Identification of $sodC$ encoding periplasmic [Cu,Zn]-superoxide dismutase in *Salmonella*

James Canvin, Paul R. Langford, Kathryn E. Wilks, J. Simon Kroll *

*Molecular Infectious Diseases Group, Department of Paediatrics, Imperial College School of Medicine at St Mary's, London W2 1PG, UK*

Received 15 December 1995; revised 20 December 1995; accepted 20 December 1995

Abstract

$sodC$, encoding [Cu,Zn]-cofactored superoxide dismutase, once thought to be virtually confined to eukaryotes, has now been described in many Gram-negative pathogens that have their primary niche of colonization in the upper respiratory tract. Their role in host–parasite interactive biology is unknown. We here show that members of the major human and animal enteric pathogenic species *Salmonella* harbour a version of $sodC$ most closely resembling that found in *Brucella abortus*. The enzyme it encodes is a novel candidate determinant of virulence in *Salmonella*, an intracellular pathogen potentially exposed to toxic oxygen free radicals within its intracellular niche.

Keywords: Superoxide dismutase; *Salmonella*; Periplasm; Bacterial [Cu,Zn]-superoxide dismutase; Bacterial virulence

1. Introduction

In bacteria and higher organisms alike, superoxide dismutase (SOD) catalyses the conversion of superoxide radical anions to hydrogen peroxide and oxygen [1] in the first of a cascade of reactions that has the net effect of neutralising cytotoxic free radicals generated during the reduction of molecular oxygen. This essential protein is virtually ubiquitous, but while eukaryotes have a copper- and zinc-cofactored enzyme ([Cu,Zn]-SOD) both for cytosol protection and in some cases as an excreted anti-inflammatory cytoprotective agent [2], the bacterial cytosol is generally protected instead by unrelated manganese- or iron-cofactored enzymes, and these are not found in an extracytosolic location. Until recently, bacterial [Cu,Zn]-SOD, which has been found located in the periplasm of only a few comparatively exotic Gram-negative microorganisms (for a recent review, see [3]), has been widely regarded as an unusual curiosity, and its function remains to be determined. However, we have recently identified the gene $sodC$, encoding periplasmic [Cu,Zn]-SOD, in many members of the *Haemophilus–Actinobacillus–Pasteurella* (HAP) family of bacteria, as well as in the upper respiratory tract (URT) invasive pathogen *Neisseria meningitidis* [4–6]. This contradiction of the conventional view that [Cu,Zn]-SOD is very uncommon in bacteria has gained strength from the observations of Benov et al. [7] and Battistoni and Rotilio [8] that a periplasmic enzyme with [Cu,Zn]-SOD activity is to be found in *Escherichia coli*. In both of these reports the purification of the enzyme has been complicated by low abundance and/or sensitivity to the action of
proteases, perhaps explaining the failure of investigators previously to detect its presence at all. In order to pursue the question of whether this activity corresponded to the presence of a sodC homologue in Enterobacteriaceae, and to advance our investigations into the possible role this enzyme might play in bacterial virulence, we elected to adapt the polymerase chain reaction (PCR) method that we used successfully in our examination of URT pathogens to the major enteric pathogens of the Salmonella family.

2. Materials and methods

2.1. Bacterial strains studied

Salmonella strains were clinical veterinary isolates, kindly provided by Dr T. Wallis (Institute for Animal Health, Compton, UK): S. typhimurium strains St4/74 and St12/75; S. choleraesuis strains Scs14/74 and ScsA50; S. dublin strains Sd3246 and Sd2229; S. enteritidis strain S13.

2.2. Recombinant DNA methods

Standard methods were used for preparation of chromosomal DNA, restriction analysis, Southern blotting, plasmid cloning and preparation of plasmid DNA [9]. Southern blots were probed to approx. 80% stringency in 0.015 M NaCl, 0.0015 M sodium citrate, 0.1% sodium dodecylsulfate (SDS) at 45°C for 1 h with three changes of buffer prior to autoradiography carried out at −70°C.

2.3. Amplification of DNA by PCR

Reactions were carried out in a total volume of 100 μl containing 100 ng of each primer, deoxynucleoside triphosphates (each at a concentration of 50 μM), gelatin (0.01% w/v), MgCl2, (2.5 mM), Tris pH 8.8 (10 mM), KCl (50 mM), and 2 U of Tbr DNA polymerase (NBL Gene Sciences Ltd, UK). This polymerase was chosen for the low frequency with which errors are introduced, and its good thermal stability. The PCR mixture was irradiated with ultraviolet light to cross-link any contaminating double-stranded DNA before template was added [10]. Ampliwax PCR Gem 100 (Perkin-Elmer Cetus, Beaconsfield, UK) was used according to the manufacturer’s instructions to minimise non-specific annealing of oligonucleotide primers to template at the start of the thermal cycling. Samples were processed through 30 cycles (2 min at 94°C (denaturation), 2 min at 42°C (annealing), 2 min 20 s at 72°C (extension)).

2.4. Nucleotide sequence determination and peptide sequence analysis

Nucleotide sequences were determined by the dideoxy chain termination method [11] using denatured plasmid templates [12]. [α-35S]dATP was used to label the growing strand. The highly processive modified T7 DNA polymerase Sequenase™ (USB Corp.) 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, UK) and oligonucleotides prepared with a model 380B DNA synthesizer (Applied Biosystems). The computer programme DARWIN (Data Analysis and Retrieval With Indexed Nucleotide/peptide sequences) [13–15] was used for multiple sequence comparisons. The programme has been developed and maintained at the Institut für Wissenschaftliches Rechnen at the Eidgenössische Technische Hochschule, Zurich and was accessed via the Internet.

3. Results

5'-univsod and 3'-univsod oligonucleotide primers, synthesized with a degeneracy allowing them to anneal to sodC from Haemophilus parainfluenzae, Brucella abortus, Caulobacter crescentus and Photobacterium leiognathi, were used as previously described [5] in a PCR strategy to attempt to isolate a segment of sodC from chromosomal DNA isolated from Salmonella typhimurium and other Salmonella species. After 30 cycles, multiple reaction products were seen over a wide size range on electrophoresis in 2% agarose gels. No product was obtained in the absence of Salmonella template DNA. There was no outstanding product around 310 bp as would be anticipated were there to be a Salmonella sodC gene
previously found to be invariant in bacterial [Cu,Zn]-SODs.

The DARWIN program Mulalignment [14]. Sequence shown corresponds to residue 88–174 of the 187-amino acid H. parainfluenzae protein [4]. The computer programme has introduced a single gap in the B. abortus sequence to optimise alignment. Spots signify identity between adjacent sequences; black arrowhead indicates an invariant Zn²⁺-ligand His; X indicates additional amino acids conserved across all known bacterial [Cu,Zn]-SOD sequences, excluding the highly divergent C. crescentus peptide sequence; open arrowheads indicate three residues previously found to be invariant in bacterial [Cu,Zn]-SODs.

Fig. 1 Alignment of [Cu,Zn]-SOD peptide sequence from S. typhimurium with B. abortus [21] and H. parainfluenzae [4], using the DARWIN programme Mulalignment [14]. Sequence shown corresponds to residue 88–174 of the 187-amino acid H. parainfluenzae protein [4]. The computer programme has introduced a single gap in the B. abortus sequence to optimise alignment. Spots signify identity between adjacent sequences; black arrowhead indicates an invariant Zn²⁺-ligand His; X indicates additional amino acids conserved across all known bacterial [Cu,Zn]-SOD sequences, excluding the highly divergent C. crescentus peptide sequence; open arrowheads indicate three residues previously found to be invariant in bacterial [Cu,Zn]-SODs.

resembling that in the other bacterial species. Nonetheless, there was a smear of products of roughly this size to be seen in the gel. A band of gel containing any PCR products of 280 bp to 350 bp was therefore excised and subjected to the same PCR protocol. At the end of this second cycle of reactions, a 310-bp product was prominent in all cases where template was included in the reaction mix (data not shown). The fragment amplified from S. typhimurium St4/74 was excised and cloned into the plasmid vector pBluescript. Two independent clones were selected and each was sequenced in both directions using the universal sequencing primers. Each gave the same sequence, which was different from any sodC previously described. The DNA and derived peptide sequences have been deposited in the EMBL database (accession number X94327). On translation, the putative Salmonella SodC segment showed 59.8% identity to the corresponding region of [Cu,Zn]-SOD in each of H. parainfluenzae and B. abortus (Fig. 1). His₈⁰, marked by an arrowhead, is a zinc-coordinating ligand invariant across all known [Cu,Zn]-SOD sequences except C. crescentus [16].

27/30 other amino acids identified as conserved among bacterial [Cu,Zn]-SOD sequences so far determined are present in the S. typhimurium sequence, including all those in positions for which functional constraints have been proposed [17].

The DARWIN program Phylotree was used to incorporate the new Salmonella sequence in an unrooted dendrogram displaying sequence relationships between all known bacterial sodC genes and selected examples from higher organisms (Fig. 2). In this diagram pairwise relationships are displayed graphically between all the prokaryotic and eukaryotic sodC sequences previously considered [5], now taking into account the addition of the enteric bacterial sequence, on the principle that the length of the path joining two peptide sequences is in proportion to their degree of similarity, calculated as the PAM distance (Percentage Accepted Mutations) separating two sequences [13,15]. Short and long PAM distances thus signify, respectively, closely similar and more divergent sequence. By this treatment it can be seen that the Salmonella sequence is more closely similar to that from B. abortus than to those found in the other species so far described. The cloned S. typhimurium fragment was used as a DNA probe in Southern hybridization experiments to assess the prevalence of sodC genes in Enterobacteriaceae. The expected strong signal was found on hybridization to chromosomal DNA from the parent S. typhimurium strain. A similar signal corresponding to an approx. 6-kb SalI fragment was obtained from all the Salmonella species examined (Fig. 3), but no signal was obtained on hybridization at similar stringency to E. coli strain DH5α [18] and the commercially available SURE strain (Stratagene). Similarly, no signal was obtained from clinical isolates of Klebsiella pneumoniae, Enterobacter aerogenes, or Pseudomonas aeruginosa. Studies are continuing to delineate the extent to which this gene is found in enteric bacteria other than Salmonella.

4. Discussion

sodC genes are clearly widespread in pathogenic Gram-negative bacteria, not only in organisms primarily located in the URT, as previously described, but also in S. typhimurium of the Enterobacteriaceae. Others have reported [Cu,Zn]-SOD activity in E.
coli, and although we have failed to demonstrate cross-hybridization of chromosomal DNA with our Salmonella probe, there may be sufficient sequence divergence to explain this. Further studies are planned in which DNA will be examined from E. coli strains known to harbour the perhaps unusual monomeric [Cu,Zn]-SOD described by Battistoni and Rotilio [8].

![Graphical representation of [Cu,Zn]-SOD peptide sequence differences computed on the basis of PAM distances](image)

Fig. 2. Graphical representation of [Cu,Zn]-SOD peptide sequence differences computed on the basis of PAM distances [14,15]. 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 follows: a, yeast; b, bovine; c, human (extracellular); d, human (intracellular); e, Onchoerca voluculus (extracellular); f, O. voluculus (intracellular); g, Schistosoma mansoni; h, C. crescentus; i, P. tetragnathi; j, B. abortus; k, H. parainfluenzae; l, H. influenzae type b; m, H. ducreyi; n, A. actinomycetemcomitans; o, A. pleuropneumoniae; p, N. meningitidis; q, P. multocida; r, S. typhimurium. All sequences are from the published literature, as referenced in Kroll et al. [5].

![Southern blot of Sall-digested chromosomal DNA hybridized to the cloned fragment of sodC from S. typhimurium](image)

Fig. 3. sodC in Salmonella. Southern blot of Sall-digested chromosomal DNA hybridized to the cloned fragment of sodC from S. typhimurium. Lanes contain: 1. 370-bp fragment of sodC flanked by vector polylinker sequence, cloned from S. typhimurium S14/74; 2–7. Sall-digested chromosomal DNA from: 2. S. enteritidis strain S13; 3. S. typhimurium S12/75; 4. S. choleraesuis Sca14/74; 5. S. choleraeuis Sca50; 6. S. dublin Sd3246; 7. S. dublin Sd2229.

Analysis of our sequence data with those of others has defined a grouped relationship of bacterial [Cu,Zn]-SOD sequences clearly distinct from eukaryotic examples. Within the family of bacterial genes, those found in URT colonists and pathogens show close sequence similarity, while the rest lie at some remove. The S. typhimurium sequence, the first to be included from an enteric pathogen, appears by contrast to be most closely related to that found in Brucella abortus (Fig. 2).

Might this have functional significance? HAP bacteria and N. meningitidis are conventionally considered to be extracellular colonists or pathogens of animals and man and we have speculated that [Cu,Zn] SOD may play a part in promoting bacterial colonization of mucosal surfaces [4]. Among the few other bacteria in which sodC genes had previously been found [19–21], B. abortus stood out not only as being a pathogen but also as a facultative intracellular parasite, one that survives and multiplies in an intracellular environment within host phagocytic cells including human polymorphonuclear leukocytes. Here, perhaps more than anywhere else, bacteria are potentially exposed to a lethal flux of oxyradicals,
and the similarity of S. typhimurium sodC to B. abortus sequence may reflect particular functional constraints arising from existence in this niche. Like other pathogenic Salmonella spp., S. typhimurium is an intracellular pathogen, sequestering within host monocyte-macrophages, and it may be that expression of sodC is of pathogenic significance in this organism. Within phagocytes, organisms must somehow abrogate the respiratory burst host defence reaction, either by inhibiting its initiation or by resisting the bactericidal action of superoxide and the other free radicals produced. In this latter scenario a periplasmic SOD, appropriately located to dismutate superoxide generated exogenously in this way, can plausibly be considered as a modulator of bacterial virulence. Evidence for such a protective role for periplasmic [Cu,Zn]-SOD has been offered in the case of B. abortus [22], and for a unique surface-associated SOD expressed by Nocardiia asteroides [23], but the issue remains controversial [24,25]. Genetic tools and model systems of infection are not well developed for the analysis of the virulence of these organisms, and the discovery of sodC in Salmonella spp., in which they are, provides an excellent opportunity to dissect the role of this novel enzyme in host–parasite interactive biology.

Acknowledgements

We gratefully acknowledge support for this work by grants from the Biotechnology and Biological Sciences Research Council and The Wellcome Trust.

References


