The role of two periplasmic copper- and zinc-cofactored superoxide dismutases in the virulence of *Salmonella choleraesuis*

Assunta Sansone,¹ Patricia R. Watson,² Timothy S. Wallis,²
Paul R. Langford¹ and J. Simon Kroll¹

Author for correspondence: J. Simon Kroll. Tel: +44 20 7886 6220. Fax: +44 20 7886 6284.
e-mail: s.kroll@ic.ac.uk

¹ Molecular Infectious Diseases Group, Department of Paediatrics, Faculty of Medicine, Imperial College, St Mary's Hospital Campus, Norfolk Place, London W2 1PG, UK

² Institute for Animal Health, Compton, Newbury, Berkshire, RG20 7NN, UK

Periplasmic copper- and zinc-cofactored superoxide dismutases ([Cu,Zn]-SODs, SodC) of several Gram-negative pathogens can protect against superoxide-radical-mediated host defences, and thus contribute to virulence. This role has been previously defined for one [Cu,Zn]-SOD in various *Salmonella* serovars. Following the recent discovery of a second periplasmic [Cu,Zn]-SOD in *Salmonella*, the effect of knockout mutations in one or both of the original *sodC-1* and the new *sodC-2* on the virulence of the porcine pathogen *Salmonella choleraesuis* is investigated here. In comparison to wild-type, while *sodC* mutants – whether single or double – showed no impairment in growth, they all showed equally enhanced sensitivity to superoxide and a dramatically increased sensitivity to the combination of superoxide and nitric oxide *in vitro*. This observation had its correlate in experimental infection both *ex vivo* and *in vivo*. Mutation of *sodC* significantly impaired survival of *S. choleraesuis* in interferon γ -stimulated murine macrophages compared to wild-type organisms, and all *S. choleraesuis* *sodC* mutants persisted in significantly lower numbers than wild-type in BALB/c (*Ity^s*) and C3H/HeN (*Ity^r*) mice after experimental infection, but in no experimental system were *sodC-1 sodC-2* double mutants more attenuated than either single mutant. These data suggest that both [Cu,Zn]-SODs are needed to protect bacterial periplasmic or membrane components. While SodC plays a role in *S. choleraesuis* virulence, the data presented here suggest that this is through overcoming a threshold effect, probably achieved by acquisition of *sodC-1* on a bacteriophage. Loss of either *sodC* gene confers maximum vulnerability to superoxide on *S. choleraesuis*.

Keywords: Superoxide dismutase, *Salmonella*, periplasm, bacterial [Cu,Zn]-SOD, bacterial virulence

INTRODUCTION

Salmonella serovars responsible for disease in various mammalian hosts can proliferate as facultative intracellular pathogens, able to survive within the phagosome and/or phagolysosome of macrophages (Buchmeier &

Heffron, 1989). *Salmonella* virulence in mice has been correlated with the net growth of *Salmonella* within these cells of the reticuloendothelial system (Fields *et al.*, 1986). This suggests a capacity to resist host defences including the superoxide radical anion ($O_2^{\cdot-}$) generated during the respiratory burst, nitric oxide (NO) produced by inducible NO synthase and a range of reactive molecules formed by reaction between these two species. These include various extremely reactive oxygen and nitrogen intermediates (ROIs, RNIs) such as the hydroxyl radical and peroxynitrite that exert a potent microbicidal effect through the indiscriminate oxidative damage they cause to lipids, proteins and DNA in cell

Abbreviations: [Cu,Zn]-SOD, copper- and zinc-cofactored superoxide dismutase; DEDC, diethyldithiocarbamate; FCS, foetal calf serum; IFN γ , interferon γ ; i.p., intraperitoneal; ROI/RNI, reactive oxygen/nitrogen intermediate; SOD, superoxide dismutase; SPER/NO, 2,2'-hydroxy-nitrosohydrazono bis-ethanamine (spermine NONOate); X, xanthine; XO, xanthine oxidase.

membranes, the periplasm and cytosol. Diverse mechanisms are beginning to be defined which contribute to the capacity of *Salmonella* strains to withstand exogenous ROI and RNI stress. The superoxide radical causes oxidative damage to iron-sulphur clusters, and the product of the *yggX* gene has been shown to exert a protective effect (Gralnick & Downs, 2001). The microbicidal effects of RNIs derived from phagocytic cell metabolism are in part vitiated by a recently described bacterial alkyl hydroperoxide reductase (AhpC) (Chen *et al.*, 1998), while the very production in the macrophage phagosome of these toxic species is impaired through the operation of a *Salmonella* pathogenicity island 2-encoded type III secretion system that mediates exclusion of NADPH oxidase assembly from the phagosome membrane (Gallois *et al.*, 2001). Overall, the superoxide radical is a key intermediate in the production of these toxic species, and bacterial superoxide dismutases might accordingly be expected to play an important part in endowing pathogenic organisms with resistance to such host defences. Recent light has been thrown on this process with the description of the role of bacterial periplasmic copper- and zinc-cofactored superoxide dismutases ([Cu,Zn]-SODs) in the virulence of various serovars of *Salmonella* (Farrant *et al.*, 1997; De Groote *et al.*, 1997).

Superoxide dismutases (SODs) are virtually ubiquitous in bacteria, catalysing the conversion of $O_2^{\cdot -}$ into hydrogen peroxide and oxygen (McCord & Fridovich, 1969) in the first of a series of reactions to remove free radicals generated during the reduction of molecular oxygen. Three types of SOD are widely found in bacteria. Two are cofactored by manganese or iron and are located in the cytoplasm where they catalyse dismutation of $O_2^{\cdot -}$ generated in the course of aerobic metabolism. A third SOD is cofactored by copper and zinc ([Cu,Zn]-SOD) and has been identified in the periplasm of a wide range of Gram-negative bacteria [*Photobacterium leiognathi* (Steinman, 1987); *Caulobacter crescentus* (Steinman, 1982); *Brucella abortus* (Stabel *et al.*, 1994); *Haemophilus* species (Langford *et al.*, 1992; Kroll *et al.*, 1995); *Legionella* species (St John & Steinman, 1996); *Actinobacillus* and *Pasteurella* species (Kroll *et al.*, 1995); *Escherichia coli* (Benov & Fridovich, 1994; Imlay & Imlay, 1996); and *Salmonella typhimurium*, *Salmonella choleraesuis* and *Salmonella dublin* (Canvin *et al.*, 1996; Farrant *et al.*, 1997)]. Within the periplasm [Cu,Zn]-SOD is inaccessible to cytosolic superoxide but is suitably located to protect the organism from exogenous superoxide and the toxic action of its further reaction products with hydrogen peroxide and nitric oxide. Studies have confirmed such a function both in *Salmonella* and *Neisseria meningitidis* (Farrant *et al.*, 1997; De Groote *et al.*, 1997; Wilks *et al.*, 1998).

The degree of dissimilarity (only 54% identity) between the translated *Salmonella sodC* sequence described by Farrant *et al.* (1997) and *E. coli* SodC, its aberrant map position with respect to the *Salmonella* and *E. coli* genomes, and its flanking regions encoding proteins resembling products of bacteriophage lambda, suggest-

ed that this gene was not the orthologue of the *E. coli* gene and that *Salmonella* may have acquired it through phage-mediated transfer from an unknown donor (Farrant *et al.*, 1997). This hypothesis has been supported by the discovery of a new *Salmonella* gene, *sodC-2*, encoding a protein 82% identical to the *E. coli* SodC (Fang *et al.*, 1999). The first *sodC* gene is now renamed *sodC-1*. Here we report an investigation of the contribution that each of these genes makes to the virulence of *S. choleraesuis*. Unexpectedly, we did not find the effect to be additive, but rather, that both genes were necessary for the contribution of [Cu,Zn]-SOD to be discerned.

METHODS

Bacterial strains and culture. *S. choleraesuis* strain A50 is a clinical veterinary isolate (Watson *et al.*, 2000). A *sodA* mutant of *S. typhimurium* was kindly provided by Dr Anjam Khan (University of Newcastle). *E. coli* S17-1 λ -pir was used as the host for conjugative transfer of plasmid DNA to *Salmonella* (Simon *et al.*, 1983). *Salmonella* strains were made resistant to nalidixic acid by standard procedures. Bacteria were grown in Luria-Bertani (LB) medium at 37 °C with shaking (130–180 r.p.m.). Where needed, antibiotics were added at: kanamycin, 50 mg l⁻¹; nalidixic acid, 30 mg l⁻¹; penicillin, 250 mg l⁻¹. Bacterial strains were stored in 10% (v/v) glycerol at -70 °C and fresh aliquots were used for each infection experiment.

Construction of *sodC-2* mutants. A 344 bp DNA fragment amplified by PCR from within the *sodC-2* ORF (nt 508–851, GenBank accession AF056931) and ligated into the suicide vector pRR10(Δ *trfA*) (Fang *et al.*, 1992) was generously provided by Ferric Fang (University of Colorado). This plasmid was mobilized from *E. coli* S17-1 into *S. choleraesuis* A50 wild-type and *sodC-1* mutant (Farrant *et al.*, 1997). Transconjugants were selected on LB agar containing penicillin for the *sodC-2* mutants or kanamycin and penicillin for the *sodC-1* *sodC-2* mutants. Homologous recombination of the plasmid into the *Salmonella* chromosome created an interruption of *sodC-2*, confirmed by Southern hybridization.

Extraction of bacterial proteins and detection of SOD activity. Periplasmic extracts were prepared from 100 ml overnight cultures of *S. choleraesuis* wild-type, and *sodC-1*, *sodC-2* and *sodC-1* *sodC-2* mutants by the method of Higgins & Hardie (1983). Proteins were precipitated with ammonium sulphate (650 g l⁻¹), concentrated by centrifugation and stored at -20 °C for later analysis. To detect SOD, proteins were separated in pre-cast wide range (pH 3–10) IEF gels (Bio-Rad). Gels were stained to reveal SOD activity using the method of Beauchamp & Fridovich (1971) as modified by Steinman (1985). [Cu,Zn]-SOD activity was identified by specific inhibition with 5 mM diethyldithiocarbamate (DEDC; Sigma), a copper chelator (Benov & Fridovich, 1994). [Fe]-SOD was identified by inhibition of its activity with hydrogen peroxide (Crapo *et al.*, 1978).

Bacterial sensitivity to superoxide and nitric oxide generated *in vitro*. Sensitivity to increased cytosolic $O_2^{\cdot -}$ flux induced by paraquat was assessed by the method of De Groote *et al.* (1995). Sensitivity to exogenous $O_2^{\cdot -}$ and NO was assessed by exposure of suspensions (10⁵ c.f.u. ml⁻¹) of stationary-phase organisms in phosphate-buffered saline (pH 7.4) (PBS) to these radicals, generated in solution respectively from reactions

between xanthine (X) and xanthine oxidase (XO) (Fridovich, 1970) and on acidification of ice cold alkaline 2,2'-hydroxy-nitrosohydrazono bis-ethanamine (spermine NONOate, SPER/NO) (De Groote *et al.*, 1997). Bacterial suspensions in these solutions were incubated with shaking at 37 °C and aliquots were removed periodically and assayed for viable counts.

Preparation of macrophages. Porcine alveolar macrophages were isolated as previously described (Farrant *et al.*, 1997). Macrophages were suspended in Iscove's modified Dulbecco's medium containing 10% (v/v) foetal calf serum (FCS), 100 mg gentamicin l⁻¹ and 100 U ml⁻¹ each of penicillin and streptomycin, and distributed into 1 ml flat-bottomed wells of tissue culture plates to give 5 × 10⁵ cells per well. Wells were incubated overnight at 37 °C in an atmosphere of 5% CO₂ to allow cells to adhere as monolayers. Two hours before infection monolayers were washed and covered with fresh antibiotic-free medium. Murine peritoneal macrophages were isolated from BALB/c mice at 4 days after intraperitoneal (i.p.) injection of 5 mM sodium periodate by peritoneal lavage. Macrophages were suspended in RPMI 1640 medium containing 10% FCS and 20 U murine interferon γ (IFNγ) ml⁻¹, and distributed into 0.1 ml flat-bottomed wells at 5 × 10⁴ cells per well. Cells were left to adhere as monolayers as before, and washed and covered with fresh medium at the start of experiments.

Infection of monolayers. Bacteria from overnight cultures were washed and resuspended in tissue culture medium at a concentration of 5 × 10⁶ c.f.u. ml⁻¹. For opsonization, bacteria were incubated with 10% normal porcine or murine serum at 37 °C for 30 min on a rolling platform at 120 r.p.m. Bacteria were added to monolayers to give a verified multiplicity of infection of 5–10 bacteria per macrophage. In experiments with IFNγ-stimulated macrophages, the infected monolayers were gently centrifuged (150 g for 10 min at 4 °C). Experiments were performed in triplicate.

Bacterial uptake and killing by macrophages. Bacterial uptake and killing were assessed by a modification of the gentamicin-protection assay of Buchmeier & Heffron (1989). Infected monolayers of IFNγ-stimulated macrophages were incubated for 15 min, followed by a wash and further incubation with Iscove's modified Dulbecco's Medium or RPMI 1640 containing 10% v/v FCS and 100 mg gentamicin l⁻¹. After 1–24 h incubation, monolayers were washed twice with PBS to remove any remaining superficially adherent viable bacteria and lysed with 0.1% (w/v) sodium deoxycholate in PBS. To assess viable counts of phagocytosed bacteria, lysates were plated on to MacConkey agar containing 30 mg nalidixic acid l⁻¹. For experiments with unstimulated macrophages, the initial incubation was extended to 1 h.

Experimental infection of mice. Mice were purchased from Charles River Laboratories. In a first experiment, groups of five 8–10 week-old BALB/c female mice were injected i.p. with 10³ c.f.u., and five C3H/HeN mice with 5 × 10⁴ c.f.u. A group of sham-inoculated mice received saline only. At 4 and 5 days post-infection, respectively, when animals infected with the wild-type *S. choleraesuis* began to exhibit symptoms of systemic disease (ruffled fur, reduced activity), all the mice were killed by CO₂ asphyxiation followed by cervical dislocation. Spleens and livers were removed, weighed and homogenized in 0.9% saline containing 1% Triton X-100. Viable counts were determined by plating serial dilutions on MacConkey agar containing nalidixic acid. Replica plating on appropriate selective media was carried out to exclude the

theoretical possibility that mutant strains might have reverted to wild-type.

In a second experiment, groups of three 8–10 week-old BALB/c female mice were infected i.p. with 100 c.f.u. *Salmonella* suspended in 0.1 ml 0.9% saline or saline only (sham infection). At 4 days post-infection the mice began to exhibit symptoms of systemic disease and were killed by CO₂ asphyxiation. Spleens and livers were removed and number of viable organisms in the organs was determined as above.

Groups of three 8–10 week-old BALB/c female mice were infected by oral administration of 10⁶ c.f.u. *Salmonella* suspended in 0.1 ml 0.9% saline. Sham-infected mice received saline only. At 5 days post-infection, as mice began to exhibit symptoms of systemic disease, they were killed by CO₂ asphyxiation. Spleens and livers were removed and the number of viable organisms in the organs was determined as above.

RESULTS

Characterization of mutants and growth characteristics

The presence of *sodC-1* and *sodC-2* was established in *S. choleraesuis* by Southern blotting (Farrant *et al.*, 1997 and further data not shown) and the set of *sodC-1* and *sodC-2* mutants of strain A50 were constructed as described in Methods. Strains were examined for SOD activity. Two bands of SOD activity, sensitive to DEDC inhibition and so characteristic of [Cu,Zn]-SOD, were present in the wild-type but one or other band, as expected, was absent in the single mutant strains (Fig. 1). SodC-2 is more acidic (pI 5–6) than SodC-1 (pI ~ 8) and runs very close to a band of [Fe]-SOD activity, sensitive to hydrogen peroxide (H₂O₂ sensitivity data not shown). *S. choleraesuis* A50 wild-type and mutant strains were grown aerobically in broth culture with shaking for 78 h, without replenishment of the LB medium. There was no significant difference in gen-

Fig. 1. Paired IEF gels stained for SOD activity (a) without and (b) with selective inhibition of [Cu,Zn]-SODs by 5 mM DEDC. The pH range of the ampholytes was 10 (top) to 3 (bottom). Lanes 1–4 contain proteins extracted from (1) *S. choleraesuis* A50 wild-type; (2) *S. choleraesuis* *sodC-1* mutant; (3) *S. choleraesuis* *sodC-2* mutant; and (4) *S. choleraesuis* *sodC-1 sodC-2* double mutant. Arrowheads mark the positions of SodC-1 (top), SodC-2 (middle) and iron-cofactored SOD (bottom).

Fig. 2. Effect of *sodC* mutations on survival of *S. choleraesuis* on exposure to O_2^- and NO generated in solution. Recovery of wild-type (●), *sodC*-1 (■), *sodC*-2 (◆) and double (▲) mutant strains in PBS+X/XO (continuous lines), PBS+SPER/NO (dotted lines) and PBS+X/XO+SPER/NO (dashed lines). A representative result of the experiment (carried out in triplicate) is shown.

eration time between wild-type and mutants during exponential growth, in maximum density achieved (10^{10} c.f.u. ml $^{-1}$) and in viability after prolonged stationary phase culture (data not shown).

Effect of mutations on sensitivity to reactive oxygen and nitrogen species *in vitro*

As expected, wild-type and *sodC* mutant strains were equally resistant to paraquat, a redox cycling reagent that penetrates the cytosol, greatly increasing superoxide production there. *S. typhimurium* Δ *sodA* (lacking cytosolic Mn-cofactored SOD) was used as the control and was shown to be more sensitive (data not shown). *S. choleraesuis* mutant strains were significantly ($P < 0.01$) more susceptible than the wild-type to killing by extracellular superoxide generated by X/XO (Fig. 2). A dramatically increased sensitivity of the mutants was observed to the synergistic killing by X/XO and the NO donor (Fig. 2). SPER/NO by itself had no antimicrobial activity against either *S. choleraesuis* wild-type or mutant strains. No additional difference in sensitivity was shown by the double mutant in any of these assays.

[Cu,Zn]-SODs and interaction of *Salmonella* with phagocytic cells *in vitro*

S. choleraesuis *sodC* mutant strains showed a significant increased susceptibility to killing by activated macrophages compared to the wild-type strain (Fig. 3). There was no difference in the susceptibility of the single mutants, or between either single and the double mutant.

Fig. 3. Effect of *sodC* mutations on the persistence of *S. choleraesuis* wild-type (●), *sodC*-1 (■), *sodC*-2 (◆) and double (▲) mutant strains in $INF\gamma$ -stimulated periodate-elicited peritoneal macrophages from BALB/c mice. Recovery of organisms from lysed cells expressed as mean ($\pm$ SEM) of triplicate wells for each time point is shown.

Macrophage activation was necessary to elicit these differences. Using non-activated murine and porcine macrophages, prepared as described by Farrant *et al.* (1997), no differences were found between wild-type and *sodC* mutant strains in either bacterial uptake or subsequent bacterial killing, whether or not bacteria were opsonized (results not shown).

[Cu,Zn]-SODs and pathogenesis of systemic disease

After i.p. infection, at the humane end point when mice began to show signs of systemic infection, the load of organisms in spleen and liver was measured as described. Whether recovered from *Ity*^s or from *Ity*^r mice, the wild-type strain was recovered in highly significantly greater numbers than the *sodC* mutant strains (Fig. 4). However, the double mutation did not attenuate any more than either single mutation. In all cases, the strains retained the expected antibiotic resistance, showing that no reversion of the *sodC* genotype had occurred. A similar result was obtained using lower i.p. inocula (100 c.f.u.) of bacteria (data not shown) and with groups of mice infected orally (Fig. 5). Once again the double mutation did not have any additional effect. We have previously demonstrated that complementation of one *sodC* mutation (*sodC*-1) with a plasmid-borne copy of the gene restored *S. choleraesuis* to wild-type virulence in this model (Farrant *et al.*, 1997).

Fig. 4. Effect of *sodC* mutations on the virulence of *S. choleraesuis* after i.p. challenge of (a) BALB/c (4 days after challenge) and (b) C3H/HeN mice (5 days after challenge). Bars show the recoveries of wild-type (wt, black bars), *sodC*-1 (C1, vertical hatched bars), *sodC*-2 (C2, horizontal hatched bars) and double (C1+C2, cross-hatched bars) mutant strains from spleen and liver. Results are expressed as c.f.u. recovered (mean+SEM) from 5 mice per bacterial strain.

Fig. 5. Effect of *sodC* mutations on the virulence of *S. choleraesuis* in BALB/c mice 5 days after oral challenge. Bars show the recoveries of wild-type (wt, black bars), *sodC*-1 (C1, vertical hatched bars), *sodC*-2 (C2, horizontal hatched bars) and double (C1+C2, cross-hatched bars) mutant strains, and virulence plasmid-cured strain (P-, white bars) from spleen and liver. Results are expressed as c.f.u. recovered (mean+SEM) from 3 mice per strain.

Fig. 6. Graphical representation of relationship between sequences of (a) *Salmonella* SodC-2 and other bacterial SodCs; (b) as (a), but including *Salmonella* SodC-1; (c) RecF from the same bacterial species.

Phylogenetic comparison of *Salmonella* SodC-1 and SodC-2 with SodC from other bacteria

The multiple sequence comparison programme UnrootedTree (at <http://cbrg.inf.ethz.ch/Server/MultAlign.html>) was used to analyse graphically the relationship between the published sequences of *Salmonella* SodCs and SodC from the disparate species *E. coli*, *Haemophilus influenzae*, *Actinobacillus pleuropneumoniae*, *Mycobacterium tuberculosis* and *C. crescentus* (Fig. 6). A similar analysis can be carried out for any set of homologous proteins, and for comparison is shown for the central metabolic (DNA repair) enzyme RecF from the same bacterial species (Fig. 6c). These phylogenetic trees are drawn on the principle that the length of the path joining loci representing protein sequences is in proportion to their degree of dissimilarity. Short and long distances thus signify, respectively, similar or divergent sequences. The striking degree of similarity between the RecF and SodC trees before *Salmonella* SodC-1 is taken into account (Fig. 6a compared to Fig. 6c) suggests that *sodC* and *recF* are not subject to different pressure of mutation in the different species analysed. *Salmonella* and *E. coli* protein sequences are generally very similar and the short distance separating the RecF sequence loci corresponds to that separating *Salmonella* SodC-2 and *E. coli* SodC. However, when the tree is reconstructed to include *Salmonella* SodC-1 (Fig. 6b), this branch diverges widely from both *Salmonella* SodC-2 and *E. coli* SodC.

DISCUSSION

Here we have shown that extracytoplasmic [Cu,Zn]-SODs in *Salmonella* protect organisms against exogenously generated O_2^- . In addition we have demonstrated the contribution of SodC-1 and SodC-2 to the virulence of *S. choleraesuis* in an experimental infection model, establishing [Cu,Zn]-SOD as a determinant of *Salmonella* virulence, reducing the effectiveness of ROI- and RNI-mediated host defence mechanisms. While the two SodCs together are associated with full virulence of *S. choleraesuis*, our data show that their contributions are not additive. Inactivation of both *sodC* genes leads to no further attenuation in the resistance of *S. choleraesuis* to O_2^- and NO generated *in vitro*, or in virulence, than is seen with either single mutant. This suggests that the contribution of [Cu,Zn]-SOD in these respects is conditional on there being a threshold level of enzyme activity. With two functional *sodC* genes, *S. choleraesuis* achieves enhanced survival under the conditions of oxidative stress encountered during experimental murine infection by the oral or parenteral route, reproduced *ex vivo* in experimental infection of activated macrophages. This observation finds a correlate in the observations of Battistoni *et al.* (2000). An *E. coli* strain engineered to increase [Cu,Zn]-SOD production was found to be much more resistant to intracellular killing than the wild-type strain, while there was very little difference in survival between the wild-type and a *sodC*-null mutant.

While a [Cu,Zn]-SOD gene has by now been found in many bacterial species, the discovery of two different, functional, versions of the gene – and very recently a third, *sodC-3* (Figueroa-Bossi *et al.*, 2001) – in the one pathogen is without precedent. The gene *sodC-2* in *Salmonella* is the true orthologue of *sodC* in *E. coli*, established by extended sequencing of the chromosomal locus in each organism. In *E. coli* a 7 kb domain contains consecutive ORFs: *slyA*–B1643–B1644–B1645–*sodC*–B1647–B1648–B1649–*nemA*. A search with the translation of each of these genes/putative genes against the *S. typhimurium* genome database (at <http://genome.wustl.edu/gsc/Projects/S.typhimurium/>) demonstrated in each case high identity (> 80%) for corresponding ORFs flanking *sodC-2*. In contrast, *sodC-1* and *sodC-3* appear to have been acquired on lysogenic bacteriophages (*Gifsy-2* and *Fels-1* respectively) (Figueroa-Bossi & Bossi, 1999; Figueroa-Bossi *et al.*, 2001). A likely origin of *sodC-1* from outside *Salmonella* is revealed by all-against-all protein sequence comparison of a set of SodC sequences. SodC-1 is as divergent in sequence from *Salmonella* SodC-2 and *E. coli* SodC as the examples of the *sodC* from such upper respiratory tract pathogens as *H. influenzae*. While an anomalously high mutation rate could theoretically be the explanation for the SodC-1 sequence divergence, the association with phage genes strongly supports the hypothesis that this gene has an exogenous origin: *sodC-1* joins a growing list of virulence genes likely to have been acquired through horizontal transfer. Carriage of *sodC-1* on lysogenized *Gifsy-2* is a characteristic of the more virulent *Salmonella* serotypes, such as *S. typhimurium*, *S. choleraesuis*, *S. dublin* and *Salmonella enteritidis*. The less virulent serotypes have only *sodC-2* (Fang *et al.*, 1999). Taken with the data presented here for *S. choleraesuis*, this suggests that selection pressure may have favoured acquisition of one or even two extra [Cu,Zn]-SOD genes by *Salmonella* serovars adapted to survive within macrophages. A mechanism to promote *sodC-1* acquisition may be proposed. *Gifsy-2* elements are released upon exposure to H_2O_2 , raising the intriguing possibility that infection of macrophages and exposure to reactive oxygen species generated in the respiratory burst may, through induction of a lytic cycle for *Gifsy-2*, potentiate transmission of the genes it carries, among them protective *sodC-1*, to other salmonellae (Figueroa-Bossi *et al.*, 1999).

The proposition that horizontally acquired [Cu,Zn]-SOD genes may make a special contribution to bacterial virulence, not conferred by endogenous *sodC*, gains support from observations in the genus *Neisseria*. Commensal neisserial species, common colonists of the human upper respiratory and genital mucosa, do not in general have *sodC* genes (our unpublished results) but *N. meningitidis*, the only neisserial species regularly causing invasive, life threatening, infection, is the exception and in this pathogen [Cu,Zn]-SOD unequivocally contributes to virulence (Wilks *et al.*, 1998). As seen for *sodC-1* in *Salmonella*, meningococcal *sodC* appears to have been acquired by horizontal transfer, in

this case probably from co-commensal *Haemophilus* species (Kroll *et al.*, 1998). In contrast, studies of other pathogens with only a single *sodC* gene and in which there is no evidence, for example from sequence anomaly at the locus, of horizontal acquisition of the gene, have generally yielded equivocal or negative evidence for the involvement of [Cu,Zn]-SOD in virulence [*B. abortus* (Tatum *et al.*, 1992; Latimer *et al.*, 1992), *A. pleuropneumoniae* (Sheehan *et al.*, 2000) and *M. tuberculosis* (Dussurget *et al.*, 2001; Piddington *et al.*, 2001)].

Fang *et al.* (1999) have studied the contribution that SodC-1 and SodC-2 make to the virulence of *S. typhimurium* and reached a different conclusion to ours. It is not, however, possible directly to compare our findings as there are significant differences in experimental design. In a different experimental model – much more prolonged murine infection to a fatal end point – they observed that only the *sodC-1 sodC-2* strain was attenuated in lethality for *Ity^s* mice (a different strain to that used here). In an *Ity^r* strain (the same as that used here), all *sodC* mutants, single and double, were slower to kill mice than the wild-type. Although the result reported for the double mutant suggested greater attenuation, the reproducibility of this single observation cannot be assessed with the data available. It may be that the contrast reflects differences in the pathogenesis of *S. typhimurium* and *S. choleraesuis* in mice. Despite being highly virulent for pigs (Watson *et al.*, 2000), *S. choleraesuis* A50 is significantly less virulent for mice than *S. typhimurium* and *S. dublin* (Farrant *et al.*, 1997).

In conclusion, in *S. choleraesuis* it seems clear that the acquisition of an additional *sodC* gene on a lysogenic bacteriophage has effectively led to a gain of function (plausibly through increased total activity) for [Cu,Zn]-SOD, contributing to virulence. Until now the contribution that *sodC* makes to bacterial virulence has seemed wholly capricious – clearly contributing in some organisms, but not at all in others. From this work we would tentatively suggest that it is in particular in those organisms in which there is evidence of horizontal acquisition of *sodC* – by bacteriophage or plasmid transfer, or through transformation with chromosomal DNA – that evidence may be found of a virulence-associated gain of function, the capacity to neutralize host defences that depend on the generation of superoxide and its further reaction products.

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REFERENCES

- Battistoni, A., Pacello, F., Folcarelli, S. & 7 other authors (2000). Increased expression of periplasmic Cu,Zn superoxide dismutase enhances survival of *Escherichia coli* invasive strains within nonphagocytic cells. *Infect Immun* **68**, 30–37.
- Beauchamp, C. O. & Fridovich, I. (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Anal Biochem* **44**, 276–287.
- Benov, L. T. & Fridovich, I. (1994). *Escherichia coli* expresses a copper- and zinc-containing superoxide dismutase. *J Biol Chem* **269**, 25310–25314.
- Buchmeier, N. A. & Heffron, F. (1989). Intracellular survival of wild-type *Salmonella typhimurium* and macrophage-sensitive mutants in diverse populations of macrophages. *Infect Immun* **57**, 1–7.
- Canvin, J., Langford, P. R., Wilks, K. E. & Kroll, J. S. (1996). Identification of *sodC* encoding periplasmic [Cu,Zn]-superoxide dismutase in *Salmonella*. *FEMS Microbiol Lett* **136**, 215–220.
- Chen, L., Xie, Q. W. & Nathan, C. (1998). Alkyl hydroperoxide reductase subunit C (AhpC) protects bacterial and human cells against reactive nitrogen intermediates. *Mol Cell* **1**, 795–805.
- Crapo, J. D., McCord, J. M. & Fridovich, I. (1978). Preparation and assay of superoxide dismutase. *Methods Enzymol* **53**, 382–393.
- De Groote, M. A., Granger, D., Xu, Y., Campbell, G., Prince, R. & Fang, F. C. (1995). Genetic and redox determinants of nitric oxide cytotoxicity in a *Salmonella typhimurium* model. *Proc Natl Acad Sci USA* **92**, 6399–6403.
- De Groote, M. A., Achsner, U. A., Michael, U. S. & 7 other authors (1997). Periplasmic superoxide dismutase protects *Salmonella* from products of phagocyte NADPH-oxidase and nitric oxide synthase. *Proc Natl Acad Sci USA* **94**, 13997–14001.
- Dussurget, O., Stewart, G., Neyrolles, O., Pescher, P., Young, D. & Marchal, G. (2001). Role of *Mycobacterium tuberculosis* copper-zinc superoxide dismutase. *Infect Immun* **69**, 529–533.
- Fang, F. C., Libby, S. J., Buchmeier, N. A., Loewen, P. C., Switala, J., Harwood, J. & Guiney, D. G. (1992). The alternative sigma factor *katF* (*rpoS*) regulates *Salmonella* virulence. *Proc Natl Acad Sci USA* **89**, 11978–11982.
- Fang, F. C., De Groote, M. A., Foster, J. W. & 8 other authors (1999). Virulent *Salmonella typhimurium* has two periplasmic Cu,Zn-superoxide dismutases. *Proc Natl Acad Sci USA* **96**, 7502–7507.
- Farrant, J. L., Sansone, A., Canvin, J. R., Pallen, M. J., Langford, P. R., Wallis, T. S., Dougan, G. & Kroll, J. S. (1997). Bacterial copper- and zinc-cofactored superoxide dismutase contributes to the pathogenesis of systemic salmonellosis. *Mol Microbiol* **25**, 785–796.
- Fields, P. I., Groisman, E. A. & Heffron, F. (1989). A *Salmonella* locus that controls resistance to microbicidal proteins from phagocytic cells. *Science* **243**, 1059–1062.
- Figuroa-Bossi, N. & Bossi, L. (1999). Inducible prophages contribute to *Salmonella* virulence in mice. *Mol Microbiol* **33**, 167–176.
- Figuroa-Bossi, N., Uzzau, S., Maloriol, D. & Bossi, L. (2001). Variable assortment of prophages provides a transferable repertoire of pathogenic determinants in *Salmonella*. *Mol Microbiol* **39**, 260–271.
- Fridovich, I. (1970). Quantitative aspects of the production of superoxide anion radical by milk xanthine oxidase. *J Biol Chem* **245**, 4053–4057.
- Gallois, A., Klein, J. R., Allen, L. A., Jones, B. D. & Nauseef, W. M. (2001). *Salmonella* pathogenicity island 2-encoded type III secretion system mediates exclusion of NADPH oxidase assembly from the phagosomal membrane. *J Immunol* **166**, 5741–5748.
- Gralnick, J. & Downs, D. (2001). Protection from superoxide damage associated with an increased level of the YggX protein in *Salmonella enterica*. *Proc Natl Acad Sci USA* **98**, 8030–8035.

- Higgins, C. F. & Hardie, M. M. (1983). Periplasmic protein associated with the oligopeptide permeases of *Salmonella typhimurium* and *Escherichia coli*. *J Bacteriol* **155**, 1434–1438.
- Imlay, K. R. C. & Imlay, J. A. (1996). Cloning and analysis of *sodC*, encoding copper, zinc superoxide dismutase of *Escherichia coli*. *J Bacteriol* **178**, 2564–2571.
- Kroll, J. S., Langford, P. R., Wilks, K. E. & Keil, A. D. (1995). Bacterial [Cu,Zn]-superoxide dismutase: phylogenetically distinct from the eukaryotic enzyme, and not so rare after all! *Microbiology* **141**, 2271–2279.
- Kroll, J. S., Wilks, K. E., Farrant, J. L. & Langford, P. R. (1998). Natural genetic exchange between *Haemophilus* and *Neisseria*: intergenic transfer of chromosomal genes between major human pathogens. *Proc Natl Acad Sci USA* **95**, 12381–12385.
- Langford, P. R., Loynds, B. M. & Kroll, J. S. (1992). Copper-zinc superoxide dismutase in *Haemophilus* species. *J Gen Microbiol* **138**, 517–522.
- Latimer, E., Simmers, J., Sriranganathan, N., Roop, I. R. M., Schurig, G. G. & Boyle, S. M. (1992). *Brucella abortus* deficient in copper/zinc superoxide dismutase is virulent in BALB/C mice. *Microb Pathog* **12**, 105–113.
- McCord, J. M. & Fridovich, I. (1969). Superoxide dismutase: an enzymatic function for erythrocuprein (hemocuprein). *J Biol Chem* **244**, 6049–6055.
- Piddington, D. L., Fang, F. C., Laessig, T., Cooper, A. M., Orme, I. M. & Buchmeier, N. A. (2001). Cu,Zn superoxide dismutase of *Mycobacterium tuberculosis* contributes to survival in activated macrophages that are generating an oxidative burst. *Infect Immun* **69**, 4980–4987.
- Sheehan, B. J., Langford, P. R., Rycroft, A. N. & Kroll, J. S. (2000). [Cu,Zn]-Superoxide dismutase mutants of the swine pathogen *Actinobacillus pleuropneumoniae* are unattenuated in infections of the natural host. *Infect Immun* **68**, 4778–4781.
- Simon, R., Priefer, U. & Puhler, A. (1983). A broad host range mobilization system for *in vivo* genetic engineering in Gram negative bacteria. *Bio/Technology* **1**, 784–791.
- Stabel, T. J., Sha, Z. & Mayfield, J. E. (1994). Periplasmic location of *Brucella abortus* Cu/Zn superoxide dismutase. *Vet Microbiol* **38**, 307–314.
- Steinman, H. M. (1982). Copper-zinc superoxide dismutase from *Caulobacter crescentus* CB15: a novel bacteriocuprein form of the enzyme. *J Biol Chem* **257**, 10283–10293.
- Steinman, H. M. (1985). Bacteriocuprein superoxide dismutases in pseudomonads. *J Bacteriol* **162**, 1255–1260.
- Steinman, H. M. (1987). Bacteriocuprein superoxide dismutase of *Photobacterium leiognathi*: isolation and sequence of the gene and evidence for a precursor form. *J Biol Chem* **262**, 1882–1887.
- St John, G. & Steinman, H. M. (1996). Periplasmic copper-zinc superoxide dismutase of *Legionella pneumophila*: role in stationary phase survival. *J Bacteriol* **178**, 1578–1584.
- Tatum, F. M., Detilleux, P. G., Sacks, J. M. & Halling, S. M. (1992). Construction of Cu-Zn superoxide dismutase deletion mutants of *Brucella abortus*: analysis of survival *in vitro* in epithelial and phagocytic cells and *in vivo* in mice. *Infect Immun* **60**, 2863–2869.
- Watson, P. R., Paulin, S. M., Jones, P. W. & Wallis, T. S. (2000). Interaction of *Salmonella* serotypes with porcine macrophages *in vitro* does not correlate with virulence. *Microbiology* **146**, 1639–1649.
- Wilks, K. E., Dunn, K. L. R., Farrant, J. L., Reddin, K. M., Gorringe, A. R., Langford, P. R. & Kroll, J. S. (1998). Periplasmic superoxide dismutase in meningococcal pathogenicity. *Infect Immun* **66**, 213–217.

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