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# Hemolysin from Listeria – Biochemistry, Genetics and Function in Pathogenesis

**Summary:** Thiol-activated hemolysins (listeriolysins) from *Listeria monocytogenes* (Sv4b) and *Listeria ivanovii* were purified to homogeneity. The N-terminal amino acid sequences of the 58 kDa listeriolysin of *L. ivanovii* and of a 24 kDa protein which may represent the CAMP-factor of *L. ivanovii* were determined. Antibodies raised against the *L. ivanovii* listeriolysin and anti-streptolysin O antibodies were used in Western blot analyses to detect listeriolysin(s) in virulent and avirulent Listeria strains. It was found that all virulent strains of *L. monocytogenes* synthesize and secrete listeriolysin (Mr 58-59 kDa), albeit in significantly variable quantities. No protein cross-reaction with anti-listeriolysin antibodies or anti-streptolysin O-antibodies was present in the supernatant of *Listeria innocua, Listeria welshimeri, Listeria grayi* and *Listeria murrayi* strains. Furthermore, the avirulent but hemolytic *Listeria seeligeri* did not cross-react with these antibodies. In a *L. monocytogenes* (strain EGD) gene bank constructed in *Escherichia coli* two types of hemolytic clones were identified. The first type carried recombinant plasmids with a common 2.0 kb fragment coding for a 23 kDa protein. This hemolytic activity was not activated by DTT and the 23 kDa protein did not cross react with anti-listeriolysin or anti-streptolysin antibodies. The other type of hemolytic clones was detected by using anti-streptolysin O antibodies to screen the gene bank. Some of these clones synthesized a protein of 61 kDa which cross reacted with anti-streptolysin O (or anti-listeriolysin) antibodies. By transposon Tn916 mutagenesis of *L. monocytogenes* two types of nonhemolytic mutants were obtained. Type I produced no extracellular protein that cross reacted with anti-listeriolysin (or anti SLO) antibodies. Instead of the 58 kDa listeriolysin protein, type II mutants released proteins which were smaller in size than listeriolysin and cross reacted with anti-SLO. These proteins probably represent truncated listeriolysins. Virulence tests in a mouse model indicated that non-hemolytic mutants of *L. monocytogenes* were avirulent. Furthermore, it was shown that these nonhemolytic mutants were unable to survive in mouse peritoneal macrophages but were still capable of entering mouse embryo fibroblast (3T6) cells. All virulent *L. monocytogenes* produced a quantitatively abundant extracellular protein of Mr 60 kDa. We isolated mutants which were still hemolytic but produced significantly reduced amounts of this protein. The ability of these mutants to enter 3T6 cells was severely impaired.

**Zusammenfassung:** *Hämolysin von Listerien – Biochemie, Genetik und Funktion in der Pathogenese.* SH-aktivierbare Hämolysine (Cytolysine) aus *Listeria monocytogenes* (Sv4b) und *Listeria ivanovii* wurden zur Homogenität gereinigt. Die N-terminalen Aminosäuresequenzen des 58 kDa großen Listeriolysins aus *L. ivanovii* und eines 24 kDa Protein, das vermutlich der CAMP-Faktor von *L. ivanovii* ist, wurden bestimmt. Mit Hilfe von Antikörpern gegen Listeriolysin aus *L. ivanovii* und Streptolysin O wurden im Western Blot virulente und avirulente Listerienstämme auf ihre Fähigkeit, Listeriolysin zu bilden, getestet. Danach synthetisieren und scheiden alle virulenten Stämme von *L. monocytogenes* Listeriolysin (Mr 58-59 kDa) aus, allerdings in sehr unterschiedlicher Menge. In den Kulturüberständen von *Listeria innocua, Listeria welshimeri, Listeria grayi* und *Listeria murrayi* konnte kein mit Listeriolysin- oder Streptolysin-O-Antikörpern kreuzreagierendes Protein nachgewiesen werden. Die avirulente, aber hämolytische Art *Listeria seeligeri* zeigte ebenfalls keine Kreuzreaktion mit diesen Antikörpern. Zwei Typen von hämolytischen *Escherichia coli* Klonen wurden in einer Genbank von *L. monocytogenes* (Stamm EGD) nachgewiesen. Der erste Typ besaß rekombinante Plasmide, die ein gemeinsames Fragment von 2 kb trugen. Dieses kodierte für ein Protein von 23 kDa, das für die hämolytische Aktivität verantwortlich ist. Diese Aktivität wurde weder mit DTT aktiviert, noch kreuzreagierte das 23-kDa-Protein mit Antikörpern gegen Listeriolysin oder Streptolysin O. Der andere Typ von hämolytischen Klonen wurde in der Genbank mit Hilfe von Streptolysin-O-Antikörpern identifiziert. Einige dieser Klone synthetisierten ein Protein von 61 kDa, das mit Antikörpern gegen Streptolysin O (oder Listeriolysin) kreuzreagierte. Durch Transposonmutagenese von *L. monocytogenes* mit Tn916 wurden 2 Typen von nichthämolytischen Mutanten erhalten. Mutanten des Typs I produzierten kein extrazelluläres Protein, das mit Antikörpern gegen Listeriolysin kreuzreagierte. Typ-II-Mutanten schieden anstelle des 58 kDa Listeriolysins Proteine mit geringerer Größe als Listeriolysin aus, die noch mit diesen Antikörpern reagierten und somit wahrscheinlich verkürzte Listeriolysinproteine darstellen. Tests auf Virulenz in einem Mausmodell zeigten, daß beide Typen von nichthämolytischen Mutanten avirulent sind. Weiterhin konnte gezeigt werden, daß diese nichthämolytischen Mutanten nicht mehr in der Lage sind, in peritonealen Mausmakrophagen zu überleben, jedoch noch in embryonale Mäusefibroblasten (3T6-Zellen) eindringen können. Alle virulenten Stämme von *L. monocytogenes* synthetisierten in relativ großer Menge ein extrazelluläres Protein von 60 kDa. Mutanten konnten isoliert werden, die zwar noch hämolytisch sind, das 60-kDa-Protein jedoch nur noch in geringer Menge produzieren. Die Mutanten haben die Fähigkeit, in 3T6-Zellen einzudringen, weitgehend verloren.

## Introduction

Most clinical isolates of Listeria belong to the species *Listeria monocytogenes*. All of these strains are also virulent in an experimental murine model (1, 2). A reduced viru-

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lence was demonstrated in this animal model for *Listeria ivanovii* (3). All known virulent strains produce hemolysin whereas non-hemolytic Listeria strains are avirulent. The hemolysin, termed listeriolysin, has therefore been implicated as an important virulence factor in infections caused by *L. monocytogenes*. Studies using non-hemolytic transposon mutants of *L. monocytogenes* support this assumption (4, 5). The existence of the avirulent, but hemolytic Listeria species, *Listeria seeligeri*, has raised the question whether only one "listeriolysin" may exist in Listeria, and recently, the presence of more than one hemolysin in clinical isolates has been postulated (6). The only hemolysin characterized biochemically is the thiol-activated listeriolysin which is a member of a group of cytolysins produced by several gram-positive bacteria. These cytolysins cross react immunologically and recognize cholesterol as common receptor (7, 8). The well studied streptolysin O (SLO) belongs to this group and it has previously been demonstrated that antibodies raised against SLO cross react with the SH-activated listeriolysin (6).

## Results and Discussion

### *Purification and Characterization of SH-activated Hemolysins (listeriolysins) from Listeria ivanovii and Listeria monocytogenes Sv4b*

Listeriolysin from *Listeria ivanovii* (ATCC 19119, SLCC 2379) and *L. monocytogenes* Sv4b (NCTC 10527) was purified essentially as described by *Geoffroy* and *Alouf* (9). SH-reactive proteins from the culture supernatants were separated from other extracellular proteins by chromatography on thiopropyl-Sepharose 6B (in PBS pH 6.0) from which they were eluted with 5 mM 1.4-dithioerythritol (DTT) in PBS pH 7.5. Active fractions were purified by gel filtration on Biogel P-100 and subsequently on FPLC-Superose 6. Figure 1 shows the protein pattern obtained by SDS-PAGE after the two initial purification steps. In the case of *L. monocytogenes* Sv4b a single protein with MW of 58 kDa was obtained, whereas in the case of *L. ivanovii* two proteins of MW 58 kDa and 24 kDa were copurified. Interestingly, the latter proteins seemed to form a rather stable complex and could not be separated from each other under non-denaturing conditions, i. e. by gel filtration on Biogel-P-100 or on FPLC-Superose 6 (data not shown). Only after addition of 0.1% SDS was separation of the two components achieved on FPLC-Superose 6 (data not shown).

The purified hemolysin preparations from *L. ivanovii* and *L. monocytogenes* Sv4b, respectively, showed all the characteristics typical for listeriolysin (7, 8), i. e. activation by SH-reagents and inhibition by cholesterol (data not shown). The isolated 24 kDa protein from *L. ivanovii*, which itself showed a slight, non-DTT-dependent hemolytic activity, exhibited a strong dose-dependent hemolytic effect on sheep erythrocytes when combined with a culture supernatant from *Rhodococcus equi*. No such reaction was observed with a supernatant from *Staphylococcus aureus* (Figure 2). This behaviour is typical for the CAMP-phenomenon observed with *L. ivanovii* and *R. equi (10)*. The N-terminal amino acid sequences of both the 58 kDa listeriolysin and the 24 kDa protein from *L. ivanovii* were determined by microsequencing in a gas phase protein sequenator (Figure 3). The amino acid sequence of the 58 kDa protein shows no homology to the N-termini of other SH-activated cytolysins (11). The comparison of the two sequences also demonstrates that the 24 kDa protein is not an N-terminal fragment of 58 kDa protein. The determination of the amino acid sequence of the 58 kDa listeriolysin from *L. monocytogenes* Sv4b and a further characterization of the 24 kDa protein from *L. ivanovii* are in progress. Rabbit antiserum raised against

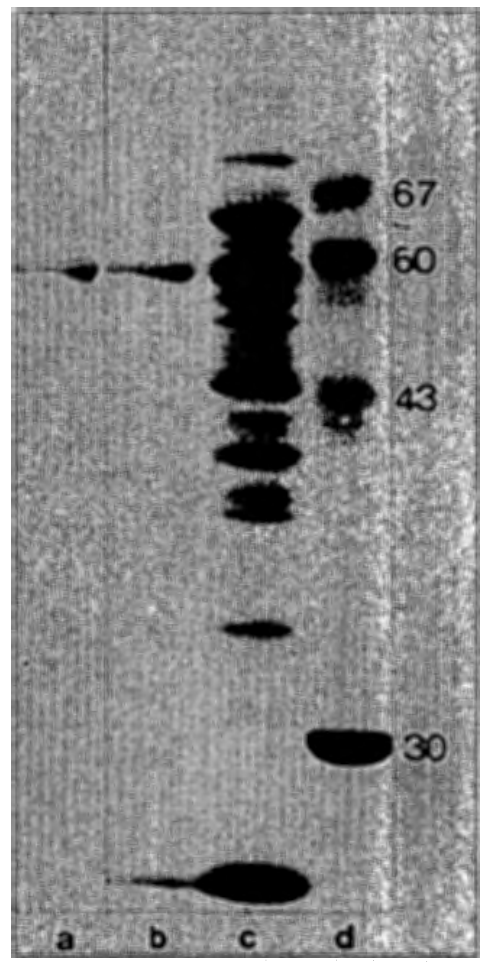


Figure 1: Analysis of the purified hemolysin from *Listeria monocytogenes* (Sv4b) (a) and *Listeria ivanovii;* (b) on SDS-PAGE after chromatography of concentrated culture supernatants on thiopropyl-Sepharose 6B and on Biogel P-100; (c) shows the protein pattern of the crude concentrated supernatant of *Listeria ivanovii* before purification; (d) marker proteins with the indicated relative molecular masses in kDa.

 

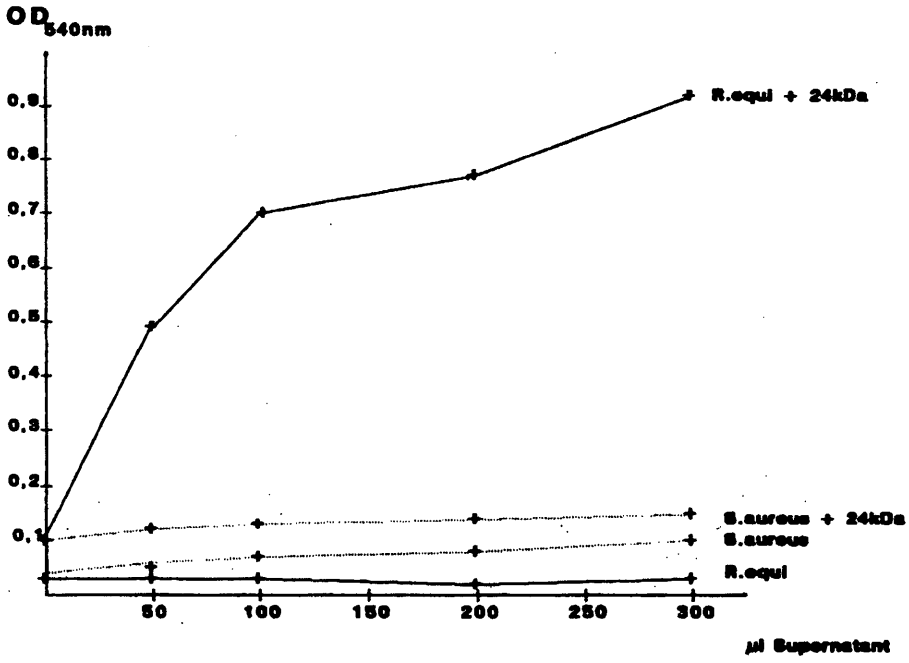


Figure 2: CAMP-effect of the purified 24 kDa protein from *Listeria ivanovii* (measured by the release of hemoglobin from sheep erythrocytes). The values were determined after combining the indicated amounts of 10 fold concentrated culture supernatants from *S. aureus* or *Rhodococcus equi* with a constant amount of the purified 24 kDa protein from *Listeria ivanovii.*

the listeriolysin preparation from *L. ivanovii* was tested by immunoblotting techniques (12) against supernatant proteins from several Listeria strains. Figure 4 shows that these antibodies react specifically with two proteins of Mr 58 kDa and 24 kDa, respectively, from *L. ivanovii* (as expected) and with one major protein from the other Listeria strains tested. These data agree with similar experiments using anti-SLO to detect listeriolysin in the supernatants of Listeria strains. The major conclusions from these data are: (a) All tested virulent strains of *L. monocytogenes* produce a protein of MW 58 to 60 kDa which cross reacts with anti-SLO and is presumed, on the basis of its biochemical characteristics, to represent listeriolysin. This protein is therefore most likely listeriolysin. The amount of this protein is, however, significantly different in the various *L. monocytogenes* strains; (b) No protein cross-reacting with anti-SLO was present in the supernatant from several strains of *L. innocua* (Sv6a and Sv6b), *Listeria welshimeri, Listeria grayi* and *Listeria murrayi.* The avirulent but hemolytic *L. seeligeri* strains did not cross react with these antibodies (Figure 4B), a finding which indicates that the hemolysin produced by *L. seeliger* is different from the listeriolysin of *L. monocytogenes.*

*Characterization of Hemolytic Clones from a Gene Library of Listeria monocytogenes*

A gene library of *L. monocytogenes* 1/2a strain EGD was constructed using the vector pUC18. The EGD strain was obtained from *S. H. E. Kaufmann* (MPI of Immunobiology, Freiburg), who originally obtained it from *G. B. Mackaness* (Saranac Lake, N.Y.). The strain is weakly hemolytic and has a $LD_{50}$ of about $10^4$ organisms for BALB/c and NMRI mice.

Ten thousand recombinants with inserts of 1 to 15 kb of Listeria DNA were screened on blood agar plates for hemolytic phenotype. A recombinant was identified that showed hemolytic activity on such agar plates after three to four days of growth. The hemolytic activity was cell-associated and could not be detected in supernatant fluids. The recombinant plasmid, designated pLM1, was found to contain an insert of 3.4 kb Listeria DNA. Using deletion mutants created by restriction endonucleases we

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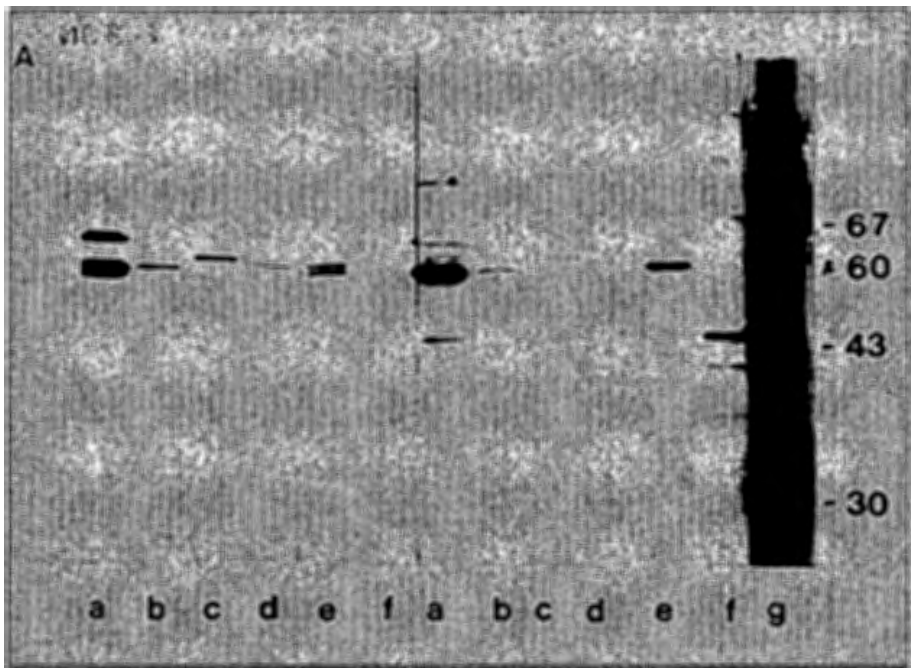
    1                                   10
A.  Asp  Ala Ser Val Tyr Ser Tyr Gln Gly Ile Ile  X  His Met Ala
                                                              (Pro)
    (Pro)Ala  X  Pro(Pro)Ala Lys ...

    1                                   10
B.  Ala Asp Ile Leu  X   Pro Met Pro Ile Asn Gln Ile Phe Pro Asp

    Pro Asp Leu Ala ...
    (Leu)
```

Figure 3: Amino acid sequence analysis of the N-terminal end of the purified 58 kDa and the 24 kDa proteins from *Listeria ivanovii.* (X) refers to amino acid positions which most likely represent Cys. Amino acids in parenthesis are possible alternatives to the amino acids shown above or their presence is uncertain.



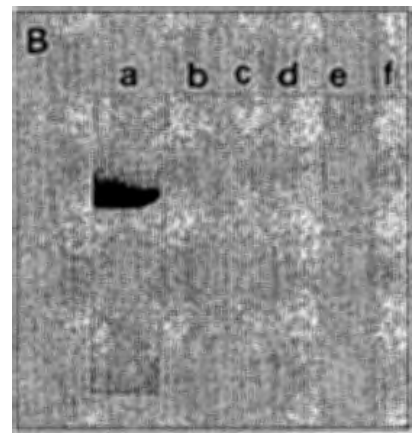




Figure 4: Western blot analysis of extracellular proteins from virulent and avirulent Listeria strains with anti-listeriolysin and anti-SLO antibodies. Left panel: reaction with anti-listeriolysin; right panel: reaction with anti-SLO. The cross reaction of proteins with a Mr of around 60 kDa with anti-listeriolysin antiserum in the lanes showing extracellular proteins from *Listeria monocytogenes* Sv1/2a EGD (c), *Listeria innocua* (d) and *Listeria monocytogenes* Sv1/2a, Mackaness (e) is most probably caused by a contamination of the anti-listeriolysin antiserum with anti 60 kDa antibodies (see Figure 11). *Listeria monocytogenes* Sv1/2a EGD produces very little listeriolysin which cannot be seen as cross hybridizing band in this figure. *Part A:* (a) *Listeria ivanovii;* (b) *Listeria monocytogenes* Sv4b; (c) *Listeria monocytogenes* Sv1/2a (EGD); (d) *Listeria innocua;* (e) *Listeria monocytogenes* Sv1/2a; (f) *S. pyogenes;* g: molecular weight markers. *Part B:* Lack of cross-reactivity of extracellular proteins from *Listeria seeligeri* with anti-SLO. SDS-PAGE and immunoblotting were performed as described (12). Lanes: (a) *Listeria monocytogenes* (strain SLCC 5764); (b–f) *Listeria seeligeri* (SLCC strains 3644, 4113, 4061, 3776, and 5921, respectively).

were able to locate the DNA to approximately 2 kb of Listeria DNA. A 23 kDa protein is unique to all recombinants producing the hemolysin. The hemolytic factor encoded by plasmid pLM1 is neither activated by reducing agents nor does it cross react with anti-SLO or antilisteriolysin antisera. We therefore conclude that the recombinant codes for a hemolytic principle are distinct from listeriolysin.

In a further attempt to identify recombinants harbouring the gene for listeriolysin we screened the gene library for clones exhibiting cross reactivity to anti-SLO. Using this approach we identified seven recombinant strains that specifically cross react with anti-SLO. Of these, two recombinants also showed cross reactivity to anti-listeriolysin (see above). DNA hybridization with these plasmids showed that they carried common sequences. One recombinant, designated pLM37, is described in more detail.

The strain harbouring the plasmid pLM37 was found to produce small zones of hemolysis after overnight growth on human blood agar plates. Spontaneous lysis of overnight cultures was often observed and may explain our inability to detect the recombinant in the initial screen for hemolytic recombinants.

The plasmid has an insert of 9 kb of Listeria DNA. A partial restriction endonuclease map was constructed and is depicted in Figure 5. Polypeptides of 61 KDa, 42 KDa

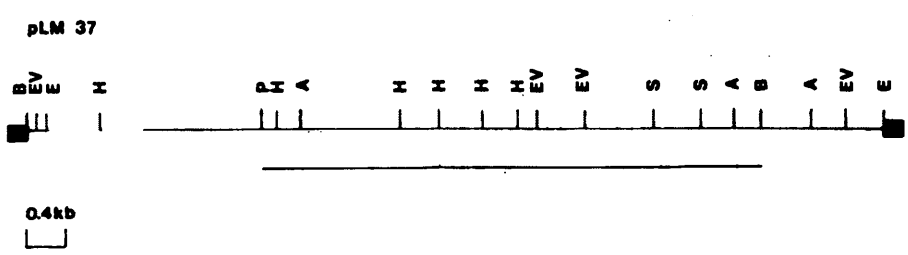



Figure 5: Restriction endonuclease map of pLM37. The closed boxes represent vector pUC18 DNA sequence. The region underlined is required for the expression and production of a hemolysin which may represent listeriolysin. Abbreviations: A = AvaI; B = BamHI; E = EcoRI; EV = EcoRV; H = HindIII; P = PstI; S = SmaI.



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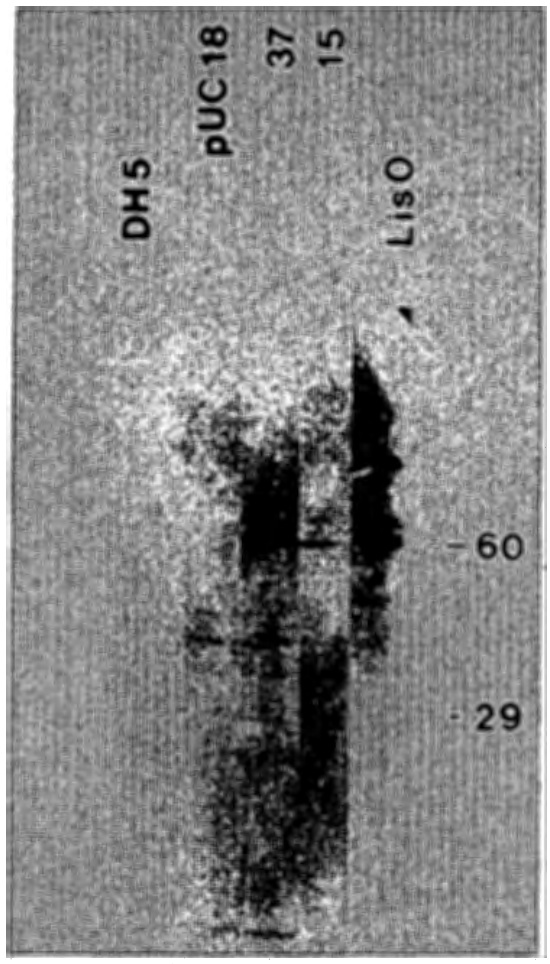


Figure 6: Immunoblots of whole cell lysates from *Escherichia coli* DH5 strains with rabbit antisera to listeriolysin. Specific reactivity to a 60 kDa protein is seen in recombinants harbouring plasmids pLM15 and pLM37 which is not present in the recipient strain DH5 with and without vector pUC18. Lane marked Lis O contains a partially purified fraction of listeriolysin from *Listeria ivanovii.*

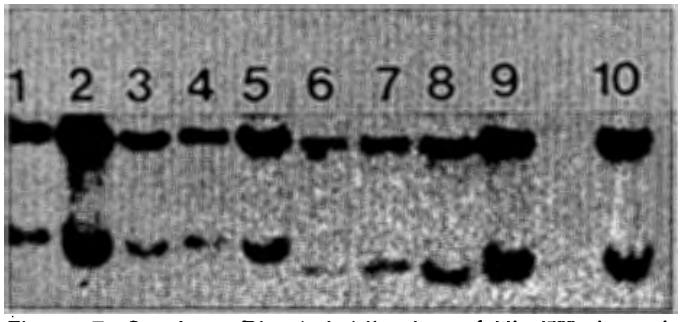


Figure 7: Southern Blot hybridization of HindIII-cleaved chromosomal DNA from type I (1–5) and type II (6–10) hemolysin-negative Tn916 insertion mutants from *Listeria monocytogenes* (Sv1/2a) with $^{32}$P-labelled Tn916 DNA (cloned in pBR 322). The sizes of the upper HindIII fragments are around 12 kb and 11 kb, respectively, the sizes of the lower HindIII fragments 6.5 kb and 5.5 kb, respectively.

and 30.5 kDa are encoded by the DNA insert of pLM37. In immunoblot assays using both anti-SLO and anti-listeriolysin antisera, the 61 kDa protein was found to be the cross reacting polypeptide (Figure 6). While the recombinant showed detectable levels of hemolytic activity in liquid assays, we have not yet been able to demonstrate convincingly enhancement of this activity using DTT or cystein.

Preliminary data using a probe obtained from the plasmid pLM37 showed that the cloned fragment is present only in *L. monocytogenes* strains. Hybridization with the plasmid pLM1, in contrast, showed that this DNA sequence is present in all Listeria species tested except *L. murrayi* and *L. grayi.*

### *Isolation of Nonhemolytic (Hly$^-$)Mutants of Listeria monocytogenes by Transposon Mutagenesis*

Several Hly$^-$ mutants of *L. monocytogenes* were isolated by Tn916 mutagenesis as described (5). These mutants have been characterized with respect to the proteins they produce extracellularly. Two types were identified, one (type I) producing no anti SLO-reacting protein of 58 kDa, and another (type II) producing smaller polypeptides, of which one, of MW 49 kDa, reacted noticeably with anti SLO antiserum and probably represented a truncated form of the listeriolysin. The absence of the 58 kDa protein in the type I mutants and the production of the 49 kDa polypeptide in the type II mutants were also observed in total cellular proteins (data not shown), suggesting that the nonhemolytic phenotype was not due to lack of transport of the protein extracellularly, but that, instead, intact, active hemolysin was not produced by these mutants.

Hybridization of HindIII-digested chromosomal DNA from Hly$^-$ mutants with $^{32}$P-labeled Tn916 indicated the presence of two hybridizing HindIII fragments, suggesting that single copies of Tn916 were inserted in the DNA of these mutants (Tn916 has a single HindIII site). Type II mutants were probably due to insertions of Tn916 within the structural gene for the hemolysin, since they produced a truncated, inactive, protein. Type I mutants may well be due to insertions of Tn916 in a regulatory sequence, the proximity of which to the structural gene is currently being studied. The sum of the two hybridizing HindIII fragments was the same for all mutants of a given type (Figure 7). Tn916 is localized in different HindIII fragments in the two types of mutants, as indicated by the fact that the sum of the two hybridizing HindIII fragments of type I mutants is different from that of type II (Figure 7). We are currently involved in the cloning of the DNA sequences immediately adjacent to the point of insertion of Tn916 in the two types of mutants.

### *Hemolysin as Virulence Factor*

Both types of hemolysin-negative Tn916 insertion mutants showed significantly reduced virulence in the mouse

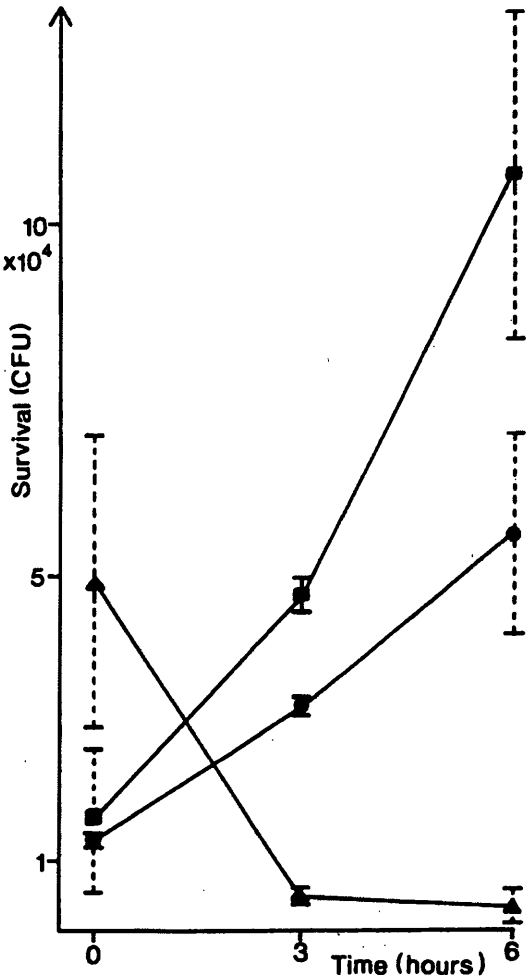


Figure 8: Survival of *Listeria monocytogenes* Sv1/2a wild-type strain and Hly⁻ mutant within mouse peritoneal macrophages. Peritoneal macrophages from three-months-old BALB/c mice were harvested and washed in PBS and resuspended in RPMI 1640 medium (Seromed) supplemented with 10% fetal calf serum and L-glutamine. $5\times10^5$ macrophages were infected with $5\times10^6$ bacteria. After 40 min of phagocytosis, the cultures were washed five times with 5 ml PBS to remove excess extracellular bacteria and reincubated for 40 min in medium containing 50 µg/ml gentamicin to kill residual or adherent bacteria not removed by the washing steps. After removal of the drug, the medium was changed every hour for the duration of the experiment (3–6 h). Each point in the figure represents an average of two experimental values. The deviations of the two values are indicated by the dashed lines. These experiments were repeated several times with different batches of peritoneal mouse macrophages and yielded qualitatively the same results.
■ *Listeria monocytogenes* (Sv 1/2a) wild-type strains Hly⁺;
● *Listeria monocytogenes* $Tc^R$ (due to a Tn916 insertion, Hly⁺;
▲ *Listeria monocytogenes* $Tc^R$ Hly⁻ (due to a Tn916 insertion in the structural hly gene).

model system as previously reported (5). Similar results have been obtained with a Tn1545 induced hemolysin-negative mutant (4).

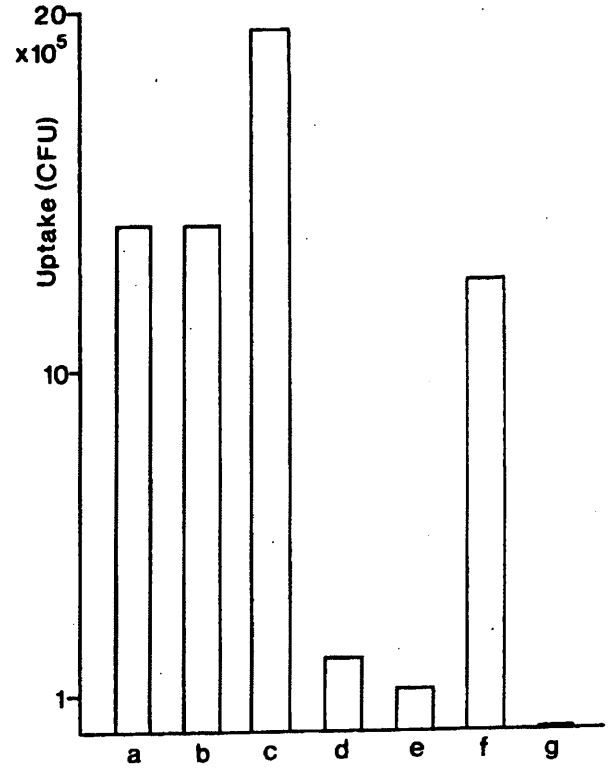


Figure 9: Uptake of Hly⁺ and Hly⁻ strains of *Listeria monocytogenes* by 3T6 fibroblast cells. Uptake assays were performed essentially as described (13). $10^6$ 3T6 cells per well were infected with $10^7$ bacteria. Each column represents the mean value of two experiments (a) *Listeria monocytogenes* (Sv 1/2a – Mackaness); (b) *Listeria monocytogenes* (Sv 1/2a-strain EGD); (c) *Listeria monocytogenes* (Sv4b); (d) *Listeria monocytogenes* (Sv3a); (e) *Listeria innocua* (Sv6a); (f) *Listeria monocytogenes* (Sv 1/2a – Mackaness) Tcr, Hly⁻ (Tn916 mutant); (g) – *Bacillus subtilis.*

To examine the possible role of hemolysin in the uptake and/or survival of *L. monocytogenes* we have been studying the interactions between the Hly⁺ and Hly⁻ *L. monocytogenes* strains and professional (peritoneal mouse macrophages) and nonprofessional phagocytic cells (3T6 mouse embryo fibroblast cell line). Unopsonized Listeria cells were mixed with macrophages in a ratio of ten bacteria per macrophage. Bacteria that remained outside the macrophages were killed by gentamicin as described (13) and the number of viable bacteria within the macrophages was determined at time 0 and 3 h and 6 h thereafter. The Hly⁺ bacteria were able to multiply and after 6 h a 10-fold increase in their number was observed. In contrast to this, the Hly⁻ bacteria failed to multiply inside the macrophages and their numbers declined steadily, so that at 6 h only 10% of the bacteria present at time 0 remained (Figure 8). These data suggest that the ability of the bacteria to survive and multiply within the macrophages is severely impaired in the Hly⁻ mutants. Hly⁺ and Hly⁻ *L. monocytogenes* are identical in terms of their ability to be internalized by non professional phagocytes (Figure 9).


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1                                   10
Asp   His Val Val Val Glu Ala Gly Asp Thr Leu Trp Gly Ile Ala
(Gly)(Gly)
                  20
Gln Ser Lys Gly Thr (Thr) Val Thr ...
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Figure 10: N-terminal amino acid sequence of the purified 60 kDa protein from *Listeria monocytogenes*. The 60 kDa protein was isolated from a Hly⁻ transposon mutant and further purified by SDS-PAGE. The protein band was eluted directly from the gel. Amino acids in parenthesis are possible alternatives to the amino acids shown in the sequence above or their presence is uncertain.

Following centrifugation with 3T6 cell monolayers Hly⁺ and Hly⁻ bacteria were able to be internalized by the cells with similar frequencies. Internalization is probably due to phagocytosis, since it was strongly inhibited by cytochalasin B, an inhibitor of microfilament function. In contrast to the Hly⁻ mutants of *L. monocytogenes* two other Listeria strains, the avirulent nonhemolytic *L. innocua* (Sv6a) and the slightly virulent, hemolytic *L. monocyto genes* (Sv3a) do not enter these host cells to any appreciable extent, similar to *B. subtilis* which was used as a noninvasive control bacterium. These data show convincingly that fully virulent Listeria must possess an active mechanism for invasion of mammalian cells. Listeriolysin is clearly not involved in this invasion step.

All virulent strains of *L. monocytogenes* capable of penetrating nonprofessional phagocytic cells, synthesize and secrete in relatively high concentration a protein of 60 kDa. It can be clearly distinguished from listeriolysin by several criteria:

(a) It is still present in both classes of Hly⁻ mutants obtained by Tn916 insertion;

(b) It is well separated from listeriolysin on 2D-gels by its alkaline isoelectric point (pH 8.5; listeriolysin pH 7.0);

(c) The N-terminal amino acid sequence of the purified 60 kDa protein (Figure 10) is totally different from the N-terminal sequence of listeriolysin from *L. ivanovii;*

(d) Antibodies raised against the 60 kDa protein do not cross-react with listeriolysin (Figure 11).

Figure 11 also shows that a protein of this size is either absent or significantly reduced in its amount in the tested avirulent strains. Most of the latter strains secrete, however, proteins of different sizes which cross-react with the antiserum against the 60 kDa protein.

Mutants were isolated from two virulent *L. monocytogenes* strains (Sv1/2a) which produced significantly lower levels of the 60 kDa protein than the corresponding wild-type strains (Figure 12A). These mutants also showed a reduced ability to penetrate 3T6 cells (Figure 12B). These lines of evidence suggest that the 60 kDa protein may play a role in the invasion process.

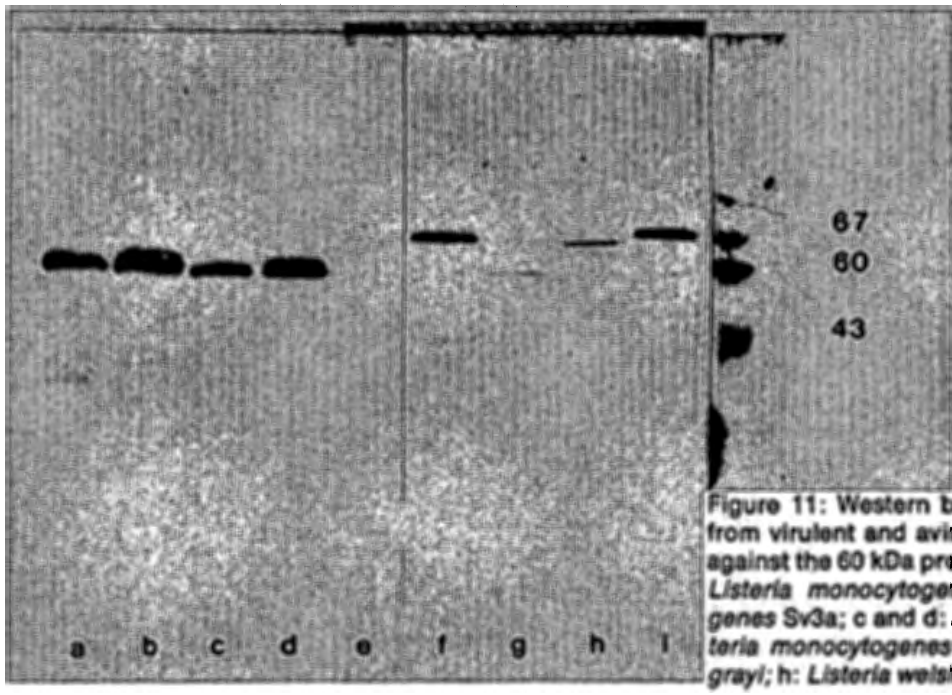


Figure 11: Western blot analysis of extracellular proteins from virulent and avirulent Listeria strains with antiserum against the 60 kDa protein from *Listeria monocytogenes*. a: *Listeria monocytogenes* Sv 1/2a; b: *Listeria monocytogenes* Sv3a; c and d: *Listeria monocytogenes* Sv4b; e: *Listeria monocytogenes* Sv4f; f: *Listeria ivanovii;* g: *Listeria grayi;* h: *Listeria welshimeri;* i: *Listeria seeligeri;* j: markers.

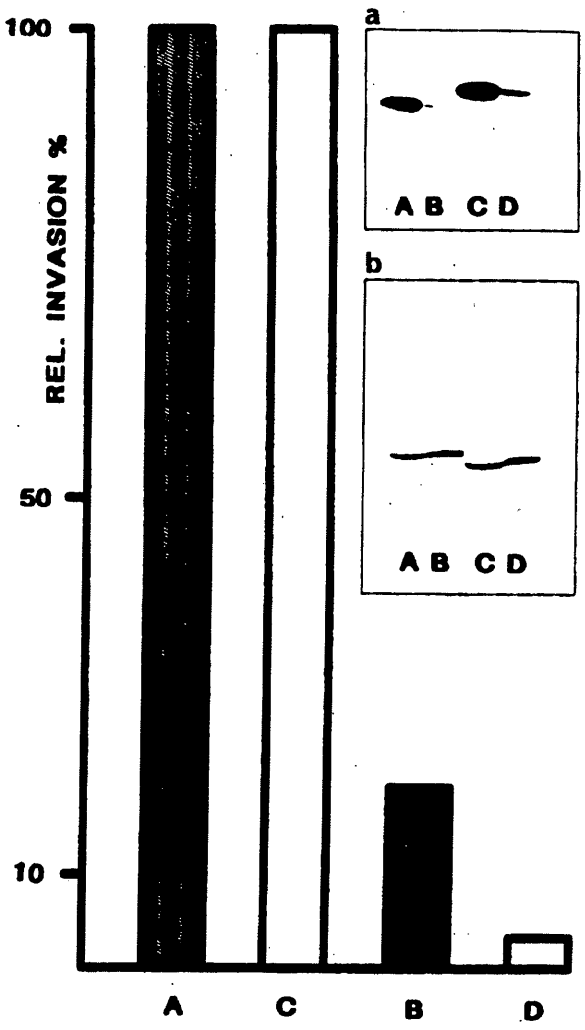


Figure 12: Correlation between reduced synthesis of the 60 kDa protein and reduced ability of mutants from *Listeria monocytogenes* 1/2a to enter 3T6 cells. a, b: Western blot analysis of extracellular proteins from wild-type (A, C) and mutants (B, D) of two *Listeria monocytogenes* Sv1/2a strains. (a): gel developed with antiserum against the 60 kDa protein; (b): gel developed with anti-SLO. Diagram shows the ability of the same wild-type and mutant strains as above to enter 3T6 cells. Experimental conditions were the same as described in Figure 9.

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