LISTERIOLYSIN O FROM

LISTERIA MONOCYTOGENES

Camilla Giammarini[1] and Mauro Magnani[2]

[1] Diatheva; [2] Centre for Biotechnology, University of Urbino, Italy.

Listeria monocytogenes

Listeriolysin O is a major virulence factor produced by the Gram-positive bacterium *Listeria monocytogenes*. The genus *Listeria* belongs to the *Clostridium* subbranch together with *Staphylococcus*, *Streptococcus*, *Lactobacillus,* and *Brochothrix* (Rocourt J. and Cossart P., 1997). This genus comprises six species (*L. grayi, L. innocua, L. ivanovii, L. monocytogenes, L. murrayi, L. seeligeri, L. welshimeri*) subdividing in 16 serotypes not species-specific (Cooper J. and Walker R. D., 1998). *L. ivanovii* and *L. monocytogenes* are the unique virulent species, but only the last is a public health concern causing a food-borne disease named listeriosis. There are 13 *L. monocytogenes* serovars which can cause disease, but 95% of human isolates belongs to 3 serovars: 1/2a, 1/2b, and 4b. Serovar 4b strains are responsible for 33 to 50% of sporadic human cases, while bacteria recovered from food mostly belong to serogroup 1/2 (Rocourt J. and Cossart P., 1997).

Human listeriosis

Listeriosis is an uncommon illness in the general population but, because of the severity of clinical signs and the high fatality rate, the medical community attention is focusing on this disease as an emerging public health problem (Gellin B. G. and Broome C. V., 1989). The majority of human infections are subclinical or characterized by mild clinical signs. In healthy individuals, consumption of contaminated food usually causes gastroenteritis, with febrile symptoms. On the contrary, *L. monocytogenes* can cause a fatal disease especially in some groups including neonates, pregnant women, the elderly, immunosuppressed transplant recipients, and other patients with impaired cell-mediated immunity (Gray M. L. and Killinger A. H., 1966; Schuchat A. et al., 1991). *L. monocytogenes* has tropism mainly for central nervous system and the most frequently observed clinical syndromes are meningitis, meningoencephalitis, and abscess formation. Sepsis is another common listeriosis form that affects prevalently immunocompromised patients rather than other hosts. Besides these systemic diseases, the bacterium causes also localized infections, such as conjunctivitis, skin infections, and lymphadenitis, with a minor population incidence (Doganay M., 2003). In pregnant women the infection of the mother can be asymptomatic or characterized by flulike illness, while the consequences for the fetus or infant are more serious including abortion, stillbirth, and neonatal meningitis or sepsis (Gellin B. G. and Broome C. V., 1989).

Animal listeriosis

Among animals, listeriosis affects especially cattle, sheep, and goats with an incubation time that varies from 2 to 6 weeks. Animals become infected by ingestion of contaminated food, particularly poor-quality silage, by pathogen inhalation or by direct contact. The pathology occurs mostly following a reduced resistance to infection caused by stress factors (i.e. pregnancy, overcrowding, transportation, and poor environmental conditions) or an exposition to very

high concentrations of the organism (Cooper J. and Walker R. D., 1998). Animals affected by listeriosis are usually febrile, anorexic, depressed, and disoriented.

The pathology may develop in different clinical forms:

- Neural (circling disease), as encephalitis and meningitis. The clinical signs include dullness, turning or twisting the head to one side, walking in circles and head pressing against solid objects (Czuprynski C. J., 1993). These manifestations are due to the formation of microabscesses in the medulla, pons, anterior spinal cord, and in the cerebellum. There may also be unilateral facial nerve paralysis, excessive salivation, and torcicollis (Dennis S. M., 1993).
- Visceral, as septicemia. It occurs primarily in birds or fetuses and neonate animals as an extension of uterine infections. The infection develops in hepatic necrosis with pin-point greyish-white lesions that could spread to the spleen (Cottral G. E., 1978).
- Reproductive, as abortions and stillbirths. This form occurs most commonly in ruminants. Bacteria reach the uterus via the vascular bed and abortions generally occur 5 to 12 days after placenta colonization (Cooper J. and Walker R. D., 1998).
- Mammary infection, as mastitis. This form usually causes subclinical infection with no evidence of mammary swelling. The excretion of the organism in milk is persistent throughout lactation increasing the risk of contamination of milk products (Fthenakis G. C. et al., 1998).
- Iritis and ketoconjunctivitis are less common (Cooper J. and Walker R. D., 1998).

Epidemiology

L. monocytogenes has unique physiological characteristics that allow the bacterium survival in different environmental conditions. It grows at very low temperature (2 to 4°C), can survive at -18°C and even at repeated freezing. The bacterium tolerates temperature up to 44°C even though its optimal growing temperature is 30 to 37°C. *L. monocytogenes* prefers neutral environmental conditions but can survive in a wide pH range, from pH 9.6 to pH 4.5. Moreover, being a facultative anaerobe and a halophilous, its multiplication is not prevented by oxygen devoid conditions and high NaCl concentration (up to 20%) (Rocourt J. and Cossart P., 1997).

The bacterium is able to survive and grow in soil and water, can attach to various kinds of surfaces (such as stainless steel, glass, and rubber) and has been found in meat and dairy processing plants.

A high percentage of animals are healthy carriers, besides *L. monocytogenes* is part of the normal flora of the distal intestinal tract of numerous animal species. There are also evidences that the use of silage in animal feeding had increased the incidence of listeriosis (Rocourt J. and Cossart P., 1997; Cooper J. and Walker R. D., 1998).

L. monocytogenes is present in a wide variety of foods, both raw and processed. Milk and dairy products are often involved in listeriosis outbreaks and, among these, soft cheeses are most frequently contaminated. A wide variety of meats and meat products, such as beef, pork, ham, smoked and fermented sausages have been associated with Listeria contamination. The pathogen is often present in high numbers on raw

and ready-to-eat seafood and fish products (Rocourt J. and Cossart P., 1997).

L. monocytogenes was first isolated from an epidemic disease in rabbits in 1926 (Murray E. G. D. et al., 1926) and in 1929 was described the first listeriosis case in man (Low J. C. and Donachie W., 1997). It's only in the 1980s that contaminated food began to be considered a primary vehicle of transmission of listeriosis (McLauchlin J., 1996). In 1981 in Canada a foodborne outbreak involved 41 patients and was probable caused by cabbages fertilized with manure from infected sheep (Schlech W. F. et al., 1983). In Boston 49 people contract listeriosis by consumption of insufficient pasteurized milk, in 1983 (Fleming D. W. et al., 1985). The largest outbreak in North America occurred in California in 1985, was traced to a soft cheese and involved 142 individuals (Linnan M. J. et al., 1988). Another contaminated cheese caused 122 listeriosis cases in Switzerland (Bille J., 1989), while in United Kingdom a patè was responsible for 300 listeric infections (McLauchlin J. et al., 1991). In France several outbreaks occurs in the 1990s caused by contaminated pork tongue, potted pork, and soft cheese (Jacquet C. et al., 1995; Goulet V. et al., 1998; Goulet V. et al., 1995). In the last years a smoked fish and a butter were involved in listeriosis cases in Sweden and Finland respectively (Tham W. et al., 2000; Lyytikainen O. et al., 2000).

The cell biology of *Listeria monocytogenes* infection

L. monocytogenes is regard to be one of the most invasive bacterium known, owing to its ability to penetrate into, survive and multiply inside cells with or without phagocytic activity. Infection usually occurs by ingestion of contaminated food. *L. monocytogenes* cross the intestinal barrier without preference for enterocytes or M cells as translocation site. Bacteria are then internalized by resident macrophages, usually in the phagocytic cells underlying the Peyer's patches. Via the lymph and the blood route, pathogens reach the spleen and the liver in which most of them are rapidly killed. When the infection control is less efficient, such as in immunocompromised patients and pregnant women, bacteria can survive and disseminate hematogeneously from hepatocytes to brain and placenta (Rocourt J. and Cossart P., 1997; Cossart P. and Lecuit M., 1998; Cossart P., 2002).

Host cell invasion starts by the interaction with the cytoplasmic membrane which progressively enwraps the bacterium (Fig. 1). Entry into cells involves several sets of bacterial and cell-surface components acting together to mediate adherence and entry. The two major bacterial invasion proteins are internalin A (InlA) and internalin B (InlB). They have different host cell specificities. InlA (also called internalin) is an 800-amino-acid protein characterized by a C-terminal hydrophobic region that serves as an anchor to peptidoglycan, and a N-terminal leucine-rich repeats region to interact with host cell surface. In particular, InlA gets in touch with E-cadherin, a protein placed on epithelial cells and involved in cells adhesion (Vazquez-Boland J. A. et al., 2001; Cossart P. and Bierne H., 2001). In contrast to internalin, InlB allows entry into a wide variety of cell types:

epithelial cells, fibroblasts, hepatocytes and endothelial cells. This is a 630-amino-acid protein with a leucine-rich repeats region, as the previous molecule, but it is loosely attached on the bacterial surface, so it could be also found in culture supernatants. Interaction of InlB with host cell is not still completely clarified but seems to involve the surface molecules gC1q-R and Met receptor tyrosine kinase (Braun L. et al., 2000; Shen Y. et al., 2000).

Following internalization, *L. monocytogenes* is inglobated into a host phagosomal vacuole, from which the bacterium escapes thanks to the secretion of listeriolysin O (LLO). LLO is a cytolytic toxin that forms pores in vacuolar membrane causing a passive flux of ions and macromolecules. As consequence, in a short time, vacuole is lysed and bacterium is released into the cytosol (Fig. 1). In this process two other molecules have a minor role: a phosphatidyl-inositol phospholipase C (PI-PLC) and the ATPase ClpC (Cossart P. and Lecuit M., 1998).

Once in the host cell cytosol, *L. monocytogenes* begins to multiply with a doubling time of about 1 hour and becomes progressively covered by actin filaments that rearrange to form a polarized "comet tail". The polymerization of globular actin (G-actin) into filamentous actin (F-actin) is induced by the bacterial protein ActA. This is a surface protein that can be artificially divided into three regions: a highly charged N-terminus; a central domain made of proline-rich repeats and a C-terminus that anchors the protein to the bacterial surface. The central domain interacts with the VASP protein that induces actin polymerization, together with the molecule profilin. Actin filaments are bound to the N-terminus of ActA by a complex that contains Arp2 and Arp3 proteins. Because of the polar distribution of ActA on the bacterial surface, the continuous monomer assembly causes the microrganism movement in the opposite direction towards the polymerization site (Fig. 1) (Cossart P. and Lecuit M., 1998).

When bacteria reach the plasma membrane, the host cell puts out long protrusions (named listeriopods), each with a bacterium inside (Fig. 1). These protrusions are then internalized by a neighbour cell, giving rise to a two-membrane vacuole. Bacterial escape from the secondary vacuole is performed by a lecithinase (PC-PLC) acting together with LLO (Gedde M. M. et al., 2000; Cossart P. and Bierne H., 2001). Thus Listeria can disseminate in tissues by a cell-to-cell spreading mechanism, circumventing host defences such as circulating antibodies and complement (Cossart P. and Lecuit M., 1998).

The genes encoding the bacterial factors involved in the different steps of the infectious process are clustered into two regions of the Listeria chromosome. *plcA*, *hlyA*, *actA* and *plcB* (encoding for the proteins PI-PLC, LLO, ActA and PC-PLC respectively) are collected in a 10 kb genetic island, absent from the non pathogenic species. The *inlAB* operon (encoding for InlA and InlB) is placed in another chromosomal region. The virulence genes are under the either absolute or partial control of a pleiotropic activator protein PrfA and their expression is modulated by the same environmental conditions, being repressed at low temperatures and maximally expressed at 37°C (Rocourt J. and Cossart P., 1997; Cossart P. and Lecuit M., 1998).

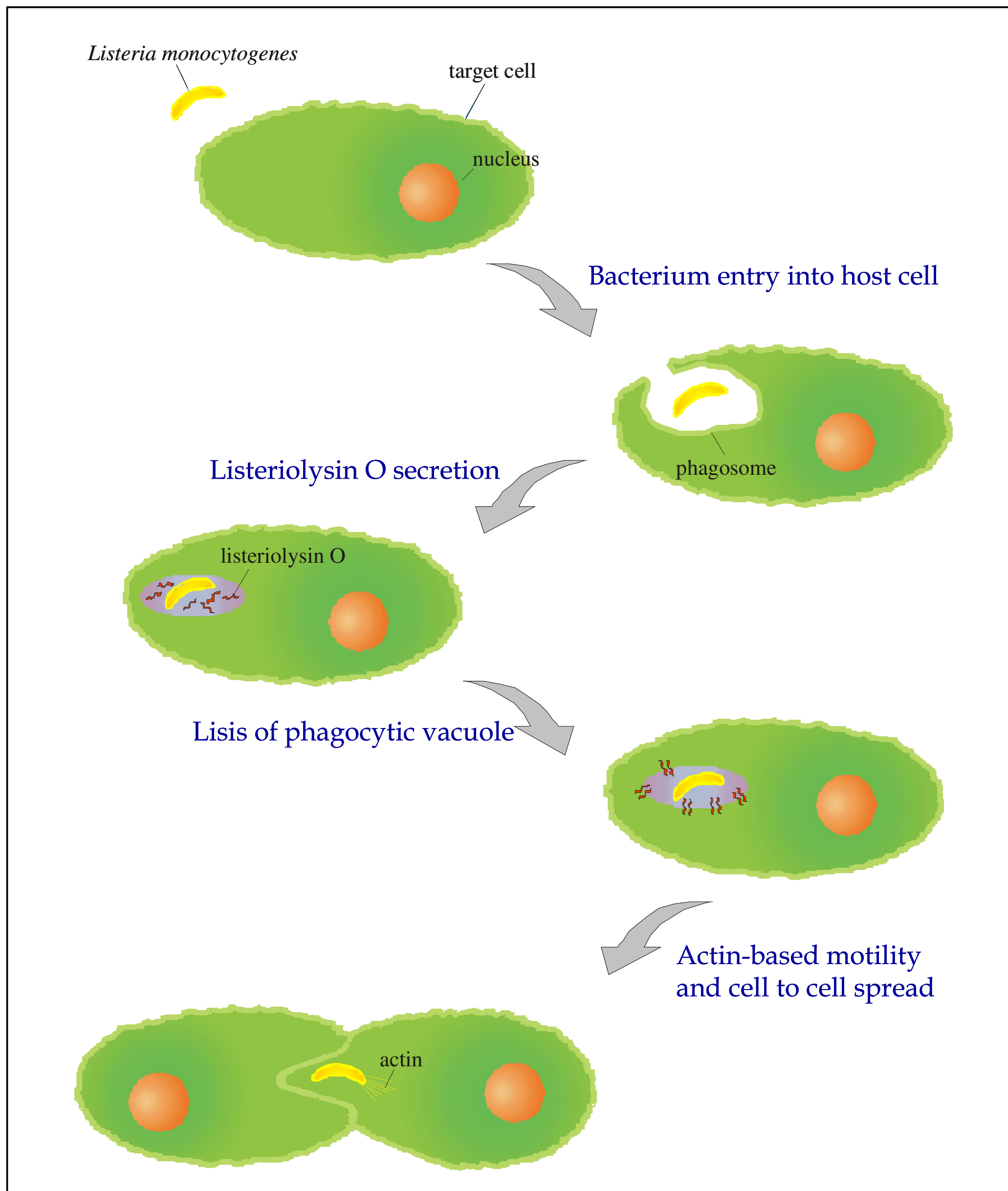

Fig. 1 - Different steps of *Listeria monocytogenes* cellular infectious process.

Listeriolysin O

Listeriolysin O (LLO) is the virulence factor that allows the *L. monocytogenes* escape from the primary phagocytic vacuole. It's a 58.6 kDa protein formed by a single polypeptide chain of 529 amino acids. The N-terminal sequence presents the characteristics of a typical Gram-positive bacterium signal sequence: the first residues, hydrophilic and positively charged, are followed by about 20 hydrophobic residues. This sequence directs the protein to the secretion pathway and is excised during the process by a cleavage after lysine 25. As consequence the secrete LLO begins from amino acid 26, is a 504-amino-acids long protein and has a molecular weight of 55.8 kDa (Mengaud J. et al., 1988).

LLO belongs to the family of thiol-activated cholesterol binding toxins produced by most of Gram-positive bacteria. Streptolysin O from *Streptococcus pyogenes* is the well characterized protein of this group, in which there are also pneumolysin from *Streptococcus pneumoniae*, perfringolysin O from *Clostridium perfringens*, cereolysin O from *Bacillus cereus*, alveolysin from *Bacillus alvei*, etc. The lytic activity of these toxins is enhanced by reducing agents and is suppressed by oxidation and by exposure to cholesterol or anti-streptolysin O antibodies (Palmer M., 2001). The thiol-activated cytolysins have a single cysteine in the C-terminal region that renders them susceptible of inactivation by oxidation, although this cysteine may be replaced with alanine without loss of lytic function (Pinkney M. et al., 1989). They form discrete pores with an internal diameter of up to 30 nm in cholesterol containing membranes. Their mechanism of action is based on interaction with cholesterol that functions not only as a binding site but also as allosteric effector, promoting the oligomerization of 20-80 toxin monomers into ring and arc-like structure (Palmer M. et al., 1998; Portnoy D. A. and Jones S., 1994).

LLO shows a peculiar mechanism of interaction with cholesterol in solution: the protein is inactivated by cholesterol but, in spite of the other toxins, retains the ability to bind membranes (Jacobs T. et al., 1998). 1 HU of LLO is inhibited by 0.2 ng of cholesterol, while other sterols such as epicholesterol and dehydroepiandrosterone are weaker inhibitor. The oxidants $HgCl_2$ and *p*-chloromercuribenzoate inactivate the toxin at 1 mM concentration. Inhibition by the mercurials is reversed by 2 mM dithiothreitol or cysteine. 2 mM iodacetic acid, 2 mM iodacetamide, 1 mM tosyllysine chloromethyl ketone and 1 mM tosylphenylalanine chloromethyl ketone are not inhibitory (Geoffroy C. et al., 1987).

The hemolytic activity of LLO is related to environmental pH: it's maximum at pH 5.5 and is almost entirely inactivated at pH 7.0. In contrast, the other thiol-activated toxins have a wider pH range of activity, with an optimum at higher pH values: pH 6.0 for pneumolysin, pH 6.5 for perfringolysin O and alveolysin, pH 7.0 for streptolysin O (Geoffroy C. et al., 1987). These findings could be explained with the role performes by LLO during the *L. monocytogenes* infection process. After phagocytosis, the environment of bacterium-containing vacuole acidifies activating the LLO. The toxin promotes vacuole lysis and bacterium release in the cytosol where a higher environmental pH reduces LLO activity (Glomski I. J. et al., 2002). Host cell membrane damage is also prevented by a rapid LLO degradation in the cytosol due to recognition of a PEST-like sequence. An amino acid sequence rich in proline (P), glutamic acid (E), serine (S) and threonine (T) is named PEST and targets proteins to degradation. In LLO N-terminus is located a region of 19 amino acids (32 to 50) showing these characteristics (Decatur A. L. and Portnoy D. A., 2000). Interestingly the deletion of the PEST-like motif not only enhances the toxicity of LLO, but also modifies the bacterial virulence. Mutant *L. monocytogenes*, lacking the LLO PEST sequence, are found to escape from the phagocytic vacuole in a difficult way suggesting that this region is essential also for the membrane disruption process (Lety M.-A. et al., 2001).

Analyzing the entire coding region of *hlyA* gene of different *L. monocytogenes* strains (GenBank AF253320; AL591974; M24199; U25443; U25446; U25449; U25452; X15127; X60035), a minimum identity of 97% at the nucleotide and of 99% at the amino acid level is revealed (Fig. 2). The majority of amino acid substitutions are found to be located at the N-terminus, into the PEST-like sequence (Fig. 2).

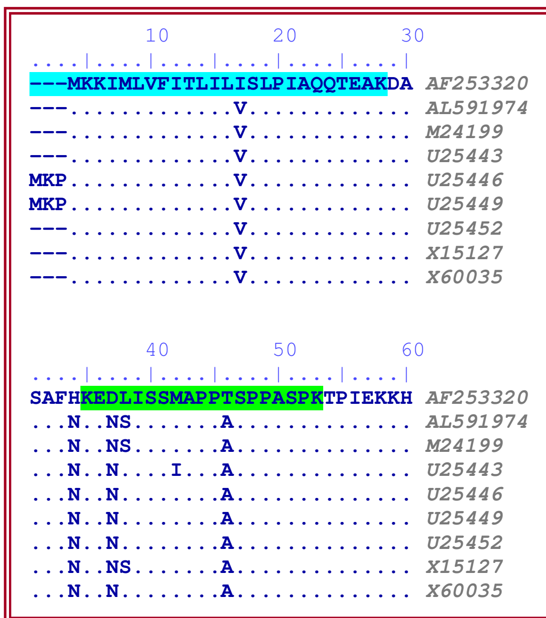

Fig. 2 - Alignment of first 60 amino acids of LLO sequence from different *L. monocytogenes* strains. Sequences are indicated with the GenBank accession number on the right. The secretion signal sequence is highlighted in light blue and the PEST-like sequence is highlighted in green. Modified from Giammarini C. et al. (2003).

A PEST sequence could be identified by the PEST-FIND program (Rechsteiner M. and Rogers S. W., 1996) that produces a score ranging from –50 to +50: a score greater than +5 defines an interesting PEST-like sequence. Submitting to the program the LLO PEST-supposed sequence of different bacterial strains, the maximum score (7.15) is obtained for the GenBank AF253320 one, corresponding to the *hlyA* gene of the ATCC 9525 *L. monocytogenes* strain.

The amino acid sequence of LLO shows also homologies with those of invanolysin (from *L. ivanovii*), perfringolysin O and streptolysin O along the whole sequence. In particular it's noteworthy that there is a high conserved region of 11 amino acids around the unique cysteine (Fig. 3).

Thanks to its low optimal pH activity, LLO is a favourable candidate as a vehicle for cytosolic drug delivery. Most of the therapeutic agents have their action sites in the cytosol or nucleus but their free diffusion into the cells is often obstructed by the plasma membrane. To avoid this obstacle, drugs can be combined to delivery system such as liposomes, inactivated bacterial cells or parts of them and viruses or viral components. Even so the use of such drug-carrier complexes often fails since endocytosis could lead to degradation of the internalized material. New strategies are now under consideration to circumvent the degradative pathway. One of them is the association of existing delivery vehicles, such as liposomes, with a bacterial hemolysin that allows the escape of internalized material into the cytosol (Provoda C. J. and Lee K.-D., 2000). Among bacterial cytolysins, LLO is the most successful one used in delivery experiments. As purified protein it has been encapsulated inside liposomes along with other molecules such as fluorescent dye (Lee K.-D. et al., 1996), ovalbumin (Lee K.-D. et al., 1996) and antisense nucleotides (Mathew E. et al., 2003). LLO is also been expressed in a wide variety of live bacterial vaccine strains promoting the access to the cytosol for bacteria, single

```
Listeriolysin O    1   DASAFHKEDLISSMAPPTSPPASPKTPIEKKHADEIDKYIQGLDYNKNNVLVYHGDAVTN   60
Ivanolysin O      25   ...VYSYQGI..H....A....K.K..V...N.AQ..Q......D...I...D.E..K.   84
Perfringolysin O   8                                      ..SG.SS.S..R.E..ASN..KIES   62
Streptolysin O   102                              .ED.TE..NDK.YS.N..ELE..AKN.ETIE..  133

Listeriolysin O   61   VPPRKGYKDGNEYIVVEKKKKSINQNNADIQVVNAISSLTYPGALVKANSELVENQPDVL  120
Ivanolysin O      85   ...KA...E..Q...................I.SLA.......................  144
Perfringolysin O  63   FV.KE.K.A..KF....RQ.R.LTTSPV..SIIDSVNDR......QL.DKA....R.TI.  122
Streptolysin O   134   FV.KE.V.KADKF..I.R...N..TTPV..SIIDSVTDR...A..QL..KGFT..K..AV  193

Listeriolysin O  121   PVKRDSLTLSIDLPGMTNQDNKIVVKNATKSNVNNAVNTLVERWNEKYAQAYPNVSAKID  180
Ivanolysin O     145   ......V.........V.H..E...Q......I.DG.....D...N..SEE...I.....  204
Perfringolysin O 123   M...KPININ.....LKGE-.S.K.DDP.YGK.SG.IDE..SK.....SSTH-TLP.RTQ  180
Streptolysin O   194   VT..NPQKIH......GDKAT-VE.NDP.YA..ST.IDN..NQ.HDN.SGG-NTLP.RTQ  251

Listeriolysin O  181   YDDEMAYSESQLIAKFGTAFKAVNNSLNVNFGAISEGKMQEEVISFKQIYYNVNVNEPTR  240
Ivanolysin O     205   ..Q.........V....A...................V.....N......T.......S  264
Perfringolysin O 181   .SES.V..K..ISSALNVNA.VLE...G.D.N.VANNEKKVMILAY...F.T.SADL.KN  240
Streptolysin O   252   .TES.V..K..IE.ALNVNS.ILDGT.GID.KS..K.EKKVMIAAY...F.T.SA.L.NN  311

Listeriolysin O  241   PSRFFGKAVTKEQLQALGVNAENPPAYISSVAYGRQVYLKLSTNSHSTKVKAAFDAAVSG  300
Ivanolysin O     265   .......S....N.........................DIFV....S....R........FK.  324
Perfringolysin O 241   ..DL.DDS..FND.KQK..SN.A..LMV.N.....TI.V..E.T.S.KD.Q...K.LIKN  300
Streptolysin O   312   .ADV.D.S..FKE..RK..SN.A..LFV.N.....T.FV..E.S.K.ND.E...S..LK.  371

Listeriolysin O  301   KSVSGDVELTNIIKNSSFKAVIYGGSAKDEVQIIDGNLGDLRDILKKGATFNRETPGVPI  360
Ivanolysin O     325   ...K..T..E...Q.A...............E....D.SK......Q..N.DKKN.....  384
Perfringolysin O 301   TDIKNSQQYKD.YE....T..VL..D.QEHNKVVTKDFDEI.KVI.DN...STKN.AY..  360
Streptolysin O   372   TD.KTNGKYSD.LE....T..VL..D.AEHNKVVTKDFDVI.NVI.DN...S.KN.AY..  431

Listeriolysin O  361   AYTTNFLKDNELAVIKNNSEYIETTSKAYTDGKINIDHSGGYVAQFNISWDEINYDPEGN  420
Ivanolysin O     385   ..........Q...V...............S.....L....A...R..VT...VS..AN..  444
Perfringolysin O 361   S..SV.....SV.AVH.KTD......TE.SK....L....A.....EVA...VS..K...  420
Streptolysin O   432   S..SV...N.KI.GVN.RT..V....TE..S....LS.Q.A....YE.L.......DK.K  491

Listeriolysin O  421   EIVQHKNWSENNKSKLAHFTSSIYLPGNARNINVYAKECTGLAWEWWRTVIDDRNLPLVK  480
Ivanolysin O     445   .V.E..K....D.D......T............IH..............V.........  504
Perfringolysin O 421   .VLT..T.DG.YQD.T..YSTV.P.EA.....RIK.R...........D..SEYDV..TN  480
Streptolysin O   492   .VITKRR.DN.WY..TSP.STV.P.GA.S...RIM.R...........K...E.DVK.S.  551

Listeriolysin O  481   NRNISIWGTTLYPKYSNSVDNPIE  504
Ivanolysin O     505   ...VC........A..DT.....K  528
Perfringolysin O 481   .I.V.........             493
Streptolysin O   552   EI.VN.S.S..S.             564
```

Fig. 3 - Alignment of amino acid sequences of some thiol-activated toxins. Numbers to the right and left of the sequences indicate the amino acid position. In yellow is highlighted a high conserved region around the unique cysteine.

protein antigens or DNA vaccine (Dietrich G. et al., 2001).

Recombinant LLO

LLO could be isolated and purified from *L. monocytogenes* culture medium. Unfortunately this method requires high culture volumes, is time-consuming and provides very low yields. Until now yields of 22.0, 13.2 or 52.0 μg of purified protein from litre of starting bacterial culture, have been reported in literature (Geoffroy C. et al., 1987; Traub V. H. and Bauer D., 1995; Baetz A. L. et al., 1995). As consequence, studies on the peculiar LLO molecular mechanism of action and protein employment in pharmaceutical application, are very difficult. A way out to the problem has been found expressing the protein in *Escherichia coli*. Significant amount of LLO was obtained by the use of the pET expression system together with a simple purification procedure. To have a recombinant LLO exactly alike to the

natural one, the *hlyA* gene of *L. monocytogenes* was cloned lacking the first 75 nt that code for the amino acid signal sequence for secretion. Thus the recombinant expressed protein started from amino acid 26 (amino acid sequence deduced from GenBank AF253320) with the addition of a methionine at the N-terminus. In the pET system the expression of the exogenous gene is carried out by the bacteriophage T7 RNA polymerase that is inducible adding IPTG to cells growing cultures. First of all the induction conditions were optimized in order to obtain the expression of the largest amount of recombinant protein in a soluble and active form. In particular, the influence of length and temperature of induction, the bacterial density and the IPTG concentration were examined. In the *E. coli* induced cells the toxin was expressed in the cytoplasmic compartment, therefore, to purify the recombinant LLO it was necessary to lyse the induced bacteria and to recover the proteins in soluble form by centrifugation. In a typical induction experiment, starting from 1 litre of induced bacterial cells the cell-free extract had a specific activity of approximately 5.6 x 10^4 HU/mg of protein corresponding to 4.0 x 10^6 HU/g of cell mass (Giammarini C. et al., in press). The high-level expressed recombinant LLO was purified to homogeneity by an adsorption chromatography on hydroxyapatite followed by an ion-exchange chromatography on SP Sepharose (Giammarini C. et al., 2003). In the first chromatographic step some problems occurred due to the compression of the matrix. These difficulties were overcome applying the sample to the matrix in batch and then pouring the mixture in a wide column. It was also necessary to wash the column with a high volume of buffer to desorb all the unbound proteins. After LLO elution, the fractions with hemolytic activity were pooled. The proteins were then concentrated by ammonium sulfate precipitation and reequilibrated in a suitable buffer. With this purification step, the specific activity increased twice, reaching 1.1 x 10^5 HU/mg of protein (Giammarini C. et al., 2003). The sample was then applied on the ion-exchange chromatography and, after washing, desorption was carried out with a linear salt gradient. Fractions with hemolytic activity were displayed on SDS-PAGE and those showing a single polypeptide chain of expected molecular weight, were pooled and concentrated by ultrafiltration. The final average yield was 4.5 mg of protein/l of cell culture with a hemolytic activity of 1.2 x 10^6 HU/mg of protein. The concentrated product appeared as a single polypeptide chain of 56 kDa on SDS-PAGE analysis, immunologically pure in terms of immunoblot reactivity with sheep immune serum (Fig. 4) (Giammarini C. et al., 2003).

This recombinant LLO was characterized and its properties were compared with those of the native one. No difference was detected in the hemolytic activity, in fact in both cases 1 HU corresponded to about 1 ng of protein (Geoffroy C. et al., 1987; Giammarini C. et al., 2003). The effect of pH on hemolytic activity was verified testing the toxin at pH ranging from 5.0 to 8.5. As expected, the hemolysin showed its maximum activity at an acidic pH, which is at pH 5.5, and the hemolytic activity rapidly decreased when the pH was raised to basic values. At neutral pH it was about 30% of maximum value and became almost irrelevant at pH values greater than 8 (Fig. 5/A). Experiments

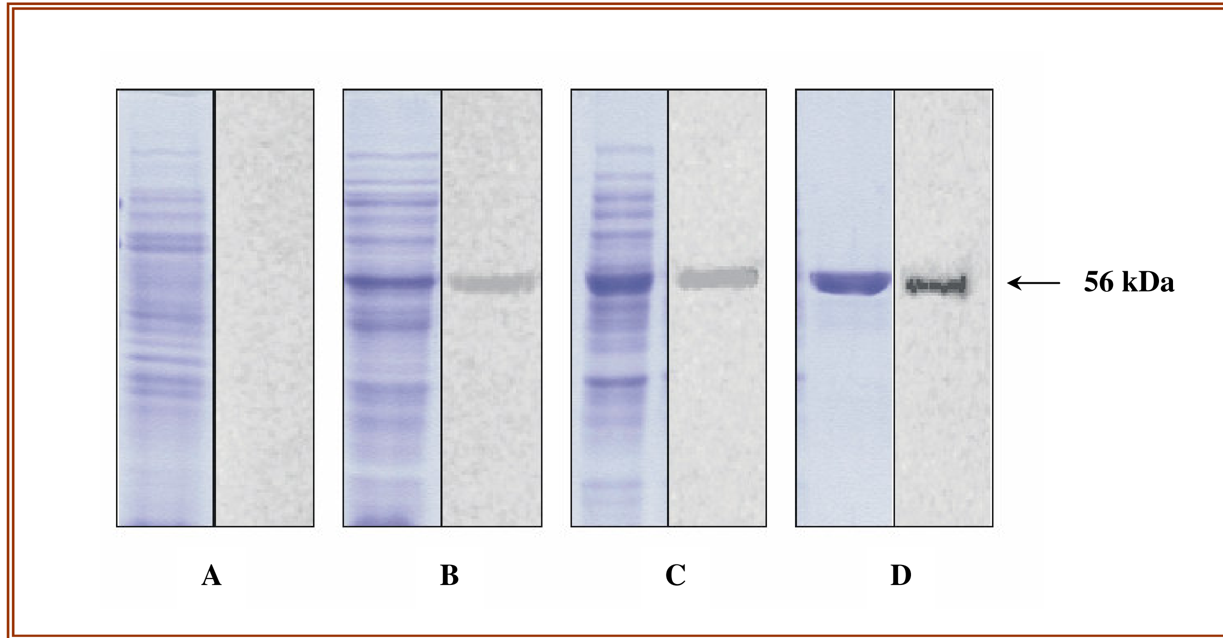

Fig. 4 - SDS-PAGE (in blue) and Immunochemical detection (in grey) of recombinant listeriolysin O at different purification steps. (**A**) 10 µg of total post-induction cell proteins of bacterial cells transformed with the expression plasmid lacking the *hlyA* gene; (B) 13 µg of total post-induction cell proteins of bacterial cells transformed with the expression plasmid with the *hlyA* gene; (C) 15 µg of proteins concentrated by ammonium sulfate precipitation and dialyzed before loading onto ion exchange column; (D) 3.3 µg of the sample at the end of the purification procedure. Modified from Giammarini C. et al. (2003).

were also made to investigate if the purified protein showed the usual properties of the thiol-activated toxins, such as hemolytic activity inhibition by oxidation and by exposure to cholesterol. The assays were performed incubating the recombinant LLO with various concentrations of specific reagents and then determining the hemolytic activity. Cholesterol caused the suppression of the recombinant LLO hemolytic activity to the extent of 20 ng of cholesterol for 1 HU (Fig. 5/B). In the same way the hemolysin was inactivated in consequence of reaction with 1.7 µM of $HgCl_2$ or *p*-chloromercuribenzoate (Fig. 5/C) and in both cases the hemolytic activity was fully restored by 2 mM dithiothreitol (Giammarini C. et al., 2003).

Since LLO is a major virulence factor and is not found in other Listeria species (Berche et al., 1990; Bourry and Poutrel, 1996), this toxin represents a suitable antigen for the development of new serodiagnostic tests. Antibodies to LLO have been already detected in the serum of goats (Bourry A. et al., 1997), sheep (Lhopital S. et al., 1993), lambs (Low J. C. and Donachie W., 1991), cows (Bourry A. and Poutrel B., 1996) and humans (Berche P. et al., 1990) by Western blot, dot-blot or ELISA analysis. Detection of anti-LLO antibodies in humans has been proved to be particularly useful for listeriosis diagnosis especially when bacteria cannot be isolated from clinical specimens, owing to the intermittent presence in blood or the inaccessible foci of bacterial replication (Gaillard J. L. et al., 1992).

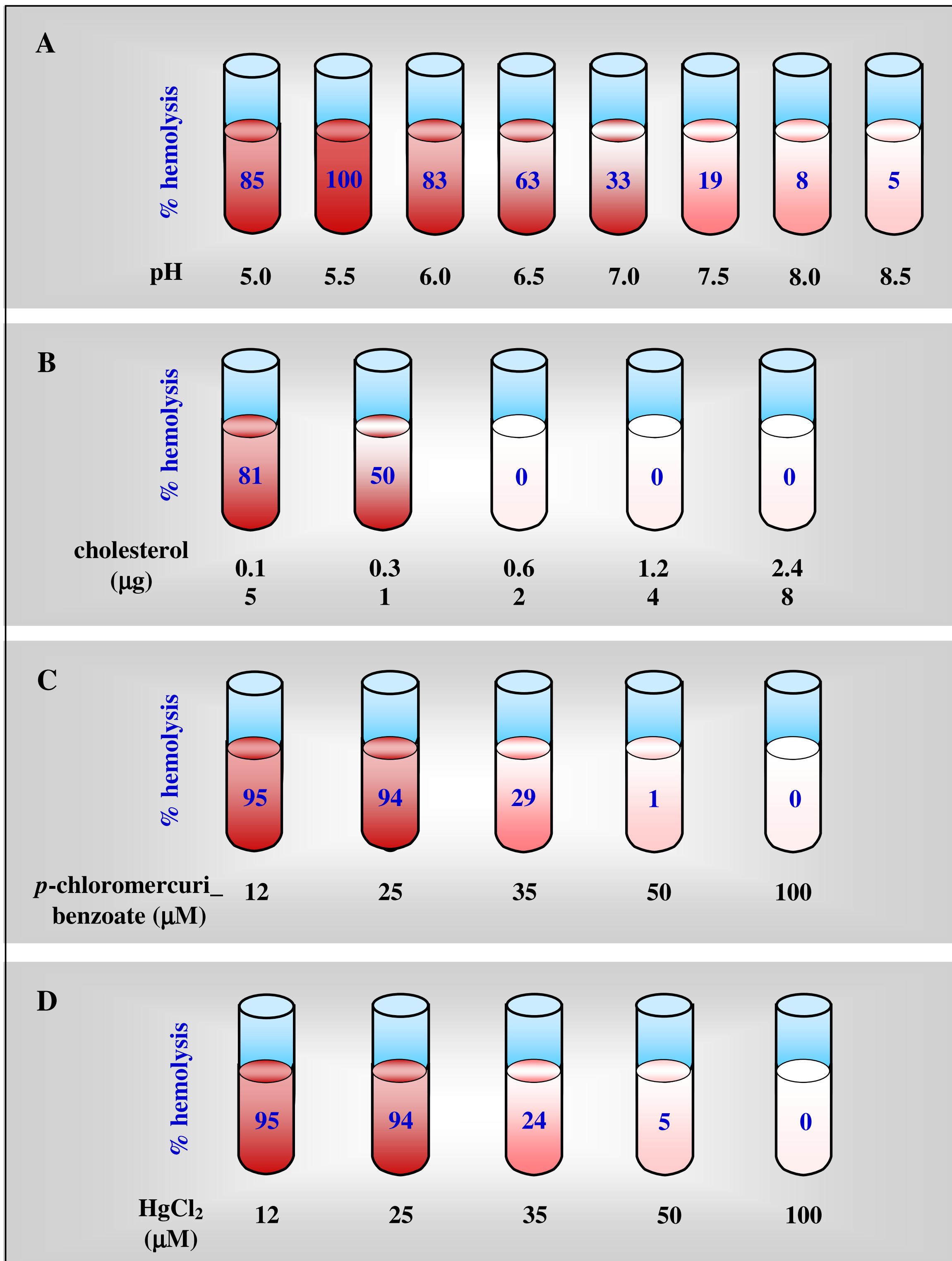

Fig. 5 - Properties of recombinant LLO. Effects of pH **(A)**, cholesterol **(B)**, *p*-chloromercuribenzoate **(C)**, and $HgCl_2$ on hemolytic activity measured as percentage of hemolysis. In all the assays the total activity was 30 HU. Modified from Giammarini C. et al., in press.

At the moment, the most common tests used, both in human and in animal diagnosis, are bacterial isolation or the serum agglutination test. The first method is laborious and time-consuming, while

the second is not specific because of cross-reactivity between *L. monocytogenes* and other Gram-positive bacteria. It is plain that *L. monocytogenes* infection diagnosis could be improved by a simple and rapid diagnostic test, such as an enzyme-linked immunosorbent assay (ELISA), but the low yields of LLO obtained from *L. monocytogenes* cultures had prevented the production of immunodiagnostic tests on a large scale till now (Baetz A. L. and Wesley I. V., 1995). Considering this, an indirect ELISA based upon the recombinant purified hemolysin was developed using serum of a sheep experimentally infected with *L. monocytogenes* (Giammarini C. et al., in press). Antigen coating and test conditions were optimized comparing data obtained from uninfected and infected serum at different experimental conditions. The optimal antigen concentration was determined by a checkerboard titration in which two-fold LLO dilutions were tested against serial serum dilutions. This method allowed to select the LLO working concentration that, at the same time, minimized the background and gave a reliable analytical signal. Moreover the binding ratios relating the infected and uninfected serum titrations were evaluated. The same criteria were applied to establish the enzyme-linked antibody working dilution. In order to obtain the maximum binding of the antigen to the plate, the coating step was also performed using LLO dissolved in different buffers and incubating the plate at various temperatures. At the end, the optimization of experimental conditions allowed to select the best sensitivity conditions (Giammarini C. et al., in press). These results suggest the possible application of the recombinant LLO for large-scale production of immunodiagnostic tests for listeriosis detection at least in sheep and likely also in other species.

REFERENCES

- **Baetz A. L. and Wesley I. V.** (1995) Detection of anti-listeriolysin O in dairy cattle experimentally infected with *Listeria monocytogenes*. *J. Vet. Diagn. Invest.* 7: 82-86.
- **Baetz A. L., Wesley I. V., and Stevens M. G.** (1996) The use of listeriolysin O in an ELISA, a skin test and a limphocyte blastogenesis assay on sheep experimentally infected with *Listeria monocytogenes*, *Listeria ivanovii* or *Listeria innocua. Vet. Microbiol.* 51: 151-159.
- **Berche P., Reich K. A., Bonnichon M., Beretti J.-L., Geoffroy C., Reveneau J., Cossart P., Gaillard J.-L., Geslin P., Kreis H., and Veron M.** (1990) Detection of anti-listeriolysin O for serodiagnosis of human listeriosis. *Lancet* 335: 624-627.
- **Bille J.** (1989) Anatomy of a food-borne listeriosis out-break *in* "Foodborne Listeriosis" (B. Behr's Gmbh & Co.,Ed.), pp. 29-36.
- **Bourry A., and Poutrel B.** (1996) Bovine mastitis caused by *Listeria monocytogenes*: kinetics of antibody responses in serum and milk after experimental infection. *J. Dairy Sci.* 79: 2189-2195.
- **Bourry A., Cochard T., and Poutrel B.** (1997) Serological diagnosis of bovine, caprine, and ovine mastis caused by *Listeria monocytogenes* by using an Enzyme-linked Immunosorbent Assay. *J. Clin. Microbiol.* 35: 1606-1608.
- **Braun L., Ghebrehiwet B., and Cossart P.** (2000) gC1q-R/p32, a C1q-binding protein, is a receptor for the InlB invasion protein of *Listeria monocytogenes*. *EMBO J.* 19: 1458-1466.
- **Cooper J. and Walker R. D.** (1998) Listeriosis. *Vet. Clin. North. Am. Food. Anim. Pract.*14: 113-125
- **Cossart P. and Bierne H.** (2001) The use of host cell machinery in the pathogenesis of *Listeria monocytogenes*. *Curr. Opin. Immun.* 13: 96-103.
- **Cossart P. and Lecuit M.** (1998) Interaction of *Listeria monocytogenes* with mammalian cells during entry and actin-based movement: bacterial factors, cellular ligands and signaling. *EMBO J.* 17: 3797-3806.
- **Cossart P.** (2002) Molecular and cellular basis of the infection by *Listeria monocytogenes*: an overview. *Int J Med Microbiol.* 291: 401-409.
- **Cottral G. E.** (1978) Manual of standardized methods for veterinary microbiology. Cornell University press, Ithaca, NY, p 432.
- **Czuprynski C. J.** (1993) Listeria. *In* Gyles C. L., Thoen C. O. (eds): Pathogenesis of bacterial infections in animals, ed 2 Iowa State University Press, Ames, Iowa, p 70.
- **Decatur A. L. and Portnoy D. A.** (2000) A PEST-like sequence in listeriolysin O essential for *Listeria monocytogenes* pathogenicity. *Science* 290: 992-995.
- **Dennis S. M.** (1993) Listeriosis (circling disease, silage sickness). *In* Howard J. L. (ed): Current veterinary therapy, ed 3. WB Saunders company, Philadelphia, p 580.
- **Dietrich G., Hess J., Gentschev I., Knapp B., Kaufmann S. H. E., and Goebel W.** (2001) From evil to good: a cytolysin in vaccine development. *Trends Microbiol.* 9: 23-28.
- **Doganay M.** (2003) Listeriosis: clinical presentation. *FEMS Immunol Med Microbiol.* 35: 173-175.
- **Fleming D. W., Cochi S. L., MacDonald K. L., Brondum J., Hayes P. S., Plikaytis B. D., Holmes M. B., Audurrier A., Broome C. V., and Reingold A. L.** (1985) Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. *N. Engl. J. Med.* 312: 404-407.
- **Fthenakis G.C., Saratsis Ph., Tzora A., and Linde K.** (1998) Naturally occurring subclinical ovine mastitis associated with *Listeria monocytogenes*. *Small Ruminant Research* 31: 23-27.

- **Gaillard J. L., Beretti J. L., Boulot-Tolle M., Wilhelm J. M., Bertrand J. L., Herbelleau T., and P. Berche** (1992) Serological evidence for culture-negative listeriosis of central nervous system. *Lancet* 340:560.
- **Gedde M. M., Higgins D. E., Tilney L. G., and Portnoy D. A.** (2000) Role of listeriolysin O in cell-to-cell spread of *Listeria monocytogenes*. *Infect. Immun*. 68: 999-1003.
- **Gellin B. G and Broome C. V.** (1989) Listeriosis. *JAMA* 261: 1313-1320.
- **Geoffroy C., Gaillard J.-L., Alouf J. E., and Berche P.** (1987) Purification, characterization, and toxicity of the sulfhydryl-activated hemolysin listeriolysin O from *Listeria monocytogenes. Infect. Immun*. 55: 1641-1646.
- **Giammarini C., Andreoni F., Amagliani G., Casiere A., Barocci S., and Magnani M.** Purification and characterization of a recombinant listeriolysin O expressed in *Escherichia coli* and possible diagnostic applications. *J. Biotechnol.*: in press.
- **Giammarini C., Andreoni F., Amagliani G., Casiere A., Barocci S., and Magnani M.** (2003) High-level expression of the *Listeria monocytogenes* listeriolysin O in *Escherichia coli* and preliminary characterization of the purified protein. *Protein Expr. Purif.* 28: 78-85.
- **Glomski I. J., Gedde M. M., Tsang A. W., Swanson J. A., and 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.
- **Goulet V., Jacquet C., Vaillant V., Rebière I., Mouret E., Lorente E., Steiner F., and Rocourt J.** (1995) Listeriosis from consumption of row milk cheese. *Lancet* 345: 1581-1582.
- **Goulet V., Rocourt J., Rebiere I., Jacquet C., Moyse C., Dehaumont P., Salvat G., and Veit P.** (1998) Listeriosis outbreak associated with the consumption of rillettes in France in 1993. *J. Infect. Dis.*177: 155-160.
- **Gray M. L. and Killinger A. H.** (1966) *Listeria monocytogenes* and listeric infections. *Bacteriol. Rev.* 30: 309-382.
- **Jacobs T., Darji A., Frahm N., Rohde M., Wehland J., Chakraborty T., and Weiss S.** (1998) Listeriolysin O: cholesterol inhibits cytolysis but not binding to cellular membranes. *Mol. Microbiol.* 28: 1081-1089.
- **Jacquet C., Catimel B., Brosch R., Buchrieser C., Dehaumont I., Goulet V., Lepoutre V., Veit P., and Rocourt J.** (1995) Investigations related to the epidemic strain involved in the French listeriosis outbreak in 1992. *Appl. Environ. Microbiol.* 61: 2242-2246.
- **Lee K.-D., Oh Y.-K., Portnoy D. A., and Swanson J. A.** (1996) Delivery of macromolecules into cytosol using liposomes containing hemolysin from *Listeria monocytogenes*. *J. Biol. Chem.* 271: 7249-7252.
- **Lety M.-A., Frehel C., Dubail I., Beretti J.-L., Kayal S., Berche P., and Charbit A.** (2001) Identification of a PEST-like motif in listeriolysin O required for phagosomal escape and for virulence in *Listeria monocytogenes*. *Mol. Microbiol.* 39: 1124-1139.
- **Lhopital S., Marly J., Pardon P., and Berche P.** (1993) Kinetics of antibody production against listeriolysin O in sheep with listeriosis. *J. Clin. Microbiol.* 31: 1537-1540.
- **Linnan M. J., Mascola L., Lou X. D., Goulet V., May S., Salminen C., Hird D. W., Yonekura M. L., Hayes P., Weaver R., Audurier A., Plikaytis B. D., Fannin S. L., Kleks A., and Broome C. V.** (1988) Epidemic listeriosis associated with Mexican-style cheese. *N. Engl. J. Med.* 319: 823-828.
- **Low J. C. and Donachie W.** (1991) Clinical and serum antibody responses to lambs to infection by *Listeria monocytogenes*. *Res. Vet. Sci.* 51: 185-192.
- **Low J. C. and Donachie W.** (1997) A review of L*isteria monocytogenes* and listeriosis. *Vet. J.* 153: 9-29.

- **Lyytikainen O., Autio T., Maijala R., Ruutu P., Honkanen-Buzalski T., Miettinen M., Hatakka M., Mikkola J., Anttila V. J., Johansson T., Rantala L., Aalto T., Korkeala H., and Siitonen A.** (2000) An outbreak of *Listeria monocytogenes* serotype 3a infections from butter in Finland. *J. Infect. Dis.* 181: 1838-1841.
- **Mathew E., Hardee G. E., Bennett C. F., and Lee K. D.** (2003) Cytosolic delivery of antisense oligonucleotides by listeriolysin O-containing liposomes. *Gene Ther.* 10: 1105-1115.
- **McLauchlin J.** (1996) The role of the Public Health Laboratory Service in England and Wales in the investigation of listeriosis during the 1980s and 1990s. *Food Control* 7: 235-239.
- **McLauchlin J., Hall S. M., Velani S. K., and Gilbert R. J.** (1991) Human listeriosis and patè-a possible association. *Br. Med. J.* 303: 773-775.
- **Mengaud J., Vicente M.-F., Chenevert J., Pereira J. M., Geoffroy C., Gicquel-Sanzey B., Baquero F., Perez-Diaz J.-C., and Cossart P.** (1988) Expression in *Escherichia coli* and Sequence Analysis of the Listeriolysin O Determinant of *Listeria monocytogenes*. *Infect. Immun*. 56: 766-772.
- **Murray E. G. D., Webb R. A., and Swann M. B. R.** (1926) A disease of rabbits characterized by a large mononuclear leucocytosis caused by a hitherto undescribed bacillus *Bacterium monocytogenes*. *J. Patho.l Bacteriol.* 28: 407.
- **Palmer M.** (2001) The family of thiol-activated, cholesterol-binding cytolysins. *Toxicon* 39: 1681-1689.
- **Palmer M., Vulicevic I., Saweljew P., Valeva A., Kehoe M., and Bhakdi S.** (1998) Streptolysin O: a proposed model of allosteric interaction between a pore-forming protein and its target lipid bilayer. *Biochemistry* 37: 2378-2383.
- **Pinkney M., Beachey E., and Kehoe M.** (1989) The thiol-activated toxin streptolysin O does not require a thiol group for cytolytic activity. *Infect. Immun.* 57: 2553-2558.
- **Portnoy D. A. and Jones S.** (1994) The cell biology of *Listeria monocytogenes* infection (escape from a vacuole). *Ann. N.Y. Acad. Sci*. 730: 15-25.
- **Provoda C. J. and Lee K.-D.** (2000) Bacterial pore-forming hemolysins and their use in the cytosolic delivery of macromolecules. *Adv. Drug. Deliv. Rev.* 41: 209-221.
- **Rechsteiner M. and Rogers S. W.** (1996) PEST sequences and regulation by proteolysis. *Trends Biochem. Sci.* 21: 267-271.
- **Rocourt J. and Cossart P.** (1997) *Listeria monocytogenes in* "Food microbiology: fundamentals and frontiers" (Doyle M. P., Beuchat L. R., and Montville T. J., Ed.), pp. 337-352, ASM Press, Washington D.C. .
- **Schlech W. F., Lavigne P. M., Bortolussi R. A., Allen A. C., Haldane E. V., Wort A. J., Hightower A. W., Johnson S. E., King S. H., Nicholls E. S., and Broome C. V.** (1983) Epidemic listeriosis-evidence for transmission by food. *N. Engl. J. Med.* 308: 203-206.
- **Schuchat A., Swaminathan B., and Broome C. V.** (1991) Epidemiology of human listeriosis. *Clin. Microbiol. Rev*. 4: 396.
- **Shen Y., Naujokas M., Park M., and Ireton K.** (2000) InIB-dependent internalization of Listeria is mediated by the Met receptor tyrosine kinase. *Cell* 103: 501-510.
- **Tham W., Ericsson H., Loncarevic S., Unnerstad H., and Danielsson-Tham M. L.** (2000) Lessons from an outbreak of listeriosis related to vacuum-packed gravad and cold-smoked fish. *Int. J. Food Microbiol.* 62: 173-175.
- **Traub V. H., and Bauer D.** (1995) Simplified purification of *Listeria monocytogenes* listeriolysin O and preliminary application in the enzyme-linked immunosorbent assay (ELISA). *Zbl.Bakt*. 283: 29-42.

- **Vazquez-Boland J. A., Dominguez-Bernal G., Gonzalez-Zorn B., Kreft J., and Goebel W.** (2001) Pathogenicity islands and virulence evolution in Listeria. *Microbes Infect.* 3: 571-584.