could alter the cleavage activity of the enzyme without altering binding to *myo*-inositol (see Supplementary Fig. S3).

The observation of actin tails for the Group III strains indicates that bacteria could escape from the single-membrane vacuole, and that this was not prevented by the lack of PI-PLC activity. Our results suggest that PI-PLC plays a role in the lysis of the double-membrane vacuole, as *inlA*_{EGDe}-complemented Group III strains did not form plaques, whereas *inlA*_{EGDe}-*plcA*_{EGDe}-complemented strains did. As the former invaded cells, multiplied intracellularly and formed actin tails, *plcA*_{EGDe} complementation should restore the lysis of the double-membrane vacuole. This is in

line with a recent report showing that PI-PLC acts synergistically with PC-PLC to dissolve the inner membrane of the double-membrane vacuole (Alberti-Segui *et al.*, 2007).

Inactivation of *InlB* appears to be related to the Ala117Thr substitution which changed the hydrophobic nature of the amino acid, unlike the Val132Ile substitution. This hypothesis could be confirmed by site-directed mutagenesis. The Ala117Thr substitution is located in the LRR domain, which is both necessary and sufficient for invasion by interaction with Met, the *InlB* receptor (Shen *et al.*, 2000).

Our results also demonstrate that the absence of *InlA* synthesis, caused by the insertion of a nonsense codon, was responsible for the low invasion rate of Group III strains into epithelial cells. Insertion of a nonsense codon leading to a truncated form of the *InlA* protein seems common, and previous studies have described 11 other different nonsense mutations (Jonquière *et al.*, 1998; Nightingale *et al.*, 2005; Felicio *et al.*, 2007; Handa-Miya *et al.*, 2007). Our PFA test on HT-29 cells combined with inoculation of mice allowed 8–20% of low-virulence strains to be detected. However, strains exhibiting only an inactive *InlA* were not detected by our test, although 35% of *L. monocytogenes* strains could have a truncated form of *InlA* and could thus be considered to be low-virulence strains (Olier *et al.*, 2003; Jacquet *et al.*, 2004). Moreover, numerous reports have demonstrated that non-haemolytic strains are avirulent, although the number of these strains is unknown, since many of them are not identified as *L. monocytogenes* strains when detection is based only on their haemolytic activity (Gaillard *et al.*, 1986; Kathariou *et al.*,

1987; Cossart *et al.*, 1989). Overall, these results suggest that the number of low-virulence strains has been underestimated in the past and that they might easily account for 50 % of *L. monocytogenes* field strains. This hypothesis changes our perception of the risk of consuming contaminated food, as only a few strains, mainly belonging to serotype 4b, may exhibit a high virulence level and therefore be responsible for clinical cases, whereas numerous strains have one or more altered virulence gene and should thus be considered low-virulence strains. These results suggest that the low virulence of *L. monocytogenes* field strains is mainly due to point mutations in several virulence genes which cannot be detected by methods such as transcriptomic analyses, genotyping or PFGE.

Our results also show that numerous strains have the same mutations. We have previously demonstrated that 42 % of low-virulence strains are mutated in PrfA (30 % had a PrfA Lys220Thr substitution and 12 % PrfAΔ174-237) (Roche *et al.*, 2005; Velge *et al.*, 2007). In the present study, 20 % exhibited the same mutations in at least three genes (*plcA*, *inlA* and *inlB*). As these five strains were unrelated in origin and isolation date, we can hypothesize that this loss of virulence represents a selective advantage, probably due to better adaptation of the bacteria to the environment. However, up to now, no increase in fitness has been detected for these low-virulence strains; they are even more susceptible than virulent strains to the selective medium used to identify *L. monocytogenes* strains (Gracieux *et al.*, 2003). Moreover, these results suggest that low-virulence strains have diverged from virulent strains, but each low-virulence group has followed a common evolutionary pathway. This hypothesis is currently being investigated, and understanding how they have evolved into several phylogenetic divisions may offer new opportunities for the detection, treatment and control of this widespread pathogen.

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