A Sco homologue plays a role in defence against oxidative stress in pathogenic *Neisseria*

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Received 15 April 2003; revised 20 May 2003; accepted 23 May 2003

First published online 11 June 2003

Edited by Vladimir Skulachev

Abstract Sco proteins are found in mitochondria and in a variety of oxidase positive bacteria. Although Sco is required for the formation of the Cu_A centre in a cytochrome oxidase of the aa_3 type, it was observed that oxidases with a Cu_A centre are not present in many bacteria that contain a Sco homologue. Two bacteria of this type are the pathogens *Neisseria meningitidis* and *Neisseria gonorrhoeae*. The *sco* genes of *N. gonorrhoeae* strain 1291 and *N. meningitidis* strain MC58 were cloned, inactivated by inserting a kanamycin resistance cassette and used to make knockout mutants by allelic exchange. Both *N. gonorrhoeae* and *N. meningitidis sco* mutants were highly sensitive to oxidative killing by paraquat, indicating that Sco is involved in protection against oxidative stress in these bacteria.

Key words: Sco; Oxidative stress; Cytochrome oxidase; *Neisseria gonorrhoeae*; *Neisseria meningitidis*

1. Introduction

Many bacteria stain positive in the 'oxidase test' indicating that they have a respiratory pathway in which ubiquinol oxidation involves electron transport via a cytochrome bc_1 complex and at least one terminal oxidase, commonly cytochrome aa_3 and/or cytochrome cbb_3 [1]. The respiratory pathway of the mitochondrion has a similar arrangement to that of 'oxidase positive' bacteria. The bacterial cytochrome aa_3 contains identical redox centres and homologous core polypeptides to those of the mitochondrial cytochrome aa_3; subunit I contains haem *a* and the haem a_3-Cu_B binuclear centre while subunit II contains the binuclear Cu_A centre [2,3]. Cytochrome cbb_3 is often found in 'oxidase positive' bacteria in addition to, or instead of, cytochrome aa_3. Subunits I and II of cytochrome cbb_3 are similar to those of cytochrome aa_3, however, subunit II of cytochrome cbb_3 lacks a Cu_A centre and the electron transfer function of this redox centre is taken by the dihaem *c*-type cytochrome [4].

Given the similarity between mitochondrial and bacterial respiratory chains it follows that some of the proteins required for assembly of the respiratory complexes will be conserved. In yeast, Sco1 (for synthesis of cytochrome *c* oxidase) has been shown to be essential for the correct assembly of a functional cytochrome aa_3, and plays an as yet undefined role in the acquisition of copper for formation of the Cu_A centre in cytochrome aa_3 [5–8]. Recently, it was shown that YpmQ, the Sco homologue of *Bacillus subtilis*, is essential for formation of a functional cytochrome aa_3 [9]. Genome sequence analysis has revealed that many 'oxidase positive' bacteria also possess a homologue of the eukaryotic Sco [10].

It has been shown that Sco from yeast (Sco1) [5,11] and *Rhodobacter sphaeroides* (PrrC) [12] are able to bind a Cu ion. This is consistent with a role for these proteins in the handling of Cu in the inter-membrane space or periplasm in the case of mitochondria and Gram-negative bacteria respectively. However, secondary structure predictions for Sco proteins have revealed that they have similarities to peroxiredoxin/thiol:disulfide oxidoreductases [13]. This has led to the proposal that these proteins may have thiol:disulfide oxidoreductase activity, and suggests that bacterial Sco proteins might have a role that is additional to or distinct from the handling of Cu ions. In this paper, we report a new biological role for a Sco homologue in *Neisseria gonorrhoeae* and *Neisseria meningitidis*: defence against oxidative stress.

2. Materials and methods

2.1. Bacterial strains and culture conditions

N. meningitidis strains used in this study were C311, MC58 [14], MC58¢3, 8013SB, MPJI11B, and MPJI18B [15]. *N. gonorrhoeae* strains used were 1291 and F62 (supplied by Dr Michael Apicella, University of Iowa, Ames, IA, USA), FA1090 (supplied by Dr Xavier Nassif, University of Paris, France), MS11 (supplied by Dr John Tainer, The Scripps Research Institute, USA), 4989 (supplied by the Australian Culture Collection, University of Queensland, Brisbane, Australia), WHO III, V, VII and clinical strains (supplied by the Queensland Health Department, Australia). Meningococcal, gonococcal and commensal *Neisseria* strains were grown on brain heart infusion agar (BHI; Acumedia) supplemented with 10% Levithal's base [16] at 37°C in 5% CO_2. Media for gonococcal strains were also supplemented with IsoVitaleX (Difco). *Escherichia coli* strain DH5α [17] containing recombinant plasmids was cultured in LB broth or on LB plates containing 1.5% bacteriological agar (Difco). Ampicillin and kanamycin were used at a final concentration of 100 μg/ml. Growth studies were conducted in supplemented BHI broth (Oxoid) at 37°C under either aerobic conditions (cultures shaken at 170 rpm) or anaerobic conditions (large volume of culture broth in an unshaken flask, containing either 0 or 5 mM $NaNO_2$). Growth was monitored by the increase in OD_{600}. Experiments were conducted in triplicate and repeated on several occasions.

2.2. Nucleotide sequence analysis

Nucleotide and protein sequence analysis were performed using MacVector (Oxford Molecular). Multiple protein alignments were

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Abbreviations: Ahp, alkyl hydroperoxidase; TSA, thiol specific antioxidant

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00632-X

conducted using MULTALIN [18]. Topology and secondary structure analyses were performed using MEMSAT [19] and PSIpred v2.3 [20] respectively. Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs.

2.3. Construction of sco knockout mutants

Most recombinant DNA techniques were performed as described in Sambrook et al. [17]. The *sco* gene was amplified from *N. gonorrhoeae* strain 1291, using the polymerase chain reaction (PCR) primers sco1 (5′-ACATTAAATCTGCCAAACGC-3′) and sco2 (5′-CACACCATGCAGGATAATGC-3′), and cloned into pGem®-T Easy (Promega). Knockout mutants of *sco* were constructed via insertion of a kanamycin resistance cassette (pUC4Kan; Pharmacia) digested with *Hin*cII into a unique *Bbs*I restriction site in the coding region of the gene. This plasmid was then linearised with *Not*I. *N. gonorrhoeae* strain 1291 and *N. meningitidis* strain MC58¢3 were transformed as described previously [21] and recombinant strains were selected by growth on appropriate BHI agar containing kanamycin (50 or 100 μg/ml). The presence of the inactive allele was confirmed by Southern hybridisation.

2.4. Southern hybridisation

*Bgl*I restriction endonuclease digested genomic DNA was separated on 1% (w/v) agarose gels and transferred to GeneScreen membrane (NEN Life Science), essentially as described by Sambrook et al. [17]. The sco probe was generated using the primers sco1 and sco2 in a PCR with *N. gonorrhoeae* strain 1291 as template. The PCR product was purified from agarose using a Qiaex gel extraction kit (Qiagen) and digoxigenin (DIG) labelled. Probe labelling and blot hybridisation and detection were performed using the DIG DNA Labelling and Detection Kit (Boehringer Mannheim) as recommended by the manufacturer. Hybridisations were performed overnight at 65°C.

2.5. Phenotypic characterisation

The naphthol and dimethylparaphenylenediamine oxidase test was performed by addition of α-naphthol and *N*,*N*-dimethyl-1,4-phenylenediammonium chloride to colonies, enabling visual detection of cytochrome *c* oxidase activity by the production of a blue compound [22]. Oxidative killing assays using paraquat were performed essentially as described by Tseng et al. [23]. Paraquat (PQ^{2+}; 1,1′-4,4′-bipyridinium dichloride) is a redox compound that is reduced to the paraquat free radical ($PQ^{\bullet+}$) by low potential electron donors within the bacterial cell. The paraquat free radical is then oxidised by dioxygen leading to generation of the superoxide anion ($O_2^{\bullet-}$). This redox cycling also depletes low potential reducing agents within the cell such as NADH [24].

3. Results

3.1. Identification of sco homologues in N. gonorrhoeae and N. meningitidis

Using yeast Sco1 (GenBank accession number NP_009593) as a query in TBLASTN searches [25], a single Sco homologue was identified in the *N. gonorrhoeae* strain FA1090 (Gonococcal Genome Sequencing Project, University of Oklahoma) (Sco_{Ng}) and *N. meningitidis* strain MC58 genomes [26] (Sco_{Nm}) with 22% sequence identity. The *N. gonorrhoeae* open reading frame (ORF) (sco_{Ng}) (nt 1 188 987–1 189 640, GenBank accession number NC_002946) and the *N. meningitidis* ORF (sco_{Nm}) (NMB1578; GenBank accession number AAF41931.1) are 654 nucleotides in length. The predicted Sco_{Ng} and Sco_{Nm} proteins share 97% identity, are 217 amino acids in length and have a calculated molecular mass of 23.1 kDa. To determine if the *sco* gene is widespread in *Neisseria* species a collection of strains was surveyed by Southern hybridisation (see Section 2). The resulting Southern blot demonstrated the presence of *sco* in all strains tested with the exception of *Neisseria elongata* (7/7 *N. gonorrhoeae*, 5/5 *N. meningitidis* and 6/7 commensal *Neisseria* strains) (data not shown).

3.2. Distribution of Sco in bacteria: no correlation with the presence of a cytochrome oxidase with a Cu_A centre

There is very strong evidence that mitochondrial Sco has either a direct or an indirect role in biogenesis of the Cu_A

Table 1
Distribution of Sco and the cytochrome oxidases aa_3 and cbb_3

Organism	Sco homologue	Cytochrome oxidase		Sco/oxidase reference
		aa_3	cbb_3	
Characterised Sco homologues				
Saccharomyces cerevisiae	Sco1 (NP_009593), Sco2 (NP_009580)	+	−	[37]/[48]
Homo sapiens	Sco1 (NP_004580), Sco2 (NP_005129)	+	−	[49]/[50]
Rhodobacter sphaeroides	PrrC (A57145)	+	+	[12]/[4,51]
Rhodobacter capsulatus	SenC (Q52720)	−	+	[10]/[52]
Bacillus subtilis	YpmQ (H69938)	+	−	[9]
Pseudomonas stutzeri	Orf193 (CAA81131)	−	+	[28]/[53]
Anaplasma marginale	MSP-5 (A49213)	N/D	N/D	[29]
Cowdria ruminantium	MAP2 (AAD40617)	N/D	N/D	[30]
Sco homologues identified by genome searches (* identified in this study)				
Neisseria gonorrhoeae	1 188 987–1 189 640 (NC_002946) (Sco_{Ng})*	−	+	a/[48,54]
Neisseria meningitidis	NMB1578 (AAF41931) (Sco_{Nm})*	−	+	[26]
Campylobacter jejuni	Cj0911 (NP_282063)*	−	+	[55]
Vibrio cholerae	Sco1/VCA0038 (NP_232439)	−	+	[56]
Pseudomonas aeruginosa	PA0114 (NP_248804)*	+	+	[57]
Rickettsia prowazekii	Sco2 (NP_220956)	+	−	[58]
Bordetella bronchiseptica	2 688 808–2 688 248 5 134 494–5 133 943 (NC_002927)*	+	+	b/[54]
Bordetella parapertussis	4 571 504–4 570 902 1 560 100–1 559 540 (NC_002928)*	+	+	b
Bordetella pertussis	1 028 043–1 027 483 3 944 153–3 943 551 (NC_002929)*	+	+	b/[54]
Bacillus anthracis	SCO1/SenC (NP_656112)	+	−	c
Legionella pneumophila	48 439–49 080 (NC_002942)*	+	−	d

Organisms containing homologues of yeast Sco1 are shown, the Sco homologues (or the genome sequence positions) are listed with their GenBank accession numbers, and the (+) presence or (−) absence of cytochrome oxidase types aa_3 (contains Cu_A and Cu_B centres) and cbb_3 (contains only a Cu_B centre) in these organisms is noted. The asterisks indicate Sco homologues identified in this study. N/D is not determined. Unfinished genomes: a, http://www.genome.ou.edu/gono.html; b, http://www.sanger.ac.uk; c, http://www.tigr.org; d, http://genome4.cpmc.columbia.edu/index.html.

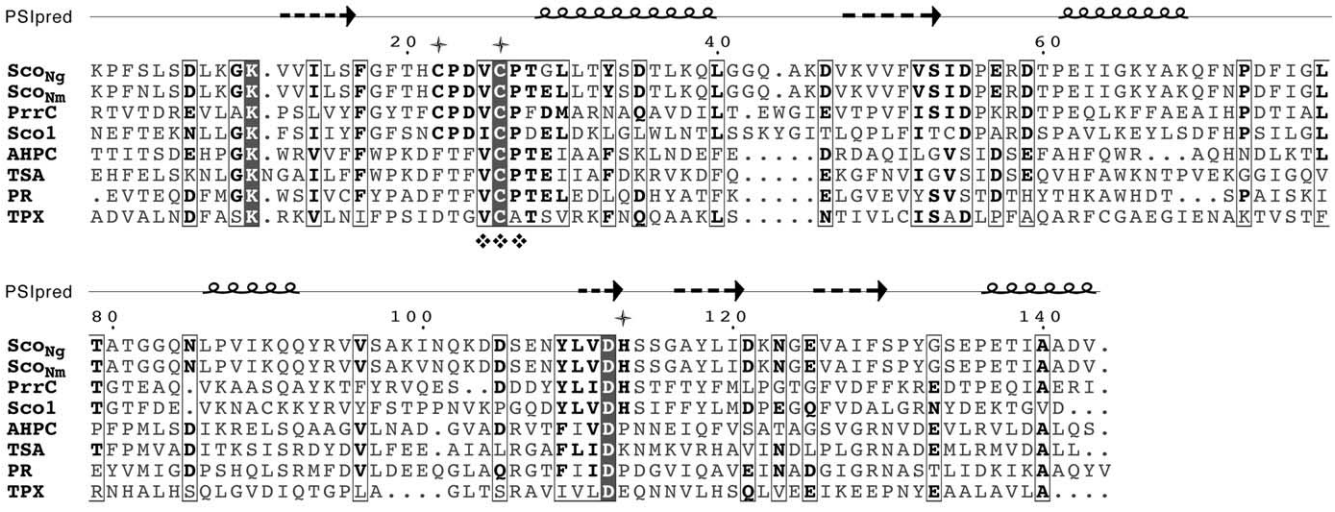

Fig. 1. Alignment of partial sequences of Sco homologues and members of the AhpC/TSA, peroxiredoxin and thiol:disulfide oxidoreductase families. The alignment includes: Sco_{Ng}, residues 71–213 of *N. gonorrhoeae* Sco homologue (nucleotides 1 188 987–1 189 640 of NC_002946); Sco_{Nm}, residues 71–213 of *N. meningitidis* Sco homologue (AAF41931); PrrC, residues 58–197 of *Rhodobacter sphaeroides* Sco homologue (A57145); Sco1, residues 127–267 of *Saccharomyces cerevisiae* Sco (NP_009593); AHPC, residues 36–170 of *Mycobacterium tuberculosis* H37Rv alkyl hydroperoxidase (NP_216944); TSA, residues 23–160 of *Helicobacter pylori* 26695 thiol specific antioxidant (NP_208354); PR, residues 23–158 of *Thermus aquaticus* peroxiredoxin (AAF82118); TPX, residues 34–165 of *Haemophilus influenzae* Rd thiol peroxidase (AAC22410). Conserved amino acids are shown in bold and boxed. ✢ represent putative active site residues of Sco homologues. ❖ indicates the conserved CVP of peroxiredoxins [36] and members of the AhpC/TSA family [34]. Above the alignment is the secondary structure of Sco_{Ng} and Sco_{Nm} predicted with PSIpred [20], → represent β-strands and ∿∿∿ represent α-helices.

centre of cytochrome aa_3 [6–8]. The widespread distribution of *sco* in bacteria has already been observed [9,12,27–30]. Although we note that all of the bacteria possessing a *sco* gene are oxidase positive (Table 1), it can also be seen that many lack the *cox* operon encoding cytochrome aa_3. Instead, these bacteria possess cytochrome cbb_3, encoded by the *cco* operon. Since cytochrome cbb_3 lacks the Cu_A centre found in cytochrome aa_3, the question was raised of the role of bacterial Sco proteins in oxidase positive bacteria that lack *cox* genes.

3.3. *Construction and analysis of* sco *mutants of* N. gonorrhoeae *and* N. meningitidis*; Sco is not required for cytochrome oxidase activity but is involved in defence against oxidative stress*

sco mutants of *N. gonorrhoeae* and *N. meningitidis* were constructed in order to determine whether Sco had a role in the function or assembly of cytochrome cbb_3. Analysis of the genomes of these *Neisseria* species revealed that all have a single oxidase, cytochrome cbb_3 encoded by the *cco* operon (Table 1). This led us to predict that if Sco was required for the assembly or function of cytochrome cbb_3 then a *sco* mutant would not be able to grow aerobically. However, the *sco* mutants of both *N. gonorrhoeae* and *N. meningitidis* grew under aerobic conditions at almost identical rates to wild type cells (data not shown). Furthermore, the *sco* mutants of both *N. gonorrhoeae* and *N. meningitidis* stained positive in the oxidase test confirming that the terminal cytochrome oxidase (COX) remains functional and that Sco is not required for its assembly (results not shown). *N. gonorrhoeae* also possesses a nitrite reductase (AniA) that terminates an anaerobic respiratory chain [31]. This enzyme is related to the multi-copper oxidase family of enzymes and contains two copper atoms [32]. A comparison of the wild type and the *sco* mutant revealed that there was no effect on the ability of cells to grow on nitrite as electron acceptor under anaerobic conditions (data not shown).

Chinenov [13] observed that Sco proteins possess a thioredoxin fold similar to those observed in peroxiredoxins and bacterial thiol:disulfide oxidoreductases. A search of the Pfam database [33] with the *N. gonorrhoeae* and *N. meningitidis* Sco sequences showed a match to the AhpC/TSA family (Pfam 00578). This family contains alkyl hydroperoxide reductases (AhpC) and thiol specific antioxidants (TSA). AhpC is responsible for directly reducing organic hydroperoxides in its reduced dithiol form [34], and TSA is a physiologically important antioxidant that constitutes an enzymatic defence against sulfur containing radicals [35]. An alignment of the predicted protein sequences of the neisserial Sco with members of the Sco, AhpC/TSA, peroxiredoxin and thiol:disulfide oxidoreductase families is shown in Fig. 1. *N. gonorrhoeae* and *N. meningitidis* Sco contain several amino acid residues conserved in these families, most notably the VCP active site motif of peroxiredoxins and AhpC/TSAs [34,36]. They also have a predicted secondary structure (see Fig. 1) that is consistent with the thioredoxin fold and known peroxiredoxin structures described by Chinenov [13] suggesting that Sco may also have an antioxidant role in *Neisseria*.

The role of Sco in protection against oxidative stress in *N. gonorrhoeae* and *N. meningitidis* was investigated via the comparison of survival of wild type and mutant strains in oxidative killing assays (see Section 2). Using paraquat as a generator of superoxide anions it was observed that *sco* mutants of both species of *Neisseria* were more sensitive to oxidative killing than wild type cells (Fig. 2).

4. Discussion

In *Saccharomyces cerevisiae*, Sco is one of several proteins required for assembly of the COX [7,37–40]. Yeast Sco binds

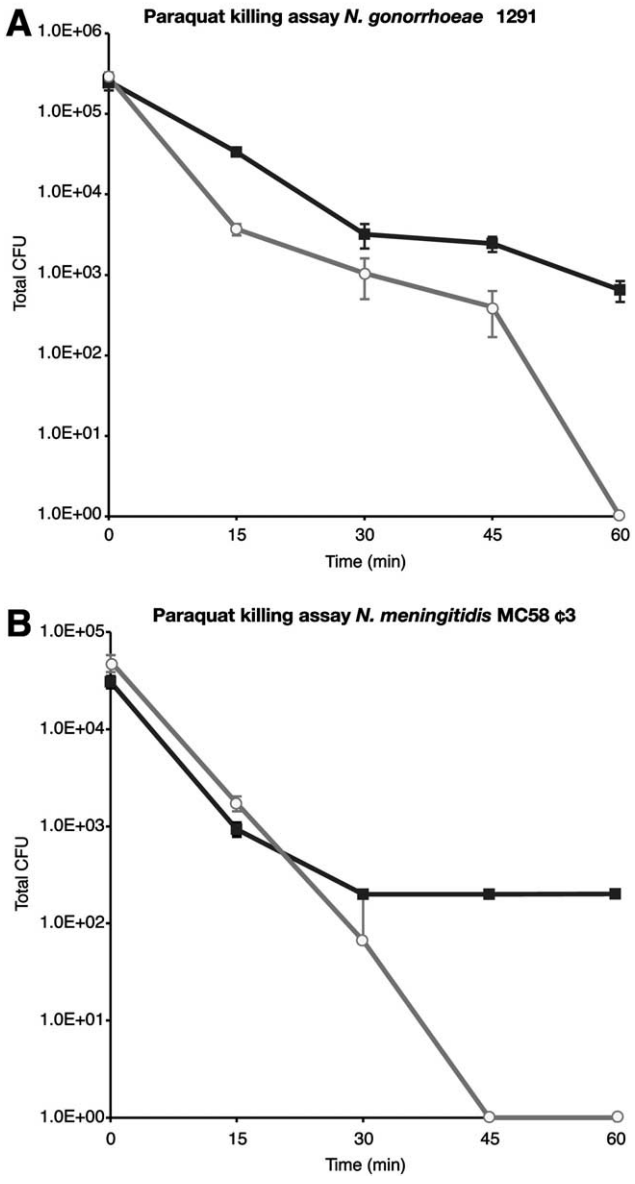

Fig. 2. Paraquat killing assays of *N. gonorrhoeae* 1291, *N. meningitidis* MC58¢3 and the *sco*::kan mutants. Experiments were performed in triplicate. Y-error bars indicate ±1 standard deviation of the mean. A: *N. gonorrhoeae* 1291 wild type strain (■); *sco*::kan mutant (○). B: *N. meningitidis* MC58¢3 wild type strain (■); *sco*::kan mutant (○).

copper [5] and has been proposed to act as either a copper storage protein [6,41] or a copper transporter [7,40]. Although it is established that Sco in mitochondria and *B. subtilis* has a critical role in the assembly of the Cu_A centre of cytochrome aa_3, our analysis shows that this protein is present in a large number of 'oxidase positive' bacteria that lack this terminal oxidase and contain only cytochrome cbb_3. A Cu_B centre is found in both cytochrome aa_3 and cytochrome cbb_3 [42] and recent results for assembly of cytochrome aa_3 in *R. sphaeroides* indicate that a distinct assembly protein, Cox11p, is required for its assembly [43]. Whatever the mechanism of insertion of the Cu_B centre in cytochrome cbb_3, our results indicate that in two *Neisseria* species Sco is not required for the assembly of cytochrome cbb_3. Nor is this protein required for assembly of a second copper protein, nitrite reductase. Recently, Zumft and co-workers also observed that Sco was not required for the assembly of nitrous oxide reductase in *Pseudomonas stutzeri*, an enzyme that contains a Cu_A centre [44]. Thus, all of the available data indicate that neisserial Sco is not involved in the assembly of Cu containing proteins associated with respiratory electron transfer. It is possible that neisserial Sco is involved in copper transport to a distinct protein with antioxidant action. However, the copper of yeast Sco is bound with high affinity [11] which further challenges the role of Sco in copper transport.

We considered the possibility that Sco proteins associated with the formation of a Cu_A centre in cytochrome aa_3 might exhibit significant differences compared to those where this role was precluded. Sequence analysis suggests that this is unlikely. In all Sco proteins the two cysteines of the CXXXCP motif and the histidine residue which have been shown to provide ligands to a Cu centre in yeast Sco1 [5,6,45] are conserved (see Fig. 1). Sco proteins are membrane anchored and face either the periplasm of Gram-negative bacteria or the mitochondrial intermembrane space [46]. Thus, a key role for Sco may be to protect proteins present in or facing the periplasm or intermembrane space from oxidative damage. It is already established that in the absence of Sco there is rapid turnover of the Cu_A containing subunit II of cytochrome aa_3 in yeast mitochondria [8]. We suggest that the failure to assemble a cytochrome aa_3 in a *sco* mutant may be due to oxidative damage to the Cu_A binding site rather than a defect in transfer of copper itself. Bacterial defences used to counter reactive oxygen species are now well characterised in many bacteria [47], however Sco may a represent a novel system for protection of proteins associated with the periplasm against oxidative stress. Our current work is aimed at determining the biochemical basis for the action of Sco proteins.

Acknowledgements: K.L.S. is supported by an Australian Postgraduate Award. We thank Dr Michael Apicella, Dr Xavier Nassif and Dr John Tainer for providing strains. We acknowledge the Gonococcal Genome Sequencing Project supported by USPHS/NIH Grant AI38399, and B.A. Roe, L. Song, S.P. Lin, X. Yuan, S. Clifton, Tom Ducey, Lisa Lewis and D.W. Dyer at the University of Oklahoma.

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