Occurrence of two superoxide dismutases in Aeromonas hydrophila: molecular cloning and differential expression of the sodA and sodB genes

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INTRODUCTION

Antioxidant enzymes are essential for living cells, providing protection from reactive oxygen species such as superoxide, which cause oxidative damage to cell structures. Superoxide dismutases (SODs; EC 1.15.1.1) constitute a ubiquitous class of antioxidant defence metalloenzymes that catalyse the conversion of superoxide radical ion into dioxygen and hydrogen peroxide.

The SODs are classified according to the metal ion cofactor required for their activity: the copper-zinc type (Cu/ZnSOD), the manganese type (MnSOD), the iron type (FeSOD) (Fridovich, 1986) and the most recently described nickel type (NiSOD) (Youn et al., 1996). Bacteria contain one to three SOD enzymes, which can be expressed simultaneously. The facultative anaerobe Escherichia coli possesses three SODs which differ in their location and temporal expression. Both FeSOD and MnSOD are cytoplasmic. FeSOD is produced at a constant rate under aerobic and anaerobic conditions, but MnSOD is only synthesized aerobically and its presence is modulated by exposure to oxygen or intracellular \( \text{O}_2^– \) or upon changes in growth phase (Demple, 1991; Compan & Touati, 1993). The third E. coli SOD, containing Cu/Zn, is located within the periplasmic space (Benov et al., 1995).

Bacteria belonging to the genus Aeromonas are Gram-negative facultatively anaerobic rods displaying catalase and oxidase activities. They are currently classified in the family Vibrionaceae, but a separate family has been proposed on the basis of the 16S rRNA cataloguing and rRNA–DNA hybridization results (Colwell et al., 1986). They are commonly found in aquatic environments and increasingly in ready-to-eat foods (Kirov, 1997). They are considered as emerging opportunistic pathogens associated with gastroenteritis and enterocolitis in humans (Merino et al., 1995).

**Keywords:** Vibrionaceae, MnSOD, FeSOD

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**Abbreviations:** CDM, chemically defined medium; DIP, 2,2'-dipyridyl; EDDA, ethylenediamine di-(o-hydroxyphenylacetic acid); PQ, paraquat; SOD, superoxide dismutase.

The GenBank accession numbers for the sequences reported in this paper are AF317226 and AF317227.
According to the description of the family Vibrionaceae in Bergey’s Manual of Systematic Bacteriology (Baumann & Schubert, 1984), Aeromonas expresses a single FeSOD and this feature tends to discriminate between the families Vibrionaceae and Enterobacteriaceae.

In this study we identified and characterized the genes encoding SODs from Aeromonas hydrophila and compared their expression to that of the E. coli SODs.

METHODS

Strains, media and culture conditions. The strains and plasmids used in this study are listed in Table 1. E. coli and A. hydrophila were grown in LB medium (per litre: tryptone 10 g; yeast extract 5 g; NaCl 5 g; pH 7.2) at 30 °C. For iron-limited cultures, the glassware was treated with 10% (v/v) nitric acid, and the cells were grown either (i) in LB medium containing iron chelators (each at 100 µM): DIP (2,2’-dipyridyl), EDDA [ethylenediamine di-(o-hydroxyphenylacetic acid)], desferrioxamine B (Desferal; Ciba-Geigy) or (ii) in CDM (per litre: glucose 5 g; (NH₄)₂HPO₄ 1 g; K₂HPO₄ 4 g; KH₂PO₄ 2.7 g) made with Chelex-100-treated distilled water and supplemented after sterilization with sulfate salts of magnesium (830 µM) and manganese (40 µM). Broth cultures were grown under high aeration (100 ml medium in a 1 litre flask with vigorous rotary shaking at 100 r.p.m.) or under low aeration (100 ml medium in a 300 ml flask with gentle swirling at 40 r.p.m.).

Cloning and sequencing of sod genes. Chromosomal DNA from A. hydrophila was prepared from an overnight culture in 5 ml LB medium. DNA was partially digested with Sau3A and fragments ranging from 2 to 8 kb were extracted from a 0.8% agarose gel using the Qiaquick Gel Extraction kit. These fragments were ligated into the dephosphorylated BamHI site of pUC19. The recombinant plasmids were transformed into the E. coli SOD⁻ mutant QC871 and plated onto LB agar containing ampicillin (50 µg ml⁻¹) and 10 µM paraquat (PQ). Chromosomal and plasmid DNA were prepared using the corresponding Qiagen kits. The inserts were sequenced by the ABI PRISM BigDye terminator cycle sequencing system (Perkin-Elmer).

Preparation of bacterial extracts and assays. The cells were harvested by centrifugation at 12000 g for 15 min at 4 °C, washed with and suspended in 50 mM sodium phosphate buffer pH 7.8, then stored at −20 °C. Cells were disrupted by sonication, and after centrifugation at 12000 g for 15 min at 4 °C, the supernatants (crude extracts) were stored at 4 °C for immediate use or frozen at −20 °C.

Protein concentration was determined using a Bio-Rad DC protein assay kit. Total SOD activity from mid-exponential-phase crude extracts was estimated using the xanthine/xanthine oxidase procedure (Beauchamp & Fridovich, 1971). The amount of SOD required to inhibit the reduction rate of nitro blue tetrazolium by 50% was defined as one unit of activity.

The number and nature of SODs were determined by the PAGE method already described (Leclère et al., 1999). Triplicate gels were soaked in riboflavine containing 5 mM H₂O₂ or 2 mM KCN to differentiate between Fe- and MnSOD (Droillard et al., 1989).

Cell fractionation by osmotic shock. Bacterial cells harvested after 30 h growth in LB medium were washed twice with 10 mM Tris. The cells were suspended in 10 mM Tris/20% sucrose and stirred at room temperature for 15 min, then suspended in the same buffer without sucrose and stirred again for 15 min in ice. The periplasmic fraction was obtained by centrifugation at 15000 g for 30 min at 4 °C and the cells were disrupted by sonication to get the cytosolic fraction.

RESULTS

Gene cloning and expression of SODs

The molecular cloning of the sod genes from A. hydrophila ATCC 7966 was achieved by genetic complementation of the E. coli QC871 sodA sodB double mutant. Two clones named EcA125 and EcA126 were selected; the corresponding plasmids pVLAh125 and pVLAh126 contained a 2.9 kb and 3.6 kb insert, respectively.

The ability of the plasmids to restore resistance to PQ to the SOD⁻ double mutant of E. coli was studied by

Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype and/or description</th>
<th>Source or ref.</th>
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</thead>
<tbody>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pUC19</td>
<td>Cloning vector; Ap⁸</td>
<td>Lab. stock</td>
</tr>
<tr>
<td>pVLAh125</td>
<td>pUC19 with 2.9 kb insert from A. hydrophila 7966; expresses an FeSOD</td>
<td>This work</td>
</tr>
<tr>
<td>pVLAh126</td>
<td>pUC19 with a 3.6 kb insert from A. hydrophila 7966; expresses a MnSOD</td>
<td>This work</td>
</tr>
<tr>
<td>E. coli</td>
<td></td>
<td></td>
</tr>
<tr>
<td>QC868</td>
<td>F⁻ leu6 thrA1 pro lacY1 supE44 bsdR(r⁻ m⁻) rpsL. (Sm⁸) tonA1 thi1</td>
<td>D. Touati</td>
</tr>
<tr>
<td>QC871</td>
<td>F⁻ leu6 thrA1 pro lacY1 supE44 bsdR(r⁻ m⁻) rpsL. (Sm⁸) tonA1 thi1 sodA25 sodB2 (Cm⁸ Kan⁸)</td>
<td>D. Touati</td>
</tr>
<tr>
<td>EcA125</td>
<td>QC871 derivative carrying pVLAh125</td>
<td>This work</td>
</tr>
<tr>
<td>EcA126</td>
<td>QC871 derivative carrying pVLAh126</td>
<td>This work</td>
</tr>
<tr>
<td>A. hydrophila</td>
<td>ATCC 7966</td>
<td>ATCC</td>
</tr>
</tbody>
</table>
growth measurements in the presence of 100 µM PQ (Table 2). *A. hydrophila* was naturally resistant to PQ at this concentration. Clones EcA125 and EcA126 showed the same PQ resistance as *E. coli* QC868, while *E. coli* QC871 (SOD−) did not grow under these conditions.

The ability of these clones to express SOD activity was tested on PAGE gels from crude cellular extracts. The mutants complemented by plasmids pVLAh125 and pVLAh126 each expressed a single SOD, while QC868 expressed two distinct activities and QC871, as expected, had no detectable SOD (Fig. 1a). The SODs expressed from EcA125 and EcA126 had different inhibition patterns and Rf values; these Rf values were 0.663 and 0.611 for *A. hydrophila* FeSOD and MnSOD respectively versus 0.583 and 0.265 for the corresponding enzymes of *E. coli* QC868 (Fig. 1b, c). Clone EcA125 produced an FeSOD as deduced by H2O2 inhibition and KCN resistance, whereas EcA126 synthesized a MnSOD as evidenced by resistance to both H2O2 and KCN.

**Sequencing and analysis of the sodA and sodB genes**

After sequencing of the 2.9 kb insert of the plasmid pVLAh125, a fragment of about 800 bp sharing high homology with *E. coli* sodB gene was suspected to bear the promoter and the coding sequence for the FeSOD of *A. hydrophila*. The promoter region included some characteristic signals necessary for transcription: an initiation sequence (+1; AACA), an AT-rich region (−10; TTATTT) and a −35 box (−33; TTTGGGC). An ATG preceded by a Shine–Dalgarno sequence (GGAGA) allowed the translation of a 194-residue protein with a theoretical pI of 6.14 and molecular mass of 21.5 kDa. The encoded protein shared 75% homology with the *E. coli* FeSOD (146/194 residues) including the amino acids implicated in the metal ligand binding (H27–H43 and D158–H162). A 12 bp inverted repeat sequence followed by a stretch of Ts was found 31 bp downstream of the stop codon and could function as a rho-independent RNA polymerase terminator.

The 3.6 kb insert of pVLAh126 contained a complete promoter and coding sequence (*sodA*) for a protein of 204 amino acids sharing 55% identity with *E. coli* MnSOD (114/206 residues). A transcription initiation sequence (+1; AAC) and a Shine–Dalgarno box (+5; GAGG) were found upstream of the ATG (+14). Three potential −35 boxes (TTG/A/TCA) could also be found within the sequence, but the role of these sequences has not been studied. An inverted 11 nt repeated sequence centred on

**Table 2. Influence of PQ on bacterial growth**

<table>
<thead>
<tr>
<th>Culture period (h)</th>
<th>Ah 7966</th>
<th>QC868</th>
<th>QC871</th>
<th>EcA125</th>
<th>EcA126</th>
</tr>
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<tbody>
<tr>
<td>0</td>
<td>0.18</td>
<td>0.04</td>
<td>0.27</td>
<td>1.73</td>
<td>1.72</td>
</tr>
<tr>
<td>24</td>
<td>4.56</td>
<td>4.99</td>
<td>5.01</td>
<td>3.58</td>
<td>3.31</td>
</tr>
</tbody>
</table>

Growth was evaluated by measuring OD600. See Table 1 for details of strains; Ah 7966, *A. hydrophila* ATCC 7966. 0, control (no PQ); PQ, 100 µM PQ added at OD600 0.2.
nucleotide 661 with a probable hairpin structure could correspond to the rho-independent terminator sequence of the mRNA. The ORF of about 700 bp encoded a protein with a theoretical pI of 6.07 and a molecular mass of 22.3. Amino acids important for the ligand binding were conserved (G$_{77}$, G$_{78}$, F$_{80}$, Q$_{150}$ and D$_{151}$) and the 12 first amino acids constituted a potential signal peptide with the most likely cleavage site between A$_{11}$ and Y$_{12}$.

Growth-phase-dependent expression of the MnSOD

When *E. coli* QC868 was grown in LB medium under high aeration to mid-exponential phase, both FeSOD and MnSOD were detected on PAGE. EcA126 expressed the MnSOD under the same experimental conditions. A further assay, using *A. hydrophila* samples withdrawn hourly during 30 h, showed that total SOD activity from crude extracts remained unchanged: 5.92 ± 1.62 units SOD (mg total protein)$^{-1}$ at 3 h (mid-exponential phase) and 5.96 ± 1.15 units SOD mg$^{-1}$ at 16 h (stationary phase). On PAGE, the FeSOD was expressed whatever the growth phase whereas the MnSOD only appeared on gels after 16 h culture, corresponding to the stationary phase (Fig. 2a).

In other experiments using *A. hydrophila* grown under low aeration, samples were regularly assayed for SOD activity and loaded on PAGE gels. The SOD activity was identical under either high or low aeration. However, only the FeSOD was continuously detectable in crude extracts from bacteria grown under low aeration during the 30 h incubation period, whereas the MnSOD was never detected even after 30 h (Fig. 2b).

**Effect of iron deficiency on MnSOD synthesis**

*A. hydrophila* was grown in LB medium in which iron was sequestered by either chemical (DIP, EDDA) or biological (Desferal) chelators. Whatever the chelator used, an identical result was obtained: the lack of iron induced the expression of MnSOD during mid-exponential phase, since the MnSOD was detectable on PAGE gels after 3 h growth (data not shown).

**Effect of PQ on expression of the MnSOD**

To test the induction of the sodA gene, *A. hydrophila*, *E. coli* EcA126 and *E. coli* QC868 (SOD$^+$) were grown in the presence of different concentrations of PQ (10 to 10$^3$ µM). PQ at any concentration did not affect the growth rate or the final biomass and the MnSOD was not detectable on PAGE gels before the stationary phase (data not shown). The total SOD activity assayed during stationary phase (16 h) remained unchanged for *A. hydrophila* and slightly enhanced for pVLAh126 cloned in *E. coli* QC871 (strain EcA126), whereas it was strongly stimulated for *E. coli* QC868 (Table 3).
Subcellular localization of the MnSOD

*A. hydrophila* cells were fractionated after 48 h growth in 100 ml LB medium under high aeration. The SOD activity measured was 5.18 ± 1.24 U (mg total protein)^−1^ in the periplasmic fraction and 1.55 ± 0.52 U mg^−1^ in the cytoplasmic fraction. For *E. coli* QC868, where the SODs are known to be cytoplasmic, the ratio between the two fractions was inverted (4.98 ± 0.32 U mg^−1^ in the cytoplasmic fraction versus 13.18 U mg^−1^ for the periplasmic space). PAGE of the *A. hydrophila* extracts showed that the MnSOD was distributed between the periplasmic and cytoplasmic fractions while the FeSOD was predominantly present in the cytoplasmic fraction (Fig. 3).

**DISCUSSION**

Two distinct SODs were identified in *A. hydrophila* ATCC 7966 and their complete genes were cloned. The sequenced insert of pVLAh126 contained an 800 bp fragment sharing high homology with the sodB gene from *E. coli* (Carlioz et al., 1988). The protein exhibited 75% identity with the *E. coli* FeSOD, and the activity expressed corresponded to an FeSOD as determined by the inhibition patterns.

A positive effect of the iron-dependent regulatory Fur protein has been observed in the expression of the sodB gene of *E. coli* (Niederhoffer et al., 1990) and recently the site of Fur regulation has been found in the promoter region. The site functioned as an extended −10 promoter containing a TGN sequence near the TA-rich region (Dubrac & Touati, 2000). Nevertheless the *A. hydrophila* FeSOD seemed to be constitutively expressed since the environmental changes such as iron limitation tested in this study did not affect its synthesis; moreover, the *A. hydrophila* sodB promoter did not show the TGN sequence and so could be unaffected by the Fur regulator.

The sequenced insert of pVLAh126 contained a 700 bp gene encoding a MnSOD displaying 55% identity with the *E. coli* MnSOD (Takeda & Avila, 1986). The amino acids implicated in the metal binding, the Y_{34} playing a catalytic role (Hunter et al., 1997), and those pinpointed as potential discriminators between the iron and manganese proteins (G_{77}, G_{95}, F_{85}, N_{149}, Q_{154}, D_{151} and V_{189}) (Parker & Blake, 1988), were conserved. A conservative change between the K_{95} responsible for electrostatic steering of the substrate and an arginine residue was observed. The amino-terminal part of the protein (65 residues) shared high homology (71%) with that of *E. coli*. Nevertheless, although 9 out of the 12 first residues were conserved (MSHTLPLAY for *A. hydrophila* vs MSYTLPSLPAY for *E. coli*), the *A. hydrophila* MnSOD contained a potential signal sequence lacking in *E. coli* Fe- and MnSODs (Nielsen et al., 1997). A similar potential sequence suggesting a periplasmic location of the enzyme has been described in *Acinetobacter calcoaceticus* MnSOD (23 amino-terminal residues) (Geißdörfer et al., 1997). In the fish pathogen *Aeromonas salmonicida* a periplasmic MnSOD has also been detected (Barnes et al., 1996) and a role in the pathogenicity was proposed by the authors. This SOD may play a role in the defence against external reactive oxygen species like the periplasmic Cu/ZnSOD found in *E. coli* (Benov & Frodovich, 1996), *Legionella pneumophila* (Saint-John & Steinmann, 1996), *Haemophilus influenzae* and *H. parainfluenzae* (Kroll et al., 1991, 1993). Recently, the molecular analysis of genetic differences between virulent and avirulent strains of *A. hydrophila* isolated from diseased fish did not indicate SODs as virulence factors (Zhang et al., 2000).

As mentioned by Barnes et al. (1996) for *A. salmonicida*, the MnSOD of *A. hydrophila* was induced when no iron was available in the medium. In *E. coli*, sodA was regulated by the Fur repressor binding to iron boxes, consensus sequences NAT^A^/πAT (Escolar et al., 1998), present in the promoter. These iron-regulatory sequences were not found in the region 300 bp upstream of the sodA gene of *A. hydrophila* although a Fur protein was present in the wild-type *A. hydrophila* 495A2 (Barghouthi et al., 1991). So it remains unclear whether the regulation is Fur-dependent, and further investigation is necessary to determine the mechanism of regulation of sodA by iron.

In LB medium, the *A. hydrophila* MnSOD was only expressed in the stationary phase under high aeration, even with an excess of iron (data not shown). These results are in accordance with the high expression of MnSOD in response to elevated oxygen levels and upon changes in growth phase in *E. coli* (Compan & Touati, 1993). Similarly increased levels of SOD activity during stationary phase were observed in other bacterial genera (Saint-John & Steinmann, 1996; Inaoka et al., 1998; Clements et al., 1999). The authors suggested that enhanced SOD levels are connected with survival of the bacterial cells under stressed conditions. The expression
of a MnSOD can also be related to quorum sensing, as was recently demonstrated for Pseudomonas aeruginosa (Bollinger et al., 2001).

The sodA gene of A. hydrophila was not regulated by addition of PQ to the medium even in E. coli EcA126 although PQ enters the cells, supported by the fact that the expression was strongly stimulated by PQ in E. coli QC868. This non-regulation by PQ may not be so surprising considering the periplasmic location of the MnSOD in A. hydrophila and the increased flux of intracellular superoxide caused by PQ (Hassan, 1984). Physiological studies are necessary to determine the precise function of the MnSOD for Aeromonas.

Lastly, considering the particular conditions of the MnSOD expression in A. hydrophila described in this paper, together with the previously reported occurrence of this enzyme in A. salmonicida (Barnes et al., 1996) it now appears to be the presence of a unique SOD in Vibrionaceae should no longer be taken into account to distinguish this family from Enterobacteriaceae.

ACKNOWLEDGEMENTS

V. Leclère is indebted to Dr D. Touati (Université de Paris VII, France) for the generous gift of double mutant E. coli QC871 and its sodA homologue QC868. This work was supported by grants from the Université des Sciences et Technologies de Lille, the Région Nord-Pas de Calais and the Fond Européen pour le Développement de la Recherche.

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SODs from *Aeromonas hydrophila*


Received 26 March 2001; revised 4 June 2001; accepted 13 July 2001.