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A role for *Haemophilus ducreyi* Cu,ZnSOD in resistance to heme toxicity

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Abstract The Cu,Zn superoxide dismutase (Cu,ZnSOD) from *Haemophilus ducreyi* is the only enzyme of this class which binds a heme molecule at its dimer interface. To explore the role of the enzyme in this heme-obligate bacterium, a *sodC* mutant was created by insertional inactivation. No difference in growth rate was observed during heme limitation. In contrast, under heme rich conditions growth of the *sodC* mutant was impaired compared to the wild type strain. This growth defect was abolished by supplementation of exogenous catalase. Genetic complementation of the *sodC* mutant in trans demonstrated that the enzymatic property or the heme-binding activity of the protein could repair the growth defect of the *sodC* mutant. These results indicate that Cu,ZnSOD protects *Haemophilus ducreyi* from heme toxicity.

Keywords Cu,ZnSOD · Superoxide dismutase · Heme · *Haemophilus ducreyi*

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Introduction

Cu,Zn superoxide dismutases (Cu,ZnSODs) catalyze the disproportionation of the superoxide anion into oxygen and hydrogen peroxide by the alternate reduction and oxidation of a metal ion which constitutes the catalytically active redox center (Battistoni 2003). Cu,ZnSOD is an important component of the antioxidant defence of aerobic organisms and can be found in all eukaryotic cells and in the periplasm of several bacteria. In view of such periplasmic location and the imperviousness of the cytoplasmic membrane to the superoxide anion (O_2^-) (Hassan and Fridovich 1979), it is believed that in bacteria Cu,ZnSOD provides a shield against exogenous oxidative stress. As phagocyte derived reactive oxygen species (ROS) comprise a physiological significant extracellular source of O_2^-, microbial Cu,ZnSOD has been proposed to provide resistance against oxidative mediated phagocyte killing. However, experimental evidence using Cu.Zn-SOD deletion mutants from a variety of bacterial pathogens in relevant animal models of infection has not been uniformly supportive of this premise (Lynch and Kuramitsu 2000; Battistoni 2003), indicating that the exact contribution of the protein to bacterial virulence has yet to be defined. Interestingly, a recent study has pointed out that significant superoxide production may occur in the periplasm of Cu,ZnSOD-expressing bacteria, suggesting that this enzyme might have a role also in

protecting bacteria from endogenous superoxide (Korshunov and Imlay 2006).

While all eukaryotic Cu,ZnSODs possess strictly conserved structural/functional properties (Bordo et al. 2001), bacterial Cu,ZnSODs show much more variable features, so that individual enzyme variants may exhibit unique properties (Lynch and Kuramitsu 2000; Bordo et al. 2001). One such example is the enzyme isolated from *Haemophilus ducreyi*, a Gram-negative heme obligate coccobacillus, which is the causative agent of chancroid, a sexually transmitted genital ulcer disease (Albritton 1989; Morse 1989). The Cu,ZnSOD from *H. ducreyi* is characterized by two properties that have not been observed in other enzymes of the same class: a metal binding N-terminal domain, possibly involved in the uptake of both Cu(I) and Cu(II) under conditions of metal starvation (Battistoni et al. 2001; D'Angelo et al. 2005), and the ability to bind a heme molecule at the interface between the two subunits (Pacello et al. 2001). Spectroscopic and structural analyses have in fact demonstrated that the enzyme contains a 6-coordinated low spin heme, with two histidines from the two subunits as axial ligands.

The role of heme in *H. ducreyi* Cu,ZnSOD remains a matter of speculation, but is likely related to the biology of *H. ducreyi*, a bacterium whose mechanism of heme acquisition remains an enigma. Although the organism expresses outer membrane proteins that specifically bind either to hemoglobin (HgbA (Stevens et al. 1996b) or HupA (Elkins et al. 1995)) or to heme (TdhA) (Thomas et al. 1998), the detailed molecular events subsequent to these surface interactions have yet to be fully elucidated. The heme-binding property displayed by *H. ducreyi* Cu,ZnSOD raises the suspicion of a participatory role for this protein in the heme uptake pathway, by perhaps shuttling heme across the periplasmic space to the cytoplasmic membrane (Pacello et al. 2001). Alternatively, Cu,ZnSOD might serve as a periplasmic heme "sink", stockpiling heme deposited in the periplasm with subsequent transfer to a dedicated heme ABC transporter responsible for conveying the molecule into the cytoplasm. To address this question, we conducted a series of biochemical and genetic studies using an *H. ducreyi sodC* mutant to examine the role of Cu,ZnSOD in *H. ducreyi* heme acquisition.

Materials and methods

Bacterial strains, plasmids and growth conditions

Bacteria and plasmids used in this study are listed in Table 1. *H. ducreyi* strains were cultivated on modified chocolate agar (CA) as described previously (Elkins et al. 1995) at 33°C in an atmosphere of 5% CO_2. Following electroporation (Totten et al. 1995), *H. ducreyi* transformants were selected on charcoal CA containing GC medium base, 1% (vol./vol.) IsoVitaleX, 1% (wt./vol.) bovine hemoglobin, 0.25% (wt./vol.) activated charcoal (Sigma, Oakville, Ont.), 35 μg/ml kanamycin. *E. coli* were cultured on Luria-Bertani (LB) agar plates or in LB broth (Difco). Antibiotics, where appropriate, were added at the following concentrations: kanamycin, 35 μg/ml; ampicillin 100 μg/ml; and chloramphenicol 2 μg/ml (*H. ducreyi*) or 50 μg/ml (*E. coli*).

Growth assays were conducted as previously described (Elkins et al. 1995) to determine the ability of *H. ducreyi* strains to use heme. Cells were passaged for four generations on clear semi-defined agar (Lee 1991) containing defined concentrations bovine hemin at 5 μg/ml (7 μM), or 50 μg/ml (77 μM). Kanamycin or chloramphenicol was added to the medium where indicated. Growth was monitored by measuring the change in A_{600} at 2 hourly intervals for 16 h. To determine the corresponding number of CFU, one ml aliquots were removed at each time point. Serial 10-fold dilutions from 10^{-3} to 10^{-7} were plated in duplicate onto CA and growth was enumerated after 48 h of incubation at 33°C. Each growth experiment was performed six times. The mean peak cell densities were compared by the Student's paired two-sided *t*-test. Analyses were performed using Sigma Stat for Windows software, version 3.00.

Production of antibody against *H. ducreyi* Cu,ZnSOD

A 6-month-old New Zealand White female rabbit (Charles River Co., St. Constant, PQ, Canada) was immunized with an 800 μl suspension comprising 100 μg of gel-purified Cu,ZnSOD and 20 μg of Gebru adjuvant (Gebru Biotechnik, Gaiberg, Germany). Four 200 μl subcutaneous and two 100 μl

Table 1 Bacteria strains, plasmids, and oligonucleotides used in this study

Strain, plasmid, or primer	Relevant characteristics	Source
Strains		
Haemophilus ducreyi		
35000	Wild-type strain isolated in Winnipeg, Manitoba, Canada	(Lee 1991)
35000δsodC	Isogenic mutant with a *kan* cassette inserted into the *Bam*HI site in *sodC*	This study
35000δsodC(pSN1-1)	35000δsodC containing pSN1-1 with the wild-type *H. ducreyi sodC* gene	This study
35000δsodC(pSN2-1)	35000δsodC containing pSN2-1 with the mutant *H. ducreyi sodC* gene His64Glu	This study
35000δsodC(pSN3-1)	35000δsodC containing pSN3-1 with the mutant *H. ducreyi sodC* gene Leu59Phe His60Gln Asp61Gly	This study
Escherichia coli		
71/78	*F- leuB6 supE44 hsdS20(r_B-m_B-) recA13 ara14 proA2 galK2 lacY1 rpsL20 xyl-5 mtl-1*	(Pacello et al. 2001)
Top10	*F- mcrA δ(mrr-hsdRMS-mcrBC) ϕ80lacZδM15 δlacX74 deoR recA1 araD139 δ(ara-leu)7697 galK rpsL (StrR) endA1 nupG*	Invitrogen
Plasmids		
pPHduSOD	Vector expressing *H. ducreyi* wild-type SodC	(Pacello et al. 2001)]
pPHduSODE64	Vector expressing mutant *H. ducreyi* SodC His64Glu	(Pacello et al. 2001)
pPHduSODF59Q60G61	Vector expressing mutant *H. ducreyi* SodC Leu59Phe His60Gln Asp61Gly	(Pacello et al. 2001)
pHDMK4	pBluescript SKII with a *kan* cartridge inserted into the *Bam*HI site within the *H. ducreyi sodC* gene[b]	(Langford and Kroll 1997)
pFP10	Cloning vector capable of replication in *H. ducreyi*; Cmr	(Pagotto et al. 2000)
pEGFP	Vector containing the enhanced green fluorescent protein (EGFP) gene	Clontech
pFP12	pFP10 with a 1126-bp fragment from pEGFP encompassing the *plac*-GFP-MCS inserted into the *Hind*III site	(Pagotto et al. 2000)
pSN1	pFP12 with the 734-kb *Kpn*I-*Not*I fragment enclosing the GFP gene removed and replaced with a 734-kb *Kpn*I-*Not*I fragment containing the wild-type *sodC* gene	This study
pSN2	pFP12 with the 734-kb *Kpn*I-*Not*I fragment enclosing the GFP gene removed and replaced with a 734-kb *Kpn*I-*Not*I fragment containing the mutant *sodC* gene His64Glu	This study
pSN3	pFP12 with the 734-kb *Kpn*I-*Not*I fragment enclosing the GFP gene removed and replaced with a 734-kb *Kpn*I-*Not*I fragment containing the mutant *sodC* gene Leu59Phe His60Gln Asp61Gly	This study
pSN1-1	pSN1 with the 230-bp *Ase*I-*Kpn*I fragment containing *plac* excised and replaced with a 230-bp PCR-derived *Ase*I-*Kpn*I fragment containing *psod*	This study
pSN2-1	pSN2 with the 230-bp *Ase*I-*Kpn*I fragment containing *plac* excised and replaced with a 230-bp PCR-derived *Ase*I-*Kpn*I fragment containing *psod*	This study
pSN3-1	pSN3 with the 230-bp *Ase*I-*Kpn*I fragment containing *plac* excised and replaced with a 230-bp PCR-derived *Ase*I-*Kpn*I fragment containing *psod*	This study

Table 1 continued

Strain, plasmid, or primer	Relevant characteristics	Source
Primers (5′ to 3′)		
5′sodCcolony	AGGATTTTATCTGCAGATGCAAATAGATTTC; 5′ oligonucleotide for colony PCR amplification of *sodC*, nucleotide positions 85 to 116[a]	This study
3′sodCcolony	ATGGCCATGGAATTATTTAATTCACCG; 3′ oligonucleotide for colony PCR amplification of *sodC*, nucleotide positions 786 to 813[a]	This study
5′sodC	AT**GGTACC**AGGAGATAAAATGAAATTAACG; 5′ oligonucleotide containing a *Kpn*I restriction site (in boldface type) for the amplification of *sodC*, nucleotide positions 184 to 213[a]	This study
3′sodC	GTTAT**GCGGCCGC**AATTATTTAATTAC; 3′ oligonucleotide containing a *Not*I restriction site (in boldface type) for the amplification of sodC, nucleotide positions 790 to 816[a]	This study
3′sodCAint	CCTAAACCTGCTACTAATTTACCGTCTTTTTC; 3′ oligonucleotide for the amplification of *sodC*, nucleotide positions 508 to 540[a]	This study
5′psod	GATTTTAT**ATTAAT**ATGCAAATAGATTTCTGGTC; 5′ oligonucleotide containing an *Ase*I restriction site (in boldface type) for the amplification of *psod*, nucleotide positions 88 to 121[a]	This study
3′psod	AATTTTACTTTATCTCCT**GGTACC**ATAAA; 3′ oligonucleotide containing a *Kpn*I restriction site (in boldface type) for the amplification of *psod*, nucleotide positions 181 to 209[a]	This study

[a] The positions correspond to the nucleotide sequences of *H. ducreyi sodC* (EMBL/GenBank DDBJ Nucleotide Sequence Data Library Accession Number U47664 (Stevens et al. 1996a)

[b] The positions correspond to the nucleotide sequences of *kan*^{R}EMBL/GenBank DDBJ Nucleotide Sequence Data Library Accession Number X06404(Langford and Kroll 1997)

intramuscular injections were administered. Two booster injections of the antigen-Gebru adjuvant mixture were subsequently given at intervals of 3 and 6 weeks. One week after the final injection, polyclonal antisera specific against Cu,ZnSOD was detected by Western immunoblot. Serum was collected and aliquots were stored at −70°C. Western blot analysis of Cu,ZnSOD was carried out using 1:2000 dilutions of the polyclonal antiserum.

Construction of an *H. ducreyi* isogenic sodC mutant

Plasmid pHDMK4, created by insertion of the 1264-bp *Tn*903 kanamycin resistance determinant from pUC4K into the *Bam*HI site of *H. ducreyi* 35000 *sodC* (Langford and Kroll 1997), was introduced into *H. ducreyi* 35000 by electroporation. Transformants were selected on CA-charcoal plates supplemented with kanamycin (35 μg/ml) and were screened by direct single colony PCR amplification. Twelve *H. ducreyi* kanamycin-resistant transformants were screened by colony PCR using oligonucleotides 5′sodCcol and 3′sodCcol to detect allelic exchange. One of these transformants, yielding a larger PCR product from the *sodC* mutant than from the wild-type strain, was selected for further study and designated 35000δsodC. To further verify that the desired gene replacement had occurred, Southern blot analysis was performed using the DIG chemiluminescent system (Boehringer Mannheim Canada). Genomic DNA purified from wild-type strain 35000 and the *sodC* mutant 35000δsodC was prepared using the Qiagen Genomic-tip system (Qiagen Inc., Mississauga, Ont., Canada) and digested to completion

with *Eco*RV. The *sodC* gene probe, a 357-bp fragment internal to the complete *sodC* coding sequence (generated by PCR amplification using primer pair 5′psodC and 3′psodCint), hybridized with a fragment of approximately 5.2-kb derived from the wild-type strain, whereas the mutant strain displayed a hybridizing band of 6.5-kb. The increase in size of the 6.5-kb fragment corresponds to the presence of the 1.3-kb *kan* cartridge in the *sodC* locus of the mutant strain, as confirmed by hybridization of this same fragment with the *kan*-specific probe. As expected, chromosomal DNA from the wild-type strain failed to bind with this probe. Finally, the absence of Cu,ZnSOD in 35000δsodC was confirmed by Western blot analyses employing the Cu,ZnSOD specific polyclonal antisera.

Challenges with catalase

To assess whether H_2O_2 was responsible for the growth impairment of *H. ducreyi* strains, bovine liver catalase 3600 U/mg of protein was added to broth cultures containing 50 μg/ml of hemin. As catalase is a recognized source of heme for *H. ducreyi* (Lee 1991), the growth promotion afforded by the addition of catalase could arise not only from the enzymatic activity of the protein, but also from the provision of additional heme. To determine the concentration of catalase that would minimize the latter possibility, preliminary experiments were conducted in which serial 2-fold concentrations of catalase were added to broth cultures of wild-type *H. ducreyi* 35000 containing 50 μg/ml of heme. The highest concentration of catalase that did not enhance growth compared to control cultures without catalase was 10 μg/ml.

Determination of Cu,ZnSOD bound heme as a nutritional source of heme

To assess whether the heme bound by SodC was available for growth, wild-type *H. ducreyi* 35000 and the *sodC* isogenic mutant were grown on clear semi-defined agar (Lee 1991) containing 50 μg/ml (77 mM) bovine hemin at 33°C in an atmosphere of 5% CO_2 through three serial transfers. Harvested cells were washed three times with 50 mM Tris–HCl to remove residual iron and heme. Cultures were suspended in sterile phosphate-buffered saline (pH 7.4) to an A_{600} of 1.0. After allowing large clumps to settle, one ml aliquots were used to inoculate 100 ml of heme-free prewarmed GC broth containing supplements as described above. Cultures were serially transferred four times at mid-log phase (A_{600} of 0.3) to assess the ability of Cu,ZnSOD bound heme to serve as a nutritional source of heme.

Assessment of heme binding

Periplasmic fractions of *H. ducreyi* were isolated by osmotic shock (Lewis et al. 1999). Cu,ZnSOD was purified by metal affinity chromatography as previously described (Battistoni et al. 2001). The quantity of heme associated with Cu,ZnSOD was determined by densitometric analysis of SDS-PAGE gels stained for heme-dependent peroxidase activity (Pacello et al. 2001), using recombinant wild-type *H. ducreyi* Cu,ZnSOD purified from *E. coli* cells grown in LB medium supplemented with 50 μg/ml heme as a standard. The amount of heme present in the recombinant enzyme preparation was determined spectrophotometrically as described (Pacello et al. 2001).

Characterization of an inactive mutant Cu,ZnSOD still able to bind heme

The expression plasmid pPHduSODF59Q60G61, expressing the inactive mutant L59F, H60Q, D61G, is a derivative of plasmid pPHduSOD (Battistoni et al. 2001; Pacello et al. 2001) and was obtained by chance during a mutagenesis experiment aimed at obtaining the single mutant H60Q (D'Angelo et al. 2005). The enzyme was isolated to homogeneity following the same procedures used for the wild type enzyme. The isolated enzyme was assayed for superoxide activity by the pyrogallol method (Marklund and Marklund 1974). One enzyme unit is defined as the amount of enzyme required to obtain 50% inhibition of pyrogallol autoxidation. The mutant enzyme, which contains heme as the wild type enzyme, displayed a specific activity of 22 U/mg compared to 5998 U/mg of wild type Cu,ZnSOD and 5882 U/mg of H64E mutant. This large fall in catalytic activity is due either to a decrease in copper binding or to distortion in copper geometry, as

evidenced by a significant alteration of the EPR spectrum (data not shown).

Construction of sodC-bearing plasmids for trans complementation in *H. ducreyi* 35000δsodC

The promotorless wild-type and the two mutant *sodC* genes were cloned by PCR amplification from plasmids pPHduSOD, pPHduSODE64 (Pacello et al. 2001), and pPHduSODF59Q60G61 (Pacello and Battistoni, unpublished data), respectively, using oligonucleotides 5′sodC and 3′sodC. Site-specific primers were engineered with *Kpn*I and *Not*I restriction sites to facilitate directional cloning of the 632-bp PCR products into the low-copy-number *E. coli–H. ducreyi* shuttle vector pFP12 (Pagotto et al. 2000) downstream of the *lac* promoter, creating pSN1, pSN2 and pSN3, respectively. To place the various *sodC* versions under the transcriptional control of the *H. ducreyi* native *sodC* promoter, a 230-bp *Ase*I-*Kpn*I fragment containing *plac* was excised from pSN1, pSN2 and pSN3. Primers 5′psod and 3′psod incorporating *Ase*I and *Kpn*I restriction sites were used to PCR amplify *psod* from pHdSOD, a recombinant plasmid which contains the full-length *sodC* gene from *H. ducreyi* 35000 (Stevens et al. 1996a, b). In-frame ligation of the resultant 121-bp amplicon into the three *sodC* constructs generated plasmids pSN1-1, pSN2-1, and pSN3-1. This strategy addressed two limitations inherent in plasmid-based *trans* complementation, in which titration effects resulting from high-copy-number vectors and heterologous promoters interfere with proper gene regulation.

All intermediate plasmid constructs were transformed into *E. coli* TOP10 by established methods (Liss 1987). *E. coli* transformants containing *sodC*-bearing plasmids were selected by using LB agar plates containing chloramphenicol (50 μg/ml). Sequencing the complement constructs verified the presence of the appropriate mutations and the absence of introduced mutations.

Results

Growth characteristics of the sodC mutant

Aerobic growth assays were conducted to determine the ability of the *sodC* mutant to use heme. All cultures were maintained until stationary phase was reached at 24 h. When heme restriction was imposed at 5 μg/ml, no growth difference was observed between the *sodC* mutant and the wild-type strain. In contrast, in the presence of 50 μg/ml of bovine hemin, the mean peak cell density attained by the *sodC* mutant was less than that of the parental strain ($4.50 \times 10^4 \pm 422$ CFU compared to $5.55 \times 10^4 \pm 250$ CFU, respectively; $P = 0.038$) indicating that the growth of the *sodC* mutant was impaired compared with that of the wild-type strain (Fig. 1). The addition of exogenous catalase (10 μg/ml, 36 U/ml) restored growth of the *sodC* mutant to the wild-type level (data not shown). This result indicates that, in the absence of Cu,ZnSOD, H_2O_2 is the chemical agent responsible for the killing of *H. ducreyi*.

Expression of Cu,ZnSOD under heme restriction

The expression of a protein engaged in heme acquisition would be predicted to be enhanced during heme deprivation. As determined by densitometric analysis of Western immunoblots, no difference in the level and in the temporal sequence of expression between periplasmic extracts prepared from the wild-type strain grown under heme sufficient and heme restrictive conditions was discerned (data not shown). In both cases, expression of Cu,ZnSOD was detected

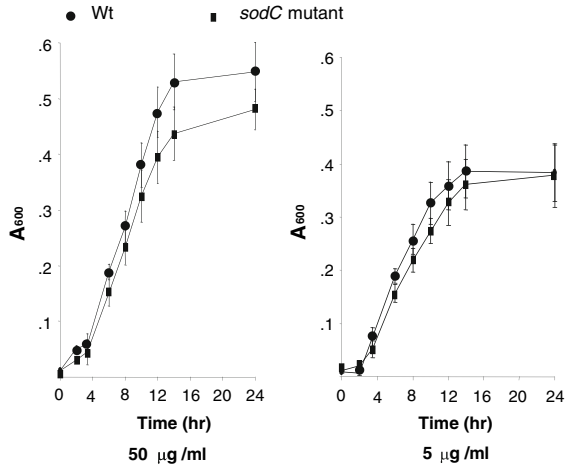

Fig. 1 Growth of the wild-type and *sodC* mutant of *H. ducreyi* 35000 in GC broth supplemented with 5 μg/ml hemin and 50 μg/ml hemin. Each value represents the mean of six replicate cultures. Standard deviations of the mean are shown by error bars

after 6 h of growth, and production remained unchanged throughout the growth cycle (data not shown).

Availability of heme bound by Cu.ZnSOD as a nutritional source of heme

To assess whether Cu,ZnSOD bound heme is available as a nutritional source, wild-type and *sodC* mutant cells were serially transferred four times into heme-free medium. There was no significant difference in the growth rate or final yield at each serial passage when comparing the parental strain with the *sodC* mutant (data not shown). This result indicated that either the heme bound by Cu,ZnSOD is not accessible for growth, or the amount of heme bound to Cu,ZnSOD is insufficient to support bacterial growth under heme limitation.

Heme accumulation by Cu,ZnSOD

To determine the heme content of Cu,ZnSOD, protein purified from periplasmic extracts of cells grown under heme limited (5 μg/ml) and heme sufficient (50 μg/ml) conditions was separated by SDS-PAGE and the gel was stained for heme-dependent peroxidase activity. Heme accumulation by Cu,ZnSOD was estimated by reference to a standard curve constructed with defined amounts of affinity-purified heme-containing recombinant *H. ducreyi* Cu,ZnSOD (Fig. 2, lanes 1–4) (Pacello et al. 2001). The enzyme sample prepared in the presence of 50 μg/ml of heme (Fig. 2, lane 6) bound approximately 2-fold more heme compared with the enzyme derived from cells grown in the presence of 5 μg/ml of heme (10 pmol vs. 5 pmol) (Fig. 2, lane 5). Despite the increased amount of heme associated with Cu,ZnSOD, the enzyme remained far from saturated as this value represented only 20% of the total heme binding capacity of the Cu,ZnSOD.

Complementation of the sodC mutation in *H. ducreyi*

As Cu,ZnSOD exhibits both SOD and heme-binding properties, the growth impairment of the *sodC* mutant

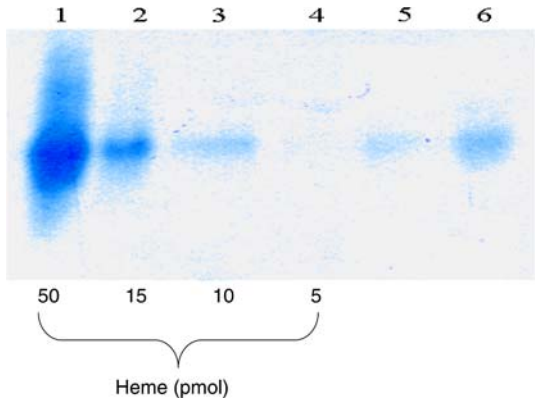

Fig. 2 Heme binding to Cu,ZnSOD. Enzyme was affinity-purified from periplasmic extracts derived from *E. coli*71/18 expressing *H. ducreyi* Cu,ZnSOD (lanes 1–4), and from wild type *H. ducreyi* 35000 grown in 5 μg/ml hemin (lane 5) and 50 μg/ml hemin (lane 6). The *E. coli* preparations contained heme at the indicated concentrations. Samples were subsequently separated by nondenaturing SDS-PAGE and the gel was stained for heme-dependent peroxidase activity and analyzed by densitometry

witnessed in the presence of 50 μg/ml of heme may have arisen from the absence of one or both of these attributes of the protein. The availability of the mutant Cu,ZnSOD E64, which is unable to bind heme due to substitution of an iron ligand (D'Angelo et al. 2005) and the isolation (see Material and methods) of a new mutant Cu,ZnSOD (F59Q60G61 Cu,ZnSOD), which demonstrated only 0.4% of the catalytic activity of the wild-type enzyme (a greater than 200-fold reduction) but conserved the ability to bind heme, facilitated the use of genetic complementation to resolve the question. The wild-type *sodC*, and the two mutated versions of *sodC* displaying these reciprocal phenotypes, were cloned into the *E. coli–H. ducreyi* shuttle vector pFP12 under the transcriptional control of the *H. ducreyi* native *sodC* promoter. Importantly, although all three complemented strains displayed an approximate 1.5-fold increase in Cu,ZnSOD production compared to the wild-type isolate, Cu,ZnSOD synthesis and export were otherwise found to be unaffected. The presence of recombinant plasmids bearing either the E64 or F59Q60G61 versions of Cu,ZnSOD reversed the growth defect of the *sodC* mutant (data not shown). In contrast, the wild-type allele in trans did not rescue the growth of the *sodC* mutant (data not shown). Catalase also fully repaired the growth deficiency of

the *sodC* strain complemented with the wild-type allele (data not shown), indicating that H_2O_2 toxicity was responsible for the growth defect. No growth enhancement was seen in the *sodC* mutants bearing plasmids carrying the two other versions of Cu,Zn-SOD (data not shown).

A comparison of the growth kinetics between the wild-type isolate and one harbouring pFP10 (Pagotto et al. 2000) (the backbone and progenitor plasmid of pFP12) disclosed no difference (data not shown). A similar outcome was obtained in analogous experiments with the *sodC* mutant (data not shown). These results indicated that no discernible metabolic cost was incurred in plasmid maintenance.

Discussion

The functional significance of *H. ducreyi* Cu,ZnSOD in bacterial survival is unclear. Experimental in vitro evidence has shown that Cu,ZnSOD imparts resistance to extracellular pyrogallol-generated superoxide (San Mateo et al. 1998). In a swine model of infection, the enzyme confers protection against neutrophil derived ROS (San Mateo et al. 1999). In contrast, expression of Cu,ZnSOD is not required for the survival and virulence of *H. ducreyi* during the initial stages of experimental infection in human volunteers (Bong et al. 2002). The Cu,ZnSOD isolated from *H. ducreyi* is the only superoxide dismutase able to bind a heme cofactor at its dimer interface. The significance of heme binding by Cu,ZnSOD is unknown, but this property is likely important in the understanding of the role of this enzyme in *H. ducreyi*.

Cu,ZnSOD could play a heme trafficking function in *H. ducreyi*, perhaps in ferrying heme across the periplasmic space to the cytoplasmic membrane. However, this assumption was not supported by evidence arising from this study, as the Cu,ZnSOD failed to fulfill two criteria expected of a protein involved in heme uptake. First, no increase in Cu,ZnSOD expression occurred when cells were subjected to hemin restriction. Second, the growth of the *sodC* mutant was not impaired during heme limitation, but rather during heme replete conditions. Results from this study, moreover, suggest that heme bound by Cu,ZnSOD is not a suitable nutritional source. In fact, the low heme saturation of Cu,ZnSOD (Fig. 2), coupled with the high heme demands of *H. ducreyi*, are not sufficient to support the growth of the organism in a heme-free environment.

In contrast, we have observed optimal growth of *H. ducreyi* under high heme concentration (50 μg/ml) required the presence of Cu,ZnSOD, and that this phenotype is abolished when bacteria are cultivated in presence of catalase. This result indicates that, in the absence of Cu,ZnSOD, H_2O_2 is the chemical agent responsible for the killing of *H. ducreyi*. A similar finding has been previously reported for *Salmonella typhimurium* (Gort et al. 1999). Such a result suggests that periplasmic O_2^- generated by endogenous metabolism may amplify the toxicity of endogenous H_2O_2 by participating in the Haber-Weiss reaction (Miller and Britigan 1997; Gort et al. 1999). In contrast, no increase in survival of the wild-type strain was seen, as scavenging of periplasmic O_2^- by Cu,ZnSOD would be expected to protect cells from this oxidative stress.

Interestingly, complementation assays indicate that the simultaneous expression of both functions of the protein was not necessary as either the catalytic or the heme binding property of Cu,ZnSOD was equally sufficient in correcting the growth defect of the *sodC* mutant. Paradoxically, the overexpression of the wild type enzyme failed to complement the growth defect of the mutant strain. As catalase fully repaired the growth deficiency of the *sodC* strain complemented with the wild-type allele, H_2O_2 toxicity was responsible for such growth defect. The precise mechanism underlying the increased vulnerability of the *sodC* mutant complemented with the wild-type gene cannot be readily explained by an accelerated formation of H_2O_2 (Scott et al. 1987), as a similar sensitivity to H_2O_2 was not seen in the *sodC* strain expressing the E64 version of Cu,ZnSOD. A potential explanation may arise from the observation that, when bound to Cu,ZnSOD, heme is significantly open to interaction with solvent (Djinovic and Battistoni, unpublished data). This exposure would facilitate the interaction of the bound heme with H_2O_2 released from the nearby enzyme active site and the generation of oxyferryl intermediates and other ROS via the Haber-Weiss scheme. Recalling that the expression of Cu,ZnSOD in the complemented strain was 1.5-fold greater than the parental isolate, such a potentially toxic property of the enzyme might reduce the viability of *H. ducreyi* when

the concentration of wild-type Cu,ZnSOD in the periplasmic compartment reaches a critical threshhold. This H_2O_2-mediated toxicity is not displayed in the other two *sodC* complemented mutants because of the absence either of the heme cofactor (E64 Cu,ZnSOD), or of the catalytic production of H_2O_2 (F59Q60G61 Cu,ZnSOD).

In a prior report, similar growth rates in exponential phase were exhibited by the *H. ducreyi* wild type strain and an isogenic *sodC* mutant (San Mateo et al. 1998), although a significant reduction in peak cell count of the *sodC* mutant compared to the wild type isolate occurred in serum-free broth. Differences in broth composition, mutant construction, and growth conditions may account for this observation. In addition, expression of a functional wild-type gene in a merodiploid strain was unable to rescue the growth defect of the *sodC* mutant. This failure was ascribed to the modulation of expression of genes upstream of the insertion arising from topological coupling by the *cat* cassette.

Heme in aqueous solutions is a powerful catalyst of ROS that promote peroxidative damage to a variety of biomolecules (Vincent 1989). This deleterious consequence of free heme is diminished by the universal association of heme with protein (Muller-Eberhard and Nikkila 1989; Dansa-Petretski et al. 1995; Wyckoff et al. 2004). By analogy, our results suggest that the attachment of heme to the Cu,ZnSOD may function to detoxify the pernicious effects of free heme. Several lines of evidence are consistent with this proposal. First, raising the extracellular heme concentration from 5 to 50 μg/ml was accompanied by an elevation in the amount of heme associated with Cu,ZnSOD without a concomitant increase in Cu,ZnSOD expression. However, the increase in heme saturation of Cu,ZnSOD (2-fold) was proportionately lower than the increase in exogenous heme (10-fold). The asymmetric relationship between Cu,ZnSOD heme accumulation and external heme concentration highlights the stringent control imposed by *H. ducreyi* on the acquisition of heme and suggests that uptake of this critical but potentially toxic molecule occurs at the stage of cell entry. Second, in contrast to other pathogenic bacteria (Imlay and Imlay 1996; St. John and Steinman 1996; Fang et al. 1999) in which Cu,ZnSOD production peaks during stationary phase, the expression of *H. ducreyi* Cu,ZnSOD was growth-phase independent. Heme-mediated free radical generation would be expected to ensue from the extensive trafficking of this molecule across the periplasmic space. The cellular response to this formidable oxidant challenge would favour a continual high-level production of the Cu,ZnSOD to sequester heme.

The results from this investigation suggest that the unique heme-binding property of the enzyme likely serves a detoxification function. As heme-obligate growth is a defining characteristic of *H. ducreyi*, the heme-binding activity displayed by the Cu,ZnSOD is predicted to occupy an important role in heme homeostasis. Elucidation of the precise contribution of the enzyme to the physiology of the organism awaits further studies.

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