

Exploring the role of the CTL epitope region of listeriolysin O in the pathogenesis of *Listeria monocytogenes*

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Listeria monocytogenes is a facultative intracellular bacterial pathogen responsible for severe opportunistic infections in humans and animals. The secreted cholesterol-dependent cytolysin, listeriolysin O (LLO), mediates phagosomal escape and allows bacterial growth in the cytosol of infected cells. In order to identify new LLO determinants participating in bacterial pathogenesis, this study focused on a major target of LLO proteolytic cleavage *in vitro*, the CTL epitope region (residues 91–99). Mutations were generated by site-directed mutagenesis in the epitope or in the two clusters of positive charges flanking the epitope. Two LLO mutants (a single mutation K103A and a double mutation R89G, K90G) were normally and stably secreted by *L. monocytogenes*. In contrast, a mutant carrying four amino acid substitutions in the epitope itself (Y92K, D94A, E97K, Y98F) was highly susceptible to proteolytic degradation. While these three LLO mutant proteins showed a reduced haemolytic activity, they all promoted efficient phagosomal escape and intracellular multiplication in different cell types, and were non-cytotoxic. The deletion of the epitope (Δ 91–99), as well as the substitution of two, three or four of the four lysine residues (K103 to K106) by alanine residues did not lead to the production of a detectable protein. These results confirm the lack of correlation between haemolytic activity and phagosomal membrane disruption. They reveal the importance of the 91–99 region in the production of a stable and functional LLO. LD₅₀ determinations in the mouse model suggest a possible link between LLO stability and virulence.

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INTRODUCTION

Listeria monocytogenes is a Gram-positive bacterium widespread in nature and responsible for sporadic severe infections in humans and other animal species (Berche, 1995; Vazquez-Boland *et al.*, 2001). This pathogen is a facultative intracellular micro-organism, capable of invading a wide variety of eukaryotic cells (Dramsı *et al.*, 1995; Gaillard *et al.*, 1986, 1996; Kuhn & Goebel, 1989), including endothelial cells (Drevets *et al.*, 1995) and macrophages (Mackanness, 1962). Each step of intracellular parasitism by *L. monocytogenes* is dependent upon the production of virulence factors (Cossart, 2002; Goebel & Khun, 2000). The major virulence genes identified so far are clustered into two distinct loci on

the chromosome and are controlled by a single pleiotropic regulatory activator, PrfA, which is required for virulence (Johansson *et al.*, 2002; Renzoni *et al.*, 1999). Among these virulence factors, listeriolysin O (LLO, encoded by the *hly* gene) plays a crucial role in the escape of bacteria from the phagosomal compartment to the cytoplasm of infected host cells. LLO-negative mutants remain trapped in the vacuole, do not grow intracellularly and are avirulent in the mouse model of infection (Gaillard *et al.*, 1986; Portnoy *et al.*, 1988).

LLO belongs to a family of cholesterol-dependent, pore-forming cytolysins (CDCs) (Alouf, 2000). The best-characterized members of the CDCs are perfringolysin O (PFO) and streptolysin O (SLO), which are both secreted by extracellular bacterial pathogens: *Clostridium perfringens* and *Streptococcus pyogenes*, respectively. The three-dimensional structure of LLO is currently unknown but that of monomeric PFO has been determined by X-ray crystallography (Rossjohn *et al.*, 1997). Replacement of LLO with PFO in *L. monocytogenes* results in a strain that is able to escape from a vacuole, albeit at a reduced efficiency, but that kills the

Abbreviations: BMM, bone-marrow-derived macrophage; CDC, cholesterol-dependent pore-forming cytolysin; CTL, cytotoxic T lymphocyte; LDH, lactate dehydrogenase; LLO, listeriolysin O; PFO, perfringolysin O; SLO, streptolysin O.

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A supplementary table of primer sequences is available with the online version of this paper.

infected cells (Jones & Portnoy, 1994a). Thus, LLO is apparently unique among the CDCs in that it can disrupt a vacuolar membrane, but does not kill the host cell upon growth in the cytosol. The molecular mechanism of LLO-dependent phagosomal escape of intracellular *L. monocytogenes* remains unknown. It is believed that a rapid degradation of LLO in the cytosol, controlled by host-cell-mediated degradation, may ensure that the host cytoplasmic membrane remains intact during infection by *L. monocytogenes* (Bubert *et al.*, 1999; Moors *et al.*, 1999). Several motifs or residues that might control intracytosolic degradation of LLO have been proposed and/or studied. In particular, a region rich in proline, glutamate, serine and threonine residues (called the PEST motif) has been identified close to the N-terminus of the mature protein. PEST motifs are thought to target eukaryotic proteins for phosphorylation and/or rapid degradation by the proteasome (Decatur & Portnoy, 2000; Lety *et al.*, 2002; and references therein).

A series of immunological studies demonstrated that the induction of acquired cellular immunity is crucial in the elimination of *L. monocytogenes*. In particular, the generation of antigen-specific cytotoxic T-cells leads to the killing of infected cells. Intracytosolic multiplication of *L. monocytogenes* induces, in mice, strong MHC class I-restricted cytotoxic T-lymphocyte (CTL) responses (Busch *et al.*, 1997; Finelli *et al.*, 1999; Sijts *et al.*, 1996). One LLO epitope (residues 91–99) was shown to elicit a very large, dominant response (Busch & Pamer, 1998; Pamer, 1994; Pamer *et al.*, 1997; Vijn & Pamer, 1997; Vijn *et al.*, 1999; Villanueva *et al.*, 1995). These studies provided the only direct evidence of a proteasome-mediated cytosolic degradation of LLO.

Correct cleavage of a CTL epitope by the proteasome is a crucial step in the formation of precursor peptides leading to MHC presentation, and a single residue exchange in the flanking region may abolish accurate proteasome-mediated cleavage (Beekman *et al.*, 2000; Yellen-Shaw & Eisenlohr, 1997; Yellen-Shaw *et al.*, 1997).

These observations led us to pursue the systematic analysis of the different motifs (or regions) comprised within the LLO sequence that may contribute to its biological activities. We addressed here the role of the CTL epitope region, focusing on the relation between LLO stability and haemolytic activity in broth; and phagosomal escape and cytotoxicity, in eukaryotic cells.

METHODS

Bacterial strains and culture conditions. EGD Δ hly is a derivative of EGD-e (serotype 1/2a), which contains an in-frame chromosomal deletion of 1080 bp in the *hly* gene (Guzman *et al.*, 1995). EGD Δ hly was transformed with the different recombinant plasmids by electroporation, as previously described (Park & Stewart, 1990). Bacteria were grown in Brain Heart Infusion (BHI) broth (Difco) at 37 °C without antibiotics, except for the pAT28-transformed strains, which were grown on BHI broth containing 60 µg spectinomycin (Spc) ml⁻¹.

Construction of the recombinant cytolysins. Chromosomal DNA, plasmid isolation, restriction enzyme analyses and amplification by PCR were performed according to standard protocols (Ausubel *et al.*, 1990; Sambrook *et al.*, 1989).

Seven LLO mutants were constructed by site-directed *in vitro* mutagenesis. Residues R89 and K90 were changed to glycine residues (denoted LLO-RK/GG), in the region upstream of the 91–99 epitope. Four mutants were constructed in the KKKK downstream region, substituting to alanine: one, two, three or all four K residues (mutations K103A; K103A, K104A; K103A, K104A, K105A; and K103A, K104A, K105A, K106A, respectively). For simplicity, the corresponding proteins were denoted LLO-K1/A1, LLO-K2/A2, LLO-K3/A3 and LLO-K4/A4, respectively. Two types of mutations were generated within the 91–99 epitope: an in-frame deletion of residues 91 to 99 (mutant protein LLO- Δ 91–99), and a substitution of the 91–99 epitope of LLO by the corresponding residues of PFO, which introduces four amino acid substitutions (Y92K, D94A, E97K and Y98F; denoted LLO-91–99P).

The LLO mutants LLO-RK/GG, LLO-K1/A1, LLO-K2/A2, LLO-K3/A3, LLO-K4/A4 and LLO- Δ 91–99 were constructed by using the GeneTailor Site-Directed Mutagenesis System (Invitrogen). Mutagenesis was performed on pAT28-*phly-hly* according to the manufacturer's recommendations.

The LLO mutant LLO-91–99P was generated by *in vitro* site-directed mutagenesis on M13mp18-*phly-hly* as described previously (Lety *et al.*, 2001). The mutation was then transferred onto pAT28, a Gram-positive/Gram-negative shuttle vector, by restriction enzyme excision from the replicative form (*Bam*HI–*Sal*I) and insertion into the corresponding sites of pAT28. All the constructs were checked by PCR sequence analysis. Recombinant pAT28 plasmids were transferred into EGD Δ hly by electroporation, as described previously (Lety *et al.*, 2001). EGD Δ hly expressing wild-type LLO (LLOwt) was used as a positive control and EGD Δ hly as a negative control.

The mature form of LLO-K4/A4 protein was expressed in *Escherichia coli* with an N-terminal His-tag. For this, the portion of the mutant *hly* gene (carried on plasmid pAT28) encoding the mature form of the protein was amplified by PCR. The amplified product was cut with restriction enzymes *Bam*HI and *Xho*I and inserted into the *Bam*HI and *Xho*I sites of the expression vector pET28a (Novagen), downstream of the His-tag cloning sequence. The recombinant protein was denoted LLO-K4/A4 His-tag. The construct was transferred by transformation into the *E. coli* recipient strain BL21star (Invitrogen). Transformed bacteria were grown on LB medium containing ampicillin. Expression of the LLO-K4/A4 His-tag protein was induced by addition of IPTG (1 mM final concentration). After several hours of induction (up to 22 h), bacteria were harvested by centrifugation. The bacterial pellets were resuspended directly into SDS-PAGE loading buffer (typically a 2 ml culture was resuspended into 50 µl loading buffer) and the samples were boiled for 5 min before loading onto gels.

The primers used to create the different LLO mutants are listed in supplementary Table S1 (available as supplementary material with the online version of this paper).

Protein preparations and analyses. Proteins were prepared from cell culture supernatants of bacteria grown in LB supplemented with 10% BHI. Concentrated culture supernatants were prepared as described previously (Lety *et al.*, 2003). Cell-free supernatants were filtered through a 0.22 µm pore size Millipore filter and concentrated by centrifugation through ultrafreez Biomax units. The LLO-mutant proteins were identified by Western blot analysis, using polyclonal anti-LLO antibody. Identical amounts of each

concentrated culture supernatant were loaded per well (except mutant LLO-91–99P, for which the concentrated supernatant was further concentrated by TCA precipitation).

Intracellular LLO analysis. LLO produced by cytosolic bacteria was detected by immunoprecipitation with anti-LLO monoclonal antibodies SE1 and SE2, essentially as described previously (Lety *et al.*, 2002). Briefly, monolayers of bone-marrow-derived macrophages (BMM) from BALB/c mice, seeded into 60 mm dishes, were infected at a bacterium:macrophage ratio of 10:1. After 30 min, monolayers were washed and reincubated for 2 h. The medium was then replaced with methionine-free RPMI minimal medium containing 10% dialysed fetal calf serum and 225 µg cycloheximide ml⁻¹. After 30 min, monolayers were pulsed-labelled for 1 h with 100 µCi (3.7 MBq) [³⁵S]methionine (Amersham). Cells were then lysed and LLO was immunoprecipitated with anti-LLO monoclonal antibodies. The immunoprecipitated material was finally subjected to SDS-PAGE and subsequent analysis on a PhosphorImager (Molecular Dynamics).

Anti-*Listeria* and anti-LLO antibodies. The polyclonal anti-LLO serum was purchased from Diatheva. It was used in Western blot at a final dilution of 1/1000, according to the manufacturer's recommendation. The polyclonal rabbit anti-*Listeria* antibody (J. Rocourt, Institut Pasteur) was used in immunofluorescence at a final dilution of 1/200. Anti-LLO monoclonal antibodies SE1 and SE2 were kindly provided by Dr A. J. Ainsworth, Mississippi State University.

Infection of macrophages and microscopic analyses. For confocal microscopy analyses, BMM from BALB/c mice were cultured and infected at a bacterium:macrophage ratio of 10–20:1 (Frehel *et al.*, 2003). Growth in J774 macrophage-like cells was performed as described previously (Jones & Portnoy, 1994b) in the absence or the presence of gentamicin (50 µg ml⁻¹ final concentration), at a bacterium:macrophage ratio of 50:1.

Processing for confocal microscopy. Double fluorescence labelling of F-actin and bacteria was performed, using phalloidin coupled to Alexa 488 (Molecular Probes) and a rabbit anti-*Listeria* polyclonal antibody revealed with anti-IgG antibody coupled to Alexa 546 (Molecular Probes). Images were scanned on a Zeiss LSM 510 confocal microscope.

Cytotoxicity. Monolayers of BMM from BALB/c mice, seeded into 60 mm dishes, were infected with the same ratios of bacteria per cell as those used for the immunofluorescence assays. Supernatants were removed from each well 4 and 6 h after infection and assayed for lactate dehydrogenase (LDH) activity by using the Cyto Tox 96 kit (Promega), as recommended by the manufacturer.

Two independent experiments were performed. In each experiment, three wells were counted per point. Percentage cytotoxicity = 100 × (experimental LDH release – spontaneous LDH release) / (maximal LDH release – spontaneous LDH release). Spontaneous LDH release was measured in supernatants of non-infected macrophages. Maximal LDH release corresponds to the macrophages lysed after treatment with 0.9% Triton X-100 (final concentration), for 45 min at 37 °C. The percentage indicated in the text corresponds to the mean of three wells of a single experiment. For example, the background of spontaneous LDH release was 348 ± 13 (mean ± SD) and the value of maximal LDH release was 9530 ± 339. With LLOwt, after 6 h, a value of 4460 ± 155 was recorded. The percentage cytotoxicity thus corresponded to 100 × (4460 – 335) / (9530 – 335) = 44.7%.

Invasion assays. The human colon carcinoma cell line Caco-2 (ATCC HTB37) and the human hepatocellular carcinoma cell line HepG-2 (ATCC HB 8065, kindly provided by P. Cossart, Institut Pasteur) were propagated in Dulbecco's modified Eagle's medium (DMEM; 25 mM glucose; Gibco). Cells were seeded at 8 × 10⁴ cells cm⁻² in 24-well tissue culture plates (Falcon). Monolayers were

used 24 h after seeding. The invasion assays were carried out essentially as described previously (Garandeau *et al.*, 2002). Briefly, cells were inoculated with bacteria at an m.o.i. of ~50 bacteria per cell. They were incubated 1 h to allow the adherent bacteria to enter and were then washed three times with RPMI and overlaid with fresh DMEM containing gentamicin (10 µg ml⁻¹) to kill extracellular bacteria. At selected intervals, cells were washed three times and processed for counting of infecting bacteria. Cells were lysed by adding cold distilled water. The titre of viable bacteria released from the cells was determined by spreading onto BHI plates. Each experiment was carried out in triplicate and repeated three times.

Infection of mice and virulence assays. The virulence of the *L. monocytogenes* mutant strains was estimated by determining the LD₅₀ in pathogen-free, 6–8-weeks-old female Swiss mice (Janvier). Groups of five mice were intravenously inoculated with bacterial suspension (0.5 ml in 0.15 M NaCl) in the lateral tail vein. Animals were pretreated with Spc (1 mg per mouse per day) in order to overcome *in vivo* instability of the recombinant plasmids, as described previously (Lety *et al.*, 2002). The mortality was followed for 7–10 days. The virulence of the strains was estimated, using the graphic Probit method (Roth, 1961). For example, the LD₅₀ of 10^{5.5}, recorded with the strain expressing LLOwt, corresponded to 100% death at a dose of 10⁶ bacteria per mouse (5/5 mice died within 1 week) and 0% death at a dose of 10⁵ bacteria per mouse; the LD₅₀ of 10^{7.5}, recorded with the strain expressing LLO-RK/GG, corresponded to 100% death at a dose of 10⁸ bacteria per mouse and 0% of death at a dose of 10⁷ bacteria per mouse.

RESULTS

Expression of the LLO mutants by *L. monocytogenes*

Constructs. Seven LLO mutants were constructed by site-directed *in vitro* mutagenesis (Fig. 1). Residues R89 and K90 were changed to glycine residues (denoted LLO-RK/GG), in the region upstream of the 91–99 epitope. Four mutants were constructed in the KKKK downstream region, changing one, two, three or all four K residues to alanine (Fig. 1b). Two types of mutations were generated within the 91–99 epitope: an in-frame deletion of residues 91 to 99 (mutant protein LLO-Δ91–99) and a substitution of the 91–99 epitope of LLO by the corresponding residues of PFO (see Methods for details).

As shown on the alignment of the N-terminal residues of the mature forms of PFO and of LLO (Fig. 1b), LLO residues 10–36 comprise a putative PEST motif (Decatur & Portnoy, 2000; Lety *et al.*, 2001) that has no counterpart in PFO. The 91–99 epitope is located 40 residues downstream of this motif. In the cytolysins secreted by two other members of the genus *Listeria*, ivanolysin O from *Listeria ivanovii* (ILO) and seeligeriolysin O from *Listeria seeligeri* (LSO), the 91–99 epitope sequence is highly conserved, with only one E97Q substitution in ILO and one N96S in LSO (as compared to six, six and four substitutions in the cytolysins secreted by *S. pyogenes* (SLO) and *Streptococcus pneumoniae* (PLY) and *C. perfringens* (PFO) respectively; Fig. 1c). The KKKK cluster downstream of the epitope is fully conserved in the three cytolysins of the genus *Listeria*. In the other cytolysins, in spite of sequence divergence, at least three positive charges

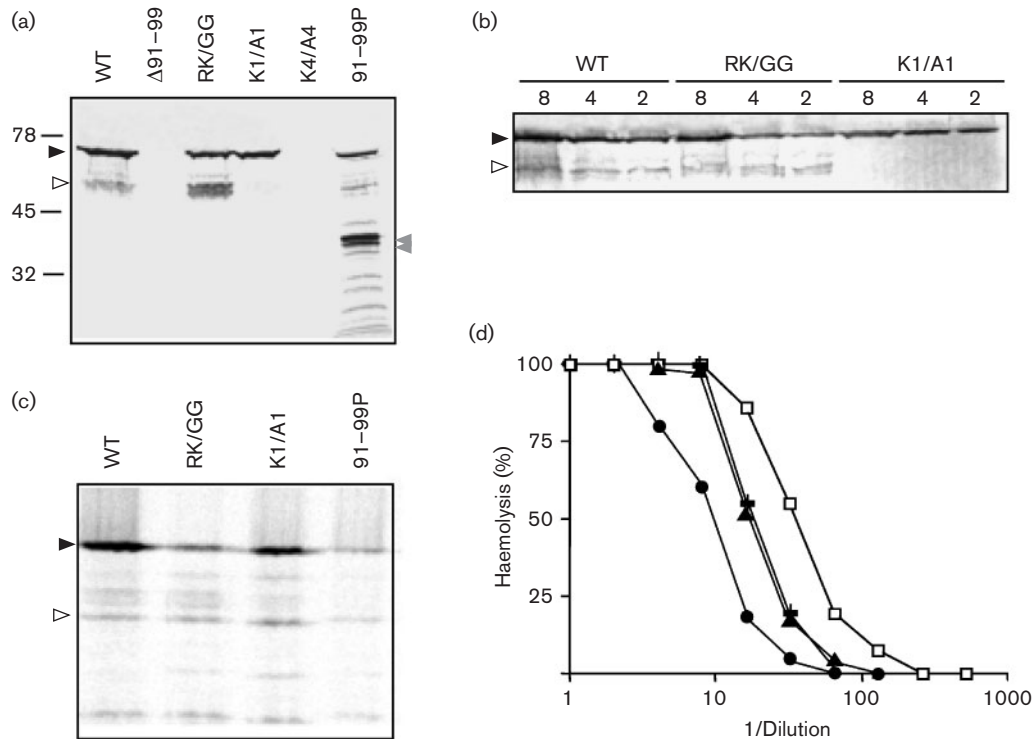


Fig. 2. Immunodetection and haemolytic activities. (a, b) Western blot analyses of LLO from *L. monocytogenes*. Concentrated culture supernatants from EGD Δ hly expressing LLOwt (WT), LLO-RK/GG, LLO-K1/A1, LLO-K4/A4 or LLO-91-99P were loaded onto SDS-10% polyacrylamide gels. Proteins were transferred electrophoretically onto nitrocellulose and detected with anti-LLO polyclonal antibody at a final dilution of 1 : 1000. (a) Ten microlitres of each concentrated supernatant from bacteria grown to stationary phase, adjusted to the same number of bacteria, were loaded per well ($\sim 4 \times 10^8$ bacteria), for LLOwt, LLO-RK/GG, LLO-K1/A1, LLO- Δ 91-99 and LLO-K4/A4. For better visualization of the numerous degradation products in LLO-91-99P, a five-fold more concentrated supernatant was prepared (the amount loaded per well corresponding to $\sim 2 \times 10^9$ bacteria). To the left of the figure, the numbers refer to apparent molecular masses (in kDa). The black triangle points to the full-size denatured monomer, the white triangle to minor LLO species and the grey triangles to the two major degradation products detected in the supernatant from LLO-91-99P producing strain only. (b) Decreasing amount of concentrated supernatant (adjusted to the same number of bacteria) from EGD Δ hly expressing LLOwt (WT), LLO-RK/GG, or LLO-K1/A1 were loaded. Numbers above correspond to the amount of concentrated supernatant (in μ l) loaded per well. (c) Immunoprecipitation of LLO secreted by intracellular *L. monocytogenes*. Proteins were metabolically labelled with [³⁵S]methionine during growth in BMM and LLO was immunoprecipitated with monoclonal anti-LLO antibody (see Methods). Cells were infected at rate of 10-50 bacteria per cell. LLOwt and derivatives were immunoprecipitated after 3 h infection. The full-length protein is indicated by a black triangle. The vertical bar below indicates the degradation products. The gel was scanned with a Molecular Dynamics PhosphorImager and the resulting image was analysed with Imagequant software. The autoradiograph corresponds to 72 h exposure. (d) Haemolytic activities. The haemolytic activity of culture supernatants from exponentially growing bacteria at 37 °C in BHI medium was tested essentially as described previously (Jones & Portnoy, 1994a). Serial twofold dilutions of each supernatant (starting from 40 μ l non-diluted sample) were tested on horse erythrocytes, at pH 7.4. The maximal OD₄₅₀ value recorded was taken as 100% haemolysis. □, LLOwt; ■, LLO-RK; ▲, LLO-K1; +, LLO-91-99P.

corresponding to the full-size protein was less abundant than that of LLOwt (or LLO-RK/GG and LLO-K1/A1) and several additional bands of lower molecular mass, most likely corresponding to degradation products, were detected by the anti-LLO antibody in the supernatant of bacteria grown to stationary phase (Fig. 2a). These low-molecular-mass bands were not detected in the supernatant of exponentially grown bacteria (Fig. 2c), suggesting that LLO-91-99P degradation occurred mainly during the stationary phase of growth.

Four mutant proteins, LLO- Δ 91-99, LLO-K2/A2, LLO-K3/A3 and LLO-K4/A4, were not detectable by the anti-LLO antibody (in exponential or stationary phase of growth). We tested whether LLO-K4/A4 could be expressed in *E. coli* with an N-terminal His-tag (see Methods for details). Whole-cell extracts from IPTG-induced cultures were loaded onto SDS-polyacrylamide gels (data not shown). Both the anti-LLO antibody and the Ni-NTA conjugate detected a band corresponding to the full-size recombinant protein. This experiment demonstrated that the

recombinant protein LLO-K4/A4 could be stably expressed in *E. coli*, confirming the assumption that it is produced but rapidly degraded in *L. monocytogenes*.

Haemolytic activities. The haemolytic activities of culture supernatants of the *EGD Δ hly* derivatives were measured on horse erythrocytes (Lety *et al.*, 2002). The activities recorded did not strictly correlate with the apparent stability of the LLO proteins in Western blot (Fig. 2d). Mutants LLO-K1/A1 and LLO-91-99P showed only a twofold lower haemolytic activity than LLOwt. In contrast, the activity recorded with mutant LLO-RK-GG was eightfold lower than that of LLOwt. No haemolytic activity was detected with the mutants LLO- Δ 91-99, LLO-K2/A2, LLO-K3/A3 and LLO-K4/A4, confirming the Western blot observations.

Phagosomal escape and intracellular survival

Phagosomal escape and intracellular multiplication was first studied in BMM from BALB/c mice, by confocal

microscopy (Lety *et al.*, 2001). Bacterial uptake was monitored up to 4 h after infection (Fig. 3a). The capacity of the mutant strains to promote phagosomal escape was determined by calculating the ratio between the number of bacteria surrounded by polymerized actin and the total number of bacteria in infected cells. These values were determined on an average of at least 50 infected cells. After 2 h infection, ~33 % of the bacteria expressing LLOwt had reached the cytosol and were surrounded by polymerized actin (Fig. 3a, d). Phagosomal escape of the three mutant strains was even more efficient, with at least 61 % escape after 2 h. No bacterial multiplication and no actin polymerization were observed with the strain expressing LLO- Δ 91-99 or LLO-K4/A4 (Fig. 3e).

We next examined, by the same method, the intracellular survival of the LLO mutant in HepG-2 hepatocytes (Fig. 3b). Bacterial uptake was monitored up to 6 h after infection. Phagosomal escape of the three mutant strains was also very efficient, confirming the BMM observations. After 2 h, the three mutant strains had already started to multiply. After

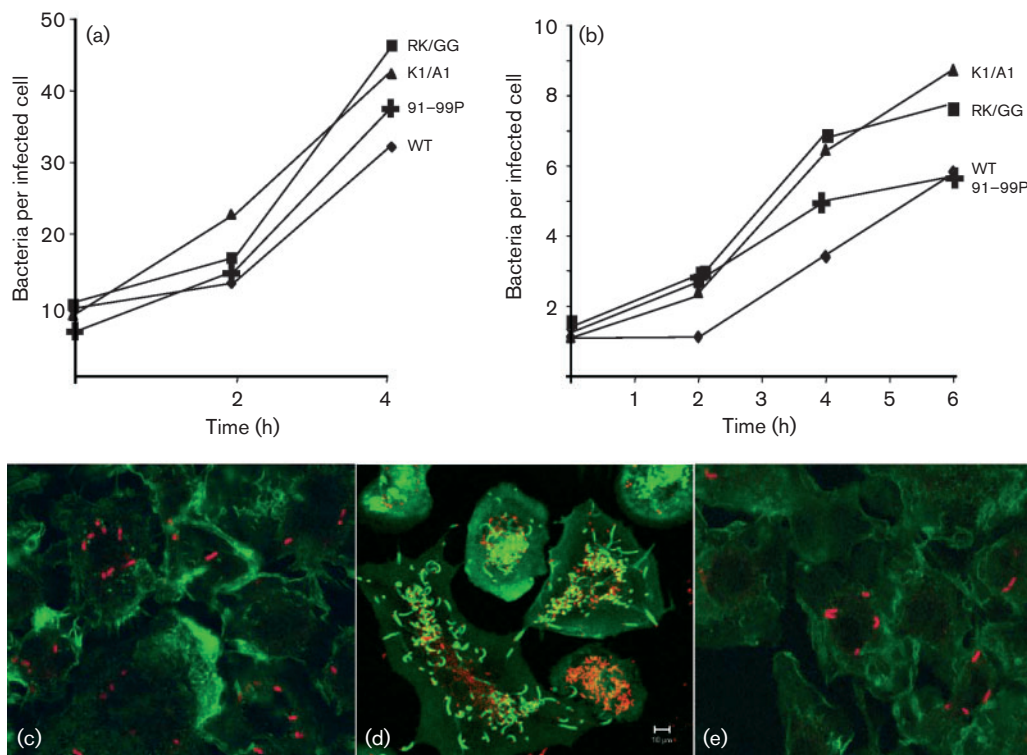


Fig. 3. Phagosomal escape and intracellular survival. Cells were exposed to *L. monocytogenes* expressing LLOwt (WT) or LLO mutants at a ratio of 50 bacteria per cell. The kinetics of infection was followed by immunofluorescence microscopy over a 4 to 6 h period. (a) BMM. (b) HepG-2 cells. Intracellular multiplication was evaluated by determining the number of bacteria per infected cell. Each point is the mean of at least 50 infected cells. The assay was performed twice; values presented correspond to a typical experiment. (c, d, e) BMM. Images were scanned on a Zeiss LSM 510 confocal microscope. F-actin is stained with phalloidin (green) and bacteria are labelled with anti-*Listeria* antibodies (red). (c) *EGD Δ hly* expressing LLO-RK/GG at t_0 (similar pictures were obtained with the other LLO-derivatives at t_0 ; not shown). (d) *EGD Δ hly* expressing LLO-RK/GG after 4 h infection (similar pictures were obtained with LLOwt, LLO-91-99P and LLO-K1/A1). (e) *EGD Δ hly* expressing LLO-K4/A4 (similar pictures were obtained with LLO- Δ 91-99 or *EGD Δ hly* alone). Bar 10 μ m.

4 h, the percentage of bacteria surrounded by polymerized actin was roughly comparable with LLOwt and with the three LLO mutants tested, ranging between 49 and 71 %. After 6 h, the mean number of bacteria per infected cell was almost twofold higher with LLO-RK/GG and LLO-K1/A1 than with LLOwt and LLO-91-99P (with 8-9 and 5-6 bacteria per cell, respectively).

Altogether, the confocal microscopy analyses showed that phagosomal escape and actin polymerization were not affected in LLO-RK/GG, LLO-K1/A1, or LLO-91-99P, in a phagocytic as well as in a non-phagocytic host cell.

We also measured the kinetics of bacterial invasion in the mouse macrophage-like cell line J774 (Fig. 4a) and in the enterocyte-like cell line Caco-2 (Fig. 4b). Cells were inoculated with bacteria, and, at selected intervals, cells were processed for counting of infecting bacteria (see Methods). In J774 cells, intracellular multiplication of LLO-K1/A1, LLO-RK/GG and LLO-91-99P was followed over an 8 h period, in the absence (not shown) as well as in the presence of gentamicin ($50 \mu\text{g ml}^{-1}$ final concentration). If any of the LLO mutants had damaged the host cell membrane, intracellular entry of gentamicin should have led to an inhibition of bacterial multiplication. The three mutants showed normal growth properties, in the absence or presence of gentamicin (Fig. 4a), thus indicating their lack of cytotoxicity. In Caco-2 cells, the three mutants also showed a normal growth over the infection period tested (Fig. 4b). After 3 h, bacteria started to multiply actively and during the next 2 h the growth rates of the mutant strains were almost identical to that of the strain expressing wild-type LLO, reflecting normal phagosomal escape and intracytoplasmic survival in these cells.

LLO expression in infected macrophages, cytotoxicity and impact on virulence

LLO produced by bacteria multiplying in infected BMM was immunoprecipitated with anti-LLO mAbs, after 3 h infection (see Methods for details). The three mutant proteins LLO-RK/GG, LLO-K1/A1, LLO-91-99P and LLOwt, were specifically immunoprecipitated by the anti-LLO MAb (Lety *et al.*, 2002). In all cases, a single major band was detected (Fig. 2c). However, the intensity of the major band detected with the three LLO mutants was weaker than that of LLOwt (37 %, 10 % and 5 % of LLOwt for LLO-K1/A1, LLO-RK/GG and LLO-91-99P, respectively: Table 1).

To test a possible cytotoxic effect of LLO-K1/A1, LLO-RK/GG and LLO-91-99P expressed by cytosolic bacteria, we measured the release of a host cytosolic enzyme, LDH, in the culture medium of infected BMM (Lety *et al.*, 2002). Less than 1 % LDH release was recorded in macrophages infected with the LLO-negative strain *EGD Δ hly*. In agreement with our earlier observations, $\sim 44.7\%$ LDH release was recorded with the strain expressing wild-type LLO after 6 h infection, reflecting cell death (Barsig & Kaufmann, 1997). A comparable value was obtained with mutant LLO-K1/A1 (53 % LDH release after 6 h). The values recorded with LLO-RK/GG and LLO-91-99P ranged between 24.3 % and 31 % LDH release (Table 1). These results indicated a lack of cytotoxicity of the three LLO mutants tested.

Finally, the virulence of the *L. monocytogenes* mutants was evaluated in the mouse infection model by determining LD_{50} , using the graphic Probit method (Roth, 1961). Groups of outbred female Swiss mice were inoculated by intravenous injection with different doses, in tenfold dilutions. As

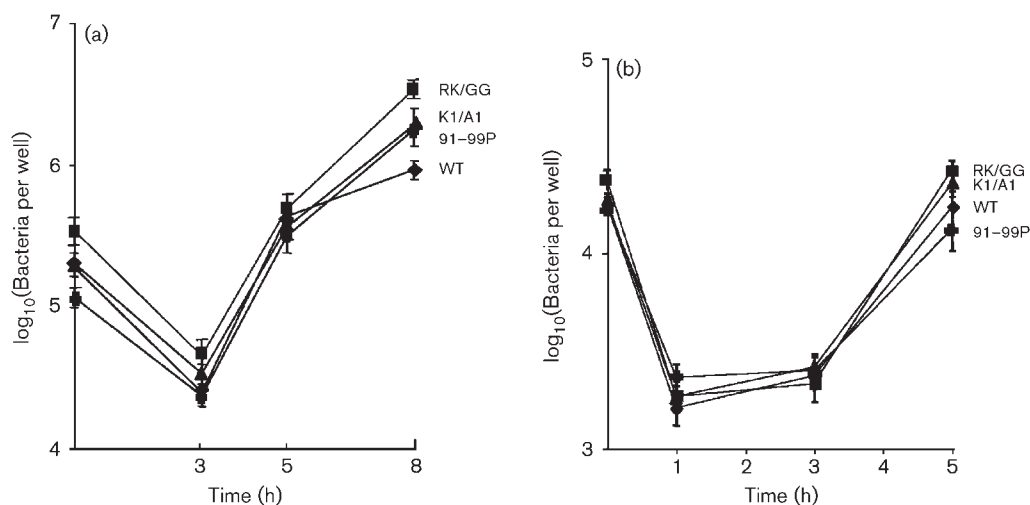


Fig. 4. Invasion assays. Cells were exposed to *L. monocytogenes* expressing LLOwt (WT) or LLO mutants (LLO-K1/A1, LLO-91-99P, LLO-RK/GG), at a ratio of 50 bacteria per cell. The number of viable bacteria was determined by spreading onto BHI plates. (a) J774 macrophages. Invasion and intracellular multiplication was monitored over a 5 h period, in the presence of $50 \mu\text{g ml}^{-1}$ gentamicin (final concentration), essentially as described by Glomski *et al.* (2003). (b) Caco-2 cells. Invasion and intracellular multiplication was monitored over a 5 h period as described previously (Bonnemain *et al.*, 2004).

Table 1. Immunoprecipitation, cytotoxicity, phagosomal escape and LD₅₀

Immunoprecipitations (Ipp) were performed in infected BMM from BALB/c mice. Intensities of the LLO bands (%) were quantified by using the ImageQuant program. The value recorded with LLOwt was taken as 100%. Cytotoxicity (LDH release; %) was determined on BMM from BALB/c mice, using the Cyto Tox 96 Kit (Promega). The values indicated correspond to 6 h infection. Phagosomal escape (%) was determined in BMM from BALB/c mice on an average of 50 infected cells. The values indicated correspond to 4 h infection. Virulence (LD₅₀) was determined by the Probit method after intravenous infection of groups of Swiss mice (five mice per point) with serial tenfold dilutions of the different strains.

LLO mutant	Ipp (%)	LDH release (%)	Phagosomal escape (%)	LD ₅₀
LLOwt	100	44.7	88.5	10 ^{5.5}
LLO-RK/GG	10	24.3	96	10 ^{7.5}
LLO-K1/A1	37	53	82	10 ^{7.7}
LLO-91-99P	5	31	83	10 ⁸
EGDΔhly	–	0	0	10 ⁹

previously shown, EGDΔhly is totally avirulent and expression of plasmid-encoded wild-type LLO restores virulence to the strain (LD₅₀ 10^{5.5}). The strains expressing LLO-RK/GG and LLO-K1/A1 showed a strong attenuation of virulence (≥100-fold decrease) and the strain expressing LLO-91-99P was almost avirulent (LD₅₀ > 10⁸) (Table 1).

DISCUSSION

Mutations in LLO that lead to inappropriate activity in the cytosol may damage the host cell and decrease virulence (Decatur & Portnoy, 2000; Glomski *et al.*, 2002, 2003; Villanueva *et al.*, 1995). The identification of a putative PEST-like sequence close to the N-terminus of the protein suggested that this region might control intracytosolic LLO degradation and therefore prevent cytotoxicity (Decatur & Portnoy, 2000; Lety *et al.*, 2001). However, the direct role of this region in cytosolic degradation of LLO is not yet established. In order to identify additional LLO motifs involved in optimal intracellular adaptation of *L. monocytogenes*, we focused here on the immunologically well characterized 91-99 CTL epitope region. Out of the seven LLO mutants constructed, only three could be detected in the supernatant of *L. monocytogenes*, indicating that the 91-99 region is important for protein stability.

Earlier studies on integral membrane protein had led to the notion of permissive and non-permissive sites in proteins (Charbit *et al.*, 1986 and references therein). A permissive site was defined as a site that accommodates local sequence modifications without complete loss of protein stability and/or functionality, whereas a non-permissive site does not tolerate changes without extensive protein degradation. According to this terminology, residues 91-99 can be considered as a non-permissive region of LLO. Due to the complex structural organization of the LLO molecule and to the conformational changes occurring upon interaction with a cholesterol-containing membrane (Schuerch *et al.*,

2005 and references therein), probably many other sites or regions of the LLO molecule are also non-permissive.

The KKKK stretch downstream of the epitope deserves particular attention since only a single amino acid substitution was tolerated. One hypothesis to account for this result could be that the loss of two positive charges in this region is not acceptable to maintain a stable structure. Based on the sequence similarities between LLO and PFO, on the folded monomer, the tip of D2 is close to the autonomously folded domain D4. Mutations at the tip of D2 may thus alter the proper interaction between these domains. The fact that the mutant protein bearing all four K residues changed to alanine (LLO-K4/A4) could be produced in *E. coli* suggests that the protein produced in *L. monocytogenes* is abnormally folded and therefore rapidly degraded by proteases (this may also hold for the other proteins that were not detectable).

While secreted as monomers by bacteria, the CDCs form oligomers upon binding to cholesterol-containing membrane. In the case of LLO, complete pore-like structures have been visualized on erythrocyte membranes (Jacobs *et al.*, 1998). However, the notion of pore formation in the acidic phagosomal compartment, where LLO expression is induced, is as yet purely speculative. The mechanisms of LLO-mediated phagosomal disruption are probably quite distinct from the pore-formation process observed on erythrocyte membranes and may not imply any oligomerization. Indeed, in spite of a two- to eightfold reduction of haemolytic activity, the three mutants LLO-K1/A1, LLO-RK/GG and LLO-91-99P allowed efficient phagosomal escape and intracellular survival of *L. monocytogenes*, in both phagocytic and non-phagocytoc cells.

In conclusion, the present work showed that three LLO mutants bearing mutations in the 91-99 region, LLO-K1/A1, LLO-RK/GG and LLO-91-99P, could grow efficiently *in vitro* (in different cell lines) but had a severe reduction of virulence in the mouse model. This novel phenotype contrasts with that of mutants in the PEST region we had

generated previously (Lety *et al.*, 2001, 2002). Indeed, all the LLO-PEST mutants were secreted by *L. monocytogenes* in normal amounts and were fully haemolytic but they were all impaired to different extents in phagosomal escape. At this stage, one may hypothesize that both the PEST and the CTL region would control cytosolic LLO stability, limiting the possible toxicity of the LLO molecule produced during intracytosolic multiplication of *L. monocytogenes*.

Mutations reducing the cytosolic stability of LLO without altering intracellular survival of *L. monocytogenes* were not expected to impair bacterial virulence. The drastic effect of the mutations in LLO on the LD₅₀ of *L. monocytogenes* could be due for example to reduced haemolytic activity. Indeed, the development of the infection leading to brain invasion requires an initial multiplication in the infected target organs and a subsequent transitory bacteraemia (Berche, 1995). Other mechanisms may also be involved *in vivo*, requiring specific LLO properties distinct from those necessary for *in vitro* multiplication.

Finally, from an immunological point of view, it would be interesting to monitor quantitatively the capacity of the stable cytolysins LLO-RK/GG and LLO-K1/A1 to be processed by the proteasome to produce CTL epitopes as well as their ability to trigger a protective immune response.

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REFERENCES

- Alouf, J. E. (2000). Cholesterol-binding cytolytic protein toxins. *Int J Med Microbiol* **290**, 351–356.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (editors) (1990). In *Current Protocols in Molecular Biology*. New York: Wiley Interscience.
- Barsig, J. & Kaufmann, S. H. (1997). The mechanism of cell death in *Listeria monocytogenes*-infected murine macrophages is distinct from apoptosis. *Infect Immun* **65**, 4075–4081.
- Beekman, N. J., van Veelen, P. A., van Hall, T. & 7 other authors (2000). Abrogation of CTL epitope processing by single amino acid substitution flanking the C-terminal proteasome cleavage site. *J Immunol* **164**, 1898–1905.
- Berche, P. (1995). Bacteremia is required for invasion of the murine central nervous system by *Listeria monocytogenes*. *Microb Pathog* **18**, 323–336.
- Bonnemain, C., Raynaud, C., Reglier-Poupet, H., Dubail, I., Frehel, C., Lety, M. A., Berche, P. & Charbit, A. (2004). Differential roles of multiple signal peptidases in the virulence of *Listeria monocytogenes*. *Mol Microbiol* **51**, 1251–1266.
- Bubert, A., Sokolovic, Z., Chun, S. K., Papatheodorou, L., Simm, A. & Goebel, W. (1999). Differential expression of *Listeria monocytogenes* virulence genes in mammalian host cells. *Mol Gen Genet* **261**, 323–336.
- Busch, D. H. & Pamer, E. G. (1998). MHC class I/peptide stability: implications for immunodominance, *in vitro* proliferation, and diversity of responding CTL. *J Immunol* **160**, 4441–4448.
- Busch, D. H., Bouwer, H. G., Hinrichs, D. & Pamer, E. G. (1997). A nonamer peptide derived from *Listeria monocytogenes* metalloprotease is presented to cytolytic T lymphocytes. *Infect Immun* **65**, 5326–5329.
- Charbit, A., Boulain, J. C., Ryter, A. & Hofnung, M. (1986). Probing the topology of a bacterial membrane protein by genetic insertion of a foreign epitope; expression at the cell surface. *EMBO J* **5**, 3029–3037.
- Cossart, P. (2002). Molecular and cellular basis of the infection by *Listeria monocytogenes*: an overview. *Int J Med Microbiol* **291**, 401–409.
- Decatur, A. L. & Portnoy, D. A. (2000). A PEST-like sequence in listeriolysin O essential for *Listeria monocytogenes* pathogenicity. *Science* **290**, 992–995.
- Drams, S., Biswas, I., Maguin, E., Braun, L., Mastroeni, P. & Cossart, P. (1995). Entry of *Listeria monocytogenes* into hepatocytes requires expression of inIB, a surface protein of the internalin multigene family. *Mol Microbiol* **16**, 251–261.
- Drevets, D. A., Sawyer, R. T., Potter, T. A. & Campbell, P. A. (1995). *Listeria monocytogenes* infects human endothelial cells by two distinct mechanisms. *Infect Immun* **63**, 4268–4276.
- Dubail, I., Autret, N., Beretti, J. L., Kayal, S., Berche, P. & Charbit, A. (2001). Functional assembly of two membrane-binding domains in listeriolysin O, the cytolysin of *Listeria monocytogenes*. *Microbiology* **147**, 2679–2688.
- Finelli, A., Kerkusiek, K. M., Allen, S. E., Marshall, N., Mercado, R., Pilip, I., Busch, D. H. & Pamer, E. G. (1999). MHC class I restricted T cell responses to *Listeria monocytogenes*, an intracellular bacterial pathogen. *Immunol Res* **19**, 211–223.
- Frehel, C., Lety, M. A., Autret, N., Beretti, J. L., Berche, P. & Charbit, A. (2003). Capacity of ivanolysin O to replace listeriolysin O in phagosomal escape and *in vivo* survival of *Listeria monocytogenes*. *Microbiology* **149**, 611–620.
- Gaillard, J. L., Berche, P. & Sansonetti, P. (1986). Transposon mutagenesis as a tool to study the role of hemolysin in the virulence of *Listeria monocytogenes*. *Infect Immun* **52**, 50–55.
- Gaillard, J. L., Jaubert, F. & Berche, P. (1996). The *inlAB* locus mediates the entry of *Listeria monocytogenes* into hepatocytes *in vivo*. *J Exp Med* **183**, 359–369.
- Garandeau, C., Reglier-Poupet, H., Dubail, I., Beretti, J. L., Berche, P. & Charbit, A. (2002). The sortase SrtA of *Listeria monocytogenes* is involved in processing of internalin and in virulence. *Infect Immun* **70**, 1382–1390.
- Glomski, I. J., Gedde, M. M., Tsang, A. W., Swanson, J. A. & Portnoy, D. A. (2002). The *Listeria monocytogenes* hemolysin has an acidic pH optimum to compartmentalize activity and prevent damage to infected host cells. *J Cell Biol* **156**, 1029–1038.
- Glomski, I. J., Decatur, A. L. & Portnoy, D. A. (2003). *Listeria monocytogenes* mutants that fail to compartmentalize listeriolysin O activity are cytotoxic, avirulent, and unable to evade host extracellular defenses. *Infect Immun* **71**, 6754–6765.
- Goebel, W. & Khun, M. (2000). Bacterial replication in the host cell cytosol. *Curr Opin Microbiol* **3**, 49–53.
- Guzman, C. A., Rohde, M., Chakraborty, T., Domann, E., Hudel, M., Wehland, J. & Timmis, K. N. (1995). Interaction of *Listeria monocytogenes* with mouse dendritic cells. *Infect Immun* **63**, 3665–3673.
- Jacobs, T., Darji, A., Frahm, N., Rohde, M., Wehland, J., Chakraborty, T. & Weiss, S. (1998). Listeriolysin O: cholesterol inhibits cytolysis but not binding to cellular membranes. *Mol Microbiol* **28**, 1081–1089.

- Johansson, J., Mandin, P., Renzoni, A., Chiaruttini, C., Springer, M. & Cossart, P. (2002). An RNA thermosensor controls expression of virulence genes in *Listeria monocytogenes*. *Cell* **110**, 551–561.
- Jones, S. & Portnoy, D. A. (1994a). Characterization of *Listeria monocytogenes* pathogenesis in a strain expressing perfringolysin O in place of listeriolysin O. *Infect Immun* **62**, 5608–5613.
- Jones, S. & Portnoy, D. A. (1994b). Intracellular growth of bacteria. *Methods Enzymol* **236**, 463–467.
- Kuhn, M. & Goebel, W. (1989). Identification of an extracellular protein of *Listeria monocytogenes* possibly involved in intracellular uptake by mammalian cells. *Infect Immun* **57**, 55–61.
- Lety, M. A., Frehel, C., Dubail, I., Beretti, J. L., Kayal, S., Berche, P. & Charbit, A. (2001). Identification of a PEST-like motif in listeriolysin O required for phagosomal escape and for virulence of *Listeria monocytogenes*. *Mol Microbiol* **39**, 1124–1140.
- Lety, M. A., Frehel, C., Berche, P. & Charbit, A. (2002). Critical role of the N-terminal residues of listeriolysin O in phagosomal escape and virulence of *Listeria monocytogenes*. *Mol Microbiol* **46**, 367–379.
- Lety, M. A., Frehel, C., Beretti, J. L., Berche, P. & Charbit, A. (2003). Modification of the signal sequence cleavage site of listeriolysin O does not affect protein secretion but impairs the virulence of *Listeria monocytogenes*. *Microbiology* **149**, 1249–1255.
- Mackaness, G. B. (1962). Cellular resistance to infection. *J Exp Med* **116**, 381–406.
- Moors, M. A., Levitt, B., Youngman, P. & Portnoy, D. A. (1999). Expression of listeriolysin O and ActA by intracellular and extracellular *Listeria monocytogenes*. *Infect Immun* **67**, 131–139.
- Pamer, E. G. (1994). Direct sequence identification and kinetic analysis of an MHC class I-restricted *Listeria monocytogenes* CTL epitope. *J Immunol* **152**, 686–694.
- Pamer, E. G., Sijts, A. J., Villanueva, M. S., Busch, D. H. & Vijn, S. (1997). MHC class I antigen processing of *Listeria monocytogenes* proteins: implications for dominant and subdominant CTL responses. *Immunol Rev* **158**, 129–136.
- Park, S. F. & Stewart, G. S. (1990). High efficiency transformation of *Listeria monocytogenes* by electroporation of penicillin-treated cells. *Gene* **94**, 129–132.
- Portnoy, D. A., Jacks, P. S. & Hinrichs, D. J. (1988). Role of hemolysin for the intracellular growth of *Listeria monocytogenes*. *J Exp Med* **167**, 1459–1471.
- Renzoni, A., Cossart, P. & Dramsi, S. (1999). PrfA, the transcriptional activator of virulence genes, is upregulated during interaction of *Listeria monocytogenes* with mammalian cells and in eukaryotic cell extracts. *Mol Microbiol* **34**, 552–561.
- Rossjohn, J., Fell, S. C., McKinstry, W. J., Tweten, R. K. & Parker, M. W. (1997). Structure of a cholesterol-binding, thiol-activated cytolysin and a model of its membrane form. *Cell* **89**, 685–692.
- Roth, Z. (1961). A graphic probit method for the calculation of LD50 and relative toxicity. *Cesk Fysiol* **10**, 408–422.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). Expression of cloned genes in *Escherichia coli*. In *Molecular Cloning: a Laboratory Manual*, pp. 17.37–17.41. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schuerch, D. W., Wilson-Kubalek, E. M. & Tweten, R. K. (2005). Molecular basis of listeriolysin O pH dependence. *Proc Natl Acad Sci U S A* **102**, 12537–12542.
- Sijts, A. J., Neisig, A., Neefjes, J. & Pamer, E. G. (1996). Two *Listeria monocytogenes* CTL epitopes are processed from the same antigen with different efficiencies. *J Immunol* **156**, 683–692.
- Tweten, R. K. (1988). Cloning and expression in *Escherichia coli* of the perfringolysin O (theta-toxin) gene from *Clostridium perfringens* and characterization of the gene product. *Infect Immun* **56**, 3228–3234.
- Vazquez-Boland, J. A., Kuhn, M., Berche, P., Chakraborty, T., Dominguez-Bernal, G., Goebel, W. & Gonzalez-Zorn, B. (2001). *Listeria* pathogenesis and molecular virulence determinants. *Clin Microbiol Rev* **14**, 584–640.
- Vijn, S. & Pamer, E. G. (1997). Immunodominant and subdominant CTL responses to *Listeria monocytogenes* infection. *J Immunol* **158**, 3366–3371.
- Vijn, S., Pilip, I. M. & Pamer, E. G. (1999). Noncompetitive expansion of cytotoxic T lymphocytes specific for different antigens during bacterial infection. *Infect Immun* **67**, 1303–1309.
- Villanueva, M. S., Sijts, A. J. & Pamer, E. G. (1995). Listeriolysin is processed efficiently into an MHC class I-associated epitope in *Listeria monocytogenes*-infected cells. *J Immunol* **155**, 5227–5233.
- Yellen-Shaw, A. J. & Eisenlohr, L. C. (1997). Regulation of class I-restricted epitope processing by local or distal flanking sequence. *J Immunol* **158**, 1727–1733.
- Yellen-Shaw, A. J., Wherry, E. J., Dubois, G. C. & Eisenlohr, L. C. (1997). Point mutation flanking a CTL epitope ablates in vitro and in vivo recognition of a full-length viral protein. *J Immunol* **158**, 3227–3234.