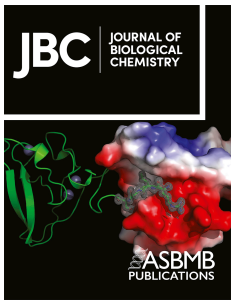


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PII: S0021-9258(24)00086-3

DOI: https://doi.org/10.1016/j.jbc.2024.105710

Reference: JBC 105710

To appear in: *Journal of Biological Chemistry*

Received Date: 11 August 2023

Revised Date: 9 January 2024

Accepted Date: 16 January 2024

Please cite this article as: Méndez AAE, Argüello JM, Soncini FC, Checa SK, Scs system links copper and redox homeostasis in bacterial pathogens, *Journal of Biological Chemistry* (2024), doi: https://doi.org/10.1016/j.jbc.2024.105710.

# Scs system links copper and redox homeostasis in bacterial pathogens

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**Running title:** Pathogen's Dsb-like proteins linking Cu and redox stress.

**Keywords**

Cu homeostasis; redox stress; Gram-negative bacteria; periplasm; thiol oxidoreductase; host-pathogen interaction

**Abstract**

The bacterial envelope is an essential compartment involved in metabolism and metabolites transport, virulence and stress defense. Its roles become more evident when homeostasis is challenged during host-pathogen interactions. In particular, the presence of free radical groups and excess copper in the periplasm causes noxious reactions, such as sulfhydryl group oxidation leading to enzymatic inactivation and protein denaturation. In response to this, canonical and accessory oxidoreductase systems are induced, performing quality control of thiol groups, and therefore contributing to restore homeostasis and preserve survival under these conditions. Here, we examine recent advances in the characterization of the Dsb-like, *Salmonella*-specific Scs system. This system includes the ScsC/ScsB pair of $Cu^{+}$-binding proteins with thiol-oxidoreductase activity, an alternative ScsB-partner, the membrane-linked ScsD, and a likely associated protein, ScsA, with a role in peroxide resistance. We discuss the acquisition of the *scsABCD* locus and its integration into a global regulatory pathway directing envelope response to Cu stress during the evolution of pathogens that also harbor the canonical

Dsb systems. The evidence suggests that the canonical Dsb systems cannot satisfy the extra demands that the host-pathogen interface imposes to preserve functional thiol groups. This resulted in the acquisition of the Scs system by *Salmonella*. We propose that the ScsABCD complex evolved to connect Cu and redox stress responses in this pathogen as well as in other bacterial pathogens.

## Introduction

The cell envelope of Gram-negative bacteria is where uptake of nutrients and efflux of unnecessary or toxic species occur. It is also the first barrier against multiple external stressors. Several physiological processes such as energy generation and cell division depend on its integrity (1). The bacterial envelope is composed of two proteolipid bilayers: the inner membrane (IM) enclosing the cytoplasm, and the outer membrane (OM) with lipopolysaccharide exposed to the external milieu. Between these two membranes is the periplasm, a protein-rich compartment that harbors a peptidoglycan layer (2). Many bacterial virulence factors reside in this compartment or depend on envelope-located systems for their secretion (3, 4). Thus, preserving its homeostasis is critical for virulence.

Pathogens are exposed to host-generated reactive oxygen and/or nitrogen species (ROS and RNS, respectively), key components of the innate immune system (5–7) (Fig. 1). ROS and RNS are also produced as common inflammation by-products in mammalian tissues (8). Two enzymes, the membrane-associated NADPH-dependent oxidase (Phox) and the inducible nitric oxide synthase (iNOS), catalyze the conversion of $O_2$ into superoxide ($O_2^-$) and the oxidation of L-arginine to L-citrulline and nitric oxide (NO), respectively, in the host (9). The reactive $O_2^-$ is rapidly converted into hydrogen peroxide ($H_2O_2$) and hydroxyl radicals (HO•) within the bacterial cell. At the same time, NO reacts with $O_2^-$ and $H_2O_2$ to produce a variety of RNS such as peroxynitrite ($ONOO^-$), perhaps the most potent non-specific biocidal agent known (10, 11). These species react with bacterial lipids and proteins, affecting the synthesis and translocation of major envelope structural components as peptidoglycans, lipopolysaccharides, and lipoproteins (12–14). This causes a rapid metabolic breakdown that eventually results in cell death.

Periplasmic enzymes such as Cu-Zn superoxide dismutases (SodC), thiol oxidoreductases of the Dsb family that repair cysteine (Cys) residues and proteases that degrade damaged proteins, are the first mechanisms used by Gram-negative bacteria to counteract oxidative stress (12, 15–19). Furthermore, some pathogens like *Salmonella enterica* and *Proteus mirabilis*, are adapted to survive in oxidative environments, and this was accomplished by acquiring additional gene-products that strengthen their response capabilities (see below). The CpxR/CpxA two-component system, together with other transcriptional regulators, coordinates cell envelope redox stress response (4, 20, 21). Instead of sensing ROS/RNS directly, these regulatory systems detect the damage caused by the reactive species. For instance, activation of CpxR/A pathway depends on

a Cys-rich sensor whose folding and/or insertion into the membrane is altered by redox stress (22).

Copper ions are required as a prosthetic group in various essential enzymes, but can also be potentially cytotoxic. For simplicity, we will refer to copper mainly as $Cu^+$, as it is the oxidation state being bound, transported, and distributed across the cell. Mechanisms of $Cu^+$ toxicity include: i) participation in Fenton-like reactions with ROS, ii) displacement of redox active Fe ions from Fe-S centers, iii) binding to non-cognate sites on macromolecules, iv) oxidation of sulfhydryl groups, particularly in the periplasm of Gram-negative bacteria v) and protein aggregation (23–28) (Fig. 1). Periplasmic $Cu^+$ overload also affects key processes such as folding and transit of lipoproteins to the OM or amino acid uptake (29, 30). In fact, $Cu^+$ is a component of the innate immune system against bacterial pathogens (31–33). Phagocytic cells actively accumulate $Cu^+$ in endocytic vesicles used to intoxicate intracellular pathogens (34). $Cu^+$ is also mobilized into the bladder to restrict colonization by uropathogens such as *Escherichia coli* or *P. mirabilis* (35, 36). Furthermore, exposure of uropathogenic *E. coli* to sub-lethal $Cu^+$ concentrations led to increased expression of the periplasmic SodC and cytoplasmic SodA enzymes, likely counteracting the metal-generated oxidative stress (37).

Although the connection of $Cu^+$ dyshomeostasis with redox stress is well known (25, 33, 38, 39), it is still unclear how the cell envelope handles and distributes this redox active metal while maintaining the required redox balance (40, 41). Defense against $Cu^+$ excess in Gram-negative species relies on the $Cu^+$ sensing cytoplasmic CueR regulator and the periplasmic-sensing two-component systems (CusR/S, CopR/S, CpxR/A) that control the expression of membrane $Cu^+$ transporters, metal chaperones, oxidases, and species-specific Dsb-like proteins such as the ScsABCD system (32, 39, 40). In particular, *Salmonella* ScsABCD is required for resistance to both $Cu^+$ and $H_2O_2$ stress, two common stressors this pathogen handles during the infection cycle (38, 42). Expression of this non-canonical Dsb system is induced by periplasmic Cu overload via activation of CpxR/A (38). Recent advances in the biochemical characterization of the ScsC/ScsB pair of thiol-oxidoreductases and identification of likely periplasmic substrates, together with a better definition of the role of associated proteins, ScsA and ScsD, support a revision of the evidence informing future studies of these proteins. In addition, we also discuss here the acquisition of this genetic locus by other Gram-negative pathogens and its putative integration into the CpxR/A regulon. Finally, we consider the relevance of the Scs system during infection.

**Periplasmic redox and Cu-stress handling by *Salmonella***

*Salmonella* encounters ROS/RNS stress during infection. Its intestinal colonization triggers inflammation with a concomitant generation of these reactive species (5). The pathogen also faces the simultaneous presence of ROS, RNS and $Cu^+$ excess (Fig. 1) in the lumen of the endocytic vesicles within phagocytic cells (5, 43). *Salmonella* employs two periplasmic Cu-Zn-Sod enzymes (Fig. 1) to remove toxic superoxide ions from the periplasm: SodCII, an *E. coli* SodC ortholog, and the horizontally acquired SodCI, encoded in the Gifsy-2 prophage along with other virulence-factors (16, 44). These homologous enzymes have different structural/functional properties and their expression depends on different transcription factors. The dimeric SodCI is tethered to the peptidoglycan and shows a catalytic rate higher than that of the soluble monomeric SodCII (45). Deletion of *sodCI* increases *Salmonella* sensitivity to both superoxide and NO (46) suggesting that SodCI prevents formation of peroxynitrite through degradation of superoxide radicals. Supporting its role in pathogenesis, *sodCI* transcription is upregulated by the PhoP/PhoQ two-component system, a major coordinator of *Salmonella* virulence (47). Thus, its deletion significantly attenuates macrophage survival and mice infection (48, 49).

The bacterial response to $Cu^+$ is also critical for *Salmonella* virulence (40, 50). It depends on the cytoplasmic $Cu^+$-sensing transcriptional regulator CueR and a set of envelope proteins expressed under its control (Fig. 1): the $Cu^+$ transporting P-type ATPase CopA, the multicopper oxidase CueO, and the $Cu^+$-binding protein CueP (50–54). CueP expression also depends on CpxR/A system (55) and this periplasmic protein is required for *Salmonella* survival and replication inside macrophages (50, 56). Some *Salmonella* serovars harbor a second $Cu^+$ transporting P-type $Cu^+$-ATPase, GolT, and a cytoplasmic $Cu^+$-chaperone GolB, both controlled by a CueR-like regulator, GolS (Fig. 1) (52, 57). *In vitro*, CueP can acquire $Cu^+$ from either CopA or GolT, and participates in the metallation of SodCI and SodCII (54, 58). However, a Δ*cueP* Δ*copA* Δ*golT* triple mutant retains fully functional SodCI and SodCII (59). On the other hand, CueP can reduce $Cu^{2+}$ to metallic $Cu^0$ (56).

*Salmonella* harbors the canonical DsbA/DsbB and DsbC-G-E/DsbD oxidoreductase systems that maintain thiol groups in the oxidative periplasmic environment (Fig. 1). The structure and biochemical function of these major complexes are discussed below. *Salmonella* also has a number of horizontally acquired genes coding for Dsb-like proteins (17). Among them, DsbL/DsbI is a DsbA/DsbB-like system required for activation of AssT, a periplasmic aryl-sulfate sulfotransferase encoded in the same chromosomal locus than *dsbL* and *dsbI* (60). SrgA is an alternative DsbA-like oxidase identified in the

*Salmonella* virulence plasmid (61). This enzyme interacts with DsbB and assists in the assembly of the plasmid-encoded Pef-fimbriae and the secretion of other virulence factors (61, 62). Accordingly, *srgA* expression is coregulated with the fimbria-coding genes (61). The Dsb-like disulfide isomerase and thiol oxidase BcfH also contributes to fimbrial biogenesis (63).

*Salmonella scsABCD* is a chromosomal operon coding for three non-canonical Dsb-like proteins, ScsB, ScsC and ScsD, and ScsA, a putative membrane-protein ScsA of unknown function (38, 42, 64) (Fig. 1). *scsABCD* transcription is induced by Cu stress and depends on CpxR/A (38). The *scs* locus was initially identified by its ability to suppress the Cu-sensitivity of an *E. coli* mutant lacking *nlpE*, the Cpx OM envelope stress sensor coding gene (64). The inclusion of both *scsABCD* and *cueP* as part of the *Salmonella* Cpx regulon (38, 55) might account for the increased sensitivity of the *cpxRA* mutant to both $Cu^{+}$ and $Au^{+}$ (65) (Checa and Soncini unpublished results). Under Cu-stress, the Scs proteins could provide feed-back control to the CpxR/A system, as deletion of the *scs* locus results in an increased Cu-dependent activation of the CpxR/A pathway (38). This resembles a phenotype observed in a *dsbA* deletion strain in *E. coli*, also a member of the Cpx regulon in this species (66).

Although the entire *scsABCD* operon is induced by Cu overload, only ScsB, ScsC, and ScsD are required for $Cu^{+}$ resistance, while they play minor roles in $H_2O_2$ tolerance (38). On the other hand, ScsA is required for $H_2O_2$ detoxification but does not provide any protection against Cu (38, 42). The presence of Scs proteins in the *Salmonella* periplasm made the DsbC/DsbD system dispensable for Cu resistance (38), differing from *E. coli,* that lacks the Scs system. Since Cu and ROS are simultaneously present in the host intracellular environment (23, 67), the Scs proteins are thought to be important under these conditions, contributing to *Salmonella* virulence. The Scs system might also compensate for the low *dsbA* expression detected at the intracellular niche (68, 69). Although the precise contribution of the Scs system to virulence has not been established and could be complex, wild-type replication levels inside macrophages were reported for Δ*scs* strains (42), except when the cellular-culture medium was supplemented with 100 μM $CuSO_4$ (30). In the presence of $Cu^{2+}$, the Δ*scs* mutant also showed a significant reduction in the expression and secretion of virulence factors required for cell invasion (42). Finally, impaired proliferation of a Δ*scsA* mutant strain was reported during host´s cortisol outburst, a phenomenon that promotes intracellular bacterial replication in the intestinal tract (70).

**The canonical Dsb system**

Cysteine is a redox active amino acid that frequently appears in pairs forming S-S bonds in periplasmic proteins (71). This stabilizes protein structure and prevents oxidation/nitrosylation of their side chains (72). Still, Cys in its reduced form is required in periplasmic proteins either for catalytic activity or to coordinate metal ions such as $Cu^{+}$ (24, 72). In this oxidative environment, the Cys redox status is usually maintained by two major thiol oxidoreductases complexes, DsbA/DsbB and DsbC/DsbD (17) (Fig. 2 and 3). DsbA and DsbC are the substrate-interacting periplasmic enzymes. DsbB and DsbD are their IM-partners that drive electrons through the membrane. These Dsb proteins harbor domains with a thioredoxin-like fold and distinctive $CX_{2/3}C$ motifs that are required for their redox activities.

Under physiological conditions, the DsbA/DsbB system assists in *de novo* disulfide-bond formation when newly synthesized proteins reach the periplasm (Fig. 2). The periplasmic DsbA functions as a monomeric oxidase that oxidizes the thiol groups of its substrates to form disulfide bonds (73, 74). As a result, the catalytic $CX_2C$ in DsbA becomes reduced. The IM component DsbB re-oxidizes the DsbA active-site residues and transfers the electrons to quinone carriers that feed the respiratory chain (75, 76) (Fig. 2). DsbB has four transmembrane segments and two periplasmic regions, each containing a pair of Cys residues (77, 78). In the N-terminal periplasmic domain of the *E. coli* protein, Cys41 and Cys44 are part of the thioredoxin-like domain that interacts with the reduced quinone carrier. Cys104 and Cys130 in the C-terminal periplasmic region perform DsbA oxidation (Fig. 2). A transient Cys41/Cys130 disulfide bond interaction enables the electron flow between both terminal regions of DsbB. *E. coli* DsbA/DsbB substrates include proteins requiring the formation of S-S bonds between non-consecutives Cys; for instance, LptD and BamA required for lipopolysaccharide or β-barrel protein insertion into the OM, or FtsN, an IM divisome component (79–82). Other DsbA/DsbB substrates participate in the assembly and/or delivery of virulence factors such as flagella, adhesins and secretion systems, or in stress signaling and activation of the major regulatory envelope homeostasis pathways (83, 84). A mechanistic example of how this Dsb system is linked to redox homeostasis is NlpE, the OM sensor of the CpxR/A system. NlpE is a large lipoprotein with five Cys and two periplasmic domains, each harboring a S-S bond in its final structure (85). Perturbation of S-S bond formation blocks NlpE trafficking to the OM. The IM-retained misfolded NlpE interacts with CpxA, inducing its autokinase activity, phosphorylation of CpxR, and expression of the Cpx regulon (29, 66). The thiol oxidase DsbA probably provides feedback control to the CpxR/A system, because its coding gene is part of the Cpx regulon in *E. coli*, but also in

*Salmonella* and other bacteria (86–88). Similarly, activation by redox stress of the Rcs system, a phospho-relay also contributing to envelope homeostasis, involves two proteins exposing Cys to the periplasm, the OM lipoprotein RcsF and the IM system repressor IgaA (89–93). RcsF usually remains in the OM trapped in a complex with BAM, the protein incorporation β-barrel assembly machinery, and porins. This prevents RcsF interaction with IgaA. Perturbation in S-S bond formation releases RcsF from the above-mentioned complex and allow it to bind IgaA. This finally results in the activation of the Rcs-regulatory pathway.

DsbC can act both as an isomerase or as a reductase (82, 94, 95). The first activity is essential to repair dysfunctional S-S bonds on periplasmic proteins having an odd number of Cys residues (Fig. 3A). Also, DsbC maintains free-SH groups preventing their oxidation/nitrosylation and catalyzes the reduction of dysfunctional S-S bonds (Fig. 3B). In fact, an *E. coli dsbC* null mutant is highly sensitive to $Cu^{+}$ (96). DsbC is a V-shaped homodimer, where each protomer contains a thioredoxin domain with a $CX_2C$ active site (97, 98). Catalysis involves the nucleophilic attack of the N-terminal Cys98 of DsbC and the formation of a mixed-disulfide complex with the substrate (97). Resolution of this intermediate and product release involves the attack of either an alternative Cys in the misfolded substrate (Fig. 3A), or the Cys101 of DsbC (Fig. 3B) (97, 99). A DsbC $CX_2S$ mutant cannot resolve the transient enzyme/substrate disulfide complex. Consequently, this mutant has been employed to trap and identify some of the DsbC target proteins (81, 100). Recycling of oxidized DsbC to its reduced form is mediated by DsbD (Fig. 3B), via its periplasmic $CX_3C$-containing α-domain (101). This large IM reductase accepts electrons from cytoplasmic thioredoxins, apparently through a cascade of redox exchange reactions involving multiple Cys in both the transmembrane helixes and the periplasmic γ-domain (101–105).

*E. coli* and *Salmonella* also harbor DsbG, a DsbC homolog, which forms an alternative redox pair with DsbD (Fig. 4). DsbG is required to preserve the catalytic Cys residues of L,D-transpeptidases involved in linking peptidoglycan to the Braun lipoprotein Lpp during peptidoglycan maturation (95). Like DsbC, the active form of DsbG is a dimer (106). Differences in a linker helix length and the pattern of hydrogen bonding at the dimer interface between these homologous proteins might contribute to substrate discrimination (98, 107). The protein environment surrounding the $CX_2C$ motifs in DsbG and DsbC also differs. This likely change the p$K_a$ and redox potentials of these enzymes, affecting their corresponding activities. Despite this, DsbC and DsbG can partially substitute each other *in vitro* and also *in vivo* (95, 108), except under $Cu^{+}$ stress (96). Another DsbD substrate is the inner membrane-anchored protein DsbE (CcmG), which

is involved in cytochrome *c* biogenesis (104, 109) (Fig. 4). Like DsbC and DsbG, DsbE has a periplasmic thioredoxin-like domain with a $CX_2C$ motif (110, 111).

**The *Salmonella* Scs system and its paralogues in other pathogens**

The *scs* locus is present in all available *S. enterica* and *S. bongori* genomes, as well as in other enterobacterial species like *Citrobacter*, *Serratia*, *Yersinia*, *Klebsiella*, *Cedecea*, *Raoultella*, *Enterobacter*, *Pantoea*, *Providencia*, *Proteus,* and a swine diarrheagenic *E. coli* TA206 strain, as well as in the gamma proteobacteria *Aeromonas* (Fig. 5). In these species, a similar operon array is conserved. The first gene, *scsA,* is followed by *scsB*, *scsC*, and *scsD*. However, *scsC* and *scsD* are inverted in *Vibrio* sp. (Fig. 5). We have reported putative CpxR-binding sites in the promoter of most *scs*-like operons (38), suggesting both the presence of Cpx-dependent regulation and a Scs-mediated feed-back control. Curiously, in *Cronobacter sakazakii* a *scsB* homolog is next to a gene coding for a protein with ~60% identity to *Salmonella* DsbG (Fig. 5). Incomplete *scs* operons, lacking either *scsA* or *scsC*, are present in *Shewanella* sp. and *Photobacterium profundum*, respectively. *Caulobacter crescentus*, a member of the alphaproteobacterial class and an opportunistic pathogen, encodes *scsB* and *scsC* homologs (112), while *Haemophilus influenzae* and *Neisseria meningitidis* only harbor a *scsD* homolog in their genomes (Fig. 5).

ScsB forms a functional redox pair with the periplasmic component ScsC in *Salmonella*, *Caulobacter crescentus,* and *Proteus mirabilis* (112–114) (Fig. 5). ScsB and its ortholog define a new group within the DsbD protein family (112, 113). These IM proteins have eight transmembrane segments and two periplasmic domains (α and γ) with the archetypical $CX_{2/3}C$ motif (Fig. 6). Considering the similarity between ScsB and DsbD at the IM region (Fig. 3 and 6), it was proposed that TrxA, the cytoplasmic enzyme that feeds electrons to DsbD, is also the electron donor of ScsB (Fig. 6). Differences between ScsB and DsbD are more evident at the periplasmic α-region. In this region, DsbD has a single immunoglobulin-like fold (Fig. 3) (115), while the ScsBα (Fig. 6) contains two of these domains in tandem (112, 116). Only the N-terminal subdomain, named α-A, includes the redox-active site ($CX_3C$ motif) that primes the ScsC activity and is conserved among the ScsB proteins (113). The other immunoglobulin-like subdomain, α-B, is more variable and has been suggested to modulate the interaction with other redox partners. For instance, *C. crescentus* ScsB interacts with ScsC and other proteins involved in the cell envelope reductive pathway such as PprX, a periplasmic peroxiredoxin, and PrxL, a peroxiredoxin-like protein (112). The possibility of crosstalk

between the *Salmonella* ScsB/ScsC and DsbD/DsbC systems was analyzed *in vitro* (114). While the DsbDα domain transfers electrons to oxidized ScsC, ScsBα cannot catalyze the reduction of DsbC.

All ScsC homologs have the characteristic thioredoxin fold, the $CX_2C$ catalytic motif, and various conserved residues (117). *Salmonella* ScsC $Gly_{186}$, $Pro_{175}$ and $Thr_{174}$ are also present in all homologs (Fig. 6), including *E. coli* DsbG. These residues are proposed to stabilize the reduced form of the protein (117). $Lys_{70}$ and $Lys_{96}$ residues that stabilize the thiolate form of the nucleophilic $Cys_{66}$ in the catalytic motif of *Salmonella* ScsC (117), are conserved in most of ScsC homologs, except in *C. crescentus*, *Shewanella* and *Pantoea* sp. However, the ScsC-like proteins show significant heterogeneity in their oligomerization state. *Proteus* and *Caulobacter* ScsC form homotrimers connected by the long N-terminal α-helix, while the *Salmonella* homolog lacking this region is a monomer (116, 118) (Fig. 6). These distinct structures seem to be associated with different roles. *Caulobacter* and *Proteus* ScsC have disulfide isomerase activity (112, 116, 118). By contrast, *Salmonella* ScsC, initially proposed as a disulfide reductase *in vitro* (117), was shown to facilitate disulfide bond formation of the periplasmic arginine sensor ArtI under stress conditions (30). Because ArtI only has two Cys, it was speculated that ScsC may also repair these oxidized residues under redox stress. SodCI and SodCII are putative *Salmonella* ScsC substrates, as are other proteins involved in amino acid sensing and import, including ArgT, GltI, and HisJ (30). Residues around the Cys in the $CX_2C$ motif likely influence the redox potential of periplasmic proteins. Both ScsC and the disulfide isomerase DsbG have identical CPYC motif at their active sites and similar low redox midpoint, -132 and -129 mV, respectively (117). Nevertheless, *Salmonella* ScsC shows a low pKa (3.4), comparable to that of the thiol oxidase DsbA (3.5). Thus, ScsC might have both oxidase and reductase activities in different conditions.

*Salmonella* ScsC and ScsB, and *C. crescentus* ScsC were shown to bind $Cu^{+}$ and $Cu^{2+}$ *in vitro* (114, 118). Both *Salmonella* ScsC and ScsB from *Salmonella* can also transfer $Cu^{+}$ to CueP with similar efficiency (114). However, neither the specificity of metal binding nor the unidirectionality of $Cu^{+}$ transfer has been tested. Also, the relevance of Cu transfer and its relationship with the redox activity of these enzymes await elucidation.

*Salmonella scsD* encodes a predicted IM-anchored protein with a periplasmic thioredoxin-fold domain. Its AlphaFold 2 structure (Fig. 6) resembles that of the membrane-linked thiol:disulfide interchange proteins such as DsbE/CcmG and TlpA, involved in cytochrome *c* and *aa3* biogenesis, respectively (110, 119). Apparently, ScsD

(Fig. 6) and its homologs have a conserved proline residue separated by 61 residues from their putative redox-active $CX_2C$ motif. Interestingly, this Pro residue stabilizes the oxido-reductase motif of DsbE/CcmG-like proteins (111). Considering that TlpA is a substrate of *Caulobacter* ScsB (112), DsbE is another DsbD partner in *E. coli* (109) (Fig. 4), and the function of both *Salmonella* ScsC and ScsD under $Cu^+$ stress depend on ScsB (38), we propose that ScsD is also a ScsB redox partner (Fig. 4).

Lastly, *Salmonella* ScsA is the only Scs protein having a $CX_2C$ motif but lacking the thioredoxin-like fold and probably the redox activity associated with this domain. Interestingly, it has no homology to any structurally characterized protein. Contrary to the other Scs proteins, its location within the bacterial envelope could not be determined (42). Some *in silico* predictions indicate that ScsA might be an integral IM protein with two transmembrane helixes (Fig. 6), while others showed a lipobox-like sequence in the N-terminal region that might direct ScsA to the OM. If ScsA is an OM protein, the $CX_2C$ motif located at the N-terminus of the putative cleavage site is expected to be removed during processing. In the hypothetical case that ScsA remains in the IM, this motif would be hidden in the membrane (Fig. 6). Interestingly, all identified ScsA homologs carry this predicted lipobox sequence, and those more closely related to the *Salmonella* ScsA also conserve the CLAC array within the $CX_2C$ motif. In addition, a peroxidase signature sequence, TAQRMAGLHAL (64), is detected in most ScsA homologs in a region predicted to be exposed to the periplasm. Taking this into account and given the role of ScsA in $H_2O_2$ tolerance (38, 42), it is tempting to speculate that this protein functions as a periplasmic peroxidase. The observation that expression of the Scs proteins avoided $H_2O_2$-mediated carbonylation of envelope proteins (42) further supports this idea. Based on the requirement of Cu stress for *scsA* transcription (38, 42), ScsA might prevent the generation of ROS species derived from Cu overload.

**Concluding remarks**

Counteracting redox and Cu stress in the envelope of bacterial pathogens is required to establish a successful infection. The collected evidence indicates that the canonical Dsb systems might not satisfy the extra demands that the host-pathogen interface imposes to preserve functional thiol groups. Then, it is logical that novel Dsb-like systems were acquired during evolution by intracellular pathogens like *Salmonella* or uropathogens like *P. mirabilis*. In fact, the *Salmonella* Scs system emerges as the linker between Cu and redox stress. The role of three of its components in disulfide bond generation and/or isomerization of envelope proteins might be assumed based on their

structural features. However, their specific chemical function and biological roles require experimental confirmation. In addition, the biochemical function of ScsA and its membrane topology remain to be determined. Importantly, the specific requirements of the ScsABCD system at the host/pathogen interface, as well as its relevance during the infection cycle of *Salmonella* and other pathogens, also need further inquiries.

## Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.

## Acknowledgments

Author contributions.

A. A. E. M., F. C. S. and S. K. C. investigation and conceptualization; A. A. E. M., F. C. S. and S. K. C. writing-original draft; A. A. E. M., J. M. A., F. C. S. and S. K. C. writing-review and editing.

Funding and additional information.

This work was supported by the Agencia Nacional de Promoción Científica y Tecnológica (PICT-2018-02122 to F. C. S.), the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) (PIP 11220200100600CO to S. K. C) and the National Institutes of Health (R01A1150784 to J. M. A.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. A. A. E. M. is a CONICET postdoctoral fellowship recipient. S. K. C. and F. C. S. are career investigators of CONICET, and F. C. S. is a career investigator of the Rosario National University Research Council.

## Abbreviations

IM, inner membrane; OM, outer membrane; ROS, reactive oxygen species; RNS, reactive nitrogen species; SodC, Cu-Zn superoxide dismutase; Phox, NADPH-dependent oxidase; iNOS, nitric oxide synthase; Dsb, Disulfide bond.

## Unpublished observations

S. K. Checa and F. C. Soncini, unpublished results.

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**Figure Legends**

**Figure 1:** Handling of copper and redox stress in *Salmonella* envelope. Cu ions, ROS and RNS are part of the host´s defense against this pathogen. Cu-transporters and chaperones are depicted in different tones of blue, cuproproteins in orange and redox enzymes in green. The regulatory systems (in red) and their regulatory networks are shown. The flux, generation and chemical transformation of ROS/RNS are indicated with arrows. Arrows with question marks represent the influx and efflux of Cu ions carried out by uncharacterized transporters. Inactivation of proteins with Cys residues involves not only Cu(I) but also ROS and RNS.

**Figure 2:** *De novo* disulfide bond formation catalyzed by the DsbA/DsbB pair. Three dimensional structures of DsbA (PDB 1A2I) and DsbB (PDB 2ZUQ) are drawn in ribbon form (in blue). All catalytic Cys residues from $CX_{2/3}C$ motifs are represented in yellow spheres. Thiol groups or S-S bonds are depicted in magenta. UQ/$UQH_2$ is the ubiquinone/ubiquinol pool. Magenta dashed arrows indicate the electron flow. Solid black arrows show the overall redox reactions.

**Figure 3:** Reactions catalyzed by DsbC. A) Isomerization of non-native disulfide bonds in envelope proteins. B) Reduction of the Cys groups on protein substrates, a reaction that requires DsbD to recycle DsbC. The structures of DsbC (PDB 1EEJ), DsbDα (PDB 1JPE), DsbDγ (PDB 2FWF) and TrxA (PDB 6H1Y) are drawn in ribbon form (in blue). The DsbDβ structure predicted by AlphaFold 2 is shown in gray. Catalytic Cys, thiol groups or S-S bonds are shown as in Fig. 2. Dashed arrows indicate either nucleophilic attack or electron flow, as shown in the figure. Solid black arrows illustrate the redox reactions.

**Figure 4:** The alternative DsbD partners, DsbG and DsbE (CcmG) and the reactions they catalyze. The structures of DsbG (PDB 1V58) and DsbE (CcmG) (PDB 2B1K) DsbDα (PDB 1JPE), DsbDγ (PDB 2FWF) and TrxA (PDB 6H1Y) are drawn in ribbon form (in blue). Structures of DsbDβ and the N-terminal region of DsbE predicted by AlphaFold 2 are shown in gray. Catalytic Cys, thiol or sulfoxide groups, S-S bonds and electron flow are shown as in Fig. 2.

**Figure 5:** The *scs* locus from *Salmonella* and paralogue species. The figure shows the chromosomal region of organisms harboring *scsABCD* homologs (analysis performed

on the “Seed Viewer”, http://pubseed.theseed.org). Only a representative strain from either *S. enterica* or *S. bongori* are included in the figure, but all *Salmonella* genomes have the *scsABCD* locus. The *S. enterica scsABCD* promoter is indicated with a black arrow. The identified (black boxes) or predicted (gray boxes) direct-repeats corresponding to the CpxR-binding sites upstream of *scsABCD* promoter are shown. Full names of analyzed strains are: *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2; *Salmonella bongori* 12149; *Citrobacter* sp. 30_2; *Cedecea davisae* DSM 4568; *Escherichia coli* TA206; *Serratia* sp. AS13; *Yersinia* sp; *Raoultella ornithinolytica* B6; *Enterobacter* sp B509; *Klebsiella* sp 1_1_55; *Hafnia alvei* ATCC 51873; *Erwinia billingiae* Eb661; *Aeromonas* sp 159; *Pantoea* sp GM01; *Proteus mirabilis* HI4320; *Providencia stuartii* MRSN 2154; *Vibrio* sp MED222; *Photobacterium profundum* SS9; *Cronobacter zakazakii* ATCC BAA-894; *Shewanella* sp W3-18-1; *Haemophilus influenzae* R2846; *Neisseria meningitidis* FAM18.

**Figure 6:** The *Salmonella* Typhimurium Scs system. The structure of ScsC (PDB 4GXZ) and TrxA (PDB 6H1Y) are shown in ribbon form (in blue). The two subdomains (A and B) from the ScsBα region predicted by AlphaFold 2 are colored in pink and purple, respectively. Structures of the β and γ domains of ScsB, ScsA and ScsD predicted by AlphaFold 2 are shown in gray. Yellow spheres represent the catalytic C composing the $CX_{2/3}C$ motifs. Inserts highlight the active sites of ScsC and ScsD with Cys residues colored in yellow. Other conserved residues important for the redox activity of ScsC are highlighted, as well as the conserved P135 residue of ScsD. Dashed arrows indicate reported (black) or hypothetical (magenta) electron transfer between Scs proteins.

**Figure 1**

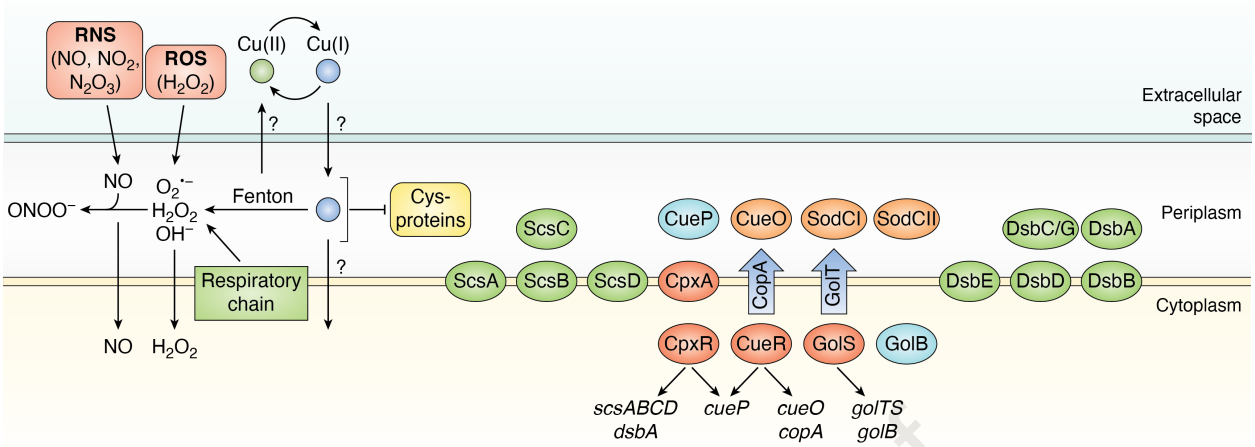


**Figure 2**

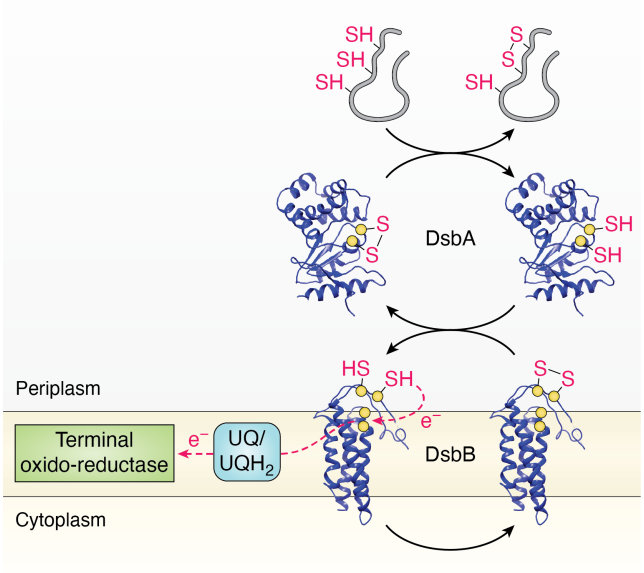


**Figure 3**

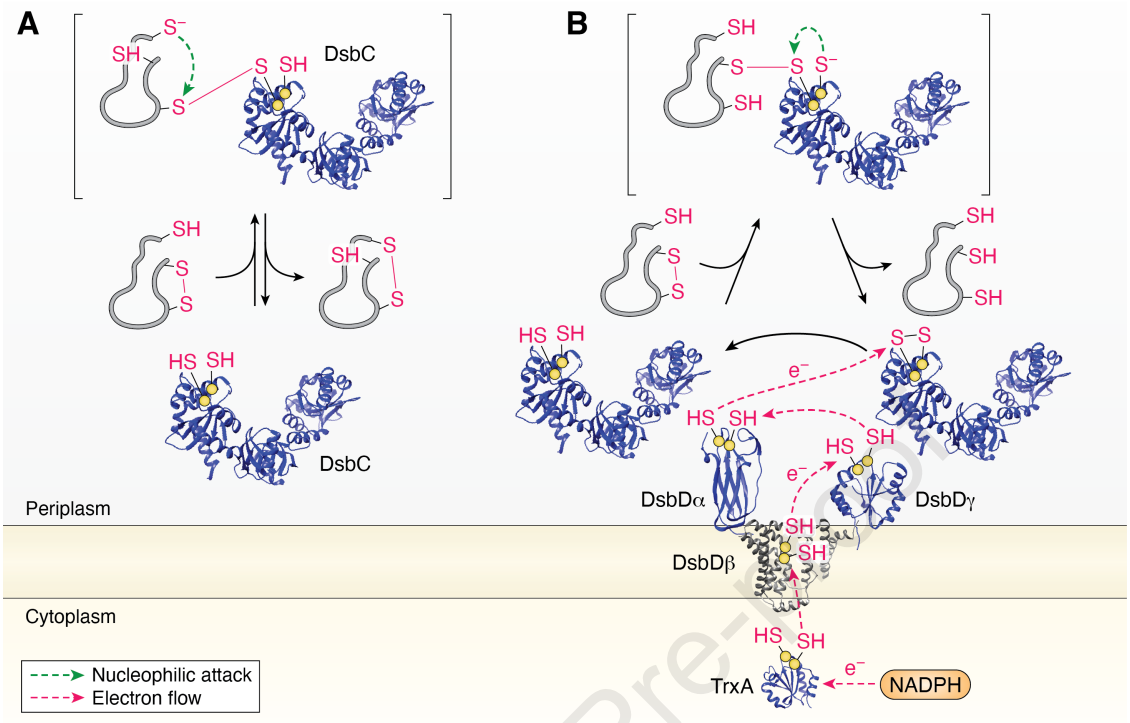


**Figure 4**

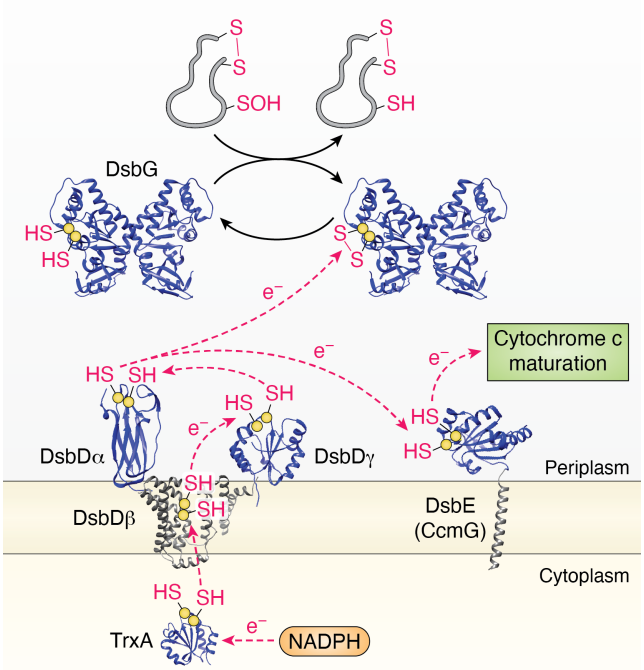


**Figure 5**

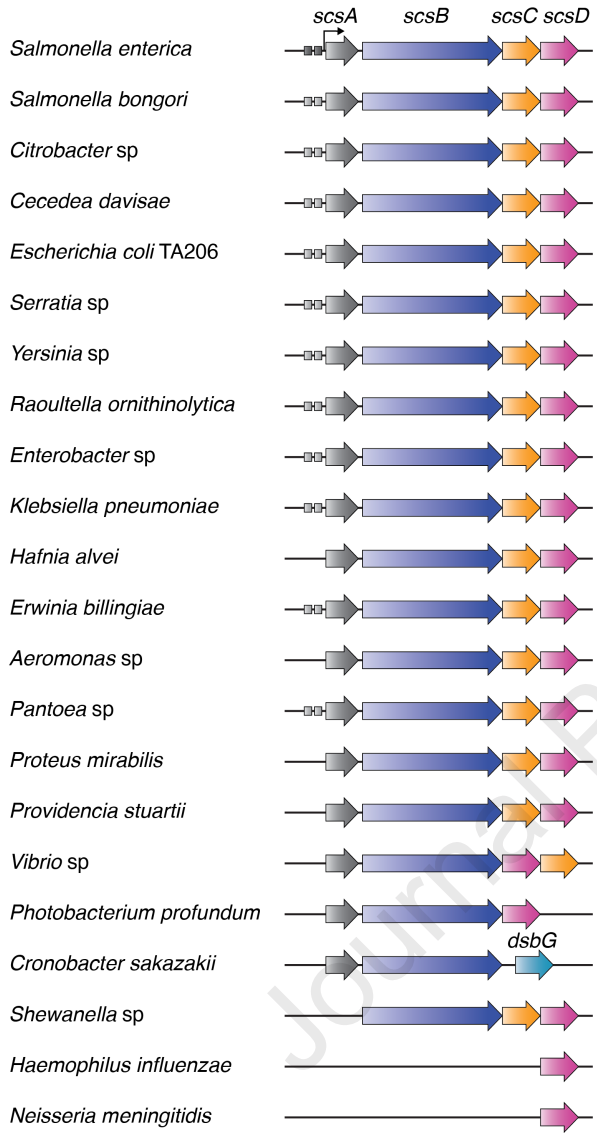
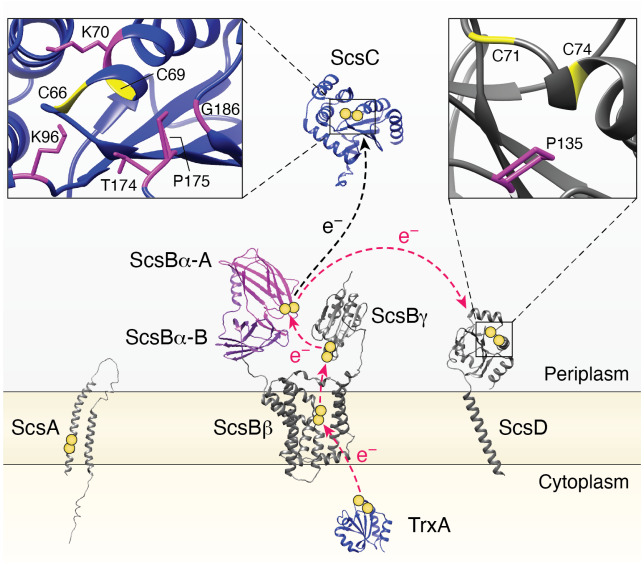


**Figure 6**



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**Declaration of interests**

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: