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CLONING AND EXPRESSION IN *ESCHERICHIA COLI* OF A GENE ENCODING SUPEROXIDE DISMUTASE FROM LISTERIA IVANOVII

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A chromosomal DNA fragment from the gram-positive bacterium *Listeria ivanovii* (ATCC 19119) encoding a superoxide dismutase (SOD) gene has been cloned in *Escherichia coli* QC779 (sodAsodB) using the plasmid vector pTZ19R. The DNA fragment inserted into the plasmid showed high structural instability in *E. coli* QC779 (recA$^+$), but turned out to be a stable 1.95 kbp DNA fragment when transformed into *E. coli* DH5α (recA$^-$). The gene is expressed in both of these *E. coli* strains at high levels. Preliminary studies showed that the activity of the recombinant SOD within *E. coli* DH5α was up to 13-times the combined activity of both *E. coli* SODs. The recombinant SOD forms active hybrid SODs with both *E. coli* SODs *in vivo*.

KEY WORDS: Molecular cloning, superoxide dismutase, *Listeria ivanovii*, heterologous gene expression, hybrid superoxide dismutase.

INTRODUCTION

Listeria ivanovii (formerly *L. monocytogenes serotype 5*, ATCC 19119) is a gram-positive bacterium which can cause severe illness in animals[1] (especially sheep and cows) and, very rarely, in man. A very interesting feature of this and other pathogenic listeriae is their ability to survive and even multiply within host cells such as macrophages (therefore, pathogenic listeriae have been termed "intracellular parasites"). Macrophages are designed to take up foreign material (such as bacteria) and to subsequently kill and digest them. Oxygen derivatives are one of the tools macrophages use for this purpose.

In this context, it has been proposed that the superoxide dismutases and/or catalases of pathogenic listeriae may play important roles in the intracellular survival.[2,3] In order to achieve a better understanding of the role of Listeria SOD in pathogenesis we attempted to isolate the SOD gene from *Listeria ivanovii*.

Listeria monocytogenes SOD previously has been suggested to be a FeSOD.[3] However, data from other groups[4,5] as well as our own investigations suggest that *Listeria sp.* most probably possess a single predominately manganese-containing SOD. Neither the molecular weights of the subunits nor the number of subunits forming active Listeria SOD have been published. Oxygen- and iron-inducibility of Listeria SOD *in vivo* has been reported[2,5] as well as a procedure for its partial purification.[2]

In the experiments described here, we transformed into *E. coli* QC779 a partial gene library of chromosomal DNA fragments inserted into the plasmid pTZ19R. *E. coli*

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QC779 which lacks both sodA and sodB activity due to insertion mutagenesis is not viable when grown aerobically in minimal medium including glucose, thiamine, paraquat and the respective antibiotics.[6] Under these conditions, the lack of SOD activity leads to impairment in the biosynthesis of several amino acids[7] (*E. coli* QC779 and *E. coli* QC781, which lacks only MnSOD activity, have generously been provided by Dr. Daniele Touati, Paris).

It is obvious that this *E. coli* strain can serve as an extremely useful tool for the cloning of SODs other than those of *E. coli*, as has also been proposed by Natvig *et al.*[6] If the gene encoding Listeria SOD is transformed into *E. coli* QC779 using an appropriate vector system the growth impairments would have to be abolished, presupposing that:

- the complete Listeria SOD gene is present in a pTZ19R plasmid and is taken up by *E. coli*;
- the regulatory Listeria DNA sequences (for transcription and translation) are present and do function within the heterologous *E. coli* host;
- the Listeria SOD gene expressed within *E. coli* has a SOD activity which is high enough to abolish growth impairments.

In the following we will show that all of these presuppositions have been fulfilled for the "suicide system" used and that *Listeria ivanovii* SOD has been functionally expressed in three *E. coli* hosts.

MATERIALS AND METHODS

Bacteria, Plasmids and Growth Media

Listeria ivanovii type strain (ATCC 19119) was from the Special Listeria Culture Collection of the Institute of Hygiene and Microbiology of the University of Würzberg. *E. coli* DH5α (F^-, endA1, hsdr17(r_{k-}, m_{k+}), supE44, thi-1, λ^-, recA1, gyrA96, relA1, Δ(argF-laczya)U169, Φ80dlacZΔM15); *E. coli* QC779 (F^-, ΔlacU169, SR^r, rpsL, Φ(sodA::MudPR13)25, Φ(sodB-kan)1-Δ_2, Cm^r, Km^r) and *E. coli* QC781 (F^-, ΔlacU169, Sm^r, rpsL, Φ(sodA::MudPR13)25, Cm^r) were used for the cloning and expression experiments (the latter two strains were from Dr. Touati, Paris).

pTZ19R (available from Pharmacia) is a pBR322 derivative (high copy number plasmid) with an *E. coli* origin of replication.

Listeria ivanovii has been grown at 37 °C in Brain Heart Infusion Broth (BHI), *E. coli* was propagated at 30 °C or 37 °C in LB medium (109 g peptose digest, 5 g yeast extract, 10 g NaCl, per liter) plus respective antibiotics. For detection of recombinant *E. coli* clones, X-Gal and IPTG were added to LB agar (for details see [8]). Minimal medium for recombinant *E. coli* QC779 was prepared as previously described,[8] supplemented with thiamin (final concentration 1 μg/ml), paraquat (final concentration 10^{-7} M), Kanamycin (50 μg/ml), Chlorpamphenicol (7.5 μg/ml) and Ampicillin (100 μg/ml).

DNA Isolation and Cloning Procedures

Chromosomal DNA was isolated from L. ivanovii as described previously.[9] Plasmid DNA from *E. coli* was isolated using slightly modified standard procedures from

previously described[8] methods. Restriction enzymes and T4 DNA ligase were purchased from commercial supliers and enzymatic reactions were carried out as recommended by the producers. Ligation has been done using a modified procedure, which has been described previously.[10] All DNA analysis and transformations have been done following standard procedures as described in [8].

Chromosomal DNA of *L. ivanovii* was partially digested using Sau3A and ligated into the compatible BAMHI site of pTZ19R. The ligation mixture was used to transform competent *E. coli* DH5α cells which were plated onto LB agar plastes containing X-Gal, IPTG and Ampicillin.[8] Approximately 4,000 "white" and 1,500 "blue" *E. coli* DH5α clones were pooled in aliquots and their plasmids were isolated using a large scale plasmid purification method. Competent *E. coli* QC779 cells were transformed using these plasmid preparations and were plated unto minimal medium agar plates prepared as described above.

Crude Extracts and Enzyme Analysis

Crude cell extracts of *L. ivanovii* were prepared by incubation of washed cells with mutanolysin and subsequent ultrasonic disruption, those of *E. coli* by ultrasonic disruption only. Cell debris were removed by centrifugation (10 min, 10.000 rpm, 4 °C). Protein analysis was done using the Bio Rad protein assay kit, with bovine serum albumin as a standard. Liquid SOD assays were according to [11], and SOD activity assays in non-denaturing acryalmide gels have been done according to [12]. All polyacrylamide slab gels were discontinuous as first described by Laemmli[13] containing 12.5 % polyacrylamide.

Induction in E. coli of lac-expression system by IPTG

Cells were grown in 50 ml LB (including antibiotics) at 37 °C to a mid-log phase (130 Klett units) and 0.5 ml 100 mM IPTG or bidestilled water were added. Then the cultures were shaken for two more hours at 37 °C and the cells harvested and lysed as described above.

RESULTS

Isolation of the Recombinant Plasmids Present in Two Isolated E. coli QC779 Clones

After 30 h at 37 °C, two recombinant *E. coli* QC779 clones which were obtained using the approach as described above were analysed for SOD activity in a non-denaturing polyacrylamide gel stained for SOD activity. Neither *E. coli* sodA nor sodB activity could be detected in the recombinant *E. coli* clones (Figure 1, lane 7). However, a single SOD activity was detected which comigrated in this system with the SOD activity from *Listeria ivanovii* cellular lysate (data not shown). The three SODs can be clearly differentiated based on migration behaviour: the R_f-values determined in a 12.5 % acrylamide gel system are 0.19 (*E. coli* MnSOD), 0.31 (*E. coli* hybrid SOD), 0.41 (*E. coli* FeSOD) and 0.56 (*L. ivanovii* SOD), respectively (see Figure 1, lanes 4 and 7). To quantify the SOD activity present in the recombinant *E. coli*, we determined SOD activity from whole cellular lysates of one SOD^+ recombinant as well as

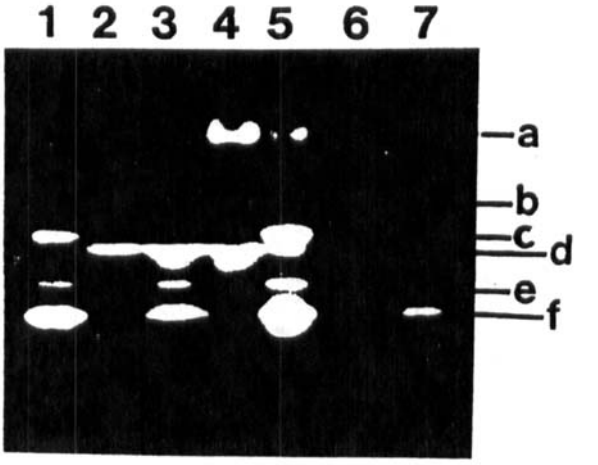

FIGURE 1 Qualitative analysis of the SOD activities present in cell lysates of the *E. coli* clones used in this study. After electrophoresis in a 12.5% non-denaturing acrylamide gel, the gel was stained for SOD activity. Lane 1, DH5α(pAHA3-1); lane 2, QC781(pTZ19R); lane 3, QC781(pAHA3); lane 4, DH5α(pTZ19R); lane 5, DH5α(pAHA3); lane 6, QC779(pTZ19R); lane 7, QC779(pAHA3). a, *E. coli* MnSOD; b, *E. coli* hybrid SOD; c. *E. coli* MnSOD/*L. ivanovii* hybrid SOD; d, *E. coli* FeSOD; e, *E. coli* FeSOD/*L. ivanovii* hybrid SOD; f, recombinant *L. ivanovii* SOD.

of *E. coli* QC779 (pTZ19R) by the ferricytochrome *c* reduction assay.[11] The measured activities are presented in Table I.

To characterize the Listeria DNA inserted in the plasmids of the two recombinant *E. coli* QC779 clones, plasmids were isolated from both clones using a large-scale purification method. In most of the plasmids isolated from both strains only a very small DNA fragment of approximately 150 bp was present, as has been detected by restriction analysis. A 150 bp DNA fragment cannot encode a SOD protein of the expected size (i.e., about 20 to 25 kDa). A few very minor further DNA fragments could also be detected in the plasmid preparations using restriction analysis. Thus, we concluded that the Listeria DNA insert must be highly unstable in this system.

TABLE I
SOD activities of the *E. coli* strains presented in this study as well as of *L. ivanovii*, determined by the cytochrome c reduction assay.

Strain	SOD activity (U/mg soluble protein)	
	− IPTG	+ IPTG
E. coli QC779(pTZ19R)	0[1,2]	N.D.
E. coli QC779(pAHA3)	107.0 (4.6)[1,3]	N.D.
E. coli DH5α(pTZ19R)	23.5 (1.0)	18.8 (0.80)
E. coli DH5α(pAHA3)	111.0 (4.7)	181.0 (7.7)
E. coli DH5α(pAHA3-1)	154.0 (6.5)	333.0 (14.1)
Listeria ivanovii	169.0 (7.2)	N.D.

[1]Overnight culture (stationary)
[2]No activity could be detected
[3]Values in parentheses indicate the factor of activity compared with *E. coli* DH5α(pTZ19R) without IPTG induction
N.D. = not determined.

To stabilize the inserted DNA, we transformed a recA⁻ strain of *E. coli* (DH5α) with the plasmid preparation mentioned above, Using a multi-step screening procedure, which will not be described in detail here, one *E. coli* DH5α clone was selected in which the Listeria DNA insert is stably maintained.

Characterization of the Recombinant Plasmid pAHA3

Restriction analysis showed that an 1.95 kbp Listeria DNA fragment has been cloned into the pTZ19R BamHI site. The plasmid DNA obtained from *E. coli* DH5α (pAHA3) showed homogeneity in its size and in restriction pattern. It is larger than the expected minimal determinant (see above). Subsequently, a HindIII-fragment subclone of pAHA3 has been established, termed pAHA3-1. It carries an approximately 1.60 kbp DNA insert which also mediates the Listeria SOD phenotype (for SOD activity see Table I and Figure 1, lane 1).

Detection and Characterization of the Recombinant Proteins in pAHA3- Containing E. coli clones

To determine which protein(s) are expressed within pAHA3-containing *E. coli* cells we analysed total cell proteins from *E. coli* QC779 (pAHA3), *E. coli* DH5α (pAHA3), and *E. coli* QC781(pAHA3) by SDS-PAGE, and compared them with the respective *E. coli* strain bearing pTZ19R (as a negative control). A 24.5 kDa protein is present in all of the *E. coli* clones bearing pAHA3, which cannot be detected (at least not in these quantities) in the pTZ19R-containing control strains (data not shown). The concentrations of this protein are directly proportional to the SOD activity of the respective *E. coli* strain. Preliminary studies show that in the case of DH5α(pAHA3-1) this protein accounts for up to 30% of the total soluble protein, depending on culturing conditions. As expected, the SOD activity of this clone is very high (see Table I).

The M_r of 24.5 kDa is identical with that of the major protein being detected when the band of *L. ivanovii* SOD activity is cut off from an SOD electropherogram, equilibrated with SDS gel buffer and run on a second denaturing polyacrylamide gel. It is also identical with the protein detected, when the cut-off band of SOD activity in *E. coli* QC779(pAHA3) or *E. coli* DH5α(pAHA3-1) are treated the same way (data not shown). This suggests that the 24.5 kDA protein is indeed the (recombinant) *L. ivanovii* SOD. No molecular weight determination for *Listeria sp.* SOD has been reported to date.

Furthermore, it could be shown by SOD electropherogramms that the recombinant *L. ivanovii* SOD builds up active hybrids of SOD with *E. coli* FeSOD as well as with MnSOD (see Figure 1). Such in vivo hybrid forms have been described for the *E. coli* SODs.[7,14]

Determination of the SOD Activities of E. coli Strains Harbouring pAHA3

SOD activities have been measured by the ferricytochrome *c* reduction assay[11] using crude cell lysates. Results are given in Table I.

DISCUSSION

In this study we have presented the following evidence that we have cloned and expressed in *Escherichia coli* an SOD gene of *Listeria ivanovii*:

(i) The recombinant gene product from *E. coli* QC779 comigrates with *L. ivanovii* SOD in native polyacrylamide gel electrophoresis, both having a R_f-value of 0.56 (*E. coli* SODs: 0.19 and 0.31 respectively) with no other SOD activity visible. Therefore, it can be excluded that the *E. coli* clones which have been obtained are simply revertants of the double mutants used.

(ii) When the band of Listeria SOD activity is cut off from an SOD electropherogram and run on a second, denaturing polyacrylamide gel, a 24.5 kDa protein is detected as the predominant protein as well as in the comigrating band of the pAHA3-1 and both pAHA3-containing *E. coli* strains, but not in the control strains harbouring pTZ19R.

(iii) Measurement of the SOD activities in the ferricytochrome c reduction assay showed an activity in the recombinant *E. coli* of up to 14-times the combined activity of both *E. coli* SODs in the control *E. coli* sodA$^+$sodB$^+$ strain (DH5α).

(iv) The instability of the recombinant DNA within *E. coli* QC779 is most probably attributable to the recA$^+$ genotype of this cell type.

Furthermore, it could be shown that the recombinant *L. ivanovii* SOD forms in *E. coli* DH5α and *E. coli* QC781 hybrid SODs with the subunits of both *E. coli* SODs. Such hybrid forms *in vivo* have been described for the *E. coli* SODs[6,14] (to be seen in Figure 1, lane 4). It could be argued that the additional bands of SOD activity visible in lanes 1, 3 and 5 are due to degradation of the recombinant protein in the heterologous host *E. coli*. However, in *E. coli* QC779(pAHA3) (Figure 1, lane 7), which lacks sodA and sodB activity, only one band of activity is detectable (even when high protein concentrations are run on the gel; data not shown). Furthermore, in *E. coli* QC781(pAHA3) the band of activity which we hypothesize as an active *E. coli* MnSOD/*L. ivanovii* SOD-hybrid is absent (figure 1, lane 3), which can be explained because of the lack of active MnSOD in *E. coli* QC781. In *E. coli* DH5α(pAHA3), which possesses both SODs in active forms, all of the postulated hybrids can be detected. Finally, in the strain producing the highest SOD activity (*E. coli* DH5α(pAHA3-1), Figure 1, lane 1), only the two *Listeria*/*E. coli* hybrid SOD-forms are seen in addition to the recombinant SOD activity (all of the cell lysates used in Figure 1 have been prepared using overnight cultures).

We suggest that in *E. coli* DH5α(pAHA3-1) the concentration of heterologous SOD is so high that it can successfully compete with the original *E. coli* SOD subunits to form active heteromers. When an *E. coli* SOD subunit is produced, the chance of an encounter and subsequent association with the recombinant Listeria SOD within the *E. coli* cell might be higher than that of finding and binding the second *E. coli* SOD subunit. This concept is in agreement with the fact that the recombinant protein in *E. coli* DH5α(pAHA3-1) represents up to 30% of the total soluble cell protein, depending on the culturing conditions (the influence of which remains to be determined).

The fact that the recombinant *L. ivanovii* SOD forms hybrid SODs *in vivo* with *E. coli* FeSOD as well as MnSOD subunits is very interesting to note from an evolutionary point of view. Obviously, the "docking" and the catalytic site-forming domains of the three different SOD subunits must be closely related in their 3-dimensional structure. Thus, the transfer of recombinant SOD genes into heterologous hosts

expressing their own SOD(s) or strains mutated in their SOD genes, expressing another SOD activity may become an interesting tool for the study of structural and functional homologies between SODs from different organisms.

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