Copper–zinc superoxide dismutase in *Haemophilus* species

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Copper-zinc superoxide dismutases ([Cu,Zn]-SODs) are ubiquitous in eukaryotes but have rarely been found in prokaryotes. A gene for [Cu,Zn]-SOD (*sodC*) has recently been cloned from *Haemophilus influenzae* type b and *H. parainfluenzae*, so other *Haemophilus* and related species were screened for the presence of [Cu,Zn]-SODs by visualization of bands of SOD activity in non-denaturing polyacrylamide gels and by gene probing. Strains of *H. aphrophilus*, *H. paraphrophilus*, *H. haemolyticus*, *H. paraphrohaemolyticus*, some non-typable *H. influenzae*, *H. haemoglobinophilus* (*canis*) and *H. parasuis* were all found to have [Cu,Zn]-SOD activity (inhibited by 2 mM cyanide) in polyacrylamide gels. In a Southern blot analysis, DNA from *H. aphrophilus*, *H. paraphrophilus*, *H. haemolyticus* and [Cu,Zn]-SOD-containing non-typable *H. influenzae* – but not the other species – hybridized to a 360 nucleotide DNA probe containing the 5′-part of *sodC* cloned from *H. influenzae* type b. Bacterial [Cu,Zn]-SODs are more prevalent than has previously been recognized.

**Introduction**

Superoxide dismutases (SODs) (EC 1.15.1.1) are metalloenzymes catalysing the conversion of superoxide radicals into hydrogen peroxide and oxygen (McCord & Fridovich, 1969). They are ubiquitous, forming an important defence against oxygen toxicity in oxygen-consuming organisms. There are two families of SODs differing in the metal(s) found at their active sites: manganese or iron, and copper/zinc. SODs containing manganese ([Mn]-SODs) occur in prokaryotes and mitochondria, while those containing iron ([Fe]-SODs) are found in prokaryotes and lower eukaryotes. Copper and zinc containing SODs ([Cu,Zn]-SODs) are principally found in eukaryotes but a few have been found in some bacteria. [Fe]- and [Mn]-SODs form one family and [Cu,Zn]-SODs another on the basis of protein sequence similarities, the two families having evolved independently under the selective pressure of oxygen toxicity (Grace, 1990).

After the first description of a bacterial [Cu,Zn]-SOD, in the fish symbiont *Photobacterium leiognathi* (Puget & Michelson, 1974), numerous bacterial species have been investigated (Britton et al., 1978; Bang et al., 1978; reviewed in Hassan, 1989). Only a small number have been found to possess [Cu,Zn]-SOD: *Caulobacter crescentus* (Steinman, 1982b), *Paracoccus denitrificans* (Vignais et al., 1982), *Pseudomonas diminuta*, *Ps. maltophilia* (Steinman, 1985) and *Brucella abortus* (Beck et al., 1990). Recently, the [Cu,Zn]-SOD gene *sodC*, encoding a protein very similar to the known bacterial [Cu,Zn]-SODs (Steinman, 1982a; Steinman & Ely, 1990; Beck et al., 1990), has been found in *Haemophilus influenzae* type b and *H. parainfluenzae* (Kroll et al., 1991). In this work, *Haemophilus* and related species have been screened for [Cu,Zn]-SOD by staining whole cell extracts separated in non-denaturing polyacrylamide gels, and by Southern hybridization to a *sodC* gene probe cloned from *H. influenzae* type b.

**Methods**

**Strains and culture conditions.** *H. influenzae* NCTC 8468 and strains identified by RM numbers were obtained from the Departmental Culture Collection. Two further *H. influenzae* strains were kindly provided by Dr P. Williams, University of Nottingham, UK. *H. aegyptius* strains were kindly provided by Dr G. M. Carlone (Centers for Disease Control, Atlanta, USA); strain Ha5 was a Brazilian purpuric fever isolate, and strain Hae5 (NCTC 8135) a conjunctivitis control (Brenner et al., 1988). *H. parasuis* 857 and *H. parasuis* 870 were kindly provided by Dr J. Musser (Pennsylvania State University, USA). *Taylorella equigenitalis* was kindly provided by Dr A. Williams (Neurophysiology Unit, AFRC/MRC, Edinburgh, UK). *Pseudomonas*...
Southern blots were probed with the pJSK114. This consists of a 360 nucleotide HindIII-NcoI fragment, the Standard methods were used for restriction digestion and Southern blotting with washing to -80% stringency (Maniatis et al., 1982). SODs. PAGE was chosen as the method for detecting their electrophoretic mobility in PAGE gels upon acrylamide concentration (Hedrick et al., 1986). Bovine erythrocyte [Cu,Zn]-SOD (Sigma) and a sonicate of Haemophilus Haemophilus known concentrations of acrylamide between 6% and 14% (w/v), and concentration (Hedrick [Mn] and hybrid [Fe,Mn]-SODs were used as appropriate controls. The PAGE conditions used were 4-5% (w/v, acrylamide) stacking gel (pH 8-3) and 10% separating gel (pH 8-9) using the buffer system of Davis (1964) except that the pH of the upper buffer was raised to 8.9 with 10 mM-NaOH. SOD activity in PAGE gels was visualized by the modification of the Beauchamp & Fridovich (1971) method as described by Steinman (1985). When used as inhibitors hydrogen peroxide or potassium cyanide were added to the riboflavin-TEMED solution to final concentrations of 5 mM and 2 mM respectively. [Cu,Zn]-SODs are characterized inactivated by cyanide, [Fe]-SODs by hydrogen peroxide, but [Mn]-SODs are resistant to both (Crapo et al., 1978; Dunlap & Steinman, 1986). Bovine erythrocyte [Cu,Zn]-SOD (Sigma) and a sonicate of Escherichia coli strain DH5\alpha containing [Fe]-, [Mn]- and hybrid [Fe,Mn]-SODs were used as appropriate controls.

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The Mₐ values of some of these [Cu,Zn]-SODs were estimated from the dependence of their electrophoretic mobility in PAGE gels upon acrylamide concentration (Hedrick & Smith, 1968). Gels were prepared containing concentrations of acrylamide between 6% and 14% (w/v), and Haemophilus extracts were compared with [Cu,Zn]-SOD standards of known Mₐ, as follows: Ps. maltophilia, 160000 and 200000 (Steinman, 1985); bovine erythrocyte [Cu,Zn]-SOD (Sigma), 31 200 (McCord & Fridovich, 1969); and Ps. diminuta, 45000 (Steinman, 1985). Sonicates of the Pseudomonas strains were used as the source of the [Cu,Zn]-SODs.

Preparation and analysis of genomic DNA. DNA was prepared from 3 ml broth cultures as previously described (Moxon et al., 1984). Standard methods were used for restriction digestion and Southern blotting with washing to ~80% stringency (Maniatis et al., 1982). Southern blots were probed with the Haemophilus DNA insert of pJSK114. This consists of a 360 nucleotide HindIII-NcoI fragment, the 5'-part of the H. influenzae type b [Cu,Zn]-SOD gene sodC (Kroll et al., 1991).

Results

SOD visualization in PAGE gels

Thirty strains of bacteria predominantly from the genus Haemophilus were screened for the presence of [Cu,Zn]-SODs. PAGE was chosen as the method for detecting enzyme activity since this has been shown to be more reliable than solution assay (Steinman, 1985). The presence or absence of SODs and their sensitivity to 2 mM-cyanide and 5 mM-hydrogen peroxide are shown in Table 1. All strains contained a single band of SOD activity not inhibited by hydrogen peroxide or cyanide, thus characteristic of a [Mn]-SOD (Crapo et al., 1978). T. equigenitalis had two such bands. As this result might have been caused by the presence of catalase co-migrating with the SOD, duplicate gels carrying extracts of H. influenzae and H. aegyptius were stained for this enzyme (Gregory & Fridovich, 1974). This demonstrated that catalase was present in all these strains except NCTC 8468 and RM107, but that it migrated differently from SOD (data not shown) and was therefore not responsible for the resistance of the putative [Mn]-SOD to hydrogen peroxide. In strains of H. parainfluenzae, some non-typable H. influenzae, H. aphrophilus, H. paraphrophilus, H. haemolyticus, H. paraparahaemolyticus, H. haemoglobinophilus and H. parasuis, a second band of SOD activity was seen. This activity, of lower mobility than the putative [Mn]-SOD, was inhibited by cyanide but not by hydrogen peroxide, characteristics that identify it as due to [Cu,Zn]-SOD (Dunlap & Steinman, 1986). Tracks from duplicate PAGE gels (±cyanide) containing extracts from ten examples with a cyanide-sensitive band are shown in Fig. 1.

The Mₐ values of some of these [Cu,Zn]-SODs were estimated by the method of Hedrick & Smith (1968) as follows: H. aphrophilus NCTC 5906, 42000; H. aphrophilus 1291, 39000; H. paraphrophilus NCTC 10557, 41000; H. haemoglobinophilus NCTC 1659, 41500; H. paraphrophilus 1041, 33000; H. parasuis 857, 34000; and H. parasuis 870, 16000.

Southern hybridization studies with the sodC probe pJSK114

Southern-blotted EcoRI digests of chromosomal DNA were probed with the Haemophilus DNA insert of pJSK114. In the case of non-influenzae strains of Haemophilus, the probe only hybridized to DNA from organisms that showed [Cu,Zn]-SOD activity in PAGE gels (Table 1). A single band of hybridization was seen with H. haemolyticus, H. aphrophilus, H. paraphrophilus, H. parainfluenzae and H. paraparahaemolyticus (Fig. 2). However, H. haemoglobinophilus and H. parasuis strains, which had activity in PAGE gels, had no hybridization signals at the 80% stringency of filter-washing employed in this study (H. haemoglobinophilus, Fig. 2; H. parasuis, data not shown). A newly prepared full-length gene probe for sodC from H. parainfluenzae 1391 in addition hybridized to chromosomal DNA from H. haemoglobinophilus NCTC 1659. The paradoxical observation of pJSK114 hybridization in capsule H. influenzae strains lacking [Cu,Zn]-SOD activity has been discussed at length elsewhere (Kroll et al., 1991).
Table 1. Cyanide and hydrogen peroxide sensitivity of SODs in PAGE gels, and hybridization of chromosomal DNA to the Haemophilus influenzae type b sodC probe cloned in pJSK114

I and II correspond to phylogenetic division I or II of capsulate H. influenzae (Musser et al., 1988). +/− indicates presence or absence of activity or probe hybridization.

<table>
<thead>
<tr>
<th>Strain</th>
<th>SOD not inhibited by 5 mM-peroxide or 2 mM-cyanide</th>
<th>SOD inhibited by 2 mM-cyanide ([Cu,Zn]-SOD)</th>
<th>Hybridization to insert of pJSK114</th>
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<tr>
<td>Haemophilus influenzae RM7109 (type a, I)</td>
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<td>Haemophilus influenzae RM6157 (type e)</td>
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<td>Taylorella equigenitalis</td>
<td>+†</td>
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* Data from Kroll et al. (1991).
† Two SOD bands.

Discussion

All the Haemophilus and related species examined in this study possess a SOD with activity resistant to cyanide and hydrogen peroxide, typical of the [Mn]-containing enzyme. A wide range of $K_F$ values (0.25-0.83 in 10% PAGE gels) was obtained for these enzymes (data not shown) suggesting structural heterogeneity as reported before for [Mn]-SODs (Steinman, 1983). In addition, strains of H. parainfluenzae, some non-typpable H. influenzae, H. aphrophilus, H. paraphrophilus, H. haemolyticus, H. paraphrophaemolyticus, H. haemoglobinophilus and H. parasuis were found to possess a second enzyme with activity characteristic of [Cu,Zn]-SOD. This study has thus considerably extended the known number of [Cu,Zn]-SODs in bacteria. In fact it has been argued before that the reported rarity of bacterial [Cu,Zn]-SODs may be more apparent than real, as although a considerable range of micro-organisms has been examined, in many cases only one strain has been investigated, and in any case, the molecular heterogeneity of these enzymes and in particular their high isoelectric point makes their detection difficult without particular attention to gel conditions (Steinman, 1985). The $M_\ell$ determinations presented here suggest a degree of molecular heterogeneity within [Cu,Zn]-SODs of Haemophilus species. With the exception of [Cu,Zn]-SOD from H. parasuis 870, all the other Haemophilus [Cu,Zn]-SODs had estimated $M_\ell$ values greater than the 31500 and 29200 reported for the enzymes from P. leigonathi (Steinman, 1982a) and C. crescentus (Steinman & Fly, 1990) respectively, although none were as high as the 45000 of Pr. diminuta (Steinman, 1985). The apparent high $M_\ell$ of [Cu,Zn]-SOD in this last species was
Fig. 1. Tracks from duplicate non-denaturing PAGE gels (a: untreated, b: with 2 mM cyanide) stained to reveal SOD activity. Tracks contain sonicates (<60 µg protein) from representative strains as follows: 1, *H. huemolyticus*; 2, *H. haemoglobinophilus*; 3, *H. aphrophilus* NCTC 5906; 4, *H. aphrophilus* 1291; 5, *H. paraphrophilus* NCTC 10557; 6, *H. paraphrophilus* NCTC 1931; 7, *H. parainjuenzae* 870; 8, *H. paraphrohaemolyticus*; 9, *H. parasuis* 857; 10, *H. parasuis* 870. In each case the upper band (faint, and therefore marked with an asterisk, in the case of *H. paraphrohaemolyticus*) is identified as [Cu,Zn]-SOD by its cyanide sensitivity. Track 7 has been reproduced previously (Kroll et al., 1991).
attributed to asymmetry of the protein altering its mobility in PAGE gels, and the same explanation may apply here. Native [Cu,Zn]-SODs are generally dimers composed of identical subunits (Steinman, 1983). The low $M_r$ (16000) estimated for the $H. parasuis$ 870 enzyme may be that of the monomeric subunit, found here due to instability of the enzyme at pH 8.9 and reassociation at pH 7.8 upon SOD visualization, as has been suggested for the $Ps. maltophilia$ enzyme (Steinman, 1985).

DNA hybridization using the $H. influenzae$ type b sodC probe pJSK114 suggests that in each of these $Haemophilus$ species, [Cu,Zn]-SOD activity is encoded by an homologous gene. Where the probe failed to hybridize to chromosomal DNA despite the presence of [Cu,Zn]-SOD activity, it may be that sequence divergence greater than the limit of 20% reliably detectable in these experiments was responsible. Such divergence has been found in a gene homologous to sodC that has now been cloned from Actinobacillus pleuropneumoniae (unpublished results).

Visualization of enzyme activity in PAGE gels has been considered the method of choice for screening for [Cu,Zn]-SODs, as it is simple and more accurate than solution assay (Steinman, 1985). The use of [Cu,Zn]-SOD gene probes like pJSK114 can greatly extend the scope of investigations, particularly where SOD activity is too low to be detected as in strains with modified or inactivated enzyme. Kroll et al. (1991) found that a CAT–TAT transition in the structural gene, mutating a critical histidine to tyrosine, was most likely responsible for the lack of [Cu,Zn]-SOD activity in $H. influenzae$ type b. [Cu,Zn]-SOD activity may not be detected where experiments are carried out under non-permissive conditions where expression of the enzyme is environmentally regulated. Such environmental regulation of bacterial [Cu,Zn]-SOD expression has been shown in the case of $P. leiognathi$ (Kobayashi et al., 1991).

DNA probes like pJSK114 will be important tools to facilitate the cloning of homologous [Cu,Zn]-SOD genes from other species in order to study the role of this enzyme in the biology of oro-pharyngeal commensals and pathogens.

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References


