Capacity of ivanolysin O to replace listeriolysin O in phagosomal escape and in vivo survival of Listeria monocytogenes

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Listeriolysin O (LLO, hly-encoded) is a major virulence factor secreted by the pathogen Listeria monocytogenes. The amino acid sequence of LLO shows a high degree of similarity with that of ivanolysin O (ILO), the cytolysin secreted by the ruminant pathogen Listeria ivanovii. Here, it was tested whether ILO could functionally replace LLO by expressing the gene encoding ILO under the control of the hly promoter, in an hly-deleted strain of L. monocytogenes. It is shown that ILO allows efficient phagosomal escape of L. monocytogenes in both macrophages and hepatocytes. Moreover, expression of ILO is not cytotoxic and promotes normal intracellular multiplication. In vivo, the ILO-expressing strain can multiply and persist for several days in the liver of infected mice but is unable to survive in the spleen. This work underscores the key role played by the cytolysin in the virulence of pathogenic Listeria.

INTRODUCTION

Listeria monocytogenes and Listeria ivanovii are the only two pathogenic species of the genus Listeria (Seeliger & Jones, 1986). Although L. monocytogenes and L. ivanovii have common virulence properties, both surviving in macrophages and non-professional phagocytes, they differ in their pathogenicity (reviewed by Vazquez-Boland et al., 2001). L. monocytogenes is responsible for sporadic severe infections in humans and other animal species, causing meningitis, encephalitis, sepsis and abortion (Berche, 1995). By contrast, L. ivanovii affects almost exclusively ruminants, especially sheep, causing abortion, stillbirth and neonatal sepsis but not encephalitis (Gonzalez-Zorn et al., 1999, and references therein). The two pathogenic species of Listeria also behave differently in the mouse model (Mainou-Fowler et al., 1988). The LD50 of L. ivanovii for mice is 2–3 logs higher (10^6–10^7) than L. monocytogenes and strikingly, it can grow in the liver but not in the spleen, whereas L. monocytogenes proliferates equally well in both organs (Rocourt et al., 1983; Gaillard et al., 1986; Hof & Hefner, 1988; Engelbrecht et al., 1998).

Each step of the intracellular parasitism by L. monocytogenes and L. ivanovii is dependent upon the production of virulence factors (Goebel & Khun, 2000; Cossart & Bierne, 2001). The major virulence gene cluster (prfA–plcA–hly–mpl–actA–plcB) of L. monocytogenes is present, with the same genetic structure and at an identical position, in the chromosome of L. ivanovii (Gouin et al., 1994; Chakraborty et al., 2000). This gene cluster is organized around hly, which encodes a pore-forming cytolyisin: listeriolysin O (LLO) in L. monocytogenes and ivanolysin O (ILO) in L. ivanovii [see Bayley (1997) and Alouf (2000)]. The hly gene product plays a crucial role in the escape of bacteria from the phagosomal compartment. LLO-negative mutants of L. monocytogenes remain trapped in the vacuole, do not grow intracellularly and are avirulent in the mouse model of infection (Gaillard et al., 1986; Kathariou et al., 1987; Portnoy et al., 1988). L. ivanovii is unique among members of the genus Listeria in possessing, in addition to the two phospholipase genes (plcA and plcB), a gene named smcL, which encodes a sphingomyelinase (SmcL; Gonzalez-Zorn et al., 1999). This enzyme has been shown to participate in the disruption of the phagocytic vacuole and in subsequent intracellular proliferation of the pathogen.

LLO and ILO, which are highly similar at both the nucleotide and protein level, belong to a large family of cholesterol-dependent cytolyisins (Alouf, 2000). This family of toxins comprises, to date, 23 members from different Gram-positive genera, including perfringolysin (PFO) whose X-ray structure has been solved (Rossjohn et al., 1997), a cytolyisin secreted by the extracellular pathogen Clostridium perfringens. Despite structural and functional similarities between LLO and PFO (44% identity at the peptide level), Portnoy and co-workers (Jones & Portnoy, 1994) have previously shown that the pfo gene, cloned under
the control of the promoter phly, was unable to complement an hly mutation in L. monocytogenes for virulence in the mouse model of infection. The resulting strain was fully haemolytic and partially able to replicate in the mouse macrophage-like J774 cell line (PFO-mediated vacuolar escape at approx. 50% of the efficiency of LLO). However, expression of PFO damaged the host cell, preventing further intracellular proliferation of the bacteria, suggesting that LLO has evolved structural features specifically adapted to the intracellular life cycle of L. monocytogenes.

Here, we tested whether ILO could replace LLO for efficient phagosomal escape of L. monocytogenes, intracellular multiplication and ultimately, for virulence in the mouse model of infection. For this, we expressed the ilo structural gene in a Δhly derivative of L. monocytogenes. We also constructed an artificial operon encoding both the smcL and the ilo gene. The properties of the recombinant L. monocytogenes strains were studied in vitro and in vivo. The implications on the role(s) of LLO and ILO in bacterial virulence are discussed.

METHODS

Bacterial strains and culture conditions. EGDAhly is a derivative of EGD (serotype 1/2a), which contains an in-frame chromosomal deletion of 1080 bp in hly (Guzman et al., 1995). Bacteria were grown in brain–heart infusion (BHI) broth (Difco) at 37°C without antibiotics, except for the pAT28 transformed strains, which were grown in BHI broth containing 60 μg spectinomycin ml⁻¹.

Constructs. Chromosomal DNA, plasmid isolation, restriction enzyme analyses and amplification by PCR were performed according to standard protocols (Sambrook et al., 1989; Ausubel et al., 1990). All the constructs were carried on the shuttle vector pAT28 (Trieu-Cuot et al., 1990), and the recombinant plasmids were transferred into EGDAhly by electroporation as described by Park & Stewart (1990). EGDAhly expressing wild-type LLO under the same conditions was used as a positive control (Dubail et al., 2000; Lety et al., 2001); EGDAhly was used as a negative control.

The recombinant plasmid pAT28-phly-ilo is a derivative of plasmid pAT28-phly-pfo (Lety et al., 2001), which carries the pfo gene encoding PFO under the control of the promoter phly. Briefly, plasmid pD1868 (kindly provided by Dr D. A. Portnoy, Department of Molecular and Cell Biology, University of California, Berkeley, CA, 94720, USA; Jones & Portnoy, 1994) was digested with SalI and Xbal, and the 1·6 kb SalI-Xbal fragment comprising phly-pfo was subcloned into the SalI-Xbal sites of pAT28, yielding plasmid pAT28-phly-pfo.

ILO. The ilo gene was amplified by PCR using primers 5'-CG GGA TCC AGG AGA GTG AAA CCC ATG AAA AAA ATA ATG GCA CTA CTT TTA ATG ACA TTG TTA TGA CTG-3' and 5'-GG GAA TAT TCC TTA ATG ATT TAC AGT ATC ACT ACT-3'. Plasmid pAT28-ilo was constructed by substitution of the BamHI–EcoRI fragment carrying the pfo gene of pAT28-pfo by a BamHI–EcoRI fragment carrying the ilo gene (the BamHI and EcoRI sites are underlined).

The 15 bases preceding the ATG start codon of the ilo gene were designed to correspond to those preceding the hly gene and thus, comprise the Shine–Dalgarno motif of hly.

SmcL–ILO. Plasmid pAT28-phly-smcL-ilo was constructed by cloning a BglII–BglII fragment carrying smcL of L. ivanovii into the BamHI site of plasmid pAT28-phly-ilo in the same orientation as ilo (checked by PCR amplification with the appropriate pairs of primers). smcL was amplified by PCR using primers 5'-CG AGA TCT AGG AGA GTG AAA CCC ATG GAA AAA TTT AAA ATT ATA AAA ACA ATA CCC-3' and 5'-CG AGA TCT TTA GTC ATT ATC AGT GAA ACC AAC TAC AGG-3'. The 15 bases preceding the ATG start codon of smcL correspond to those preceding hly. A BglII site was created immediately upstream of the Shine–Dalgarno sequence; a second BglII site was created immediately downstream of the stop codon. We checked, by RT-PCR with appropriate primer pairs, that both the smcL and the ilo gene were transcribed in this construct (not shown).

Preparation of proteins and analyses. Proteins were prepared from supernatants of strain EGDAhly transformed with the different pAT28 derivatives. Concentrated culture supernatants from bacteria grown in Luria–Bertani rich medium were prepared essentially as described previously (Lety et al., 2001). Cell-free supernatants were filtered through a 0·22 μm pore-size Millipore filter. The filtered supernatants were concentrated by centrifugation through ultrafree Biomax units (cut-off 30 kDa). Cytolysins were identified by Western blot analysis with anti-pneumolysin mAb PLY-5, which was kindly provided by Dr De los Toyos, Area de Immunologia, Facultad de Medicina, Universidad de Oviedo, Spain (Jacobs et al., 1999). mAb PLY-5 was used at a final dilution of 1/200, as described previously (Lety et al., 2001). Identical volumes of each concentrated culture supernatant were loaded into each well.

Haemolysis. Haemolytic phenotypes were visualized by spreading bacteria onto horse- or sheep-blood agar plates containing Columbia base medium (BioMérieux). CAMP-like tests were performed on horse- and sheep-blood agar plates, as described by Ripio et al. (1995). Haemolytic activity of bacterial culture supernatants was measured by lysis of horse or sheep erythrocytes, as described by Jones & Portnoy (1994). The values corresponding to the reciprocal of the dilution of culture supernatant required to lyse 50% of horse erythrocytes were used to compare the haemolytic activities of the different supernatants.

Infection of mice and virulence assays. The virulence of strains was estimated by determining the LD₅₀ using the Probit method and by the bacterial survival in tissues. Specific pathogen-free, 6- to 8-week-old female Swiss mice (Janvier, Le Genest St Isle, France) were used. Bacteria were grown for 18 h in BHI broth, centrifuged, washed once, appropriately diluted in 0·15 M NaCl and then inoculated intravenously into the mice via the lateral tail vein (0·5 ml). Groups of five mice were challenged with various doses of bacteria (10⁶, 10⁵, 10⁴ or 10³ bacteria per mouse), and mortality was observed for 10 days.

Kinetics studies. Twenty mice were inoculated per mutant. At days 1, 2, 3 and 6 after inoculation, groups of five mice were killed and the organs (spleen and liver) were aseptically removed and separately homogenized in 0·15 M NaCl. Bacterial numbers in organ homogenates were determined at various intervals on BHI plates containing spectinomycin. Five-hundred microlitres of bacterial suspension were injected into each mouse (containing 5 x 10⁵ bacteria for EGDAhly expressing LLO, or 10⁶ bacteria for EGDAhly expressing ILO alone or co-expressing SmcL and ILO).

Assays were carried out on animals that had been pre-treated with spectinomycin (1 mg spectinomycin per mouse per day), in order to overcome in vivo instability of the recombinant plasmids (except for EGDAhly alone).

Culture of cell lines and microscopy analyses

Cell cultures and infections. Bone marrow (BM)-derived macrophages from BALB/c mice were cultured as described previously.
(De Chastellier & Berche, 1994) and then infected as follows. Overnight-grown bacteria were diluted in cell culture medium to give a bacterium-to-macrophage ratio of 10:1. Bacteria were allowed to adhere to cells by incubation on ice for 15 min, then to enter cells by placing the cells at 37°C for 15 min. After thorough washings, infected cells were re-fed with fresh medium.

Human hepatocellular carcinoma cell line HepG-2 (ATCC HB 8065) was obtained from S. Dramsi and P. Cossart (Institut Pasteur, Paris, France). HepG-2 cells were cultured using RPMI 1640 medium supplemented with 10 % FCS at 37°C under a 5 % CO₂ atmosphere seeded at 2 × 10⁴ cells per culture dish. Cells were infected 48–72 h after seeding with overnight-grown bacteria, at a ratio of 50:1 for 1 h at 37°C. As for macrophages, non-ingested bacteria were removed by washing, and infected cells were fed with fresh culture medium.

**RESULTS**

Expression of ILO by *L. monocytogenes*

The amino acid sequence of LLO shows a high degree of similarity to that of ILO, secreted by *L. ivanovii*, with 80 % identity. The *ilo* structural gene was expressed under the control of the *hly* promoter (Fig. 1a,b). We also constructed a strain co-expressing the *smcL*-encoded sphingomyelinase of *L. ivanovii* (Gonzalez-Zorn et al., 1999) and ILO (see Methods for details). All the constructs were carried on the Gram-positive/Gram-negative shuttle vector pAT28 (Trieu-Cuot et al., 1990), and the recombinant plasmids were transferred by electroporation into EGDΔ*hly*.

**Immunodetection and haemolytic activities.** Secretion of ILO in the culture supernatant of recipient strain EGDΔ*hly* was tested by Western blot, using mAb PLY-5, an mAb raised against the pneumolysin of *Streptococcus pneumoniae*, which specifically recognizes the conserved undecapeptide in the C-terminal portion of cytolsins. The ILO protein secreted by the strain expressing ILO alone was very well detected by mAb PLY-5 (Fig. 2a). We monitored quantitatively the amount of ILO secreted by the strain expressing ILO alone and compared it to that of the LLO-expressing strain, by SDS-PAGE followed by immunodetection and Coomassie blue staining (see Methods for details). As shown in Fig. 2(b), the amount of ILO produced was roughly comparable to that of LLO (approx. 57 %), as determined by densitometry scanning of the Western blot (using the NIH image software, version 1.61). This result was further confirmed by Coomassie blue staining of the culture supernatants (Fig. 2c). By contrast, in the strain co-expressing SmcL and ILO, the amount of ILO detected in the supernatant was only 7 % of that of the ILO-expressing strain. Thus, although the *smcL* and *ilo* genes were co-transcribed in this construct (determined by RT-PCR, not shown), the insertion of the *smcL* gene between *phly* and *ilo* significantly decreased the expression of ILO.

The EGDΔ*hly* derivatives showed a clear haemolytic activity.

**Table 1.** Properties of the LLO mutants in BM-derived macrophages

<table>
<thead>
<tr>
<th>Protein(s)*</th>
<th>Escape from phagosome† (%)</th>
<th>Positive cells‡ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 h</td>
<td>4 h</td>
</tr>
<tr>
<td>EGDΔ<em>hly</em> (control)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>LLO (wild-type)</td>
<td>59</td>
<td>90</td>
</tr>
<tr>
<td>ILO</td>
<td>61</td>
<td>87</td>
</tr>
<tr>
<td>SmcL + ILO</td>
<td>55</td>
<td>85</td>
</tr>
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*The constructs were expressed on pAT28 derivatives in EGDΔ*hly*.

†The percentage (%) of bacteria that had escaped from the vacuole was determined indirectly based upon the percentage of bacteria coated with actin filaments, as determined by phalloidin staining as described by Jones & Portnoy (1994).

‡The percentage (%) of positive cells corresponded to the cells that contained bacteria surrounded by polymerized actin (after 2 and 4 h of re-infection).
on horse-blood agar, confirming their production of active cytolysin (not shown). The haemolytic activities of culture supernatants were measured on horse erythrocytes, as described previously (Lety et al., 2001). The haemolytic activity of EGD\textit{Dhly} expressing ILO was about fivefold lower than that of EGD\textit{Dhly} expressing LLO (Fig. 2d). For comparison, with EGD\textit{Dhly} expressing PFO from the \textit{pfo} gene placed under \textit{phly} control on pAT28 (see Methods), the haemolytic activity recorded in the culture supernatant was about fourfold higher than that of EGD\textit{Dhly} expressing LLO (not shown).

On blood agar, \textit{L. ivanovii} differs from \textit{L. monocytogenes} in that it causes a bi-zonal haemolytic effect and when grown close to \textit{Rhodococcus equi}, gives rise to a characteristic shovel-shaped patch of synergistic haemolysis on sheep-blood agar – also called the 'CAMP-like' phenomenon – due to the expression of the sphingomyelinase (SmcL) encoded by \textit{smcL} (Gonzalez-Zorn et al., 1999). As shown in Fig. 2(e), a strong activity could be visualized on sheep-blood agar plates, demonstrating the production of SmcL by the \textit{L. monocytogenes} derivative carrying both the \textit{smcL} and the \textit{ilo} gene.

**Expression of ILO allows intracellular multiplication and restores in vivo survival**

The ability of the ILO-expressing strain to disrupt phagosomal membranes and to multiply in the cytosol of infected cells was evaluated both in BM-derived macrophages and in hepatocytes. Virulence was studied by measuring the ability of the strain to multiply and persist in the target organs of infected Swiss mice after intravenous inoculation. The \textit{in vitro} and \textit{in vivo} properties of the ILO-expressing strain were then compared to those of a strain co-expressing SmcL and ILO.

**In vitro properties.** The intracellular fate of EGD\textit{Dhly} expressing ILO was examined by confocal microscopy in BM-derived macrophages from BALB/c mice, after double staining with an anti-\textit{Listeria} antibody and with \textit{β}–phalloidin to visualize the F-actin (Gaillot et al., 2000). EGD\textit{Dhly} expressing LLO and EGD\textit{Dhly} alone were used as controls. We monitored quantitatively the capacity of the mutant strain to promote the disruption of the phagosomal membrane and to multiply intracellularly, by immunofluorescence microscopy. We calculated: (i) the percentage of phagosomal escape (i.e. the number of bacteria surrounded by polymerized actin/total number of bacteria in cells); (ii) the number of infected cells containing bacteria surrounded by polymerized actin; and (iii) the number of bacteria per infected cell (on a mean of 50–100 infected cells), at the different times of the infections.

As shown in Table 1 and Fig. 3, EGD\textit{Dhly} expressing ILO was able to efficiently disrupt the phagosomal membranes of infected macrophages. After 4 h post-infection, the percentage of bacteria surrounded by polymerized actin (reflecting the percentage of phagosomal escape) reached 87 %, a value...
very similar to that recorded with the strain expressing LLO. As in the case of the LLO-expressing strain, actin polymerization was visible in 100% of the cells (Table 1). As shown in Fig. 4, the intramacrophagic multiplication of the ILO-expressing strain was similar to that of the LLO-expressing strain, while EGDhly alone failed to multiply. Confirming the confocal analyses, electron microscopy analyses showed that, after 4 h infection, all the bacteria expressing ILO had multiplied and were visible inside the cytoplasm of infected BM-derived macrophages, surrounded by a mesh-work of polymerized actin (Fig. 5).

To test the possible cytotoxicity of ILO, we measured the release of a host cytosolic enzyme, LDH, into the culture medium of infected BM-derived macrophages. After 4 h infection with EGDhly expressing LLO, approximately 30% of LDH release was recorded. By contrast, less than 1% of LDH release was recorded in BM-derived macrophages infected with EGDhly alone, confirming the complete lack of cytotoxicity of the LLO-negative mutant of L. monocytogenes. These results are in agreement with earlier data which showed that murine BM-derived macrophages infected by L. monocytogenes die by necrosis without the characteristic hallmarks of apoptosis (Barsig & Kaufmann, 1997). LDH release is thus likely to reflect the cell lysis induced by bacterial multiplication. The LDH release recorded with EGDhly expressing ILO remained very low, at approximately only 10% after 4 h infection (one-third lower than with EGDhly expressing LLO), demonstrating the lack of cytotoxicity of ILO.

We next studied infection and intracellular multiplication of the ILO-expressing strain in the human hepatocellular carcinoma cell line HepG-2, by confocal microscopy.
Bacterial content was monitored at 2 and 4 h after infection. Confirming the behaviour in BM-derived macrophages, the ILO-expressing strain could disrupt the phagosomal membranes of hepatocytes and multiply intracellularly as efficiently as the LLO-expressing strain. At 4 h after infection, the percentage of bacteria surrounded by polymerized actin ranged between 78 and 82% and with both strains, actin polymerization was visible in most cells (Fig. 6a, b) and bacterial counts increased from approximately 5 bacteria per cell (at $T_0$) to approximately 25 bacteria per cell.

Thus, in phagocytic and non-phagocytic cells, ILO could replace LLO for efficient phagosomal escape and intracellular multiplication of L. monocytogenes.

**In vivo properties.** EGDΔhly is totally avirulent, and expression of plasmid-encoded LLO restores virulence to the strain (Dubail et al., 2000). EGDΔhly expressing ILO remained avirulent at a dose of $10^8$ bacteria per mouse (at a 10-fold higher dose, the strain became toxic and mice died with convulsions within 2 h of infection). The ability of EGDΔhly expressing ILO to multiply in the spleen and liver of infected mice was evaluated by infecting mice intravenously with the dose of $10^8$ bacteria per mouse (at which all the mice recovered from infection). As shown in Fig. 7(a), the kinetics of survival in the liver showed an increase of bacterial counts up to day 2 after infection, reaching $7.7 \times 10^6$ bacteria per liver. The bacterial counts remained comparable at day 3 (approx. $7 \times 10^6$ bacteria per liver); at day 6, a significant number of bacteria still survived in the infected livers ($1.8 \times 10^4$ bacteria per organ). By contrast, bacterial multiplication was drastically affected in the spleen. Bacterial counts dropped from $6.3 \times 10^5$ bacteria per spleen at day 1 after infection to only $2 \times 10^3$ at day 3, and bacteria were eliminated from...
the spleens by day 6 (\( <10^2 \) bacteria per spleen). Thus, at
day 3 after infection, the number of bacteria expressing
ILO was approximately 3500-fold higher in the liver
than that in the spleen. By contrast, confirming earlier
observations (Gaillard et al., 1986; Berche, 1995), with
the LLO-expressing strain the number of bacteria in the
spleen paralleled that in the liver (not shown).

Co-expression of SmcL and ILO. We observed that
EGD\( \Delta \)hly co-expressing SmcL and ILO was also able to effi-
ciently disrupt the phagosomal membranes of infected BM-
derived macrophages (Table 1). After 4 h post-infection,
85 % of the bacteria were surrounded by polymerized
actin and polymerization was visible in 100 % of the cells
(values similar to those recorded with the strain express-
ing ILO or LLO). In mice, EGD\( \Delta \)hly co-expressing SmcL
and ILO was also avirulent, and all the mice survived to a
challenge of \( 10^8 \) bacteria (at a dose of \( 10^9 \) the strain was
toxic). However, the kinetics of bacterial survival in the
spleen and liver were more drastically affected than with
the ILO-expressing strain (Fig. 7b). Bacteria were very
rapidly eliminated from the spleen (counts dropped from
8\( \cdot 9 \times 10^2 \) bacteria per spleen at day 1 to \( <10^2 \) by day 2).
In the liver, bacterial elimination was slower and bacteria
were still detected up to day 3 after infection (7\( \cdot 9 \times 10^3 \)
bacteria per organ). Thus, this strain behaved like an
LLO-negative strain (EGD\( \Delta \)hly; Dubail et al., 2000).

DISCUSSION
We have shown that ILO can functionally replace LLO
in vitro for efficient phagosomal escape of \( L. \) monocytogenes
and intracellular multiplication in BM-derived macro-
phages as well as in hepatocytes. In vivo, the ILO-expressing
strain can multiply and persist for several days in the liver of infected mice but is unable to multiply in the spleen.

**ILO can replace LLO for phagosomal escape and allows in vivo survival.** Expression of ILO promoted efficient disruption of the phagosomal membranes of BM-derived macrophages, and intracellular multiplication of *L. monocytogenes* expressing ILO was essentially indistinguishable from that of the LLO-expressing strain. Moreover, expression of ILO appeared to be poorly cytotoxic. These data are strikingly different from those observed when the extracellular cytolysin PFO was expressed by *L. monocytogenes* in place of LLO, which revealed a high cytotoxicity of the PFO protein (Jones & Portnoy, 1994). To test whether the observed lack of virulence was mainly due to the high cytotoxicity of PFO, Portnoy and co-workers developed an elegant genetic procedure to select variants that would support normal intracytosolic growth (Jones et al., 1996). In that study, all the non-cytotoxic PFO mutants selected, corresponding to single amino acid substitutions, were capable of mediating phagosomal escape and intracytoplasmic growth. However, they all remained avirulent in the mouse model. These data indicate that LLO possesses specific properties, and in particular a lack of cytotoxicity, which are important for the development of infection by *Listeria*.

In this respect, it is worth mentioning that a putative PEST sequence, suspected of controlling the intracytosolic half-life of ILO, has been identified close to the N terminus of the LLO protein (Decatur & Portnoy, 2000; Lety et al., 2001). The deletion of this motif did not affect secretion and haemolytic activity of LLO but it did abolish bacterial virulence. We have demonstrated (Lety et al., 2002) that the susceptibility of LLO to intracellular proteolytic degradation is not related to the presence of a high PEST score sequence. Furthermore, we have shown that single amino acid substitutions in the distal portion of the PEST motif are sufficient to impair phagosomal escape and to abolish the virulence of *L. monocytogenes*, unravelling the critical role of this region of LLO in the pathogenesis of *L. monocytogenes*. Although the peptide sequence of ILO is highly similar to that of LLO, this proximal region is less conserved than the rest of the protein and no PEST motif can be identified by the PEST-FIND program (http://www2.ibiopasteur.upmc.fr/seqanal/interfaces/pestdfind-simple.html).

Remarkably, the kinetics of survival of the ILO-expressing strain in infected mice revealed that the mutant strain could multiply in the liver up to 2 days after infection (reaching approx. $10^7$ bacteria per organ). The persistence of a significant number of ILO-expressing bacteria in the liver 6 days after infection demonstrated a greater resistance to host defence mechanisms than an LLO-negative mutant (Gaillard et al., 1986). By contrast, bacterial multiplication was drastically affected in the spleen and the mutant strain remained avirulent. The inability of *L. monocytogenes* expressing ILO to persist in the spleen could be due to an increased susceptibility to proteolytic degradation of the ILO molecule in spleen cells. Other explanations are also possible. For example, an efficient infection by an ILO-expressing *L. monocytogenes* strain may require a higher level of expression of ILO. Alternatively, since LLO was shown to contribute to cell-to-cell spread (Dancz et al., 2002), the avirulence of the ILO-expressing strain might be due to a defect of ILO which fails to disrupt efficiently the double vacuolar membrane entrapping the bacteria during their passage to adjacent cells. Interestingly, it has previously been shown (Conlan & North, 1994) that neutrophils are not as important in anti-listerial defence in the spleen as in the liver during the first days of the infectious process. Neutrophils fail to substantially restrict *L. monocytogenes* multiplication in the spleen because the cells in which the bacterium resides in this organ (essentially monocytes and macrophages) are less permissive for its growth than are hepatocytes; even in the absence of neutrophils, *L. monocytogenes* is not found in large numbers inside individual spleen cells.

As mentioned earlier, in the mouse model, *L. ivanovii* is able to colonize the liver but not the spleen. It is thus tempting to suggest that the capacity of *L. monocytogenes* to multiply in the spleen might be mediated, at least in part, by specific properties of the LLO molecule that ILO does not possess. Conversely, ILO may have developed specific properties that allow *L. ivanovii* to develop in the livers but not in the spleens; these properties are favourable for the development of an infectious process in ruminants, their usual hosts.

Finally, the inability of *L. monocytogenes* expressing ILO to be virulent up to a dose of $10^8$ bacteria per mouse (a higher dose being toxic) might be linked to the high LD$_{50}$ of *L. ivanovii* (approx. $10^7$ per mouse) in this animal model (Rocourt et al., 1983; Hof & Hefner, 1988; Engelbrecht et al., 1998). It is thus not excluded that, in other animal hosts, the recombinant strain might show some virulence.

In *L. monocytogenes*, the two phospholipases, PlcA (a phospholipase C specific for phosphatidylinositol) and PlcB (a broad-substrate-range phospholipase), contribute to the disruption of the phagosomal membrane in synergy with the cytolysin (Geoffroy et al., 1991; Vazquez-Boland et al., 1992; Marquis et al., 1995). In *L. ivanovii*, an additional phospholipase, the *smcL*-encoded sphingomyelinase, has been shown to participate in the disruption of the phagocytic vacuole and subsequent intracellular proliferation. The fact that *L. ivanovii* possesses an additional phospholipase suggests that the haemolysin and the PlcA/PlcB pair of *L. ivanovii* might be less efficient in disrupting the phagocytic vacuole and require the help of sphingomyelinase. We therefore tested whether the co-expression of SmcL and ILO by EGD*hly* would restore virulence. We found that, in spite of a significant reduction in the production of ILO, the co-expression of SmcL and ILO did not alter the ability to escape from the phagosomes of BM-derived macrophages and intracellular multiplication. However, *in vivo* kinetics studies revealed that the strain was very rapidly eliminated from both the liver and spleen of infected mice. Production of SmcL is specific to *L. ivanovii* strains.
and is not normally required by L. monocytogenes to produce an infection. The mechanism by which SmcL impairs in vivo survival of L. monocytogenes has yet to be elucidated. At this stage, it might be due only to too low a level of ILO production.

In conclusion, the present work shows that the cytolsin produced by L. ivanovii can replace LLO for phagosomal escape, intracellular multiplication and in vivo survival of L. monocytogenes. Although the precise mechanisms of phagosomal escape are not yet understood, it has been proposed that cholesterol-dependent cytolsins may act as mediators of protein delivery to the host cytosol, like type III secretion systems of Gram-negative bacteria (Madden et al., 2001). In this respect, it has already been demonstrated that LLO is responsible for the induction of a series of signalling events (Kayal et al., 1999; Vazquez-Boland et al., 2001) in infected cells. Whether ILO or other related cytolsins display similar functions is unknown, but it is likely that LLO has probably gradually evolved to acquire unique features that allow optimal multiplication and survival of L. monocytogenes in the broad variety of cell types, tissues and hosts that this pathogen can infect.

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