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ISOLATION AND CHARACTERIZATION OF GENES CODING FOR PROTEINS INVOLVED IN THE CYTOLYSIS BY *LISTERIA IVANOVII*

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We established a library of chromosomal DNA of *Listeria ivanovii* in the pTZ19R plasmid system, using *Escherichia coli* DH5alpha as the host. One recombinant clone reacted strongly with a polyclonal antiserum raised against the listeriolysin O and a second exoprotein (24kDa) of *L. ivanovii*, which is most probably also involved in cytolytic processes. The recombinant *E. coli* clone may contain part of the listeriolysin O gene of *L. ivanovii*.

Analysis of bacterial cytolysins by genetic manipulation is of special interest because the genes encoding cytolytic factors can be removed from their natural genetic background and transferred to a different one, e.g., an *Escherichia coli* cell. Thus, detailed biochemical analysis becomes possible. Here we present a study using methods of molecular biology to isolate genes coding for cytolytic factors of the strongly haemolytic *Listeria ivanovii*.

Materials and methods

Bacteria. L. ivanovii strain SLCC 2379 and *E. coli* strain DH5alpha were used.

Isolation of chromosomal DNA of L. ivanovii has been carried out according to [1] with some minor modifications.

Isolation of plasmid-DNA. Large scale plasmid-preparation and cesium chloride density-gradient centrifugation was performed as described in [2], with an additional proteinase and RNase treatment after alkaline lysis. Small scale plasmid isolation was performed according to [3].

DNA-ligations, digestions with restrictions enzymes, DNA/DNA-hybridizations, formation of completent cells were performed as described in [2]. For DNA/DNA-hybridization assays the nitrocellulose was incubated in hybridization buffer for 2 h at 50 °C, the denatured DNA probe was added, and the nitrocellulose incubated overnight at 50 °C. The filter was washed twice with 5 × SSC/0.1% SDS at 40 °C for 20 min each. Following autoradiography, the nitrocellulose was washed again with 1 × SSC/0.1% SDS at 40 °C for twice 20 min and again used for autoradiography. DNA was labelled using the Boehringer Mannheim random primed labelling kit.

Screening of the library has been performed according to [4] using a polyclonal antiserum raised in rabbits against a mixture of 58kDa (LLO) and 24kDa proteins of *L. ivanovii* (dilution of the antiserum 1 : 1000). The 24kDa protein co-purified by thiodisulfide exchange chromatography of culture supernatants of *L. ivanovii* and could not be separated from the LLO under native conditions.

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Immuno (Western-) Blots were performed as described in [5] using an anti-SLO-serum (generous gift of Dr. J. Alouf, Paris: Charge 94) or anti-LLO-serum (as described above), at a dilution of 1 : 1000 and 1 : 100, respectively. To reduce non-specific binding, all sera were preadsorbed to cellular lysates of *E. coli* DH5 alpha harbouring the pTZ19R plasmid without a DNA insert.

Haemolytic assays. Cellular lysates of *E. coli* were first treated with 10 mM dithiothreitol during 15 min at 37 °C. Then they were incubated with 1% erythrocytes in phosphate-buffered saline (pH 6.0) at 37 °C for 30 min and centrifuged. In the supernatant, haemolysis was measured spectrophotometrically at 543 nm.

Results

A total of 4500 recombinant *E. coli* clones were obtained by ligation of Sau3A partially digested *L. ivanovii* chromosomal DNA into the BamHI digested vector pTZ19R and subsequent transformation into *E. coli* DH5alpha. Ampicillin-resistant *E. coli* transformants were first phenotypically screened for a *Listeria* DNA insert by colour reaction of LB agar containing 0.2 mM IPTG and 40 mg/l X-Gal (recombinant *E. coli*-clones appear colourless [2]). Fifty-one of these clones were randomly chosen for insert DNA size analysis. The molecular weight of *Listeria* insert DNA varied from 0.3 kb to 12.0 kb, with an average of 3.9 kb.

If one presupposes that the chromosome of *L. ivanovii* is of about the same size as the *E. coli* chromosome, and that all DNA fragments are ligated

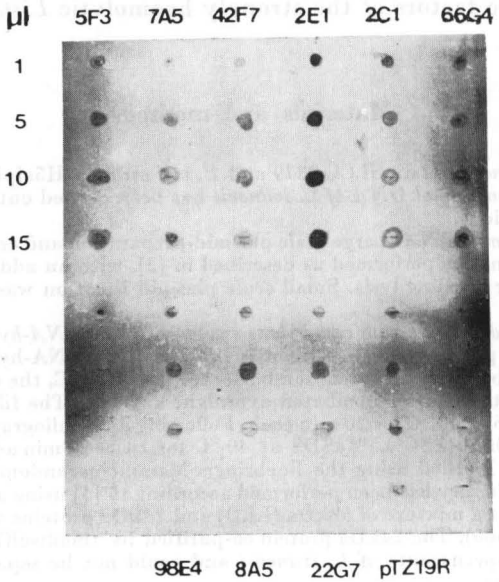

Fig. 1. Dot-blot immunoassay: cellular lysates of those recombinant *E. coli*-clones which reacted strongly in the colony-immunoblot were dotted onto nitrocellulose. The antigenic proteins were detected with anti-LLO-serum (1 : 1000), followed by visualization of primary antibody binding by a second, peroxidase conjugated swine-anti-rabbit-antibody (1 : 1000). "pTZ19R" marks the negative control

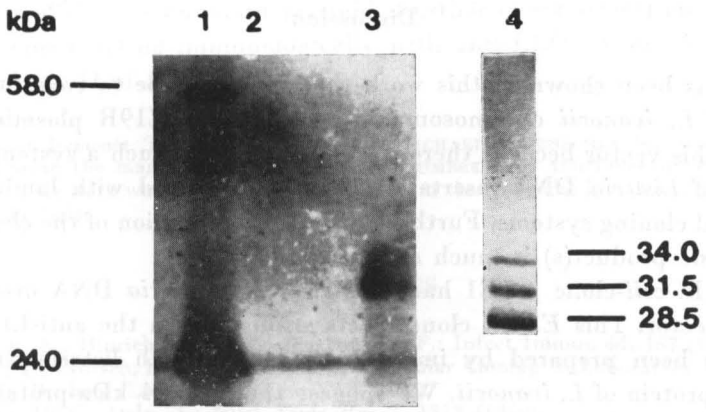

Fig. 2. Western-blot immunoassay using anti-LLO-serum, visualization as in Fig. 1. Lane 1: culture supernatant of *L. ivanovii;* lane 2: cellular lysate of *E. coli* DH5alpha (pTZ19R); lanes 3 and 4: cellular lysate of *E. coli* 2El. The proteins were probed with an anti-LLO-serum (lanes 1, 2 and 3 at a dilution of 1 : 1000, lane 4 at 1 : 100)

and stably transformed with the same frequency, then the probability that any gene is included in the library is 98% [6].

Out of the 4500 recombinant *E. coli* clones 9 were strongly positive in the colony-blot immunoassay with anti-LLO-serum. Using a dot-blot immunoassay of cellular lysates of these 9 recombinants, one clone remained strongly positive (Fig. 1). This *E. coli* clone was called #2E1.

In a Western-blot assay using the anti-LLO-serum (described in the methods section), 3 proteins were detected in cellular lysates of #2E1 which reacted immunologically, having molecular weights of 34.0, 31.5 and 28.5 kDa, respectively (Fig. 2, lane 4). Using the anti-LLO-serum at a dilution 1 : 1000, only one of these proteins (having a molecular weight of 31.5 kDa) was detected (Fig. 1, lane 3). The 34.0 kDa protein seems to be the primary gene product, and both other proteins may be degradation products, as proteolysis of recombinant proteins occurs rather often in *E. coli* [7]. Surprisingly, none of these proteins reacted immunologically in a Western-blot analysis with anti-streptolysin O (SLO-) serum, but there was a reaction with another protein having a molecular weight of 24. OmkDa.

The *Listeria*-DNA insert of #2E1 is 4.5 kb and hybridizes with three distinct fragments of *L. ivanovii* chromosomal DNA digested with EcoRI (1.3 kb, 5.4 kb and 7.0 kb molecular weight, respectively). It also hybridizes with chromosomal DNA from *L. monocytogenes* 1/2a EGD, digested with EcoRI and HindIII (molecular weights of 2.4 kb and 2.65 kb, respectively [8].

Cellular lysates of #2E1 do not show enhanced haemolytic activity compared with a vector-containing strain of *E. coli* DH5alpha. This result was also found after preincubation of the lysates in 10 mM dithiothreitol, which elevates the haemolytic potential of listeriolysin O [9].

Discussion

It has been shown in this work that it is possible to construct a gene library of *L. ivanovii* chromosomal DNA in the pTZ19R plasmid system. We used this vector because there is evidence that in such a system a higher stability of *Listeria* DNA inserts is achieved compared with lambda phage and cosmid cloning systems. Furthermore, characterization of the cloned DNA and its gene product(s) is much simplified.

The *E. coli*-clone #2E1 harbours a 4.5 kb *Listeria* DNA insert in the pTZ19R vector. This *E. coli* clone reacts strongly with the anti-LLO-serum, which has been prepared by immunizing rabbits with listeriolysin O and a 24 kDa-protein of *L. ivanovii*. We suggest that the 24 kDa-protein is most probably involved in the regulation of cytolytic processes or is a cytolysin itself. The 24 kDa-protein binds to erythrocyte membranes and studies with transposon mutants of *L. ivanovii* revealed that it may be regulated in a coordinated fashion with the sphingomyelinase and/or lecithinase genes. Apparently, a primary gene product, having a molecular weight of 34.0 kDa, is expressed in #2E1 from the inserted *Listeria* DNA. The gene for the 24.0 kDa-protein cannot encode a protein of this molecular weight, neither as a protein having a signal sequence for transport, nor as a fusion protein with the vector's beta-galactosidase.

Therefore, we suggest that the recombinant *L. ivanovii* DNA in #2E1 consists at least partially of the genetic information corresponding to the amino terminal part of listeriolysin O of *L. ivanovii*. This *Listeria* DNA fragment apparently bears its own transcription and translation signals, because the partial gene is expressed in *E. coli* without induction of transcription. Of course, we cannot exclude the possibility that there exists a protein in *L. ivanovii* which cross-reacts with the anti-LLO-serum used in our studies and which is only slightly expressed in *L. ivanovii*, but which, upon cloning into a multi-copy-plasmid, such as pTZ19R, is expressed at high levels in *E. coli*. Such a gene product would then be detected by the colony-immunoassay. One can speculate that the epitope(s) of listeriolysin O reacting with the anti-SLO-serum lie(s) within the carboxyterminal part of the protein. This suggestion is supported by the fact that amino acid sequence comparisons between the SH-activated cytolysins reveal significant homologies only in the carboxyterminal part of these proteins [10]. This region is, according to our hypothesis, not expressed in *E. coli* #2E1 and therefore no cross-reaction of the recombinant protein from this partial gene with anti-SLO-serum would be expected.

The recombinant 24 kDa-protein which is expressed in #2E1 and which cross-reacts immunologically with the anti-SLO-serum used in our experiments is most probably the product of another gene lying within the *Listeria*

DNA insert of this recombinant plasmid. Further characterization will reveal if the proteins reacting immunologically with anti-LLO-serum are related to the listeriolysin O of *L. ivanovii*.

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