PURIFICATION AND CHARACTERIZATION OF CYTOLYSINS FROM LISTERIA MONOCYTOGENES SEROVAR 4b AND LISTERIA IVANOVII

J. KREFT, D. FUNKE, R. SCHLESINGER, F. LOTTSPEICH and W. GOEBEL

Institute of Genetics and Microbiology, University of Würzburg, and Max-Planck-Institute for Biochemistry, Martinsried, FRG.

Several exoproteins from Listeria monocytogenes serovar 4b (NCTC 10527) and Listeria ivanovii (ATCC) 19119, SLCC 2379), respectively, have been purified to homogeneity by thiol-disulfide exchange chromatography and gel filtration. Both strains produce a haemolytic/cytolytic protein of Mr 58 kDa, which has all the properties of a SH-activated cytolysin, the prototype of which is streptolysin O (SLO), and this protein has therefore been termed listeriolysin O (LLO). In addition a protein of Mr 24 kDa from culture supernatants of L. ivanovii co-purified with LLO. The N-terminal aminoacid sequences of both proteins from L. ivanovii have been determined. By mutagenesis with transposons of Gram-positive origin (Tn916 and Tn1545), which have been introduced via conjugation into L. ivanovii, several phenotypic mutants (altered haemolysis on sheep blood agar or lecithinase-negative) were obtained. Results on the properties of these mutants will be presented.

It is generally accepted that listeriolysin O, a SH-activated exotoxin produced by several pathogenic Listeria, plays an important role in the pathogenesis of these facultative intracellular bacteria [1, 2]. In order to elucidate if Listeria strains other than Listeria monocytogenes serovar 1/2a produce such toxins, we undertook a search for the detection and subsequent purification/characterization of such exoproteins from different Listeria strains and species.

The use of conjugative transposons from Gram-positive bacteria (transposons Tn916 and Tn1545) has proven to be helpful in the identification of virulence factors from Listeria [2, 3], therefore we tried to adapt this method to Listeria ivanovii.

Materials and methods

Bacteria. L. ivanovii (ATCC 1919, SLCC 2379), L. monocytogenes serovar 4b (NCTC 10527) nad Rhodococcus equi (NCTC 1621) were from the strain collection of the Institute for Hygiene and Microbiology, University of Würzburg. Streptococcus faecalis CG110 (with Tn916) was donated by D. B. Clewell (Ann Arbor, USA), L. monocytogenes BM4140 (with Tn1545) and Escherichia coli BM2962 (with pAT93) by P. Courvalin (Paris). Bacteria were grown on brain heart infusion broth (BHI), Gibco/ or blood agar base (BAB No. 2, Oxoid), supplemented as described below, at 37 °C.
Purification of listeriolysin was done by thiol-disulfide exchange chromatography on thiopropyl-Sepharose 6B (Pharmacia) and gel filtration of Biogel P-100 (Biorad) as described [4]. SDS polyacrylamide gel electrophoresis of proteins was performed with TCA-precipitated culture supernatants of 12.5% slab gels [5].

Chromosomal and plasmid DNAs were isolated according to published procedures [6, 7] with minor modifications.

Transposon mutagenesis of L. ivanovii was performed essentially as described [2] by filter matings of Streptococcus (with Tn916) or L. monocytogenes (with Tn1545) and a streptomycin-resistant (SmR) mutant of L. ivanovii.

Procedures for DNA-(Southern-) hybridization and immunoblotting have been previously described [2].

Haemolysin assays were performed as described in the adjacent paper [8].

In enzymatic tests for sphingomyelinase (Smase) and phospholipase (Plase) TNPAL-sphingomyelin or p-nitrophenyl-phosphorylcholine, respectively, were used as substrates [9, 10].

Results

Purification and characterization of listeriolysin 0 and the 24 kDa protein.

By thiol-disulfide exchange chromatography and gel filtration [4] we could purify from BHI-culture supernatants (concentrated about 50-fold by ultrafiltration) of both L. ivanovii and L. monocytogenes serovar 4b a single protein of Mr 58 kDa, which fulfilled all the requirements for a SH-activated cytolysin of the streptolysin O (SLO) type [11], namely haemolytic activity, inactivation by oxygen or micromolar amounts of cholesterol, activation by SH-reagents (dithiothreitol, DTE) and immunological cross reaction with anti-SLO (unpublished [12]). These cytolysins were therefore identified as listeriolysin O (LLO). In the case of L. ivanovii a second major supernatant protein of Mr 24 kDa copurified with LLO. The possible role of this protein has still to be elucidated. The N-terminal aminoacid sequences of LLO and the 24 kDa protein from L. ivanovii have been determined (Fig. 1).

Transposon mutagenesis. L. ivanovii was mutagenized by the conjugative transposon Tn916 [2] and Tn1545 [13], respectively. Tn916 proved to be

![Fig. 1. N-terminal aminoacid sequence of LLO from L. ivanovii, compared to LLO from L. monocytogenes serovar 1/2 [14] and of the 24 kDa protein from L. ivanovii. X means an amino acid which could not be determined precisely (most presumably serine). Residues homologous to LLO from L. monocytogenes are underlined.](image-url)
rather inefficient when looking for altered haemolytic/phospholipolytic phenotypes of the transconjugants. Tn1545 was much more successful, yielding five mutants of a relevant phenotype among 3000 recipients. The screening of all transconjugants was done on BAB/blood agar plates +/− DTE or with erythrocytes pretreated with R. equi culture supernatant or on egg yolk agar plates.

L. ivanovii mutants with relevant phenotypes were analyzed by Southern hybridization of their chromosomal DNAs to probes specific for Tn916 or Tn1545, thus demonstrating the presence of the respective transposon in the chromosome of the mutant transconjugants (data not shown). Furthermore the exoprotein pattern, the enzymatic (Smase and Plase) activities in concentrated culture supernatants and the behaviour in the classical CAMP-test of the mutants were compared with the wildtype strain. Smase and Plase could be differentiated by the use of specific substrates (see above). The results from the most interesting mutants are shown in Table I.

**Table I**

*Relevant properties of Tn1545 mutants*

<table>
<thead>
<tr>
<th>Strain</th>
<th>WT</th>
<th>20/24</th>
<th>8/6</th>
<th>44/2</th>
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<tbody>
<tr>
<td>58 KDa</td>
<td>+</td>
<td>−</td>
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<tr>
<td>24 KDa</td>
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<td>Smase</td>
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<td>+</td>
<td>+</td>
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<td>Plase</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>CAMP</td>
<td>+</td>
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Smase: Sphingomyelinase C activity
Plase: Phospholipase C (lecithinase) activity

**Discussion**

From the results described above several conclusions can be drawn:
(i) L. ivanovii (ATCC 19119, SLCC 2379) and L. monocytogenes serovar 4b (NCTC 10527) both produce and secrete a haemolysin/cytolysin comparable to streptolysin O (SLO), therefore termed listeriolysin O (LLO), with a Mr of 58 kDa. The N-terminal aminoacid sequence of LLO from L. ivanovii shows significant homology to LLO from L. monocytogenes serovar 1/2a [14].
(ii) Transposon mutagenesis of L. ivanovii proved to be possible and useful, in particular with Tn1545. Several mutant types were obtained, where one or several exoproteins were no longer secreted, pointing to a possible coor-
ordinate regulation of the genes responsible for these proteins. Comparison of the phenotypes, protein patterns and enzymatic activities showed that most presumably the sphingomyelinase present in the wild type L. ivanovii is the factor which interacts with the R. equi exosubstance in the CAMP-reaction.

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REFERENCES