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

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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 muntants 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.

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Purification of listeriolysin was done by thiol-disulfide exchange chromatography on thiopropyl-Sepharose 6B (Pharmacia) and gel filtration of Biogel P-100 (Biorad) as decribed [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 decribed 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 O and the 24 kDa protein. By thiodisulfide 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

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LLO L. Ivanovii	Asp	Ala	Ser	Val	Tyr	Ser	Tyr	Gln	Gly	Ile	Ile	X (Ser)	His	Met	Ala	
LLO L. monocyt.	Asp	Ala	Ser	Ala	Phe	Asn	Lys	Glu	Asn	Ser	Ile	Ser	Ser	Met	Ala	

				20			
LLO L. Ivanovii	Pro	Pro	Ala	X (Ser)	Pro	Pro	Ala	Ser
LLO L. monocyt.	Pro	Pro	Ala	Ser	Pro	Pro	Ala	Lys

24 KDa   Ala Asp Ile Leu X Pro Met Pro Ile Asn Gln Ile Phe Pro Asp

Pro/Leu   Asp   Leu   Ala

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

rather inefficient when looking for altered haemolytic/phospholipolytic pheno-
types 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

Table I

Relevant properties of Tn1545 mutants

Strain	WT	20/24	8/6	44/2
58 KDa	+	−	−	+
24 KDa	+	+	−	−
Smase	+	+	+	−
Plase	+	+	−	+
CAMP	+	+	+	−

Smase: Sphingomyelinase C activity
Plase: Phospholipase C (lecithinase) activity

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 con-
centrated 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.

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-

dinate 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.

REFERENCES

1. Gaillard, J. L., Berche, P., Sansonetti, P.: Infect Immun **52,** 50 (1986).
2. Kathariou, S., Metz, P., Hof, F., Goebel, W.: J Bacteriol **169,** 1291 (1987).
3. Berche, P., Gaillard, J. L., Sansonetti, P. S.: J Immunol **138,** 2266 (1987).
4. Geoffroy, C., Alouf, J. E.: J Biol Chem **258,** 9968 (1983).
5. Laemmli, U. K.: Nature **227,** 680 (1970).
6. Birnboim, H. C., Doly, J.: Nucl Acids Res **7,** 1513 (1979).
7. Flamm, R. K., Hinrichs, D. J., Thomashow, M. F.: Infect Immun **44,** 157 (1984).
8. Haas, A., Kreft, J., Goebel, W.: Acta Microbiol Hung **36,** (1989).
9. Gatt, S., Dinur, T., Barenholz, Y.: Biochem Biophys Acta **530,** 503 (1978).
10. Kurioka, S., Matsuda, M.: Anal Biochem **75,** 281 (1976).
11. Smyth, C. J., Duncan, J. L.: In Jeljaszewicz, J., Wadström, T. (eds): Bacterial Toxins and Cell Membranes. Academic Press, New York 1978. p. 129.
12. Goebel, W., Kathariou, S., Kuhn, M., Sokolovic, Z., Kreft, J., Köhler, S., Funke, D., Chakraborty, T., Leimeister-Wächter, M.: Infection **16,** 149 (1988).
13. Collatz, E., Carlier, C., Courvalin, P.: J Bacteriol **156,** 1373 (1983).
14. Mengaud, J., Vicente, M. F., Chenevert, J., Pereira, J. M., Geoffroy, C., Gicquel-Sanzey, B., Baquero, F., Perez-Diaz, J. C., Cossart, P.: Infect Immun **56,** 766 (1988).