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A gene encoding a superoxide dismutase of the facultative intracellular bacterium *Listeria monocytogenes*†

(Recombinant DNA; metalloenzyme; paraquat; oxidative stress; genetic complementation; pathogenic bacteria; hybrid enzyme formation)

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SUMMARY

A gene (*lmsod*) encoding superoxide dismutase (SOD; EC 1.15.1.1) of the facultative intracellular pathogen, *Listeria monocytogenes*, was cloned by functional complementation of an SOD-deficient *Escherichia coli* mutant. The nucleotide sequence was determined and the deduced amino acid (aa) sequence (202 aa) showed close similarity to manganese-containing SOD's from other organisms. Subunits of the recombinant *L. monocytogenes* SOD (re-SOD) and of both *E. coli* SODs formed enzymatically active hybrid enzymes in vivo. DNA/DNA-hybridization experiments showed that this type of recombinant re-*sod* gene is conserved within the genus *Listeria*.

INTRODUCTION

L. monocytogenes is a Gram$^+$ bacterial pathogen causing severe opportunistic infections in man and animals. It is able to survive and to multiply within phagocytic host cells such as macrophages and thus has been named a 'facultative intracellular bacterium'. The uptake of *L. monocytogenes* by phagocytes induces a significant oxidative metabolic burst in these cells, resulting in the release of bactericidal superoxide radicals into the phagosome (McGowan et al., 1983). Superoxide dismutase (SOD; EC 1.15.1.1) converts superoxide into hydrogen peroxide, which then is metabolized by catalases and peroxidases. On the one hand, this enzyme is part of the common defense mechanisms of aerobic bacteria against endogenous oxidative stress. On the other hand, listerial SOD can counteract the oxygen-dependent defense mechanisms which play an important role in the killing of bacteria by phagocytic cells. Therefore, SOD is considered to be a putative virulence factor of *Listeria* (Chakraborty and Goebel, 1988).

Recently the *sod* gene has been cloned from the animal pathogen *L. ivanovii* using genetic complementation of an *E. coli sodA/sodB* double mutant, grown under aerobic conditions on minimal medium containing paraquat (Haas and Goebel, 1992). *L. monocytogenes* is clearly distinct from *L. ivanovii* with regard to biochemical and serological characteristics (Rocourt, 1988). Among these two pathogenic, facultative intracellular *Listeria* species, *L. monocytogenes*

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Abbreviations: aa, amino acids(s); Ap, ampicillin; *B.*, *Bacillus*; bp, base pair(s); Δ, deletion; Fe-SOD, iron-containing SOD; kb, kiloase(s) or 1000 bp; *L.*, *Listeria*; *lisod*, *L. ivanovii sod* gene; *lmsod*, *L. monocytogenes sod* gene; Mn-SOD, manganese-containing SOD; nt, nucleotide(s); ORF, open reading frame; PA, polyacrylamide; PAGE, PA-gel electrophoresis; ^{R}, resistant/resistance; RBS, ribosome-binding site(s); re-, recombinant; SOD, superoxide dismutase; *sod*, gene encoding SOD; Sv, serovar; [ ], denotes plasmid-carrier state.

exhibits a significantly higher virulence than *L. ivanovii*. To address the question whether differences in the structure and regulation of SOD may contribute to the different pathogenic potential of these two listerial species, we have cloned and characterized the *sod* gene from *L. monocytogenes*. In addition, the isolation of this gene will enable us to construct defined mutations in the *sod* gene for in vivo virulence tests.

EXPERIMENTAL AND DISCUSSION

(a) Cloning of the *L. monocytogenes sod* gene and analysis of *Escherichia coli* recombinants

A genomic library of *Hin*dIII-digested chromosomal DNA from *L. monocytogenes* Sv1/2a EGD (S.H.E. Kaufmann, Ulm, Germany) was established using plasmid pTZ18R (Ap^R, *lacZ'*; Mead et al., 1986) as a vector and *E. coli* DH5α (*recA1*, *lacZ*ΔM15, *hsdR*; Bethesda Res. Labs.) as a host. Plasmid DNA prepared from 4000 recombinant clones of this library was then used to transform *E. coli* QC779 (*sodA25*, *sodB*Δ*2*; Carlioz and Touati, 1986). In contrast to *E. coli* DH5α, strain QC779 is restriction proficient and thus not suitable as a primary host for heterologous DNA. The resulting *E. coli* QC779 transformants were plated onto selective LB agar containing 100 μg Ap/ml and 0.05 mM paraquat (methyl viologen, Sigma Chemical Co., St Louis, MO), which induces intracellular superoxide generation (Hassan, 1988), and grown aerobically at 37°C. Under these conditions *E. coli* QC779 cells which do not harbour a re-*sod* gene cannot grow. After 15 h of incubation, 18 clones resistant to paraquat-induced superoxide generation were isolated all containing a re-plasmid with an identical 2.17-kb insert of *L. monocytogenes* DNA. Retransformation of *E. coli* QC779 with these re-plasmids reproducibly yielded transformants which were resistant to paraquat-induced oxidative stress.

Cell lysates from the positive clones were electrophoresed on nondenaturing PA gels and stained for SOD activity (Beauchamp and Fridovich, 1971). All clones showed a SOD activity band which comigrated with the authentic *L. monocytogenes* SOD (R_f value 0.64 relative to bromophenol blue on 12.5% PA gels) (Fig.2). The *E. coli* SODs migrated at clearly distinct positions (R_f values 0.18 for Mn-SOD, 0.51 for Fe-SOD and 0.34 for hybrid *E. coli* SOD; Fig.2). This suggested that a full *lmsod* gene has been selectively cloned by this procedure. The re-plasmid from one of the isolated clones (pAHA8) was chosen for further analysis.

Selection procedures reported so far for the isolation of re-*sod* genes by genetic complementation in *E. coli* always used paraquat-containing minimal media, and positive clones were detected within three days after transformation (Nakayama, 1990; Van Camp et al., 1990; Haas and Goebel, 1992). Our improved method gave a higher yield of positive clones which can be detected after one day of incubation.

(b) Nucleotide sequence analysis of pAHA8

The nt sequence of the complete insert of pAHA8 was determined. It comprised 2170 bp with a G+C-content of 36%, typical for *Listeria* DNA. The ORF of the *lmsod* gene spanned 606 bp and is shown in Fig.1, together with the 5'- and 3'-noncoding regions. It coded for a protein of 202 aa with a calculated M_r of 22631, which is in a good agreement with the 24 kDa determined by SDS–PAGE (not shown) of cell lysates and of *L. monocytogenes* SOD purified as previously described (Haas and Goebel, 1992). No putative prokaryotic export-mediating signal sequence or cytoplasmic membrane associated regions could be detected in the deduced aa sequence of *L. monocytogenes* SOD by computer analysis (Devereux et al., 1984). Comparison of the *lmsod* gene with the previously characterized *lisod* gene revealed 89.5% nt sequence identity in the coding region and 95% identity for the deduced aa sequences. Identity ratios on the aa sequence level between the SODs of *L. monocytogenes* and other microorganisms were: 71% with *Bacillus stearothermophilus* Mn-SOD, 63% with *E. coli* Mn-SOD, 50% with *E. coli* Fe-SOD and 42% with *Mycobacterium leprae* Mn-SOD. In *L. monocytogenes* SOD all five aa residues which can be used as 'primary candidates' to differentiate between Mn-SODs and Fe-SODs (Gly^{76}, Gly^{77}, Phe^{84}, Gln^{154}, Asp^{155}; Fig.1) and 14 out of 17 'secondary candidates' (Parker and Blake, 1988a) correspond to the Mn-SOD type. These findings suggest that *L. monocytogenes* SOD most probably is a Mn-containing enzyme, as has already been shown by enzymatic and structural analysis for the closely related *L. ivanovii* SOD (Haas and Goebel, 1992).

(c) SOD activity of various *Escherichia coli sod* mutants containing pAHA8

It has previously been shown that subunits of the re-*L. ivanovii* SOD form enzymatically active hybrids in vivo with subunits of both *E. coli* SODs (Haas and Goebel, 1992). Here we show that such a hybrid formation also occurs in *E. coli* harbouring the re-*lmsod* gene on pAHA8 (Fig.2).

As has been mentioned above, in cell lysates prepared from *E. coli* QC779*sodA25sodB*Δ*2*[pAHA8] only one SOD activity band comigrating with *L. monocytogenes* SOD could be detected (Fig. 2, lane 5). This protein was overexpressed in *E. coli*, amounting to about 15% of the total soluble cell proteins (not shown). However, in *E. coli* DH5α*sodA*$^+$ *sodB*$^+$[pAHA8] two activity bands in addition to *E. coli* Fe-SOD, Mn-SOD and re-*L. monocytogenes* SOD could be detected (Fig.2, lane 7). One of these ac-

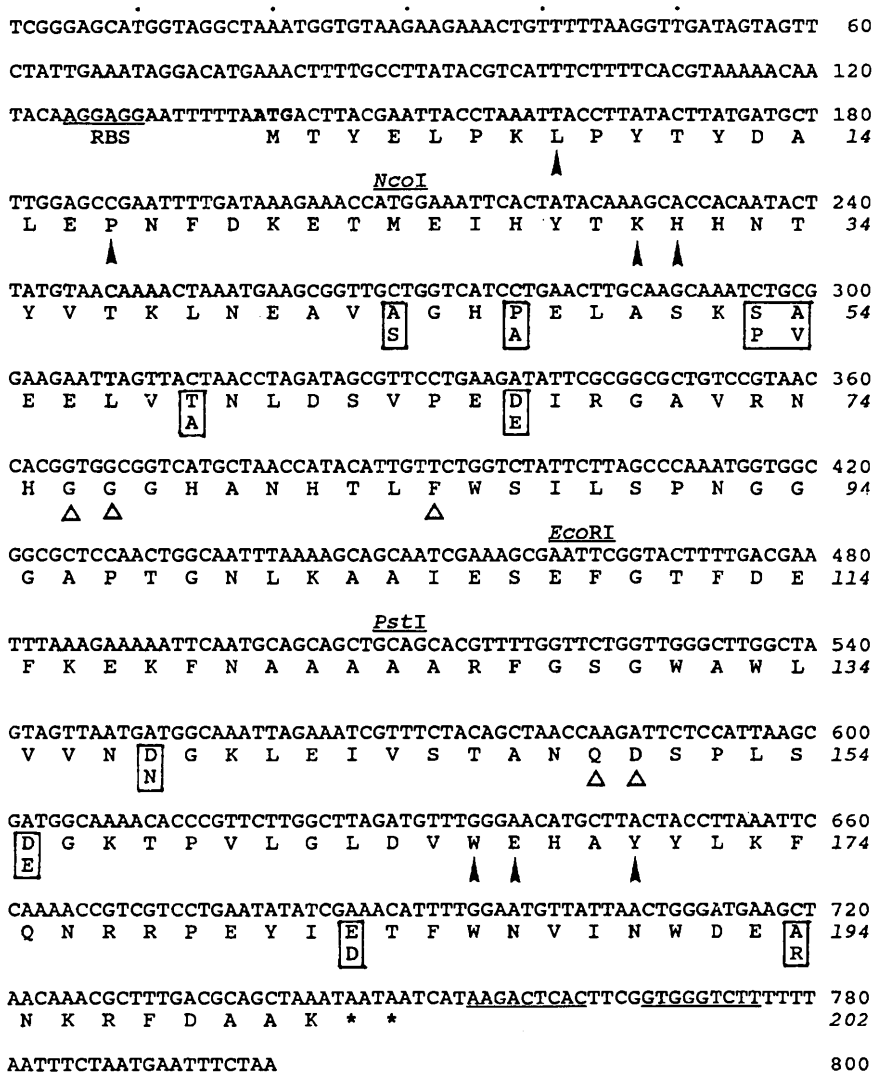

Fig. 1. Nucleotide sequence of the *lmsod* gene and deduced aa sequence. The GenBank/EMBL accession No. is M80526. A putative RBS, stop codons and restriction sites for *Eco*RI, *Nco*I and *Pst*I are indicated. A possible weak, Rho-independent transcription terminator is underlined. Those aa which differ in *L. ivanovii* SOD are shown below the aa sequence of *L. monocytogenes* SOD (boxed). The aa which are primary candidates to differentiate between Mn-SODs and Fe-SODs (Parker and Blake, 1988a) are indicated by open triangles below the sequence, and those which are involved in subunit contact in *B. stearothermophilus* Mn-SOD (Parker and Blake, 1988b) by blackened arrowheads. **Methods.** Chromosomal DNA from *Listeria* was isolated according to Flamm et al. (1984). Plasmids were isolated from *E. coli* by an alkaline lysis procedure, DNA manipulation and analysis were performed according to Maniatis et al. (1982), except for the ligation during construction of the library (Pečenka et al., 1988). Recombinant *E. coli* DH5α with DNA inserts in pTZ18R were identified by the β-galactosidase complementation assay (Maniatis et al., 1982). Restriction fragments from the pAHA8-insert were subcloned into pTZ18R and the nt sequences from both strands were determined by the dideoxy chain-termination method (Sanger et al., 1977), using a plasmid-sequencing protocol (Pharmacia DNA sequencing kit, 8% PA-14% urea (w/w) gels). M13 universal and reverse primers as well as oligodeoxyribonucleotide primers (approx. 20 nt) were synthesized on a 380A DNA synthesizer (Applied Biosystems).

tivities (R_f value 0.61) also appeared in *E. coli* QC781 (*sodA*, Mn-SOD-deficient; Carlioz and Touati, 1986). This band migrated between *E. coli* Fe-SOD and *L. monocytogenes* SOD. The other activity (R_f value 0.47) correlated with the expression of the *E. coli* Mn-SOD in strain QC870 (*sodB*, Fe-SOD-deficient; Carlioz and Touati, 1986) and migrated between Mn-SOD and *L. monocytogenes* SOD (Fig.2, lane 1). From these results we concluded that these activities constitute enzymatically active hybrids between one subunit of the *L. monocytogenes* SOD and one of the *E. coli* Fe-SOD or Mn-SOD, respectively. This assumption is supported by the fact that seven out of the eight aa residues involved in subunit contact of *B. stearothermophilus* SOD (Parker and Blake, 1988b) are conserved in both *E. coli* SODs and in the *L. monocytogenes* enzyme (see also Fig.1).

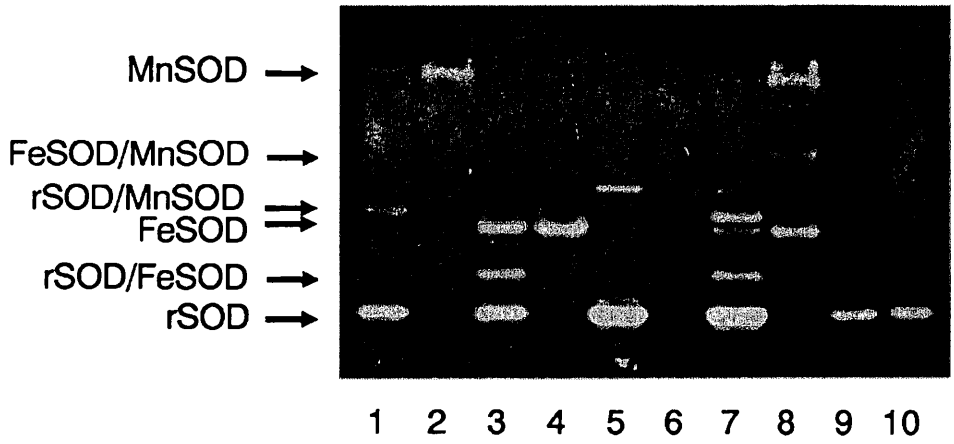

Fig. 2. Functional expression of re-*lm sod* in *E. coli* and in vivo formation of active SOD hybrids. Cleared cell lysates from the strains indicated below were electrophoresed on nondenaturing 12.5% PA gels and stained for SOD activity. The positions of *E. coli* Mn-SOD (**MnSOD**) and Fe-SOD (**FeSOD**), *L. monocytogenes* re-SOD (**rSOD**) as well as the hybrid SOD forms (rSOD/MnSOD; rSOD/FeSOD; **FeSOD/rSOD; FeSOD/MnSOD**) are indicated. Lanes: **1** and **2**, *E. coli* QC870[pAHA8 or pTZ18R, respectively]; **3** and **4**, *E. coli* QC781[pAHA8 or pTZ18R]; **5** and **6**, *E. coli*QC779[pAHA8 or pTZ18R]; **7** and **8**, *E. coli* DH5α[pAHA8 or pTZ18R]; **9**, *L. monocytogenes*; **10**, purified rSOD. **Methods**. Cleared cell lysates were obtained after sonication of cells from overnight cultures of *E. coli* carrying pAHA8 (containing *lmsod*) or the vector pTZ18R, respectively (LB broth, 37°C) and of cells from *L. monocytogenes* (BHI broth, 37°C; before lysis cells were incubated with 50 μg/ml of mutanolysin; Sigma Chem.). For each lysate, 90 μg of soluble proteins (lanes **1** and **9**) or 5 μg of purified rSOD (lane **10**) were electrophoresed. Negative staining for SOD activity was done by the nitroblue-tetrazolium method (Beauchamp and Fridovich, 1971).

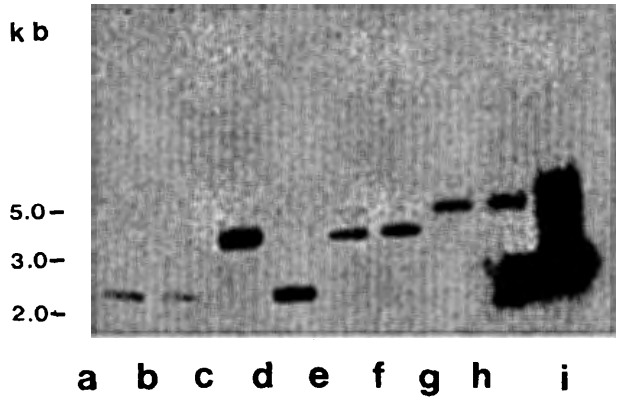

Fig. 3. DNA/DNA-hybrization of *Hin*dIII-digested chromosomal DNAs from *Listeria* to a labeled internal fragment of *lmsod* (nt 174–756, Fig.1). The size (in kb) of hybridizing fragments is indicated on the left margin. Lanes: **a-b**, *L. monocytogenes* strain EGD or Mackaness (SLCC5764), respectively; **c**, *L. ivanovii* (ATCC19119); **d**, *L. seeligeri* (SLCC3379); **e**, *L. innocua* Sv6a; **f**, *L. welshimeri*; **g**, *L. murrayi*; **h**, *L. grayi*; **i**, pAHA8, digested with *Hin*dIII. All *Listeria* strains were from the Special Listeria Culture Collection (SLCC), Würzburg, Germany. **Methods**. DNA fragments were electrophoresed on TAE–1% agarose slab gels (Maniatis et al., 1982), denatured and transferred onto nitrocellulose (Schleicher & Schuell, Germany). The DNA probe was eluted from a TAE-gel using the Geneclean Kit (Bio 101, Inc., La Jolla, CA), and randomly labeled with [α-^{32}P]dATP (Amersham Ltd.) using a commercial kit (Pharmacia). Prehybridization (4 h) and hybridization (16 h) were in 6 × SSC at 57°C, filters were washed twice for 30 min in 1 × SSC/0.1% SDS at 57°C and autoradiographed for 24 h at −20°C. SSC is 0.15 M NaCl/0.015 M Na_3-citrate pH 7.6; TAE is 0.04 M Tris·HCl/0.02 M Na-acetate/0.02 M EDTA/0.018 M NaCl pH 8.0.

(d) DNA/DNA hybridization experiments

To investigate whether DNA sequences homologous to *lmsod* are also present in other species of the genus *Listeria*, we performed DNA/DNA hybridization experiments with an internal fragment of *lmsod* (Fig.1, nt 174-756) as a probe. Under high stringency conditions, related nt sequences could be detected in *L. monocytogenes* Sv1/2a strain Mackaness as well as in all other species of the genus (*L. ivanovii, L. seeligeri, L. innocua, L. welshimeri, L. murrayi, L. grayi*), indicating the conservation of this type of the *sod* gene within the genus (Fig.3).

(e) Conclusions

(*1*) Using an improved protocol for the genetic complementation of an *E. coli sodA/sodB* double mutant, the *sod* gene from *L. monocytogenes* was selectively cloned from a plasmid library. The aa sequence of the *L. monocytogenes* enzyme is almost identical to the previously characterized SOD from *L. ivanovii* and closely related to Mn-SODs from other organisms.

(*2*) *L. monocytogenes* SOD and both *E. coli* SODs form enzymatically active hybrids in vivo, probably due to the high homology of aa in the contact region of the enzyme subunits. The nt sequences homologous to *lmsod* could be detected in all species of the genus *Listeria*.

(*3*) Survival of *L. monocytogenes* in phagocytic cells is a crucial step in the development of listerial infections. SOD may play an important role in this process since it contributes strongly to the bacterial defense against toxic oxygen

metabolites generated by the phagocyte. Recently it has been shown that SOD contributes to the pathogenicity of two other facultative intracellular bacteria, *Nocardia asteroides* (Beaman and Beaman, 1990) and most probably also *Shigella flexneri* (Franzon et al., 1990). The availability of the *sod* gene from *L. monocytogenes*, the most virulent *Listeria* species, will enable investigations on the synthesis and function of this enzyme within the infected cell. Recently, using the cloned gene, we have constructed by insertional mutagenesis a *sod*-deficient mutant of *L. monocytogenes*. Experiments with this mutant are in progress and will help to clarify the role of SOD in listerial infections.

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