Association of Listeriolysin O with the Cell Surface
of Listeria monocytogenes

JACEK BIELECKI
Institute of Microbiology, Warsaw University
Nowy Świat 67, 00-046 Warsaw, Poland
Received 3 September, 1994

Abstract

The single amino acid substitutions were created in the C-terminal region of listeriolysin O. Mutations cys484 to Ala and Ser showed a close relationship between surface activity of LLO and presence of cysteine in this polypeptide. We demonstrated that Cys484 is necessary for surface haemolytic activity. LLO with Cys484 is strongly connected to the surface of the cell but LLO Ala484 or Ser484 is easy removed by the washing. Listeriolysins O secreted by the mutants were active at low pH and inhibited by cholesterol. Mutated hemolysin was still active at high pH without cysteine in reaction mixture whereas wild hemolysin was not. Surface haemolytic activity was blocked in Ala484 strain at high pH. The finding that mutants Ala484 and Ser484 have changed surface activity suggests that cysteine in LLO may play a significant role for surface haemolytic activity necessary in late stages of Listeria monocytogenes cell cycle.

Introduction

Listeriolyisin O (LLO) is an essential determinant of pathogenicity. It is a sulphhydril-activated hemolysin. We previously reported that Bacillus expressing listeriolysin O can grow in the cytoplasm of J774 macrophage-like cells (Bielecki et al., 1990). LLO is not unique in its ability to mediate lysis of the host vacuole. Bacillus subtilis strains expressing PFO (perfringolysin O) grew within a macrophage cell line to P ortsnoy et al., 1992b). These thiol-activated cytolysins belong to family of related haemolysins. Common features shared among these haemolysins include inhibition by free cholesterol, the presumed receptor and the presence of the single unique cysteine that renders the haemolysins susceptible to reversible inactivation by oxidation. There is a conserved undecapeptide ECTGLAWEWWKR in the C-terminal region of the protein. It was demonstrated (Pinkey et al., 1989; Saunders et al., 1989) that the cysteine residues of SLO (streptolysin O) and PLO (pneumolysin O) are not essential for activity. Four isogenic mutant strains differing by single amino-acid substitution in the conserved
region of LLO were constructed and analysed (Michel et al., 1990). The thiol group of Cys484 was not essential for either haemolytic activity in vitro or virulence in vivo. The authors have found that only mutation Trp492 appears to be required for both hemolytic activity and virulence. Listeriolysin O is the most prominent of a set of the PrfA-dependent proteins (PdPs). All PdPs are either secreted or are localised at the cell surface (Portnoy et al., 1992a; Sokolovic et al., 1993). It was shown that the expression of the five clustered genes of \textit{L. monocytogenes} plcA, hly, mpl, actA and plcB is under the control of the positive regulation factor PrfA. Stress conditions lead to the induction of the additional PdPs: ActA and PlcA (Sokolovic et al., 1993). There was a strong induction of LLO under heat-shock conditions.

In this communication, I tried to explain the role of the unique cysteine of the LLO for the activity of the LLO as the surface protein of \textit{L. monocytogenes}. We have created single amino acid substitutions and Cys484 was mutated to Ala or Ser. LLOs secreted by mutants were active at low pH and inhibited by cholesterol as the wild type. At high pH listeriolysin O with Ala484 was, unlike to wild hemolysin, still active without reducing agent in reaction. Both mutated listeriolysin O were easily washed from the cell surface of \textit{L. monocytogenes} cells. There were no surface hemolytic activity of the washed pellets of Ala484 and Ser484 strains at high pH without reducing agents. We suggest that the unique cysteine in LLO can be required for its hemolytic activity at pH higher than 7.0 and for the activity of LLO on the surface of the cell of \textit{L. monocytogenes}.

Experimental

Materials and Methods

\textbf{Bacterial strains and growth conditions.} The bacterial strains and plasmids used in this study are listed in Table I. Bacteria were grown on brain heart infusion agar (BHI – Difco Laboratories, Detroit, Mich.) or in BHI broth at 37° or 42°. \textit{Escherichia coli} strains were grown in LB (Davis, 1980) broth or agar. These media were supplemented with ampicillin (50 μg/ml), chloramphenicol (20 μg/ml) or tetracycline (12.5 μg/ml) purchased from Sigma. All stock cultures were stored as suspensions of cells at -70° in 50% glycerol.

\textbf{DNA isolation and transformation.} Plasmid DNA was isolated as described by Maniatis et al. (1982). For large scale plasmid isolation bacteria were grown in 100 ml of BHI broth and a plasmid enriched fraction was isolated by a large scale version of plasmid screening procedure. Plasmid DNA was further purified in CsCl-ethidium bromide density gradient. Total DNA of \textit{Listeria} was isolated as described by Flamm et al. (1984). \textit{E. coli} was transformed by the CaCl\textsubscript{2} heat shock method (Maniatis et al., 1982).

\textbf{Restriction endonuclease analysis.} The restriction endonucleases BamHI, HindIII, HincII and EcoRI were used under conditions recommended by the supplier (Gibco BRL, Berlin, Germany). Restricted DNA (approximately 0.2 μg) was subjected to electrophoresis in a horizontal 0.7% agarose gel in TBE buffer (Maniatis et al., 1982). DNA fragments were purified by recovery of DNA from low-melting-temperature agarose (BRL) gels or electroelution into dialysis bags.
Table I
List of bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype or Relevant Properties</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>I0403S</td>
<td><em>Listeria monocytogenes</em>; wild type</td>
<td>Bishop and Hinrichs, 1987</td>
</tr>
<tr>
<td>JB1182</td>
<td>1040S (Cya+484Ala)</td>
<td>This study</td>
</tr>
<tr>
<td>JB1183</td>
<td>1043S (Cya+484Ala)</td>
<td>This study</td>
</tr>
<tr>
<td>MC10061</td>
<td><em>E. coli</em>, hsdR mcrB araD139 Δ (araABC-leu) 7679 Δ lacX74 galU galK rpsL thi.</td>
<td>Casadaban et al., 1983</td>
</tr>
<tr>
<td>CJ 236</td>
<td>E. coli, dtr+ “ung”</td>
<td>Kunkel et al., 1987</td>
</tr>
<tr>
<td>DH5 ntF</td>
<td>E. coli, endA1 hsdR17 supE44 thi-1 recA1 gyrA9, relA1</td>
<td>Hanahan, 1983</td>
</tr>
</tbody>
</table>

Transformation of *L. monocytogenes*. Protoplasts of *L. monocytogenes* were prepared and transformed with plasmid DNA by using a procedures described by Camilli et al. (1990). For transformation, 1 μg of pLJV was added. Protoplasts were plated onto DM3 plates (Chang and Cohen, 1979), 0.5M sodium succinate (pH 7), 0.5% Casamino Acids, 0.5% yeast extract, 0.35% K₂HPO₄, 0.15% KH₂PO₄, 0.5% glucose, 0.02 M MgCl₂, 0.01% bovine serum albumin, 0.8% agar containing 12.5 μg of tetracycline per ml and incubated at 30°. Tetracycline resistance transformants formed colonies after 3 to 4 days.

Assay for hemolytic activity. Listeriolysin O activity was assayed as described Kingdon and Sword (1970). Briefly, two-fold serial dilutions of supernatant were made in phosphate buffered saline containing 6M cysteine (pH 5.8). After a 30 min incubation at 37°, a 1/10 volume of a 10% solution of sheep red blood cells was added to the sample. Sheep red blood cells (RBC), less than three weeks old, were washed three times in phosphate buffered saline, pH 7.4. They were diluted just before use into PBS with addition of cysteine when necessary, pH 5.6. After an additional 30 min incubation at 38° the tubes were subjected to centrifugation and were scored for visible haemolysis. Hemolytic units were expressed as the reciprocal of the highest dilution showing complete haemolysis.

Analysis of the surface proteins. Bacterial isolates were grown in brain heart infusion (BHI) broth for 18h at 37°C. Cells were collected by centrifugation and washed three times in phosphate-buffered saline (PBS). The resulting pellet was resuspended in PBS containing chloramphenicol (20 μg/ml) and used for hemolytic assay according to standard procedure described for supernatant. When it was necessary, cells from the pellet were additionally incubated with 5M lithium chloride or other compounds and washed again in PBS. For determination of the protein content on the cell surface the pellet was resuspended in sample buffer FSB (2%, w/v, SDS, 10%, v/v, glycerol, 5%, v/v, β-mercaptoethanol, 0.002% bromophenol blue, 0.02M Tris/HCl), boiled for 5 min at 100°C and centrifuged again in Eppendorf tube. SDS-extracted proteins on this way were checked on SDS-PAGE according to Laemmli (1970). For determination of the presence of LLO on the surface of the washing cells, the cells were resuspended in 25 mM Tris-HCl buffer containing EDTA (10 mM), glucose (50 mM), lysosome (4 mg/ml), incubated 30 min at 37°, diluted 1:1 with 2× FSB, boiled 5 min and analysed by SDS-PAGE and Western blotting.

SDS-PAGE and Western immunoblotting. Culture supernatants were prepared for electrophoresis by precipitation with 10% TCA on ice for 1h., suspended 20 μl of Laemmli sample buffer (Laemmli, 1970) and heated to 95°C for 3 min. Protein separation was achieved by SDS-polyacrylamide gel electrophoresis in 12.5% polyacrylamide gels as described by Laemmli (1970). Surface proteins were obtained after washing the cell pellet three times with PBS, shaking the cells with sample buffer and boiling by 5 min. The sample buffer extracts were examined on SDS-PAGE gels. For Western blotting, proteins were transferred to nitrocellulose (Schleicher and Schuell, Keene, N.H.) with a Transphor electroblotter Hoefer Scientific Instrument, San Francisco, Calif.). All subsequent steps were performed in
50 mM Tris (pH 7.5) – 2 mM EDTA – 0.15M NaCl – 0.5% Nonidet P-40 – 1% calf serum. The nitrocellulose filter was first treated overnight in 1% bovine serum albumin and then incubated for 1 h with a mouse anti-LLO monoclonal antibody (kindly provided by William Bibb). The nitrocellulose filter was washed and reacted with 1251-labelled sheep antimouse immunoglobulin G (Amersham Corp., Arlington Heights, Ill.) After extensive washing, the filter was exposed to X-ray film at -70°C in the presence of two intensifying screens.

DNA sequencing. The dideoxy chain-terminator sequencing technique was used on M13 single stranded DNA (Sanger et al., 1977) using 32P-dATP (Amersham, 800 Ci/mmol) and Sequenase Version 2.0 kit. Sequencing was performed according to procedure recommended by the supplier (USB). All sequencing reactions were run according to Step-By-Step Protocols from US Biochemical Corporation.

Hybridizations. The filters from Southern blots were hybridized with DNA probes that had been labelled in vitro with 32P-dCTP at 3000 Ci/mM (Amersham). Hybridizations were performed under conditions of high stringency at 37°C (Camilli et al., 1989). Autoradiography with XAR-5x-ray films (Eastman Kodak) was done for revealing of hybridization.

In vitro mutagenesis. Oligonucleotide site-directed mutagenesis in M-13 was performed using the Protocols of Kunkel (Kunkel, 1985; Kunkel et al., 1987). Oligonucleotides used for the mutagenesis: 5'CCAGTGCGCTCTTTAGGCG-3’ for mutation Cys484Ala and 5’-CCAGTGCGTTCTTTAGGCG-3’ for mutation Cys484Ser (changes from the wild type sequence are underlined). Single stranded bacteriophage vector with insert (Hind III fragment from hly A gene) containing a small number of uracil residues in place of thymine was prepared from E. coli CI 236 (dut, ung) strain. A synthetic oligonucleotide containing the mutation of interest was annealed to the template and treated with T7 DNA polymerase (USB) and T4 DNA ligase (BRL) to produce a double-stranded circular molecule. Introduction of this heteroduplex molecule into a wild type (dut ung) strain allowed the efficient recovery of mutant DNA. Mutated fragment Hind III from RF of the bacteriophage M13 was cloned to the thermosensitive for DNA replication (Villafane, 1987) plasmid pHIV and transformed to L. monocytogenes 1040S. Allelic exchange between plasmid and chromosome was obtained after integration of the plasmid in the chromosome by selecting for Cm' transformants at 42°C. Isolation of plasmid-chromosome cointegrates and identification of resolution products was done according to Hamilton et al. (1989).

Results

Construction of pHIV derivatives used for allele replacement. The first stage of mutagenesis procedure involved constructing the plasmid pHIV, harbouring the 410 bp HindIII fragment containing the 3' end of hly A with Cys484 mutated to Ala and Ser. To do that we first cloned fragment HindIII from plasmid pDP102 containing a 3.2 kb BamHI fragment insert with the LLO structural gene, hlyA (Camilli et al., 1989) in M13mp19 and mutagenized as described in Materials and Methods. The inserts were completely sequenced to verify that only the desired mutations were present. The sequences of the mutated regions are shown in Fig. 1. Codon Cys484 was mutated to an alanine and to a serine. The 410 bp HindIII fragments carrying mutated sequences were cloned in the E. coli – Listeria shuttle vector pHIV and introduced into L. monocytogenes. After recombination between plasmid and chromosome DNA cells were grown without selection and screened for antibiotic resistance. The clones, where allele exchange
Fig. 1. Nucleotide sequence determination of the DNA fragments. Wild type (panel A), mutant Cys484Ser (panel B) and mutant Cys484Ala (panel C).

had occurred were analysed by Southern blot analysis. Chromosomal DNA isolated from various clones was hybridized with probes which were oligonucleotides used for oligo-directed mutagenesis. A comparison of the hybridization pattern of the chromosomal DNA allowed for selection of the mutants. Such a cross hybridization between changed oligonucleotide and changed sequence in hlyA had been observed. The homology was additionally checked by washing of the hybridization filters in different temperatures above 50°C. An analysis of the mutations Cys484Ala and Cys484Ser allowed to choose desire mutations. The mutant strains derived from *L. monocytogenes* 1040S were verified on this way and were subjected for further analysis.

**Hemolytic activity of the supernatants of the mutants.** Based on data that mutations Cys484Ala and Cys484Ser are present on the chromosome of *Listeria*, strains we prepared supernatant proteins from the wild-type and the mutants and tested for the presence of listeriolysin by SDS-PAGE and immunoblotting with anti-listeriolysin antiserum. Based on the position of the hemolysin from wild type we showed that haemolysins produced by mutant strains have the same molecular weight (Fig. 2). These both listeriolysins had the same hemolytic activity in supernatants assayed in standard conditions. Hemolytic activity of the supernatants from the wild type and the mutants determined at pH 5.6 with addition of the cysteine as the reducing agent (Kingdon and Sword, 1970) were about 80 units. Mutated haemolysins like wild LLO were maximally active
Fig. 2. Western blot analysis of culture supernatants of the wild type and mutated strains.

Proteins were precipitated from culture supernatants fluids with 10% trichloroacetic acid and separated on an SDS – 7% polyacrylamide gel. Each lane represents approximately 2 ml of culture supernatant fluid. Lane 1: strain L. monocytogenes 10403S, Lane 2: mutant Cys484Ala; Lane 3: mutant Cys484Ser.

at low pH and nearly inactive at pH 7.4. Additionally to above presented experiments we have checked the hemolytic activity of the toxins at high pH without reducing agents. It was surprising result that hemolytic activities of the mutated LLOs at pH 7.4 without cysteine added into reaction were much more higher than the activity of the LLO from the wild type. The results presented in Table II did not exclude the role of the cysteine residue for full hemolytic activity of the listeriolysin O from L. monocytogenes.

**Table II**

Maximal hemolytic activity in culture supernatants of strain 10403S and mutant derivatives measured at different pH.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Hemolytic activity</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>with cysteine</td>
<td>pH 5.6</td>
<td>pH 7.4</td>
<td>pH 5.6</td>
<td>pH 7.4</td>
</tr>
<tr>
<td>10403S</td>
<td>80</td>
<td>20</td>
<td>80</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Cys484Ala</td>
<td>80</td>
<td>20</td>
<td>80</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Cys484Ser</td>
<td>60</td>
<td>20</td>
<td>60</td>
<td>40</td>
<td></td>
</tr>
</tbody>
</table>

Surface associated hemolysin in L. monocytogenes. In order to define more than only one function of listeriolysin O we have compared electrophoretic patterns of the cell surface components isolated from the wild type and both mutant
strains and we have checked surface hemolytic activity of the cells washed in PBS and the cells washed in lithium chloride. Western blott analysis showed that LLO from the wild type was not released from the surface when the bacteria were incubated in PBS (Fig. 2, lane 4) and in other media at different temperatures (data not shown). As was shown in Fig. 3, LLO from wild type was detected in the large amounts on the surface of the wild type even after washing in 5M lithium chloride (Fig. 3 lane 1). LLOs Cys484Ala and Cys484Ser were easy washed in the same conditions (Fig. 3 lanes 2 and 3). Mutated LLOs were easy washed out the surface in any case. The effect of the mutations on the hemolytic activity of the surface of *L. monocytogenes* was evaluated by estimation a number of the hemolytic units in washed pellets. Hemolytic activity was then measured in pellets taken from different growth phases. The pellets from 18h cultures the highest activity. As shown in Table 3 the wild-type strain pellet had a hemolytic titer in solution of 80 units/ml. The same hemolytic activity we have found in the pellet of the mutant strains in standard conditions (pH 5.6 with cysteine). The pellet of the wild type was still active in pH 7.4 without cysteine (40 units) whereas mutants Cys484Ala and Cys484Ser had no detectable hemolytic titer in solution inder these conditions (Table 3). The mutant strains formed small zones of haemolysis on blood agar plates that were revealed by lightly scraping away single colonies. We did not observe hemolytic activity of the pellet from the mutant strains washed in lithium chloride whereas wild type had still detectable hemolytic titer (20–40 units). Hemolytic titer was expressed as the reciprocal of the highest dilution of culture supernatant or pellet at which complete lysis of
Table III
Maximal hemolytic activity in the pellets of strain 10403S and mutant derivatives measured at different pH.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Hemolytic activity</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>with cysteine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>pH 5.6</td>
<td>pH 7.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10403S</td>
<td>80</td>
<td>40</td>
<td>80</td>
<td>40</td>
<td></td>
</tr>
<tr>
<td>Cys484Ala</td>
<td>80</td>
<td>30</td>
<td>80</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Cys484Ser</td>
<td>80</td>
<td>30</td>
<td>60</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

.....

erythrocytes was observed. It was shown that the loss of the surface hemolytic activity of the mutated strains was always connected with its loss on the cell surface of *L. monocytogenes*.

**Characterization of the surface hemolytic activity.** Because cholesterol inhibition is a characteristic property of thiol activated haemolsins first we tried to check the influence of the cholesterol on the haemolysis performed by the supernatants or the pellets of the mutants and the wild strain. Haemolysis titrations were done in the presence of various concentrations of cholesterol to evaluate the concentration which inhibited 50% of the hemolytic activity. Inhibition of the supernatants occurred at almost the same concentration of the cholesterol for both mutants and wild type (about $3 \times 10^{-7}$ M) were not inhibited at pH 5.6. We have never seen any differences in hemolytic activity between the mutants and the wild type if the hemolytic activity of the pellet treated with cholesterol was assayed with or without cysteine at pH 5.6. The pellets of both mutants treated by cholesterol were not active at pH 7.4 when the wild *Listeria* still had hemolytic activity about 40 units. Results from these experiments are summarised in Table 2. In this paper we did not described experiments with the restoration of the hemolytic activity by the supernatants of the mutants and wild type in the *Listeria* strains deprived of the listeriolysin O. These experiments are carried out by our laboratory independly.

**Discussion**

The first allele replacement experiments in *L. monocytogenes* were described by Michel *et al.* (1990). They obtained isogenic strains differing by a single amino acid in the sequence of the secreted listeriolysin. Their results demonstrated that thiol group of the cysteine was not absolutely required for activity, in agreement with results obtained with pneumolysin and streptolysin (Pinkley
et al., 1989; Saunders et al., 1989). Altering the single Cys residue in SLO to either Ala or Ser did not abolish its cytolytic activity. The Cys-428Ala modified pneumolysin was almost indistinguishable from the wild-type toxin in terms of hemolytic activity and lytic and inhibitory effects on human polymorphonuclear leucocytes. The Cys-428Ser and Cys-428Gly pneumolysins had reduced activity on erythrocytes, being 20 times less active than the wild type. Saunders et al. (1988) suggested that their reduced activity could be due to forming oligomers that were not functional. It was shown in our experiments that Cys484 in LLO was not essential for toxin hemolytic activity when supernatants of \textit{L. monocytogenes} and its mutants were assayed at standard conditions. To examine directly the role of Cys we have checked activity of the hemolysin from the supernatants of \textit{L. monocytogenes} and both mutants at pH 7.4 without cysteine. We were surprised seeing some differences in hemolytic activity. Mutated haemolysins assayed without cysteine at pH 7.4 were about twofold more active than wild LLO. Earlier suggestions that a Cys residue is essential for LLO cytolytic activity make sense. These results show that Cys484 may play important role as a regulation site in LLO. We still know too less about mechanisms of a chemical regulation of the bacterial toxins. It seems to be very probable that hemolytic activity of the listeriolysin O \textit{in vivo} depends from Cys residue. It can be regulated by different chemical compounds in natural environment of cell cycle of \textit{L. monocytogenes}. Our results indicated another possible role of Cys484 for full activity of listeriolysin O. We are sure that Cys residue is necessary for connection of LLO to the surface structures of \textit{L. monocytogenes} cells. We have shown that there is a hemolytic activity on the surface of the listerial cells. This activity was easy removed by washing the cells in the PBS in the mutated strains. Cell surface proteins have been poorly characterised so far, but they could be involved in the virulence of \textit{L. monocytogenes}. In order to define a hemolytic activity of the cell surface we have compared electrophoretic patterns of cell surface components from wild type and the Cys484 mutants. We have demonstrated that listeriolysin O is located on the surface of the cell together with other proteins like actA gene product (Portnoy et al., 1992). An attempt to detect binding of LLO to the surface by Western blotting was successful. We have shown that listeriolysin O is anchored on the cell surface. We believe that surface hemolysin is necessary during the late stages of the life cycle of \textit{L. monocytogenes}. The LLO is expressed on the bacterial surface by the mutated strains. In this case LLOs expressed on the bacterial surface by the mutated strains. In this case LLOs were easy removed from the surface by washing in PBS or 5M lithium chloride whereas wild hemolysin was not washed from the surface. It has not yet been shown whether the surface listeriolysin is involved in virulence of \textit{L. monocytogenes}. The role of cysteine for surface activity of listerial hemolysin has not been suggested. It can not be excluded that the surface listeriolysin is associated with the \textit{Listeria} surface by other means than the cysteine direct binding, e.g.,
interaction with other proteins. There is probably more than one bacterial product involved in surface hemolytic activity, because, besides the mutants described here, another mutant has been described in our lab which easy loses the surface hemolytic activity, i.e., 60 kDa – protein minus strain (data not shown). The 60 kDa protein is expressed on the bacterial surface and could be a surface hemolysin activator or regulator. Future physical characterization and the use of new mutants received in our laboratory may provide an insight into the exact nature of the surface hemolytic activity. We still are not sure if surface hemolytic activity depends on the protein anchored to the cell surface or the protein released from the bacterial surface. Could the surface hemolysin directly interact with the cell membrane? We have not enough data to answer right now. We need more time for explanation of this complicated mechanism. We hope to explain the role of the C-terminal residue for surface hemolytic activity by complementation tests with other surface components in near future.

**Acknowledgements.** Author is very grateful to Dr Daniel Portnoy of the University of Pennsylvania, Philadelphia, USA in whose laboratory part of this work was performed and Dr Z. Bielecka for help and advice on DNA sequencing. This study was supported by the grant 0530/P2/93/04 from KBN (Poland) and Biomol 8 Program (FNP).

**Literature**


