Bacterial [Cu,Zn]-superoxide dismutase: phylogenetically distinct from the eukaryotic enzyme, and not so rare after all!

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Copper- and zinc-containing superoxide dismutases ([Cu,Zn]-SODs) are generally considered almost exclusively eukaryotic enzymes, protecting the cytosol and extracellular compartments of higher organisms from damage by oxygen free-radicals. The recent description of a few examples of bacterial forms of the enzyme, located in the periplasm of different Gram-negative micro-organisms, prompted a re-evaluation of this general perception. A PCR-based approach has been developed and used successfully to identify bacterial genes encoding [Cu,Zn]-SOD in a wide range of important human and animal pathogens – members of the *Haemophilus*, *Actinobacillus* and *Pasteurella* (HAP) group, and *Neisseria meningitidis*. Comparison of [Cu,Zn]-SOD peptide sequences found in *Haemophilus ducreyi*, *Actinobacillus pleuropneumoniae*, *Actinobacillus actinomycetemcomitans*, *Pasteurella multocida*, and *N. meningitidis* with previously described bacterial proteins and examples of eukaryotic [Cu,Zn]-SOD has shown that the bacterial proteins constitute a distinct family apparently widely separated in evolutionary terms from the eukaryotic examples. The widespread occurrence of [Cu,Zn]-SOD in the periplasm of bacterial pathogens, appropriately located to dismutate exogenously derived superoxide radical anions, suggests that this enzyme may play a role in the interactive biology of organisms with their hosts and so contribute to their capacity to cause disease.

Keywords: [Cu,Zn]-superoxide dismutase, bacterial pathogenicity, phylogenetic tree, *Haemophilus-Actinobacillus-Pasteurella*, *Neisseria*

INTRODUCTION

The metalloenzyme superoxide dismutase (SOD) catalyses the conversion of superoxide radical anion to oxygen and hydrogen peroxide (McCord & Fridovich, 1969) in the first of a series of protective reactions that remove cytotoxic free radicals generated during the reduction of molecular oxygen. Virtually all cells have one

or more cytosolic SODs to scavenge radicals released during aerobic metabolism, while higher eukaryotes produce in addition an extracellular form of the enzyme (Hjalmarsson *et al.*, 1987) presumed to have a cyto-protective function. The enzymes which prokaryotes and eukaryotes produce are, however, strikingly different. Of the many bacterial SODs which have been characterized, two groups can be distinguished on the basis of their central metal cation – manganese [Mn] or iron [Fe]. Both groups exhibit extensive primary sequence and structural similarity, suggesting a divergent evolutionary path from a common ancestor (Grace, 1990). Both are confined to the cytosol. In contrast, while eukaryotes harbour a [Mn]-SOD in mitochondria, a copper- and zinc-[Cu,Zn]-containing enzyme (constituted as a dimer of identical subunits) is found in the cytosol, while a second, different, [Cu,Zn]-SOD is found as the extracellular form. [Cu,Zn]-SODs show no sequence similarity to [Fe]- or [Mn]-SOD, suggesting that the two classes of enzyme arose in-

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Abbreviations: [Cu,Zn]-SOD, [Cu,Zn]-superoxide dismutase; DARWIN, Data Analysis and Retrieval With Indexed Nucleotide/peptide sequences; HAP, *Haemophilus-Actinobacillus-Pasteurella*; PAM, Percentage Accepted Mutations; SOD, superoxide dismutase; TEMED, *N,N,N',N'*-tetramethylethylenediamine.

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dependently (Grace, 1990), perhaps reflecting convergent evolution on an important phenotype.

The discovery that the bacterial endosymbiont of the ponyfish, *Photobacterium leiognathi*, produced a [Cu,Zn]-SOD (Puget & Michelson, 1974) generated considerable controversy. An early hypothesis of horizontal gene transfer to the bacterium from the host (Bannister & Parker, 1985) has become increasingly hard to sustain as [Cu,Zn]-SOD genes and/or activities have been discovered in a slowly expanding list of organisms including *Caulobacter crescentus* (Steinman, 1982), *Paracoccus denitrificans* (Vignais *et al.*, 1982), some pseudomonads (Steinman, 1985), *Brucella abortus* (Beck *et al.*, 1990), and *Haemophilus influenzae* and *Haemophilus parainfluenzae* (Kroll *et al.*, 1991). Most recently, a SOD, judged to be a copper- and zinc-containing enzyme on the basis of inhibition by cyanide and inactivation by hydrogen peroxide, has been detected in an *Escherichia coli* strain (Benov & Fridovich, 1994).

The observation that the genes cloned from *Pbo. leiognathi*, *C. crescentus* and the *Haemophilus* species encode proteins with N-terminal sequences characteristic of leader peptides, suggesting localization outside the cytosol (confirmed as periplasmic in *C. crescentus* and *H. parainfluenzae*) (Steinman, 1987; Steinman & Ely, 1990; Kroll *et al.*, 1991), further suggests that bacterial [Cu,Zn]-SODs might fulfil a different biological role to the Fe- or Mn-containing enzymes, more analogous to eukaryotic extracellular [Cu,Zn]-SODs in dismuting exogenously derived superoxide. This might be of particular benefit to organisms colonizing a well-oxygenated environment, or for pathogens exposed to the burst of oxygen free-radicals released in the course of phagocytic cell host defence activity, and in the latter could be conceived as a determinant of virulence. Such generalizing hypotheses cannot, however, be developed while the catalogue of bacterial species making the enzyme remains so small, and the examples so diverse. Our discovery of [Cu,Zn]-SOD in different *Haemophilus* species that colonize or invade the human respiratory tract (Kroll *et al.*, 1991; Langford *et al.*, 1992) led us to speculate that the enzyme may be much more widely distributed among bacteria than until now appreciated. Here we report the result of a systematic examination of the diverse *Haemophilus-Actinobacillus-Pasteurella* (HAP) group of bacteria in an attempt to delineate the species range and diversity of this enzyme as a first step to defining its role in the interactive biology of these human and veterinary bacterial pathogens and their hosts.

METHODS

Bacterial strains and growth conditions. Strains were selected as clinical or veterinary isolates previously characterized as typical of the species and used in studies of pathogenicity. *Actinobacillus pleuropneumoniae* strains of different serotypes and a strain of *Actinobacillus lignieresii* were kindly provided by Professor Ian Smith, Royal Veterinary College, London, UK. *Actinobacillus actinomycetemcomitans* strain 925 is a clinical isolate kindly provided by Eileen Anderson, Public Health Laboratory Service, Oxford, UK. Strains analysed in greater depth in this

Table 1. Strains used in this work

Species	Strain	Reference
<i>H. parainfluenzae</i>	1391	Kroll <i>et al.</i> (1991)
<i>H. ducreyi</i>	35000	Purcell <i>et al.</i> (1991)
<i>H. influenzae</i> (non-typable)	15N	Quentin <i>et al.</i> (1990)
<i>A. pleuropneumoniae</i>	S1421	Kilian <i>et al.</i> (1978)
<i>A. actinomycetemcomitans</i>	Y4	Tsai <i>et al.</i> (1979)
<i>Pas. multocida</i>	T5	Gift from Professor I. Smith, Royal Veterinary College, London, UK
<i>N. meningitidis</i>	MC58	McGuinness <i>et al.</i> (1991)
<i>E. coli</i>	DH5 α	Hanahan (1983)

work are identified further in Table 1. Brain-heart infusion, where necessary supplemented with 2 μ g NAD ml⁻¹ and 10 μ g haemin ml⁻¹ (for *Haemophilus* strains) or with NAD alone (for *Actinobacillus* strains), was used in broth form for liquid and (supplemented with 1%, w/v, agar) solid bacterial culture. Broth cultures were propagated by shaking at 200 r.p.m. in air at 37 °C.

Extraction of bacterial cell proteins, gel electrophoresis and detection of SOD. The cell pellet from 25 ml exponentially growing aerobic cultures was broken up by freeze-thawing followed by sonication as previously described by Kroll *et al.* (1991).

PAGE conditions were 4.5% (w/v) stacking gel (pH 8.3) and 10% (w/v) 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 M NaOH.

SOD activity in PAGE gels was visualized by the method of Beauchamp & Fridovich (1971) as modified by Steinman (1985). When used as an inhibitor of SOD activity, potassium cyanide was added to the riboflavin-TEMED solution to a final concentration of 2 mM.

Recombinant DNA methods. Standard methods were used for preparation of chromosomal DNA, restriction analysis, Southern blotting, plasmid cloning and preparation of plasmid DNA (Sambrook *et al.*, 1989). Southern blots were probed to ~80% stringency in 0.015 M NaCl, 0.0015 M sodium citrate, 0.1% SDS at 45 °C for 1 h with three changes of buffer prior to autoradiography carried out at -70 °C.

Amplification of DNA by PCR. Reactions were carried out in a total volume of 100 μ l containing 100 ng of each primer (Fig. 1), deoxynucleoside triphosphates (each at a concentration of 200 μ M), gelatin (0.01%, w/v), MgCl₂ (2.5 mM), Tris, pH 8 (10 mM), KCl (50 mM), and 2 U *Taq* polymerase (Perkin-Elmer Cetus). The PCR mixture was irradiated with ultraviolet light to cross-link any contaminating DNA before template was added (Sarkar & Sommer, 1990). Samples were overlaid with mineral oil and processed through 30 cycles [2 min at 94 °C (denaturation), 2 min at 42 °C (annealing), 2 min at 72 °C (extension)].

Nucleotide sequence determination. Nucleotide sequences were determined by the dideoxy chain-termination method (Sanger *et al.*, 1977) using denatured plasmid templates (Hettari & Sakakai, 1986). [³⁵S]dATP α S was used to label the growing strand. The highly processive modified T7 DNA polymerase

Sequenase (USB) was used with a sequencing kit according to the manufacturer's instructions. Oligonucleotide primers were the universal forward and reverse sequencing primers (New England Biolabs) and oligonucleotides were prepared with a model 380B DNA synthesizer (Applied Biosystems). The Computer program DARWIN (Data Analysis and Retrieval With Indexed Nucleotide/peptide sequences) (Gonnet *et al.*, 1992; Gonnet, 1992, 1994) was used for multiple sequence comparisons.

RESULTS

Initial attempts to identify cyanide-inhibitable SOD activity (characteristic of [Cu,Zn]-SOD) in whole cell protein extracts of examples of different HAP species gave unreproducible results, appearing to depend critically on the conditions under which the organisms were grown. Southern hybridization probing with the cloned [Cu,Zn]-SOD gene (*sodC*) from *H. parainfluenzae* under conditions that permitted up to ~20% sequence mismatch gave faint signals with most of the different chromosomal DNAs, suggesting that the species under consideration harboured a version of the gene (data not presented), but these were too weak to allow the probe to be used as a selection tool in a cloning strategy. We therefore developed an approach based on PCR to isolate *sodC* genes.

The published amino acid sequences of [Cu,Zn]-SOD from *H. influenzae*, *H. parainfluenzae*, *C. crescentus*, *B. abortus* and *Pho. leiognathi* (Kroll *et al.*, 1991; Steinman, 1982, 1987; Beck *et al.*, 1990) were used to identify conserved regions in the middle (around the enzymically/structurally important His and Cys residues) (Getzoff *et al.*, 1989), and near the C-terminus of the enzyme. From these, degenerate oligonucleotide primers (5' univsod and 3' univsod) were designed to match the corresponding regions of *sodC*. *Haemophilus* codon bias (Kroll *et al.*, 1990) was applied to contain oligonucleotide degeneracy within manageable bounds (8-fold for 5' univsod, 128-fold for 3' univsod, Fig. 1).

5' Univsod and 3' univsod were used in PCR reactions with chromosomal DNA from *H. influenzae* and *H. parainfluenzae* strains known to contain *sodC*; they amplified a 310 bp fragment as expected from the published sequence (Kroll *et al.*, 1991). Representative strains of HAP bacteria (Table 1) were then examined in an attempt to amplify intervening nucleotide sequence from a [Cu,Zn]-SOD gene. Amongst the multiple products inevitably obtained with degenerate primers of this sort, prominent bands of around 310 bp were seen in each case. No amplification product was found when no template was present, or when chromosomal DNA from *E. coli* strain DH5 α was used (Fig. 2). In view of the success of this approach, DNA was prepared from other bacterial pathogens of the upper respiratory and genital mucosa and examined in the same way. Strains of *Neisseria meningitidis* (a Gram-negative pathogen of major medical importance responsible for life-threatening meningococcal septicaemia and meningitis) yielded a comparable amplification product (Fig. 2). No such product was obtained using DNA from examples of the Gram-negative

pathogens *Neisseria gonorrhoeae* (three strains examined), *Bordetella pertussis* (two strains) or *Moraxella catarrhalis* (three strains), from the Gram-positive pathogen *Streptococcus pneumoniae* (three strains) or from *Mycobacterium tuberculosis* (four strains).

Five of the new candidate *sodC* PCR fragments were cloned and sequenced, and the corresponding deduced amino acid sequence segments compared with the known examples of bacterial [Cu,Zn]-SOD (Figs 3 and 4). The close similarities strongly suggested that in each case a *sodC* homologue had been identified. In order to assess whether these genes encoded active [Cu,Zn]-SOD (Kroll *et al.*, 1991), *Actinobacillus* species were examined in more detail. The cloned fragment from *A. pleuropneumoniae* strain S1421 (with 80.1% sequence identity to the original *Haemophilus* probe) was used as a Southern hybridization probe against DNA extracted from a range of serotypes of that species and DNA from other pathogenic *Actinobacillus* spp. It hybridized strongly to a single chromosomal fragment in all cases, indicating the presence of *sodC* DNA in each (data not presented). A range of these strains was grown aerobically in rapidly shaking liquid culture, harvested in the late-exponential phase, and whole cell extracts were examined for SOD activity by non-denaturing PAGE. Under the gel conditions described by Steinman (1985), a cyanide-sensitive band of SOD activity characteristic of [Cu,Zn]-SOD was identified in each case (Fig. 5). In similar experiments, the presence of active [Cu,Zn]-SOD was confirmed in all the species with *sodC* sequence considered here (data not shown).

The set of five new bacterial [Cu,Zn]-SOD sequence segments, all five previously known examples, and a selection of intracellular and extracellular [Cu,Zn]-SOD sequence segments from eukaryotes ranging from yeast to humans were compared all-against-all using the interactive system for sequence matching and data analysis (DARWIN) (Gonnet *et al.*, 1992; Gonnet, 1992, 1994), developed and maintained at the Institut für Wissenschaftliches Rechnen at the Eidgenössische Technische Hochschule, Zurich, and accessed via the Internet. Using the computer program Mulalignment to demonstrate optimal alignments, it is clear that there are substantial differences between the prokaryotic and eukaryotic examples of this enzyme (Fig. 4). While there are strong similarities between peptide sequences within each phylum, there is otherwise substantial divergence of sequence between the kingdoms, though the sequence from *C. crescentus* is exceptional in aligning rather poorly to any of the rest. There is a suggestion from the sequence alignments that specific peptide domains are characteristic of prokaryotic or eukaryotic [Cu,Zn]-SODs, with the implication that secondary and tertiary structures may differ. Clarification of these observations awaits the crystallographic determination of one or more bacterial [Cu,Zn]-SOD structures, currently in hand in the cases of the *A. pleuropneumoniae* and *Pho. leiognathi* proteins (K. Forest, E. Getzoff & J. Tainer, personal communication).

The DARWIN program Phylotree was used to put the differences between peptide sequences on a quantitative

Fig. 1. Design of oligonucleotide primers 5' univsod and 3' univsod and their location (indicated by dashed lines) within the *H. parainfluenzae* gene *sodC* (arrow). Primers were synthesized by reverse translation of consensus peptide sequences identified by comparison of published sequences from *C. crescentus* (Steinman, 1982), *B. abortus* (Beck et al., 1990), *Pho. leiognathi* (Steinman, 1987), and *H. parainfluenzae* (Kroll et al., 1991), corresponding (5' univsod) and complementary (3' univsod) to the peptide sequence. Nucleotide degeneracy is indicated by IUPAC code: R = A or G; Y = C or T; M = A or C; S = C or G).

Fig. 2. Ethidium-bromide-stained agarose gel showing electrophoretically separated PCR products generated with 5' univsod and 3' univsod and DNA templates as follows: Lanes: 1, no chromosomal DNA; 2, *H. parainfluenzae*; 3, *A. pleuropneumoniae*; 4, *A. actinomycetemcomitans*; 5, *N. meningitidis*; 6, *Pas. multocida*; 7, *H. ducreyi*; 8, biotype IV nontypable *H. influenzae*; 9, *E. coli*; 10, DNA size markers (sizes indicated in bp). The position of the 310 bp fragment corresponding to *sodC* sequence is indicated.

basis. In the resulting unrooted dendrogram (Fig. 6), pairwise relationships are displayed according to the principle that the length of the tree branch path joining two peptide sequences is in proportion to their degree of homology, calculated as the PAM distance (Percentage Accepted Mutations) separating two sequences (Gonnet et al., 1992; Gonnet, 1994). Short PAM distances thus signify close similarity of sequence, and long distances, correspondingly divergent sequence, and this treatment demonstrates clearly that the prokaryotic and eukaryotic sequences fall into two widely separated lineages, with the unique *C. crescentus* sequence highly divergent from all the other examples.

DISCUSSION

Our results suggest that the capacity to make [Cu,Zn]-SOD, encoded by *sodC*, is far more widely present in bacteria than has previously been recognized, occurring in diverse colonists and pathogens of the respiratory and genital tracts. Two factors may have contributed to frustrate previous attempts to detect the enzyme in prokaryotes. The first is the selection of conditions under

which bacteria are to be cultured, as we have found that the level of [Cu,Zn]-SOD activity can be considerably increased when organisms are grown in shaking liquid media rather than on nutrient agar plates. In agreement with this observation, production of the *E. coli* putative [Cu,Zn]-SOD is strongly induced during aerobic growth (Benov & Fridovich, 1994). While the simplest inference is that *sodC* transcription is positively regulated by aerobiosis, this awaits further investigation. The second is the selection of conditions under which to run non-denaturing gels to detect [Cu,Zn]-SOD activity. In gels prepared with pH 7.8 buffer, the protein migrated very slowly, producing a band of activity that is diffuse and hard to separate from background. Increasing the pH of the stacking gel to 8.3 and that of the separating gel to 8.9 as suggested by Steinman (1985) has allowed somewhat clearer resolution, though it is possible in some cases that proteins may be denatured and so not detected under these conditions (Steinman, 1985).

The PCR approach using the univsods as oligonucleotide primers has proved a powerful means, complementary to enzymological methods, for finding these genes, successfully allowing amplification of a part of *sodC* from many organisms and so making straightforward the cloning of whole genes and their flanking sequences. While by this method the range of examples of bacterial [Cu,Zn]-SOD has been greatly widened, failure in amplifying a homologous sequence from a particular organism is of course no proof of its absence. Our lack of success, for example, in finding a *sodC* gene in *E. coli* strain DH5 α , or in strains of *S. pneumoniae* or *M. tuberculosis*, may simply reflect critical differences of nucleotide sequence in the part of the gene selected to define the primers in those species. Interesting examples of bacterial species that merit further investigation include *Legionella pneumophila* and *N. gonorrhoeae*. [Cu,Zn]-SOD activity has been reported to be present in *L. pneumophila* (unpublished result of the author cited in Steinman, 1992) but attempts to clone the gene for this have so far failed. We too have so far been unsuccessful in finding *sodC* in our examination of one strain with the univsod primers, but with a much more substantial choice of sequences to compare as the new bacterial *sodC* genes are fully sequenced, new primers selected with a different appropriate codon bias may prove successful in the future. The interest in *N. gonorrhoeae* lies

Fig. 3. Nucleotide sequences of *sodC* DNA fragments generated with 5' univod and 3' univod from *Pas. multocida* (PM), *H. ducreyi* (HD), *A. actinomycetemcomitans* (AA), *A. pleuropneumoniae* (AP) and *N. meningitidis* (NM), aligned with *H. parainfluenzae* (HP) *sodC* sequence. Base number 1 is the first following 5' univod, the sequence ending with the last base before 3' univod.

Fig. 4. DARWIN alignment of [Cu,Zn]-SOD peptide sequences (Gonnet, 1992). The new bacterial [Cu,Zn]-SOD sequence fragments are compared to all other published prokaryotic examples and a selection of intracellular and extracellular eukaryotic [Cu,Zn]-SOD sequences from the corresponding part of the protein as follows (acknowledging published sources of sequence): a = yeast (Johansen et al., 1979); b = bovine (Steinman et al., 1974); c = human (extracellular) (Hjalmarsson et al., 1987); d = human (intracellular) (Barra et al., 1980); e = *Onchocerca volvulus* (extracellular) (James et al., 1994); f = *O. volvulus* (intracellular) (Henkle et al., 1991); g = *Schistosoma mansoni* (Simurda et al., 1988); h = *C. crescentus* (Steinman, 1982); i = *Pho. leiognathi* (Steinman, 1987); j = *B. abortus* (Beck et al., 1990); k = *H. parainfluenzae* (Kroll et al., 1991); l = *H. influenzae* type b (Kroll et al., 1991); m = *H. ducreyi*; n = *A. actinomycetemcomitans*; o = *A. pleuropneumoniae*; p = *N. meningitidis*; q = *Pas. multocida*. Within peptide sequence, continuous lines signify gaps introduced by the computer program to optimize alignment. At the ends of the peptide sequence, dashes signify amino acids for which no meaningful alignment can be made. Vertical lines below the sequence alignment indicate completely conserved amino acids, the arrow indicates a Zn-ligand His invariant except in *C. crescentus*, and asterisks indicate additional amino acids conserved across all bacterial examples (not including the highly divergent *C. crescentus* peptide sequence). Numbering is based on the human intracellular [Cu,Zn]-SOD as in Getzoff et al. (1989).

in the intriguing observation, long established, that many strains produce only very low levels of SOD despite their efficient survival in an oxygen-free-radical-rich environment (Norrod & Morse, 1979). This raises the question of whether they make a novel, hard-to-detect [Cu,Zn]-SOD

— perhaps a cytoplasmic version — instead of the usual [Mn]- or [Fe]-enzyme. Although our preliminary studies have failed to amplify *sodC* DNA from a few strains, this is clearly a problem to be revisited once further primers are available.

Fig. 5. Paired non-denaturing polyacrylamide gels stained to show SOD activity as an achromatic zone: without selective [Cu,Zn]-SOD inactivation (left) and in the presence of 2 mM KCN (right). Lanes contain whole cell protein extracts from bacterial species as follows: 1, *A. pleuropneumoniae* serotype 3 (strain S1421); 2, *A. pleuropneumoniae* serotype 5; 3, *A. pleuropneumoniae* serotype 7; 4, *A. pleuropneumoniae* serotype 8; 5, *A. lignieresii*; 6, *A. actinomycetemcomitans* strain NCTC 09710; 7, *A. actinomycetemcomitans* strain 925. The well-defined achromatic band of relatively low mobility, absent in the presence of KCN, represents [Cu,Zn]-SOD; the more diffuse zone, unaffected by KCN, represents [Fe]- or [Mn]-SOD.

Pairwise comparison of the peptide sequence segments now available from ten prokaryotic [Cu,Zn]-SODs with each other and with a selection of eukaryotic sequences divides the whole set cleanly into two lineages (Figs 4 and 6). The separation is so clear-cut that it seems safe to draw several conclusions.

First, there is no hint based on peptide sequence that any example of bacterial [Cu,Zn]-SOD has arisen as the result of a gene transfer event from a eukaryote. This hypothesis, proposed at the time of the discovery of [Cu,Zn]-SOD in the fish endosymbiont *Pho. leiognathi* (Flohe, 1984), has been regarded with increasing scepticism as other examples have emerged in free-living bacteria. The sequence data presented here, though limited, clearly support the alternative hypothesis that the two *sodC* families are the descendants – now highly diverged in sequence – of an ancient, ancestral, gene.

Second, while all the bacterial [Cu,Zn]-SODs that have been characterized appear to be extra-cytoplasmically located (Steinman & Ely, 1990; Kroll *et al.*, 1991; Stabel *et al.*, 1994; and our unpublished results for the *Actinobacillus* spp. and *N. meningitidis*), this does not reflect any discernible closer relationship – at least in terms of sequence relatedness or secondary structure – to the

extracellular than to the intracellular forms of the eukaryotic enzyme.

Third, of all the bacterial [Cu,Zn]-SODs so far described, that made by *C. crescentus* is the most divergent in terms of peptide sequence, containing as it does unique additional peptide domains not found in either prokaryotic or eukaryotic examples (Fig. 4). So far this has been the only bacterial [Cu,Zn]-SOD subjected to functional analysis (Steinman, 1993) and our results suggest that considerable caution should be used in extrapolating conclusions as to the possible function of periplasmic [Cu,Zn]-SOD in this organism to other bacteria.

With the extensive range of bacterial sequences available here, the identification of residues that are invariant across different [Cu,Zn]-SODs, which can therefore be considered as potential fixed points in the determination of protein structure, can be made somewhat more secure. A previous comparison of a set of eukaryotic [Cu,Zn]-SOD protein sequences with a single bacterial example (*Pho. leiognathi*) (Getzoff *et al.*, 1989) classified 23 residues as apparently invariant, in three categories: active site (15 residues), dimer interface (4 residues) and β -barrel (4 residues). Inclusion of *Pho. leiognathi* sequence in their comparison allowed Getzoff *et al.* (1989) to reject 25

Finally, the discovery that [Cu,Zn]-SOD is quite widespread in mucosal colonists and pathogens rather than a great rarity has biological implications. In each of the organisms that has been found to produce [Cu,Zn]-SOD, a conventional [Fe]- or [Mn]-SOD is also present (Fig. 4; Langford *et al.*, 1992; Steinman, 1992, 1985, 1982; Vignais *et al.*, 1982; and our unpublished results). In those new examples of [Cu,Zn]-SOD genes that we have cloned in entirety (the *Actinobacillus* spp. and *N. meningitidis*; data not presented), the nucleotide sequence, like those previously published, in each case encodes a protein starting with an N-terminal leader peptide motif, suggesting that the enzyme is extra-cytosolic. As superoxide radical anion generated in the cytosol cannot diffuse across the bacterial inner membrane (Hassan & Fridovich, 1979), this strongly suggests that while the conventional enzyme disposes of metabolically generated superoxide, the [Cu,Zn]-SOD has a distinct role in the interactive biology of the organism with its environment. A role for surface-associated SOD in microbial pathogenicity has been identified for *Nocardia asteroides* (Beaman & Beaman, 1990), and suggested for *B. abortus* (Tatum *et al.*, 1992), while production by *M. tuberculosis* of an extracellular [Fe]-SOD (Zhang *et al.*, 1991) may contribute to the capacity of that organism to survive within macrophages. Pathogens like *A. pleuropneumoniae*, *H. ducreyi* and *N. meningitidis* colonize mucosal surfaces conventionally regarded as aerobic and further excite production of superoxide by phagocytic cells in the course of the inflammatory reaction they provoke. Extra-cytosolic bacterial [Cu,Zn]-SOD may promote their survival by enhancing resistance to the cytotoxic effects of oxygen free-radicals through modulation of the concentrations of superoxide and hydrogen peroxide generated spontaneously in their environment or during the respiratory-burst phase of host defence.

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Fig. 6. Graphical representation of peptide sequence differences computed on the basis of PAM distances (Gonnet 1992, 1994). PAM distances are shown as the relative lengths of each line on the figure (drawn to scale), originating at the centroid (small unlabelled circle). Sequences are identified by single-letter labels as in Fig. 4. Among the bacterial sequences, close similarity, and thus short separating PAM distances, has led to the labels k, l, p and n, in that order top-to-bottom, overlying one another.

residues which had previously been considered invariant. Eleven of their 23 residues lie within the region considered here. Metal ligands His₆₃, His₇₁, Asp₈₃ and His₁₂₀ are present in all examples, as is Asp₁₂₄, which is involved in maintaining the correct orientation of Cu and Zn liganding residues. However His₈₀, identified as a zinc-coordinating residue in all previous examples, is replaced with Asp in *C. crescentus*. As Bordo *et al.* (1994) observe, though, His is substituted for Asn/Asp 16 residues nearer the N-terminal end of the protein, and together with other changes nearby, this may constitute a 'rescue' mutation in the *sodC* gene of this organism. Two glycines in the segment under consideration, identified by Getzoff *et al.* (1989) as involved in stabilization of the structure of the active site (in positions 61 and 82), remain invariant, but Pro₆₆, considered important in orienting Zn ligands towards the cation, loses invariant status, being replaced by Ser and Thr in *H. influenzae* and *C. crescentus*, respectively. Gly₁₁₄ is preserved (making allowance for

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