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The *Listeria monocytogenes* LPXTG surface protein Lmo1413 is an invasin with capacity to bind mucin

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ABSTRACT

Many Gram-positive bacterial pathogens use surface proteins covalently anchored to the peptidoglycan to cause disease. Bacteria of the genus *Listeria* have the largest number of surface proteins of this family. Every *Listeria* genome sequenced to date contains more than forty genes encoding surface proteins bearing anchoring-domains with an LPXTG motif that is recognized for covalent linkage to the peptidoglycan. About one-third of these proteins are present exclusively in pathogenic *Listeria* species, with some of them acting as adhesins or invasins that promote bacterial entry into eukaryotic cells. Here, we investigated two LPXTG surface proteins of the pathogen *L. monocytogenes*, Lmo1413 and Lmo2085, of unknown function and absent in non-pathogenic *Listeria* species. Lack of these two proteins does not affect bacterial adhesion or invasion of host cells using in vitro infection models. However, expression of Lmo1413 promotes entry of the non-invasive species *L. innocua* into non-phagocytic host cells, an effect not observed with Lmo2085. Moreover, overproduction of Lmo1413, but not Lmo2085, increases the invasion rate in non-phagocytic eukaryotic cells of an *L. monocytogenes* mutant deficient in the acting-binding protein ActA. Unexpectedly, production of full-length Lmo1413 and InlA exhibited opposite trends in a high percentage of *L. monocytogenes* isolates obtained from different sources. The idea of Lmo1413 playing a role as a new auxiliary invasin was also sustained by assays revealing that purified Lmo1413 binds to mucin via its MucBP domains. Taken together, these data indicate that Lmo1413, which we rename LmiA, for *Listeria*-mucin-binding invasin-A, may promote interaction of bacteria with adhesive host protective components and, in this manner, facilitate bacterial entry.

Introduction

The *Listeria* genus comprises Gram-positive bacteria with ubiquitous distribution in nature including soil, vegetables, fruit, and dairy food products (Hain et al., 2006). Among these bacteria, the foodborne pathogen *Listeria monocytogenes* is of outmost clinical relevance due to its capacity to cause serious and often fatal systemic diseases in humans. The fatality rate of human listeriosis is estimated in 30%, despite of antibiotic treatment. The difficulty in eradicating *L. monocytogenes* is mostly due to its ability to cross host defence barriers such as the intestine, the placenta or the blood–brain barrier causing rapid dissemination of the bacteria to deep organs (Swaminathan and Gerner-Smidt, 2007). These features are shared by *Listeria* pathogenic species that infect livestock (Vazquez-Boland et al., 2001).

L. monocytogenes is an invasive bacterium that enters, survives and multiplies inside phagocytic and non-phagocytic cells (Cossart and Toledo-Arana, 2008; Stavru et al., 2011). Once inside the host cell, intracellular bacteria hijacks cytoskeleton components to move in the cytosol and to spread from cell to cell escaping humoral immune responses (Stavru et al., 2011). Entry of *L. monocytogenes* into non-phagocytic cells follows a 'zipper-like' mode mainly orchestrated by two surface proteins, internalin A (InlA) and internalin B (InlB). These two invasins engage as host receptors the adhesion molecule E-cadherin and the hepatocyte growth factor receptor Met, respectively (Bonazzi et al., 2009). Other *L. monocytogenes* surface proteins such as Auto, Vip, LapB, ActA, and the lipoprotein LpeA promote entry of the pathogen into

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non-phagocytic cells (Cabanes et al., 2004, 2005; Reglier-Poupet et al., 2003; Reis et al., 2010). Recently, it has been reported that host membrane perforation by listeriolysin O can also trigger *L. monocytogenes* uptake (Vadia et al., 2011).

A feature shared by all *Listeria* species is the high content of genes (>40) encoding surface proteins covalently anchored to the peptidoglycan (Bierne and Cossart, 2007; Mariscotti et al., 2012). In *Listeria*, these proteins are in two groups differing in their C-terminal sorting motifs, LPXTG and NAKTN/NPKSS, which are recognized by sortase A (SrtA) and sortase B (SrtB), respectively (Bierne et al., 2004, 2002; Mariscotti et al., 2009). In the first *L. monocytogenes* genome completely sequenced, that of the strain EGD-e, a total of 41 genes encoding LPXTG proteins and two genes encoding SrtB substrates were found (Bierne and Cossart, 2007; Cabanes et al., 2002). Some of these 41 LPXTG proteins are associated to virulence, as the aforementioned invasins InlA, Vip and LapB (Camejo et al., 2011). Further genome comparisons have revealed that about one-third of the arsenal of LPXTG proteins carried by every pathogenic *Listeria* species is absent in non-pathogenic *Listeria* species (Doumith et al., 2004; Hain et al., 2012). The biological function of many of these LPXTG surface proteins remains undefined.

To unravel the putative contribution to virulence of new *L. monocytogenes* LPXTG proteins, we focused in Lmo1413 and Lmo2085, two LPXTG surface proteins absent in non-pathogenic *Listeria* that were identified by proteomics in the cell wall of the EGD-e strain growing in laboratory medium (Calvo et al., 2005; Pucciarelli et al., 2005). Proteomics has also revealed that Lmo1413 is produced by intracellular *L. monocytogenes* whereas Lmo2085 is not detected in this infection condition (García-del Portillo et al., 2011). Lmo1413 and Lmo2085 are predicted to contain MucBP and CnaB domains, respectively (Bierne and Cossart, 2007). Surface proteins containing MucBP domains are present in some Gram-positive bacteria (Juge, 2012) and facilitate adhesion to the host cell via interactions with the mucus secreted by epithelial cells (McGuckin et al., 2011). Examples include the proteins MUB of *Lactobacillus reuteri*, InlJ of *L. monocytogenes*, and SP1932 of *Streptococcus pneumoniae* (Bumbaca et al., 2007; Juge, 2012; Roos and Jonsson, 2002; Sabet et al., 2008). CnaB-type domains similar to those found in Lmo2085 are present in collagen-binding surface proteins, pilins and other cell-surface proteins of Gram-positive bacteria that recognize host adhesive molecules (Kang and Baker, 2011).

In this study, we report that, unlike Lmo2085, Lmo1413 has the capacity to bind mucin in vitro and to promote entry into non-phagocytic cells of *L. innocua*, a non-invasive and non-pathogenic *Listeria* species. Likewise, Lmo1413 proved to be efficient in assisting the entry of a *L. monocytogenes* mutant defective in the actin-binding protein ActA.

Materials and methods

Bacterial strains, eukaryotic cell lines and growth conditions

The bacterial strains used are listed in Table 1. *L. monocytogenes* and *L. innocua* strains were grown at 37 °C in brain heart infusion (BHI) broth. The chemically defined minimal medium IMM (Phan-Thanh and Gormon, 1997) supplemented with 17.9 μM ferric citrate ($C_6H_5O_7Fe$), was also used. *Escherichia coli* strains were grown in Luria Bertani (LB) broth at 37 °C. Media were supplemented with erythromycin 1.5 μg/mL or ampicilin 100 μg/mL when appropriate. NRK-49F fibroblasts (ATCC CRL-1570) were propagated in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% foetal bovine serum (FBS). The epithelial cell lines JEG-3 (ATCC HTB-36), HeLa (ATCC CCL-2), and Vero (ATCC CCL-81) were grown in Eagle's Minimum Essential Medium (MEM) containing 4 mM L-glutamine and 10% FBS. For bacterial infection (see below), eukaryotic cells were cultured at a density of 2.5×10^5 cells/mL using 24-well plates.

Table 1
Bacterial strains used in this study.

Species and strain	Relevant genotype	Source or reference
L. monocytogenes		
EGD-e		Glaser et al. (2001)
MD2306	*ΔinlA*	Trinad Chakraborty
MD1672	*Δlmo2085*	This work
MD1679	*Δlmo1413*	This work
MD1695	*Δlmo1413* pP1-*lmo1413*	This work
MD1696	*Δlmo2085* pP1-*lmo2085*	This work
MD2929	*ΔinlA* pP1	This work
MD2930	*ΔinlA* pP1-*lmo1413*	This work
MD2931	*ΔinlA* pP1-*lmo2085*	This work
MD2932	*ΔinlB* pP1	This work
MD2933	*ΔinlB* pP1-*lmo1413*	This work
MD2934	*ΔinlB* pP1-*lmo2085*	This work
MD2935	*ΔactA* pP1	This work
MD2936	*ΔactA* pP1-*lmo1413*	This work
MD2937	*ΔactA* pP1-*lmo2085*	This work
MD2938	*ΔinlA Δlmo1413*	This work
MD2939	*ΔinlA Δlmo2085*	This work
PML1	Serotype 1/2b	Poultry farm (B. González-Zorn)
PML2	Serotype 1/2b	Poultry farm (B. González-Zorn)
PML3	Serotype 4b	Slaughterhouse (B. González-Zorn)
PML4	Serotype 1/2c	Slaughterhouse (B. González-Zorn)
PML5	Serotype 1/2c	Slaughterhouse (B. González-Zorn)
PML6	Serotype 1/2a	Slaughterhouse (B. González-Zorn)
PML7	Serotype 1/2b	Slaughterhouse (B. González-Zorn)
PML8	Serotype 1/2b	Slaughterhouse (B. González-Zorn)
PML9	Serotype 1/2c	Processed poultry products (B. González-Zorn)
PML10	Serotype 1/2a	Processed poultry products (B. González-Zorn)
PML11	Serotype 1/2a	Slaughterhouse (B. González-Zorn)
RyC1	Serotype 1/2a	Human clinical isolate (J.C. Pérez-Díaz)
L. innocua		
CLIP 11262		Glaser et al. (2001)
MD1239	pP1	This work
MD1693	pP1-*lmo1413*	This work
MD2913	pP1::*lmo1413ΔMucBP*	This work
MD2914	pP1::*MucBPΔlmo1413*	This work
MD1228	pP1-*lmo2085*	This work
E. coli		
BL21	F⁻ *dcm ompT hsdS gal* (*malB*⁺)	Lab stock
MD2704	pET22::*lmo0320*-6×His	Cabanes et al. (2005)
MD1697	pET22::*lmo1413*-6×His	This work
MD1691	pET22::*mucBPΔlmo1413*-6×His	This work
MD1622	pET22::*lmo1413*ΔmucBP-6×His	This work

Bacterial infection assays

Bacteria were grown overnight in either BHI or IMM media at 37 °C without aeration to reach an OD_{600} of ~0.9 to 1.0 or ~0.8, respectively. These bacteria were washed and diluted in tissue

culture medium and added to eukaryotic cells at a multiplicity of infection (MOI) of 10:1. Incubation with bacteria ranged from 30 min (JEG-3 cells) to 60 min (Vero, HeLa and NRK-49F cells). After extensive washing with phosphate-buffered saline (PBS), pH 7.4, cell-associated bacteria were enumerated by lysing of the cells in a solution containing PBS, pH 7.4, 1% TritonX-100, and 0.1% sodium dodecyl sulfate (SDS). These extracts were plated onto BHI plates and cell-associated bacteria calculated by colony counting. To determine invasion rates, eukaryotic cells were infected as above, washed three times with pre-warmed PBS buffer and, incubated in fresh culture medium containing 100 μg/mL gentamicin until 2 h post-infection. Infected cells were lysed to determine number of viable intracellular bacteria as described (García-del Portillo et al., 2011).

Generation of L. monocytogenes Δlmo1413 and Δlmo2085 deletion mutants

Fragments of ~500-bp DNA flanking the *lmo1413* and *lmo2085* genes were amplified by PCR using chromosomal DNA of *L. monocytogenes* strain EGD-e as template. Oligonucleotide primers used are listed in Table S1 of supplementary information. These ~500-bp fragments were digested and then ligated in the BamHI and EcoRI cloning sites of the thermo-sensitive plasmid pMAD (Arnaud et al., 2004). The Δ*lmo1413* and Δ*lmo2085* deletions were verified by PCR. For complementation purposes, *lmo1413* and *lmo2085* wild-type alleles were cloned in the pP1 plasmid (Dramsi et al., 1995) using primers listed in Table S1 and verified by sequencing. Relative expression of the transgene was estimated by semi-quantitative RT-PCR.

Purification of Lmo1413, Lmo1413ΔMucBP and MucBP variants

Fragments of the *lmo1413* gene encoding amino acid residues 30–439 (Lmo1413 variant), 30–132/342–439 (Lmo1413ΔMucBP variant) or 133–341 (MucBP variant), were PCR-amplified using primers incorporating NdeI and XhoI restriction sites (Table S1). PCR products were then cloned into the C-terminal six-histidine tag expression vector pET22b (Novagen). Recombinant proteins were expressed and purified by immobilized metal affinity chromatography as recommended by the manufacture (Clontech Laboratories, Inc.).

Binding of recombinant Lmo1413 protein variants to immobilized mucin

Type II mucin from porcine stomach (Sigma M2378) and bovine serum albumin (BSA) were dissolved in PBS buffer pH 7.4 at 100 μg/mL and immobilized in microtiter wells (Maxi-sorp plates, Nunc). The wells were then blocked for 2 h at room temperature with 0.2 mL of PBS containing 3% skim milk. Blocking solution was then discarded and 100 μL of purified protein at different concentrations was added. The plate was incubated for 1 h a room temperature (RT) and then washed four times with PBST (0.1% Tween 20 in PBS pH 7.4 buffer). Fifty microliters of the monoclonal anti-6×His antibody (1:1000 dilution in PBS-3% skim milk) were added. Plates were incubated for 1 h at RT, washed with PBST, and then incubated for 1 h at RT with secondary antibody anti-mouse peroxidase (1:1000) in PBS 3% milk. After washing, the conjugated enzyme was allowed to react with *o*-phenylendiamine dihydrochloride (Sigma) and absorbance at 490 nm was measured using a microplate reader (Bio-Rad).

Antibodies, cell wall extracts containing LPXTG proteins, western assays and immunofluorescence microscopy

Polyclonal rabbit anti-Lmo1413 and anti-Lmo2085 sera were obtained using full-length 6×His-tagged recombinant proteins. Mouse monoclonal anti-InlA (Mengaud et al., 1996a) was also used. Goat anti-mouse and goat anti-rabbit antibodies conjugated to horseradish peroxidase (Bio-Rad) were used as secondary antibodies. Proteins recognized by the antibodies were visualized by chemoluminescence using luciferin-luminol reagents. Cell wall extracts containing LPXTG surface proteins were obtained by mutanolysin treatment of intact bacteria as described (Jonquieres et al., 1999) with increased incubation of bacteria with the enzyme, 4 h (Mariscotti et al., 2012). In all experiments, bacteria were grown overnight in BHI broth in non-shaking conditions (final OD_{600} ~0.8 to 1.0). For immunofluorescence microscopy, infected cells were fixed and labelled with polyclonal rabbit anti-*Listeria* antiserum 839 (Bierne et al., 2002), 1 μg/mL phalloidin conjugated to Alexa-488 and goat anti-rabbit conjugated to Alexa-594, as described (Aiastui et al., 2010; Bierne et al., 2004).

Mouse infections

Six-week-old female BALB/c mice were injected with a sub-lethal amount of bacteria, ~10^4 colony-forming units (CFUs), previously grown overnight in BHI liquid broth. For oral infections, mice were challenged with ~5×10^9 bacteria. Liver and spleen were obtained aseptically at 24, 48 and 72 h after infection (four mice in each group) and CFUs counted by plating of the organ homogenates onto BHI agar plates. Statistical analyses were performed using the student *t* test. *P* values ≤0.05 were considered statistically different.

Statistical analysis

Data were analyzed with GraphPad Prism version 5.0 software (GraphPad Inc., San Diego, CA), using one-way analysis of variance (ANOVA) with Tukey's post-test. A *P* value lower than 0.05 was considered significant.

Results

Search for new virulence factors in the L. monocytogenes LPXTG protein family

Comparative genome analyses reveal that *L. monocytogenes* strain EGD-e contains twenty LPXTG surface proteins that are absent in the non-pathogenic species *L. innocua* (Buchrieser et al., 2003; Glaser et al., 2001). Of those with a function assigned, InlA, InlH, InlJ, InlK, Vip, Lmo2026 and LapB, all have been related to virulence [reviewed in (Bierne and Cossart, 2007; Camejo et al., 2011)]. We focused in new *L. monocytogenes* LPXTG proteins having no ortholog in *L. innocua*. To search for phenotypes, we selected LPXTG proteins produced in laboratory conditions. Two of these, Lmo1413 and Lmo2085, were identified by proteomics in the cell wall of *L. monocytogenes* EGD-e growing in BHI broth (Calvo et al., 2005; Pucciarelli et al., 2005). Lmo1413 is also present in the cell wall of intracellular *L. monocytogenes* upon infection of epithelial cells (García-del Portillo et al., 2011). Genome analysis shows that *lmo1413* is flanked by genes conserved in *L. monocytogenes* and *L. innocua* (Fig. 1A). Three genes, *lin1450-lin1451-lin1452*, replace *lmo1413* in the *L. innocua* genome (Fig. 1A). None of these genes, which encode putative proteins of unknown function, has ortholog in *L. monocytogenes*. This genomic region may therefore represent a hot spot for horizontal gene transfer. In the case of *lmo2085*, it has in the vicinity a tRNA-Leu-encoding gene (Fig. 1A). The *L. innocua* genome contains this tRNA-Leu gene and all flanking genes

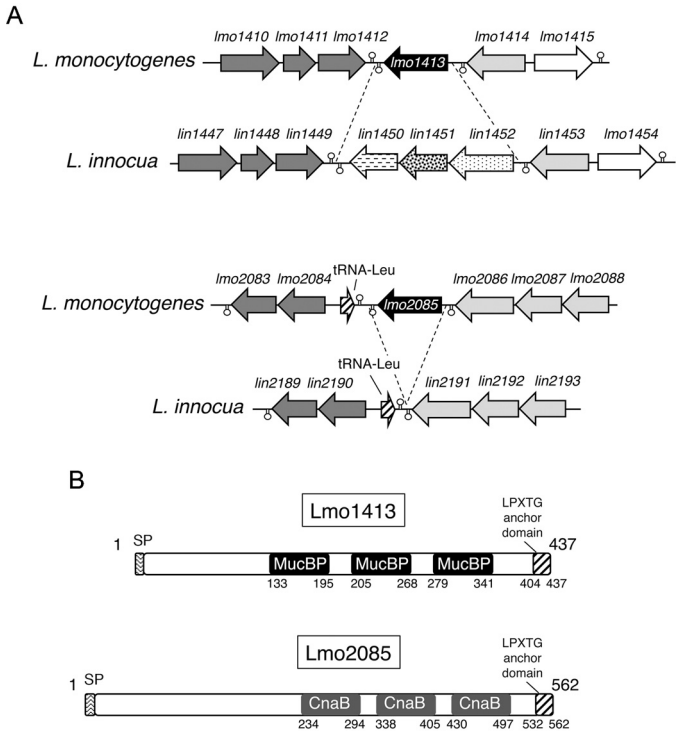

Fig. 1. *lmo1413 and lmo2085* are *L. monocytogenes*-specific genes that encode LPXTG surface proteins with MucBP and CnaB domains, respectively. (A) Comparison of the genomic regions of the *L. monocytogenes* EGD-e strain containing the *lmo1413* and *lmo2085* genes with the respective regions in the genome of the non-pathogenic *L. innocua* species. Note that in *L. innocua* the *lmo1413* is replaced by three genes having no ortholog in *L. monocytogenes* while *lmo2085* has no substitute in the *L. innocua* genome. Putative terminators are indicated. (B) Scheme of the domain architecture predicted for Lmo1413, consisting in three MucBP domains positioned in tandem followed by the C-terminal LPXTG anchoring domain; and, for Lmo2085, with three CnaB domains and the C-terminal LPXTG anchoring domain. SP, signal peptide.

but there is no gene substituting *lmo2085* (Fig. 1A). The Lmo1413 primary structure shows the presence of three domains conferring capacity of binding to mucin (MUCin-binding protein domain, MucBP, Pfam06458) (Fig. 1B) (Bierne and Cossart, 2007). MucBP domains are annotated in the PFAM database within the Pfam clan named 'Gram-pos_anchor', which includes other Pfam domains as 'C-term anchor' (Pfam13461) and 'Gram_pos_anchor' (Pfam00746). This latter domain contains the LPXTG sorting motif recognized by SrtA. This motif in Lmo1413 is a canonical LPKTG sequence with the rest of its 'Gram_pos_anchor' domain highly conserved respect Pfam00746 (E-value of 6.5e−07). In the case of Lmo2085, the protein harbours three CnaB domains followed by a Gram_pos_anchor domain with a canonical LPQTG sequence (Fig. 1B). CnaB-domains have been described in Gram-positive surface proteins interacting with collagen, in pili, and other surface proteins (Kang and Baker, 2011).

Lmo1413 and Lmo2085 are dispensable for L. monocytogenes to invade non-phagocytic cells using in vitro infection models

To test whether Lmo1413 and Lmo2085 contribute to *L. monocytogenes* invasion of cultured eukaryotic cells, we constructed defective isogenic mutants and the respective complemented strains. A Δ*inlA* mutant was also included as control. Prior to the infection assays, cell wall extracts were obtained from the different bacterial strains grown in BHI broth to estimate Lmo1413 and Lmo2085 relative levels (Fig. 2A). These assays revealed that the lack of Lmo1413 or Lmo2085 does not affect InlA levels and vice versa (Fig. 2A). The complemented strains produced and anchored to the cell wall large amounts of Lmo1413 and Lmo2085 (Fig. 2A). None of these strains exhibited noticeable changes in growth rate compared to the parental wild-type strain EGD-e (data not shown). Non-phagocytic eukaryotic cells such as JEG-3 human epithelial cells and NRK-49F rat fibroblasts were infected with these strains. Lack of InlA caused a ∼10 to 100-fold decrease in the rate at which *L. monocytogenes* invaded these cells (Fig. 2B). The effect was more pronounced in human epithelial cells than in rat fibroblasts, which may account for the weak recognition of murine E-cadherin by InlA (Lecuit et al., 1999; Mengaud et al., 1996b). Western assays revealed that E-cadherin is produced in JEG-3 epithelial cells and NRK-49F fibroblasts, although in the latter case it appeared mostly as processed forms (Fig. S1, supplemental material). Processing of E-cadherin by metallo-proteinases (MMPs) has been reported in pathophysiological processes (Covington et al., 2006). Of interest, NRK-49F fibroblasts are also known to produce MMPs in culture (Rankin et al., 2013). Unlike the phenotype exhibited by the *L. monocytogenes* Δ*inlA* mutant, no changes in invasion rate were observed for the Δ*lmo1413* and Δ*lmo2085* mutants or the complemented strains (Fig. 2B). These data indicated that neither Lmo1413 nor Lmo2085 are essential in vitro for *L. monocytogenes* invasion of non-phagocytic cell lines. In vivo experiments consisting in oral and intravenous challenge of BALB/c mice revealed that Δ*lmo1413* and Δ*lmo2085* mutants proliferate in liver and spleen at a similar extent as wild-type bacteria (Fig. 3). These later observations indicate that Lmo1413 and Lmo2085 are dispensable for *L. monocytogenes* to cross the intestinal epithelium or to colonize the liver and spleen.

Lmo1413, but not Lmo2085, promotes entry of L. innocua into non-phagocytic eukaryotic cells

Proteomics has revealed that *L. monocytogenes* produces many surface proteins bound to the peptidoglycan in laboratory media and the intracellular niche of eukaryotic cells (Calvo et al., 2005;

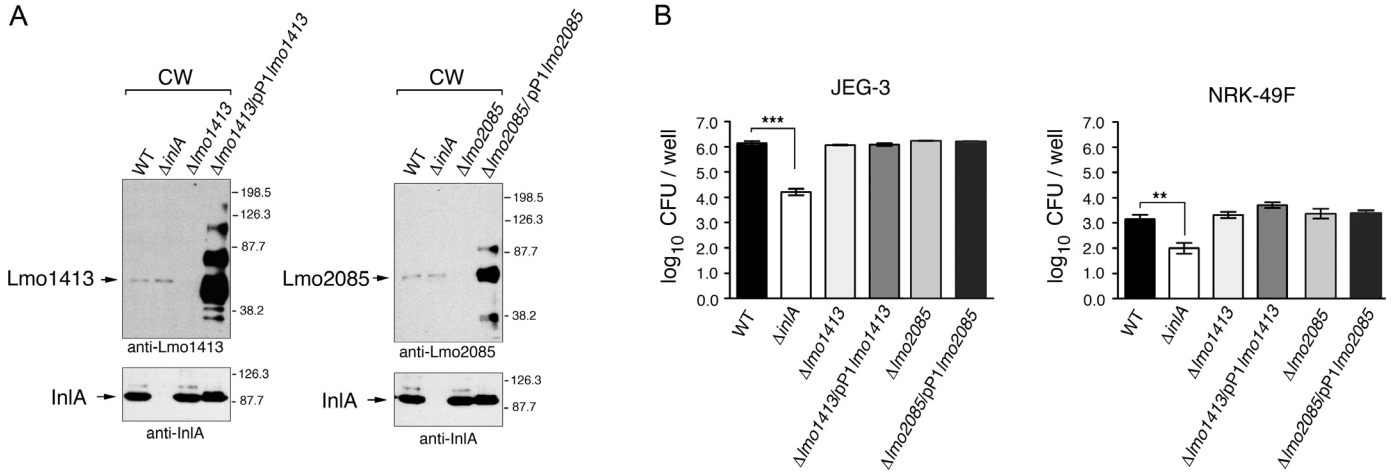

Fig. 2. Lmo1413 and Lmo2085 are dispensable for *L. monocytogenes* invasion of epithelial cells and fibroblasts. (A) Western assays revealing the presence/absence of Lmo1413 and Lmo2085 in cell wall extracts of *L. monocytogenes* wild-type, Δ*inlA*, Δ*lmo1413*, Δ*lmo2085* and the complemented strains Δ*lmo1413*/pP1-*lmo1413* and Δ*lmo2085*/pP1-*lmo2085*. Levels of internalin A (InlA) were also determined as control. (B) Invasion rate of the indicated *L. monocytogenes* strains monitored at 2 h post-infection in JEG-3 epithelial cells and NRK-49F fibroblasts. Note that bacterial entry in these cell lines does not require Lmo1413 or Lmo2085 and is dependent on the production of InlA. Shown are the means and standard deviation of three independent experiments. **, $P = 0.001$ to 0.01; ***, $P = 0.0001$ to 0.001 by one-way ANOVA with Tukey's post-test.

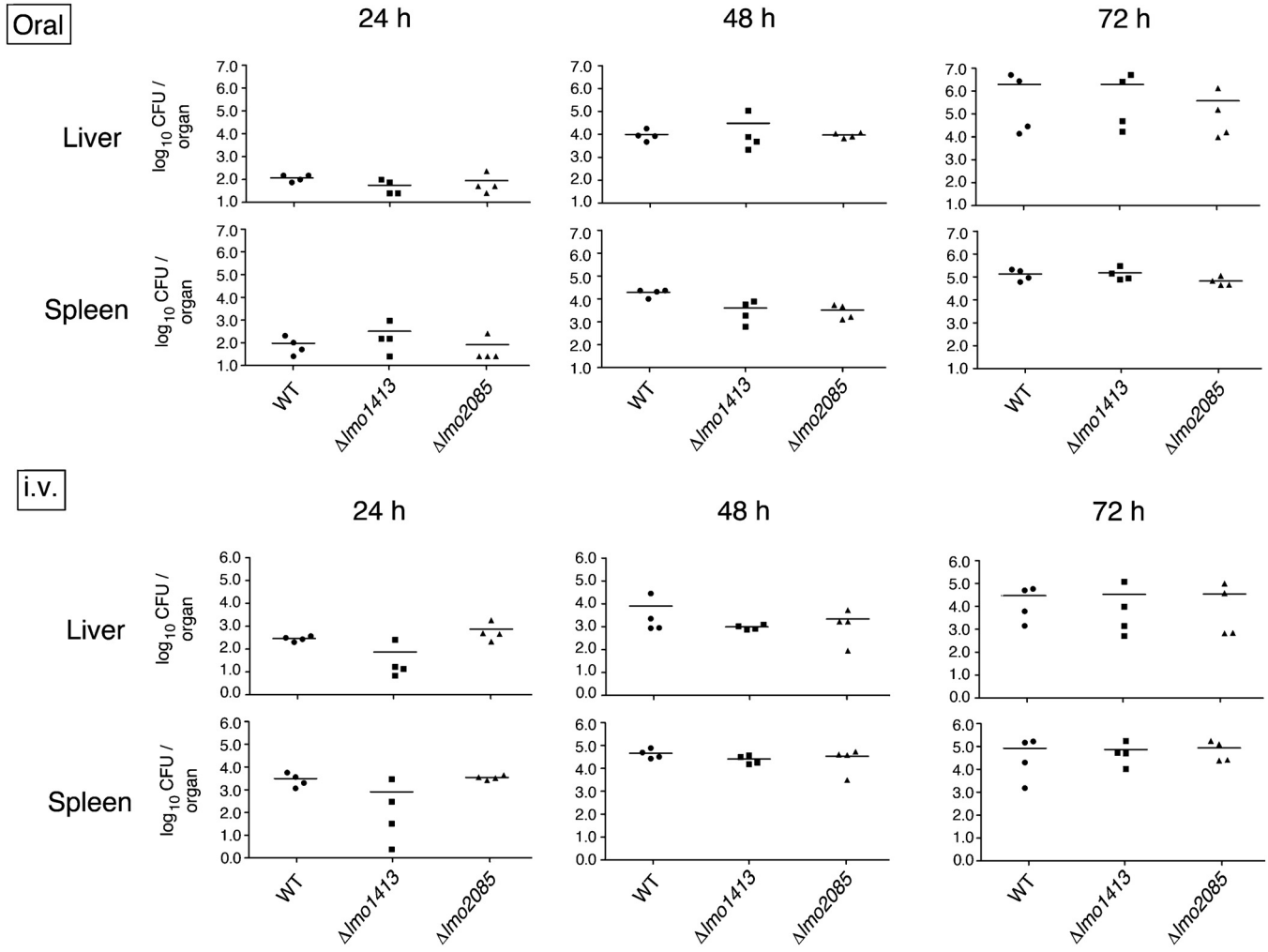

Fig. 3. Lmo1413 and Lmo2085 are not involved in colonization of liver and spleen of mice upon oral or intravenous administration of *L. monocytogenes*. Shown are the viable bacteria counted in these organs at different post-infection times (24, 48, and 72 h). No statistically significant differences were found between wild-type strain and the Δ*lmo1413* or Δ*lmo2085* mutants.

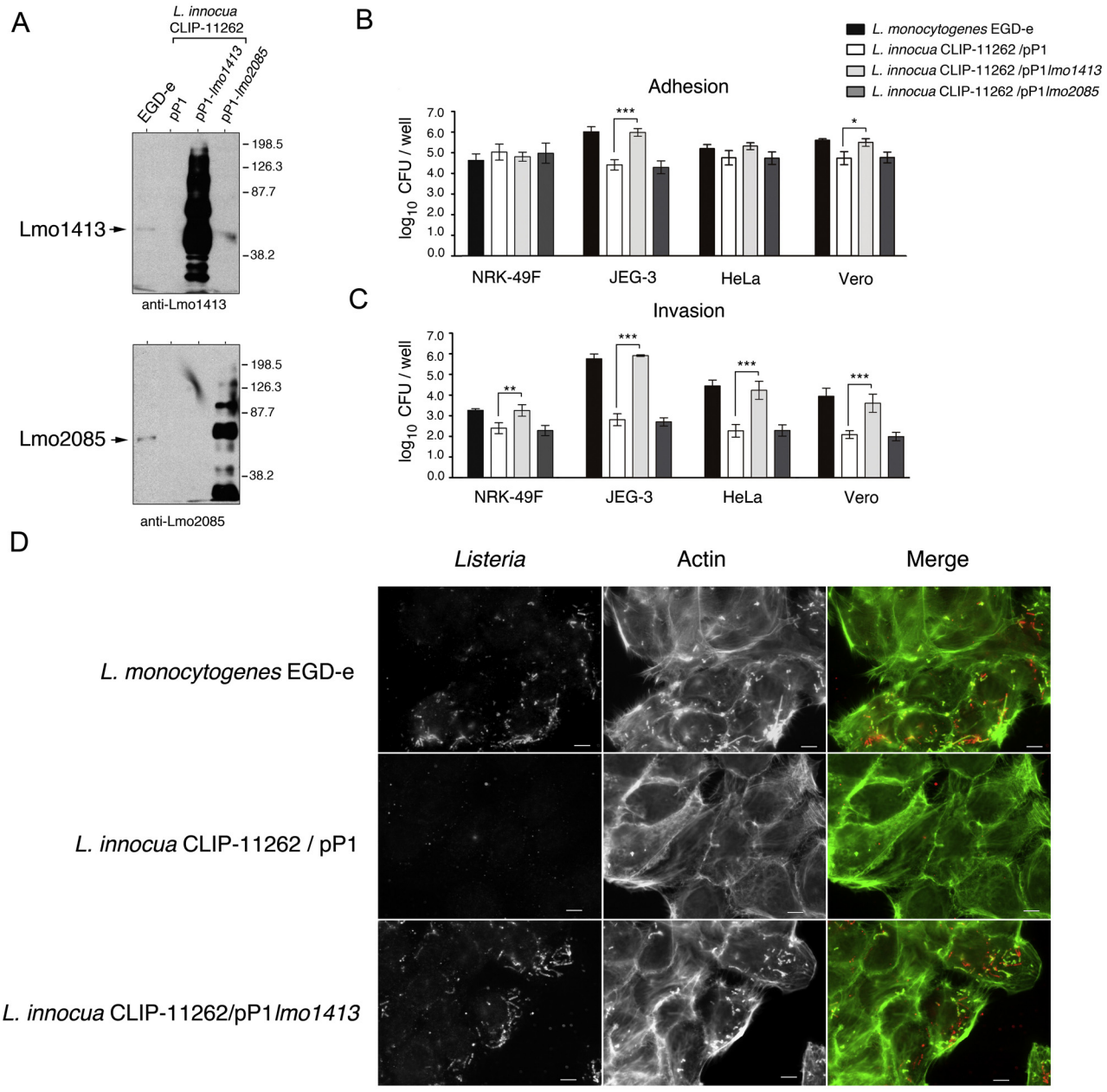

Fig. 4. Expression of Lmo1413, but not of Lmo2085, confers capacity to the non-invasive *L. innocua* to adhere and to enter into non-phagocytic cells. (A) Western assays performed with anti-Lmo1413 and anti-Lmo2085 antibodies showing the levels of these two proteins in cell wall extracts of *L. monocytogenes* wild-type strain EGD-e and in *L. innocua* wild-type strain CLIP11262 carrying the expression plasmids pP1-*lmo1413* or pP1-*lmo2085*; (B) adhesion rates of *L. monocytogenes* and *L. innocua* recombinant strains in different epithelial cells and fibroblasts. Note how expression of Lmo1413, but not Lmo2085, increases adherence of *L. innocua* up to the levels of *L. monocytogenes* wild-type EGD-e strain in JEG-3 and Vero cells; (C) invasion rates of *L. innocua* expressing Lmo1413 or Lmo2085 in distinct non-phagocytic cells; Shown in (B) and (C) are the means and standard deviation of three independent experiments. *, $P = 0.01$ to 0.05; **, $P = 0.001$ to 0.01; ***, $P = 0.0001$ to 0.001 by one-way ANOVA with Tukey's post-test; (D) microscopy analysis of JEG-3 cells infected with the indicated strains. Infected cells were fixed at 6 h post-infection. Intracellular bacteria and actin were labelled with rabbit anti-*Listeria* antiserum 839 (Bierne et al., 2002) recognized by secondary anti-rabbit Alexa-594 and phalloidin conjugated to Alexa-488, respectively. Bar: 10 μm.

García-del Portillo et al., 2011; García-del Portillo and Pucciarelli, 2012; Pucciarelli et al., 2005). Some of these surface proteins include the invasins InlA, InlB, and ActA. Taking in account these observations, we hypothesized that a putative role of Lmo1413 or Lmo2085 as invasins could be masked in wild-type *L. monocytogenes* by other 'major' invasins. We then expressed these two LPXTG proteins in *L. innocua*. Western assays showed that, as *L. monocytogenes*, the recombinant *L. innocua* strains produced Lmo1413 and Lmo2085 and anchored large amounts of these proteins to the peptidoglycan (Fig. 4A). Expression of Lmo1413, but not of Lmo2085, increased significantly the adherence of *L. innocua* to some non-phagocytic cell lines, such as JEG-3 and Vero (Fig. 4B). No such enhanced adhesion was observed in NRK-49F fibroblasts or HeLa epithelial cells (Fig. 4B). Remarkably, the expression of Lmo1413 rendered *L. innocua* highly invasive in all non-phagocytic cell lines tested (Fig. 4C). Increases of up to ~1000-fold in the invasion rate were registered in JEG-3 cells for the *L. innocua* strain producing Lmo1413 compared to the strain bearing the empty vector (Fig. 4C). Interestingly, Lmo1413 overexpression in *L. innocua* allowed this non-pathogenic bacterium to reach invasion rates close to those exhibited by *L. monocytogenes* EGD-e (Fig. 4C). Similar effects have been reported for *L. innocua* expressing InlA or InlB (Braun et al., 1999; Gaillard et al., 1991) Noteworthy, the *L. innocua* strain overproducing Lmo2085 did not exhibit increased invasiveness in any of

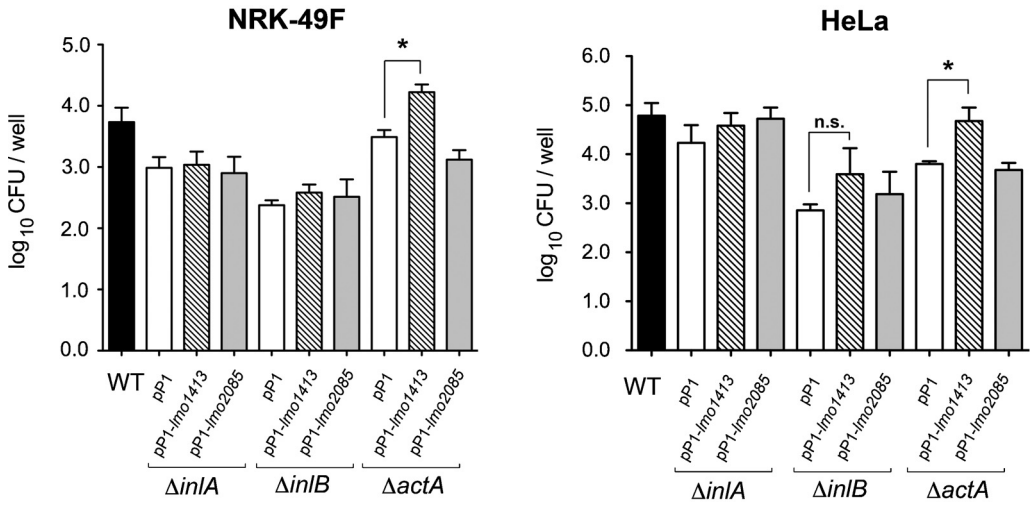

Fig. 5. Expression of Lmo1413 confers invasiveness to an *L. monocytogenes* Δ*actA* mutant in epithelial cells and fibroblasts. Shown are the invasion rates obtained upon infection of the indicated non-phagocytic cell lines, NRK-49F fibroblasts and HeLa epithelial cells. Infection conditions were as described (García-del Portillo et al., 2011). Data represent the means and standard deviation of three independent experiments. *, $P = 0.01$ to 0.05; n.s, not significant by one-way ANOVA with Tukey's post-test.

the experiments (Fig. 4B and C). Microscopy analyses confirmed the ability of *L. innocua* expressing Lmo1413 to invade non-phagocytic cells (Fig. 4D). Altogether, these data revealed that Lmo1413 is capable of promoting bacterial adhesion and invasion.

Lmo1413 promotes invasiveness of a L. monocytogenes mutant lacking the actin-binding protein ActA

Although *L. monocytogenes* strains lacking Lmo1413 or Lmo2085 displayed no defect for invasion in vitro (Fig. 2), the data obtained in *L. innocua* prompted us to assess whether any of these two LPXTG proteins could exert an auxiliary role in *L. monocytogenes* entry. Such hypothetical function could be visualized at a better extent in the absence of major invasins, such as InlA and InlB (Bierne and Cossart, 2007). Attempts to generate double mutants lacking simultaneously InlB and either Lmo1413 or Lmo2085, were unsuccessful (data not shown). However, we were able to construct the double mutants Δ*inlA* Δ*lmo1413* and Δ*inlA* Δ*lmo2085*. These double mutants behaved as the Δ*inlA* mutant when tested in invasion assays with non-phagocytic cell lines (Fig. S2, supplemental material). These data ruled out a *L. monocytogenes* invasion pathway strictly dependent on either Lmo1413 or Lmo2085. Similarly to the experiments performed in *L. innocua* (Fig. 4), we asked whether overproduction of either Lmo1413 or Lmo0285 could restore invasiveness of *L. monocytogenes* mutants lacking invasins as InlA, InlB, or the actin-binding protein ActA. Others and we have reported that ActA is required for efficient *L. monocytogenes* entry into certain cell lines (García-del Portillo et al., 2011; Suarez et al., 2001). Under the overexpression conditions used, Lmo1413, but not Lmo2085, restored invasiveness of Δ*actA* mutants in both epithelial and fibroblast cell lines (Fig. 5). No such effect was observed for Δ*inlA* and Δ*inlB* mutants overproducing either Lmo1413 or Lmo2085 (Fig. 5). These observations provided additional support for the potential of Lmo1413 in assisting *L. monocytogenes* entry into non-phagocytic cells.

Expression profiles of Lmo1413 and InlA in natural isolates of L. monocytogenes

The in vitro infection assays shed some light on the potential role of Lmo1413 as invasin promoting *L. monocytogenes* entry into host cells. We hypothesized that this role of Lmo1413 as invasin could also be inferred if differences in its sequence were observed in natural isolates of *L. monocytogenes*. Polymorphisms affecting the virulence of *L. monocytogenes* have been reported for other major invasins, especially InlA (Van Stelten and Nightingale, 2008). Many of these polymorphisms consist in mutations causing premature stop codons, shown in some cases to be responsible for loss of anchorage of InlA to the peptidoglycan and virulence attenuation (Nightingale et al., 2008). Interestingly, premature stop codons in *inlA* are more frequent in food isolates (about 30%) than in human clinical strains (<2%) (Jacquet et al., 2004; Nightingale et al., 2005). Considering these previous findings, we monitored the production of Lmo1413 and InlA in a series of twelve *L. monocytogenes* isolates belonging to diverse serotypes and obtained from different sources such as farms, food products, slaughterhouses and cases of human listeriosis (Table 1). Strikingly, western blot analyses of cell wall extracts revealed that the six isolates that produced full-length InlA had an unstable Lmo1413 protein that seemed to undergo partial proteolysis (Fig. 6A). A few exceptions were the human clinical isolate RyC1, the isolate PML11 (collected from a slaughterhouse), and the reference strain EGD-e (Fig. 6A). Conversely, most isolates producing a stable full-length Lmo1413 did not have InlA anchored to the peptidoglycan (Fig. 6A). Such opposite trend, exhibited by nine of the 12 isolates examined, suggests that the simultaneous production of Lmo1413 and InlA could be counter-selected by *L. monocytogenes* when adapting to environmental niches distinct from the human host. Intriguingly, sequencing of the *lmo1413* and *inlA* alleles harboured by these isolates confirmed the presence of premature stop codons in *inlA* in those isolates in which no InlA protein was detected in the cell wall extracts. These isolates associated with specific alleles in the Lmo1413 protein sequence (Fig. 6B and C), but remain non-invasive (Fig. 6D). This latter finding discards the idea of Lmo1413 as invasin that could functionally replace InlA in particular isolates. On the other hand, like InlA, Lmo1413 displays polymorphism and the two alleles detected in our strain collection match with different InlA sequences (Fig. 6B and C). Such association supports a certain degree of coevolution between these two surface proteins.

Adhesion of Lmo1413 to mucin

In silico analyses show that Lmo1413 is one of the twelve *L. monocytogenes* LPXTG proteins containing MucBP domains (Bierne and Cossart, 2007). Unlike Lmo1413, which carries exclusively MucBP domains, other LPXTG proteins contain in addition

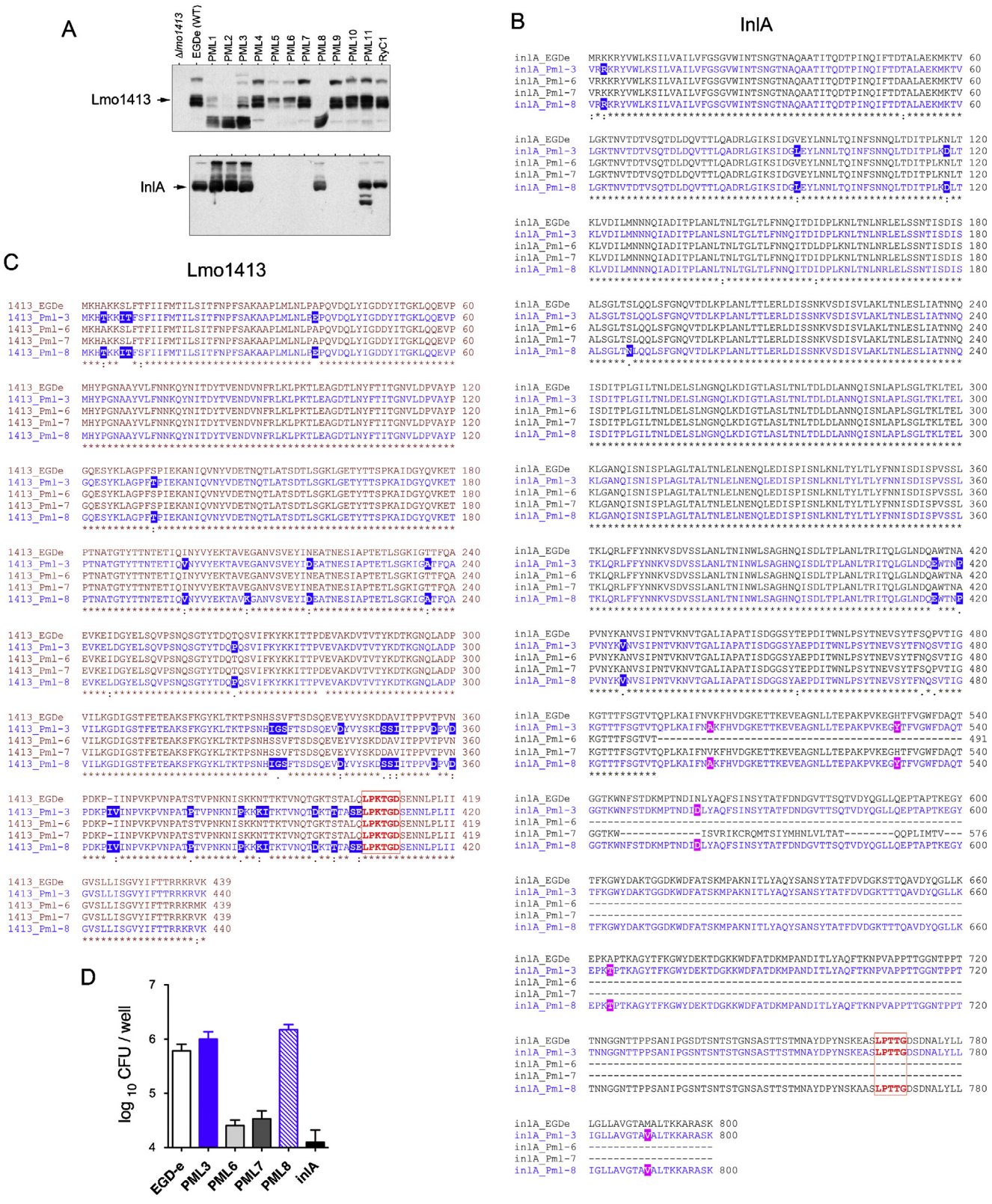

Fig. 6. Distinct expression profile of Lmo1413 and InlA in natural isolates of *L. monocytogenes* obtained from diverse sources. (A) Western assays with anti-Lmo1413 and anti-InlA antibodies used in cell wall extracts obtained from the indicated natural isolates of *L. monocytogenes*. The exact genotype and sources are indicated in Table 1. Note the opposite trends in production of these two surface proteins exhibited by most of the isolates. (B and C) protein sequences corresponding to Lmo1413 and InlA in isolates PML3, PML6, PML7 and PML8. Note the presence of premature stop codons in the InlA sequence that correlate with the production of a particular Lmo1413 allele. Highlighted in blue are those residues that display polymorphism among isolates and in red the LPXTG motifs. (D) Invasion assays in JEG-3 human epithelial cells of the indicated *L. monocytogenes* strains. (For interpretation of the references to color in this legend, the reader is referred to the web version of the article.)

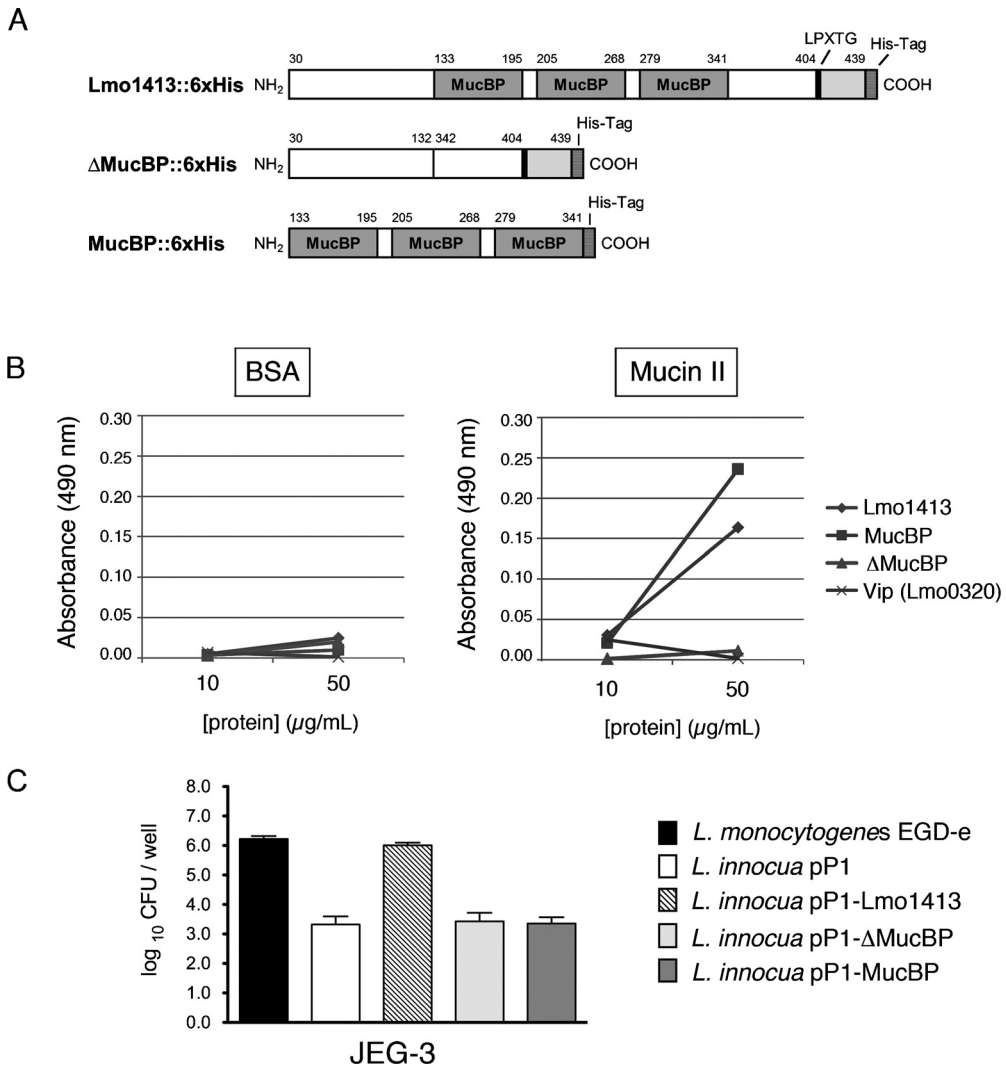

Fig. 7. The predicted MucBP domains of Lmo1413 promote binding to mucin type II. (A) 6×His-epitope tagged variants of Lmo1413 constructed for purification purposes and further used in a series of in vitro binding assays to immobilized mucin type II; (B) ELISA-assays in which plates were coated with two different proteins (BSA or mucin type II) at 100 μg/mL. Coated plates were then incubated with purified full-length and truncated Lmo1413 variants and with Vip (Lmo0320) as control of an LPXTG protein binding to a different host protein (Gp96) (Cabanes et al., 2005). Note that only full-length Lmo1413 and the truncated form carrying the MucBP domains exhibited capacity of interacting with mucin type II. (C) Invasion assays in JEG-3 epithelial cells of *L. innocua* strains expressing full length Lmo1413 or the indicated truncated variants. Data represent the means and standard deviation of three independent experiments. No significant differences were found between *L. innocua* strains carrying the empty vector and those expressing the truncated variants.

immunoglobulin-like (IR) domains and leucine-rich repeats (LRR), this latter a common feature of the 'internalin group' of proteins (Bierne and Cossart, 2007; Cabanes et al., 2002). The adhesin InlJ, which has a domain configuration of 15 LRR repeats followed by one IR domain and four MucBP domains, typifies this second type of multi-modular proteins (Bierne and Cossart, 2007). LRR domains have also been reported to bind mucin (Linden et al., 2008).

Lmo1413 lacks any recognizable LRR domain, so we sought to determine whether the MucBP domains present in this protein could bind to mucin. We generated recombinant Lmo1413-6×His-tagged variants carrying exclusively the three MucBP domains (variant "MucBP") or the remaining part of the protein (variant "ΔMucBP") (Fig. 7A). In vitro binding assays revealed that the MucBP variant exhibited even higher capacity than the full-length Lmo1413 protein for binding to mucin type II, the major component of intestinal mucus (Fig. 7B). Interestingly, the ΔMucBP construct lost completely its ability for binding to mucin (Fig. 7B). Controls providing specificity for these binding reactions included BSA and another LPXTG protein, Vip (Fig. 7B). Overall, these data supported the idea that MucBP domains of Lmo1413 could mediate binding of this LPXTG surface protein to mucin. To address whether this mucin-binding capacity of Lmo1413 was related to the capacity of this protein to confer invasiveness, we expressed in *L. innocua* the MucBP and ΔMucBP truncated versions but containing the native signal peptide and the LPXTG anchoring motif required for cell wall anchoring. Unlike full-length Lmo1413, none of these two truncated variants increased the invasion rate of *L. innocua* in JEG-3 or NRK-49F cells (Fig. 7C). Globally, these data demonstrate the capacity of Lmo1413 to bind mucin II, which depends on the MucBP domains, and indicate that these domains are required but not sufficient to promote bacterial internalization into host cells.

Discussion

In this work we explored the function of two *L. monocytogenes*-specific LPXTG proteins, Lmo1413 and Lmo2085, produced and anchored to the peptidoglycan by this pathogen in microbiological media (Pucciarelli et al., 2005) and, for the case of Lmo1413, also inside host cells (García-del Portillo et al., 2011). In vitro infection assays revealed that neither Lmo1413 nor Lmo2085 are essential

for the entry of wild-type *L. monocytogenes* into cultured non-phagocytic cells. These data initially discarded these two surface proteins as *bona fide* invasins, as reported for other surface proteins such as Vip, LapB, ActA, Auto, and LepA (Camejo et al., 2011). We however found that *L. innocua* expressing Lmo1413 renders this normally non-invasive bacterium as highly efficient for entering into non-phagocytic cells (Fig. 4). This effect was not seen with *L. innocua* having Lmo2085 anchored to the peptidoglycan. It is generally accepted that a protein able to make non-pathogenic bacteria invasive for non-phagocytic cells should be considered as 'invasin'. Such conclusion has been reached in studies that document in normally non-invasive bacteria an increase in invasion rates upon InlA (Gaillard et al., 1991; Innocentin et al., 2009) or InlB (Braun et al., 1999; Muller et al., 1998) expression. Heterologous expression of the adhesin InlJ in *L. innocua* also increases bacterial adherence (Sabet et al., 2008). Based on the data obtained with Lmo1413, we considered the possibility of this LPXTG protein acting as an accessory invasin in *L. monocytogenes* entry, a role that could be masked in vitro by the presence of other 'major' invasins such as InlA and InlB. In addition, we cannot rule out that Lmo1413 could be strictly required for invasion of concrete non-phagocytic cell lines or cell types distinct of those used in this study. Such preference of *L. monocytogenes* for using distinct invasins to trigger uptake by varied cell types as hepatocytes, fibroblasts, epithelial or endothelial cells, has been demonstrated for InlA, InlB and Vip (Cabanes et al., 2005; Gaillard et al., 1996; García-del Portillo et al., 2011; Parida et al., 1998). Nonetheless, the fact that Lmo1413 overexpression in *L. innocua* resulted in gain of invasiveness favours the idea that its putative receptor is present in the cell lines used in our study. In this scenario, the uptake mechanism directed by Lmo1413 might not be as efficient as the promoted by InlA or InlB with their respective receptors (Bonazzi et al., 2009). Intriguingly, Lmo1413 is able of restoring the invasion capacity of a Δ*actA* mutant (Fig. 5). The defect in invasion exhibited by the Δ*actA* mutant in certain cell lines is equivalent to the defect displayed by mutants lacking either InlA or InlB. An example is the NRK-49F fibroblasts, in which Δ*actA*, Δ*inlA* and Δ*inlB* mutants all behave as non-invasive (Fig. 5). These observations allow us to hypothesize a probable role of ActA in stabilizing protein interactions in which both InlA and InlB are involved and that may be required for bacterial entry into specific cell lines. Lmo1413 could also fulfil such hypothetical function in assisting 'major' invasins in the absence of ActA, at least under overexpression conditions. Such postulate would also be consistent with the lack of phenotypic complementation when Lmo1413 is overexpressed in Δ*inlA* and Δ*inlB* mutants.

Our studies directed to get insights into the host molecule(s) recognized by Lmo1413 proved that Lmo1413 regions with predicted MucBP domains bound efficiently to mucin type II. Based on this feature and the invasiveness exhibited by *L. innocua* expressing this LPXTG surface protein, we renamed it as LmiA, for Listeria-mucin-binding invasin-A. To our knowledge, our study represents the first report demonstrating that MucBP domains of a *L. monocytogenes* LPXTG protein bind to mucin, as it was previously shown for MUC domains in surface proteins of the commensal *Lactobacillus reuteri* (Boekhorst et al., 2006; Juge, 2012; Roos and Jonsson, 2002). Invasion assays performed with *L. innocua* expressing truncated versions of the Lmo1413 protein, either MucBP domains or the remaining part of the protein, were unsuccessful in terms of enhancing bacterial entry. These findings demonstrated that MucBP domains, although necessary for invasion, are not sufficient to promote this event. Other regions of the protein may be required in whole bacteria to stabilize MucBP–mucin interaction and/or to play additional roles leading to bacterial uptake. Our data, although provide clues on the type of host molecule probably recognized by LmiA, do not shed light on the exact host location where LmiA could interact with its receptor. A recent transcriptomic study reported that *lmo1413* expression is down-regulated in the mouse intestine (Toledo-Arana et al., 2009). Based on this, it is tempting to speculate on an extraintestinal function for LmiA (Lmo1413). Mucin type II is a glycoprotein secreted to the intestinal lumen (Kawashima, 2012) but all mammalian cells produce surface glycoproteins carrying carbohydrates structurally similar to those found in mucins (Marth and Grewal, 2008). These surface glycoproteins could also be potential targets of LmiA in deeper tissues. Further studies assessing the identity of the hypothetical host receptor engaged by LmiA will require purified protein and binding assays to distinct subcellular host extracts. We must also consider that among the 41 LPXTG proteins of the *L. monocytogenes* strain EGD-e (Glaser et al., 2001), twelve of them are predicted to contain from one up to fourteen MucBP domains (Bierne and Cossart, 2007; Cabanes et al., 2002). Of these proteins, some are in the 'internalin family' since contain LRR domains (Lmo2026, Lmo0732, Lmo0171, InlJ, Lmo2396, Lmo0327, InlI) while the remaining contain either MucBP domains only (Lmo0835, Lmo1413, and Lmo2179) or additional domains (Lmo0175, Lmo2178). A recent study showed that the *L. monocytogenes* internalins InlB, InlC and InlJ bind to mucin II, and such property was linked to the LRR domain (Linden et al., 2008). This was especially relevant in the case of InlJ (Sabet et al., 2005), harbouring both LRR and MucBP domains. Remarkably, when produced as a LRR-IR variant, the LRR domains of InlJ bind to mucin II in the absence of the rest of the protein (Linden et al., 2008). The same study did not report however analogous tests with the isolated MucBP domain of InlJ. Our findings with LmiA support a model involving MucBP domains as regions that could mediate bacterial interaction to the intestinal mucus without discarding the binding to mucin-like glycoproteins present on the surface of the infected cell. Direct binding of MucBP domains to the host cell surface is supported by a study showing that isolated MucBP domains of a *Lactobacillus plantarum* surface protein can block bacterial adhesion to Caco-2 epithelial cells (Du et al., 2010). The same work also reported the capacity of these isolated MucBP domains for binding human mucus (Du et al., 2010).

In summary, with the available information we can only speculate about the function of LmiA in vivo. The fact that the simultaneous production of full-length LmiA and InlA was not favoured in a high percentage of the *L. monocytogenes* strains isolated from varied sources point out to probable co-evolution between these two surface proteins. Our virulence assays discarded a prominent role of LmiA in bacterial passage of the intestinal epithelium and colonization of spleen and liver, but further studies are required to investigate whether other potential sites of action such as the placenta and/or the brain–blood barrier, may exist. It would be also of interest to test whether this *L. monocytogenes*-specific LPXTG protein could promote residence of the bacteria in the intestinal lumen by enhancing the attachment of the bacteria to the mucus. Such a role could promote *L. monocytogenes* residence in the human intestine and favour asymptomatic carriage, a phenomenon reported in several outbreaks (Dalton et al., 1997). Nonetheless, *L. monocytogenes* is known to exhibit lower adhesive rate to human mucus than other bacterial enteric pathogens such as *E. coli*, *Salmonella enterica*, *Enterobacter sakazakii*, or *Clostridium difficile* (Collado et al., 2005). These considerations should also take in account that besides LmiA, *L. monocytogenes* has the potential of producing simultaneously many other LPXTG proteins carrying MucBP domains, whose function is still unknown.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijmm.2014.01.003.

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