Salmonella ScsB and ScsC protect against toxic copper

The Scs disulfide reductase system cooperates with the metallochaperone CueP in Salmonella copper resistance

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**ABSTRACT**

The human pathogen *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) contains a complex disulfide bond (Dsb) catalytic machinery. This machinery encompasses multiple Dsb thiol-disulfide oxidoreductases that mediate oxidative protein folding, and a less characterized suppressor of copper sensitivity (Scs) gene cluster, associated with increased tolerance to copper. To better understand the function of the *Salmonella* Scs system, here we characterized two of its key components, the membrane protein ScsB and the periplasmic protein ScsC. Our results revealed that these two proteins form a redox pair in which the electron transfer from the periplasmic domain of ScsB (n-ScsB) to ScsC is thermodynamically driven. We also demonstrate that the Scs reducing pathway remains separate from the Dsb oxidizing pathways and thereby avoids futile redox cycles. Additionally, we provide new insight into the molecular mechanism underlying Scs mediated copper tolerance in *Salmonella*. We show that both ScsB and ScsC, can bind toxic copper(I) with femtomolar affinities and transfer it to the periplasmic copper metallochaperone CueP. Our results indicate that the *Salmonella* Scs machinery has evolved a dual mode of action, capable of transferring reducing power to the oxidising periplasm and protecting against copper stress by co-operating with the cue regulon, a major copper resistance mechanism in *Salmonella*. Overall, these findings expand our understanding of the functional diversity of Dsb-like systems, ranging from those mediating oxidative folding of proteins required for infection to those contributing to defence mechanisms against oxidative stress and copper toxicity, critical traits for niche adaptation and survival.

The bacterial cell envelope harbours disulfide bond forming (Dsb) proteins, a group of redox active enzymes characterized by a thioredoxin (TRX)-like domain and a conserved CxxC catalytic motif (1). Dsb enzymes introduce disulfide bonds to diverse substrates, including secreted toxins, bacterial adhesins and nutrient acquisition and secretion systems, which are required for bacterial fitness and virulence (1,2). Typically, Dsb proteins form two separate redox pathways, the oxidative DsbA/B pathway, which oxidizes thiols in substrate proteins, and the reductase/isomerase DsbC/D pathway, which corrects non-native disulfide bonds (3).

*Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) is a main cause of food borne illnesses such as acute gastroenteritis and can cause life-threatening bacteremia in children and immunocompromised individuals (4-6). This Gram-negative facultative intracellular pathogen displays an unusual complexity in the Dsb machineries it encodes. In addition to the disulfide bond forming DsbA/B system (7), and a DsbC/D isomerization pathway (8), *S. Typhimurium* contains a separate DsbL/I redox pair (9,10), a plasmid encoded DsbA homologue SrgA (9,11) and a set of related and less characterized proteins encoded by the Suppressor of copper sensitivity (Scs) locus (Figure 1A) (12).

The Scs locus encompasses four proteins (ScsA-D). ScsA and ScsB are predicted integral membrane proteins; ScsA has 2 membrane-spanning alpha-helices (12) and ScsB contains two periplasmic domains (n- and c-ScsB) that frame a central transmembrane domain (t-ScsB) (13). ScsC is a periplasmic soluble protein and ScsD has a predicted N-terminal membrane anchor joined to a periplasmic domain (12). Scs proteins contain the hallmarks that characterize Dsb proteins; they all incorporate a putative catalytic CxxC motif which in the case of ScsC, c-ScsB and ScsD, is imbedded in a TRX-fold (12,14). The conservation of the Dsb protein features in the Scs family suggests that they are participants in dithiol/disulfide interchange reactions.

The Scs proteins were first identified for their ability to confer resistance to copper stress in *Salmonella* (12). Copper plays a central antibacterial role in the innate immune system of many organisms, through its oxidising effects on damaging cell membranes and generating reactive oxygen species along with its ability to displace enzyme iron-sulfur complexes (15). During systemic infection, *Salmonella* is exposed to elevated copper concentrations within the macrophage phagosome and copper tolerance is a critical adaptive trait for its survival (16-18). Copper resistance mechanisms in *Salmonella* include the cue/gol regulon, which contains a cytoplasmic sensor CueR that upon stimulation by toxic Cu(I), induces the expression of...
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Plasma-membrane P-type copper-transporting P1B-type ATPases such as CopA and GolT, which remove excess Cu(I) from the cytoplasm; the periplasmic cuprous oxidase CueO, and the metallochaperone CueP (17,19-21). The latter is a major periplasmic Cu(I) binding protein required for Cu tolerance and Cu delivery to the stress response Cu/Zn-superoxide dismutase (Cu/Zn-SOD) (22,23). CueP is also regulated by the Cu-dependant CpxR/CpxA two-component regulatory system, which additionally regulates the transcription of the scs operon (23,24). The co-regulation of CueP and Scs proteins suggests a concerted action in copper homeostasis, however, the mechanism underlying Scs mediated copper resistance had remained unknown.

In this study we have investigated the Salmonella Scs electron transfer pathway. Through redox and kinetics studies we demonstrate that ScsB and ScsC form a redox pair and that this system is maintained separate from the DsbA oxidizing machinery but cross-talks with the DsbD reductase system. We also investigated the molecular basis of ScsB/ScsC mediated copper tolerance. Combination of quantitative copper binding and copper transfer experiments revealed that both ScsB and ScsC tightly bind Cu(I) and are able to deliver it to periplasmic CueP. These results provide an unprecedented biochemical insight into the mechanism underlying the ScsB/ScsC mediated copper resistance trait which involves an interplay between the Scs system and the cue regulon, a major copper resistance machinery in Salmonella.

RESULTS

Distribution of Scs systems across bacteria

Although the scs cluster was first identified in S. Typhimurium (12), genes encoding for Scs proteins, are widespread across Proteobacteria (13). Using the STRING analysis (25), we re-evaluated the distribution of the scs operon across the ever-increasing number of publicly available bacterial genomes. Analysis of the 1,678 bacterial genomes showed that the components of the scs operon are more frequently found in alpha- and gamma-Proteobacteria and are less common in beta- and delta-Proteobacteria (Table S1). The full scs operon appears to be limited to some Enterobacteriaceae including Salmonella enterica, Serratia proteamaculans, Citrobacter koseri and Klebsiella pneumoniae along with Aeromonas hydrophila (Aeromonadaceae family) and Photobacterium profundum (Vibrionaceae family). Other species in the Proteobacterium phylum contain all scs genes but scsA, while most alpha-Proteobacteria primarily encode ScsB and ScsC proteins, which also occur in Fibrobacteres, Bacteroidetes and Planctomycetes (Table S1). The widespread distribution of ScsB and ScsC is important given the role these two proteins play in bacterial fitness (24). In this study we have investigated the Salmonella ScsB and ScsC redox system and the molecular basis for their role in reducing copper toxicity.

Salmonella ScsC structurally resembles DsbA but lacks dithiol oxidase activity

The recent characterization of ScsC proteins from different organisms has uncovered a remarkable diversity in structure and function. Structural and biochemical studies of S. Typhimurium ScsC revealed that this protein resembles the DsbA-like thiol oxidase, both structurally and in redox properties (14) (Figure 1B). Given the similarities between Salmonella ScsC and DsbA, we investigated whether ScsC displayed thiol oxidase activity in a peptide oxidation assay where we employed a fluorescently labelled peptide that is known to be oxidized by DsbA-like proteins (26). The oxidation activity was monitored by the increment of europium fluorescence resulting from peptide cyclization, via disulfide bond formation between the terminal cysteines (Figure 1C). While DsbA was able to efficiently oxidize the standard peptide substrate as indicated by the increase in fluorescence intensity over time, equivalent concentrations of ScsC showed a fluorescence profile comparable to that of background oxidation (Figure 1C). These results indicate that despite the similarities between ScsC and DsbA in their structures and reduction potentials, they have different oxidoreductase functions probably with different substrates.

ScsB and ScsC form a redox pair in S. Typhimurium

Previous work has shown that ScsC exists in the reduced state in the Salmonella
Salmonella ScsB and ScsC protect against toxic copper periplasm (14). We assessed the ability of ScsB, the predicted thiol reductase encoded immediately upstream of ScsC, to catalyze its reduction. A gel-shift assay was used to monitor the redox state of ScsC upon incubation with the n-terminal domain of ScsB (n-ScsB). Stoichiometric amounts of reduced n-ScsB (n-ScsBred) and oxidized ScsC (ScsCox) were incubated and samples were taken from the reaction mixture at 15, 120, and 300 second time points. Determination of the redox state of ScsC was done by alkylating free thiols with 4-acetoamido-4′-maleimidystilbene-2,2′ disulphonic acid (AMS), which increased the mass by 500 Da for each free cysteine (1 kDa total) that could be detected by SDS-PAGE.

These experiments showed that ScsCox (Figure 2A, lane 2) was rapidly reduced by n-ScsBred (Figure 2A, lane 6), as shown by the SDS-PAGE analysis of AMS derivatized samples (Figure 2A, lanes 3-4) as well as western immunoblotting using anti-ScsC antibody (Figure S1A). These data show that Salmonella ScsC and ScsB form an efficient redox relay. We also investigated the reverse electron flow by mixing n-ScsCox and ScsCred (Figure 2B, lanes 6 and 2, respectively) and showed that reduced ScsC was unable to transfer electrons to oxidized n-ScsB (Figure 2B, lanes 3-5 and Figure S1B).

**Kinetic characterization of the ScsB – ScsC interaction**

To further characterize the interaction between n-ScsB and ScsC, we used surface plasmon resonance (SPR) to determine binding affinity ($K_D$) of these two proteins in different redox states. We first analyzed the n-ScsBred-ScsCox electron transfer complex by immobilizing n-ScsBox onto a CM5 sensor chip and injecting a concentration series of ScsCox over the chip surface, which gave a $K_D$ of $310 \pm 46 \mu M$ (Figure 2C, Table 1). This micromolar binding affinity is comparable to the previously reported $K_D$ of $310 \pm 46 \mu M$ for $E. coli$ n-DsbDox and e-DsbDred ($K_D$ of $86 \mu M$ (27)) and characteristic of the transient protein-protein interactions between Dsb-like proteins. Interestingly, a similar $K_D$ value of $287 \pm 34 \mu M$ (Table 1, Figure S2B) was obtained for the reverse interaction between ScsCred and n-ScsBox, which, as shown before, does not favour an electron transfer reaction. SPR experiments were also carried out for the two proteins in the same redox state, which yielded $K_D$ values of $233 \pm 56 \mu M$ and $361 \pm 12 \mu M$ for n-ScsBred-ScsCred and n-ScsBox-ScsCox respectively (Table 1, Figure S2A-2C). To validate these binding data, we immobilized ScsC on the chip and injected n-ScsB, which yielded similar results (data not shown). These kinetic studies show that the interaction between n-ScsB and ScsC is oxidation state independent, as evidenced by the similar $K_D$ values obtained for all investigated complexes (Table 1).

**The interaction between ScsB and ScsC is thermodynamically driven**

The reduction potential of n-ScsB in equilibrium with different concentrations of oxidized and reduced dithiothreitol (DTT) was determined by monitoring the relative amounts of oxidized and reduced protein using AMS alkylation of free thiols. Protein-AMS adducts with increased molecular mass were detected by SDS-PAGE analysis (Figure 2D). An equilibrium constant of $(1.24 \pm 0.15) \times 10^{-2}$ was calculated by plotting the relative amount of reduced n-ScsB versus [DTT$_{red}$]/[DTT$_{ox}$]. This $K_{eq}$ value corresponds to a standard reduction potential of $-256 \pm 10$ mV, which is substantially more reducing than that of ScsC (-132 mV) (14). Collectively, these results show that although kinetics factors may contribute to the interaction between n-ScsBred and ScsCox (Table 1), thermodynamic factors favour the electron flow between these proteins and prevent the unproductive reverse electron transfer from n-ScsBox to ScsCred.

**The ScsB-ScsC reducing system interacts with DsbA less efficiently**

Given the similarity in three dimensional structures and reduction potentials between ScsC and DsbA, we wondered whether n-ScsB could also reduce the thiol oxidase DsbA. Using the AMS gel-shift assay, we could only detect a partial reduction of DsbA (Figure 3A, lane 3-5) when DsbAox (Figure 3A, lane 2) was incubated with n-ScsBred (Figure 3A, lane 6). Indeed, while n-ScsBred reduced 75% of ScsCox after 15 seconds, only 28% of DsbA was reduced by n-ScsBred in the same timeframe (gel band intensity analysis ImageJ 1.45). This indicates a comparatively slower electron transfer process between n-ScsBred to DsbAox. Furthermore, although the amount of reduced DsbA increased as time progressed, reduction of the protein did not go to completion after 5
minutes incubation (57% DsbA reduction after 5 minutes incubation vs 100% of ScsC reduction). We also attempted to determine the dissociation constant for the interaction between n-ScsBred and DsbAox by SPR (Figure S2D). DsbA has a comparable molecular weight to ScsC, however the binding response for DsbA at 500 µM was only 11 RU compared to ~400 RU binding response for ScsC at 585uM. These results suggest that the interaction between DsbA and ScsB is too weak to be detected by SPR and to be biologically relevant.

As oxidized DsbA and reduced ScsC co-exist in the periplasm, the cross talk between these proteins was also tested. When DsbAox (Figure 3B, lane 2) was mixed with ScsCred (Figure 3B, lane 6), no reduction of DsbA was detected even after 5 minutes incubation. Together, these results support the hypothesis that in the periplasm of Salmonella the reducing Scs system is kept separate from the DsbA oxidative pathway probably by means of enzyme specificity.

**Cross talk between Salmonella ScsC/B and DsbC/D reducing systems**

We investigated the interaction between the Salmonella ScsB / ScsC and DsbD / DsbC reducing pathways, by assessing the ability of the two membrane bound reductases, n-DsbD and n-ScsB, to reduce ScsC and DsbC, respectively. AMS-based gel-shift assays showed that reduced n-DsbD efficiently transfers electrons to oxidized ScsC (Figure 3C), whereas the reverse reaction did not result in any apparent change in the redox state of the proteins (Figure S1C). The n-DsbDred / ScsCox interaction was also analyzed by SPR, which yielded a $K_D$ of 422 ± 26 µM, which is similar to the $K_D$ of the n-ScsBred / ScsCox interaction (Table 1). With regard to the n-ScsBred and DsbCox electron transfer, this occurred less readily under the conditions tested (Figure 3D), although the two proteins seem to interact by SPR yielding a $K_D$ of 334 ± 26 µM (Table 1).

**n-ScsB, ScsC and CueP bind Cu(I) with affinities in the sub-picomolar to femtomolar range**

The scs operon, particularly ScsB and ScsC provide copper tolerance to Salmonella (14,24). To understand the mechanism underpinning the scs-mediated increased copper tolerance, the copper binding properties of n-ScsB and ScsC were quantified and compared with that of CueP, a Salmonella specific copper chaperone required for the activation of Cu/Zn-SOD in the periplasm (17). CueP together with the scs cluster are co-regulated by the CpxR/CpxA envelope stress response (22,24).

We used a competition assay involving the chromophoric Cu(I) chelator bicinchoninic acid (Bca) to accurately determine the Cu(I) binding affinities to each protein (28). Titration of increasing concentrations of n-ScsBred into a series of solutions containing the same amount of [Cu(I)(Bca)_2]^3- with 0.25 fold excess Bca, led to an initial linear decrease in absorbance at 562 nm. This decrease is known to occur when copper is removed from the [Cu(I)(Bca)_2]^3- (Figure 4A(i)). This indicates a non-competitive Cu(I) transfer from the probe complex [Cu(I)(Bca)_2]^3- to the protein domain n-ScsB during the early titration that becomes more competitive at a later titration stage due to the accumulation of free Bca ligand. A plot of the concentration of [Cu(I)(Bca)_2]^3- versus total [n-ScsB]/[Cu(I)] for those titration points with non-competitive Cu(I) transfer generated a straight line intercepting at [n-ScsB]/[Cu(I)] = 1.0 (Figure 4A(ii)). Curve-fitting of the experimental data to a model of a single Cu(I) site derived a stoichiometry of one Cu(I) per n-ScsB protein domain. An increase of [Bca]tot to 300 µM induced an effective competition for Cu(I) between Bca and n-ScsB (Figure 4A(ii)).

An equivalent titration of fully oxidized n-ScsB caused little concentration change of [Cu(I)(Bca)_2]^3- (Figure 4A, red empty circles), but upon further addition of 1.0 mM of the strong reductant sodium dithionite (29), the observed concentrations of [Cu(I)(Bca)_2]^3- dropped to the same level as those for the fully reduced protein (Figure 4A, red crosses). Addition of the same amount of dithionite into a control solution without protein caused no change in [Cu(I)(Bca)_2]^3- concentration (see the red cross point at [n-ScsB]/[Cu(I)] = 0 in Figure 4A).

These experiments suggest that the two Cys sidechains in n-ScsB are the key Cu(I) ligands.

Equivalent experiments for the ScsCred generated two binding curves for the conditions of [Bca]tot = 80 and 300 µM (Figure 4B (i, ii)). Both were non-linear and represented competitive Cu(I) binding between Bca and ScsC. It is difficult to estimate the Cu(I) binding...
stoichiometry from these experiments. However, a control experiment using \([\text{Bca}]_{\text{tot}} = 80 \ \text{µM}\) with \(\text{SscS}_{\text{cox}}\) detected no Cu(I) binding while addition of reductant sodium dithionite into the solution induced concentration changes of \([\text{Cu}^+ (\text{Bca})_2]^3^-\) identical to that of the assay using \(\text{SscS}_{\text{red}}\) (Figure 4B, red empty circles versus red crosses). This again demonstrated that the two Cys sidechains in \(\text{SscS}\) are the key binding sites for Cu(I) and consequently a binding stoichiometry of one Cu(I) per \(\text{SscS}\) can be assumed. Fitting of the two sets of experimental data in curves (i) and (ii) to a one-site binding model derived \(K_D = 10^{-13.1}\) M and \(>10^{-13.5}\) M for \(\text{SscS}\), respectively. The flatness of the binding curve (ii) suggested non-effective competition for \(\text{SscS}\) under the condition of \([\text{Bca}]_{\text{tot}} = 300 \ \text{µM}\), where only an upper-limit affinity was estimated. However, the competition with \([\text{Bca}]_{\text{tot}} = 80 \ \text{µM}\) in (i) was effective, allowing a more reliable estimation of affinity for \(\text{SscS}\) with a \(K_D = 10^{-13.1}\) M (Table 2).

Equivalent experiments with \(\text{CueP}_{\text{red}}\) generated two very different binding curves (i) and (ii) for the conditions \([\text{Bca}]_{\text{tot}} = 80 \ \text{and} \ 300 \ \text{µM}\), respectively (Figure 4C). The binding curve (i) was largely non-competitive with a linear relationship between the concentration of \([\text{Cu}^+(\text{Bca})_2]^3^-\) and total \([\text{CueP}]/[\text{Cu(I)}]\) at low \([\text{CueP}]/[\text{Cu(I)}]\) range, allowing a reliable estimation of binding stoichiometry of two Cu(I) per CueP protein molecule. The binding curve (ii) was non-linear, indicating competitive Cu(I) binding under higher concentrations of Bca. This allows a satisfactory curve-fitting of the experimental data to a two-site binding model and derivation of a \(K_D = 10^{-15.9}\) M for CueP assuming identical affinity for the two sites (Table 2).

The above experiments demonstrate that CueP possesses a Cu(I) binding affinity comparable to that of n-ScsB but with doubling Cu(I) binding capacity. On the other hand, the Cu(I) affinity of \(\text{SscS}\) is ~100 times weaker than that of CueP or n-ScsB.

**SscS binds Cu(II) with sub-nanomolar affinity**

The environment of periplasm is generally more oxidizing than that of cytosol and the copper ions in periplasm may exist in redox equilibrium between Cu(I) and Cu(II) (21). Therefore, the possibility of periplasmic proteins \(\text{SscS}\) and CueP to interact with Cu(II) was investigated. The first experiment was designed to test whether Cu(II) may be reduced by the free Cys thiols in \(\text{SscS}\) and CueP upon interaction. \(\text{SscS}_{\text{red}}\) and \(\text{CueP}_{\text{red}}\) were each incubated with one equivalent of Cu(II) (added as \(\text{CuSO}_4\) solution). After half an hour incubation under anaerobic conditions, unreacted Cys thiols were trapped with excess iodoaceticamide (IAA, 100 equivalents) in the presence of BCS (bathocuproinedisulfonic acid) and EDTA (each 5 equivalents). BCS and EDTA exhibit high affinity for Cu(I) and Cu(II) \((\log \beta_2 = 19.8\) for \([\text{Cu}^+(\text{Bcs})_2]^3^-\) and \(\log K_a = 15.9\) for \(\text{Cu}^{11}\text{-EDTA at pH 7.4}\) (28) and can free Cys thiols in each protein from copper binding allowing an effective trapping by IAA.

The reaction mixture was analyzed for Cu oxidation state by electrospray ionization mass spectrometry (ESI-MS). Control samples of \(\text{SscS}_{\text{red}}\) and \(\text{CueP}_{\text{red}}\) without added Cu(II) were also analysed. Approximately 15% of the reduced \(\text{SscS}\) was oxidized by Cu(II) to the disulfide form, less than the predicted 50% oxidation for a full Cu(II) reduction. In contrast, the reduced CueP was oxidized stoichiometrically by one equivalent of Cu(II) to produce about 50% oxidized form which contained one disulfide bond (Figure 5A, Figure 5B, Table S2). These experiments demonstrate that reduced \(\text{SscS}\) is somewhat resistant to oxidation by Cu(II) but reduced CueP reacts with Cu(II) and therefore is not compatible for Cu(II) binding studies.

The interaction of \(\text{SscS}\) with Cu(II) was evaluated quantitatively with the two dansyl peptide (DP) probes DP1 and DP2 recently reported (30). These probes fluoresce intensively at \(\lambda_{\text{max}} \sim 550\) nm upon excitation at ~330 nm, but Cu(II) binding induces fluorescence quenching. They each bind one equivalent of Cu(II) with different affinities \((K_D = 10^{-8.1}\) M for DP1 and \(10^{-10.1}\) M for DP2 at pH 7.4) and are effective probes for detection of Cu(II) binding to proteins with comparable affinity. \(\text{SscS}_{\text{cox}}\) was tested initially to avoid potential complication of Cu(II)-induced protein thiol redox chemistry. Titration of Cu(II)-taken as \(\text{CuSO}_4\) into a solution containing an equal molar concentration of DP1 and \(\text{SscS}_{\text{cox}}\) induced initially minor fluorescence quenching with two apparent turning points at \([\text{Cu(II)}]/[\text{DP1}] \sim 1.0\) and 2.0, respectively, while a control titration of DP1 probe generated only one titration turning point at
[Cu(II)]/[DP1] ~ 1.0 (Figure 5C). This indicates that ScsCox can bind one equivalent of Cu(II) with affinity considerably higher than that of DP1 which was estimated to be $K_D = 10^{-9.5}$ M at pH 7.4 (Table 2). This value was matched by an equivalent independent estimation with the DP2 probe that derived a $K_D = 10^{-9.6}$ M for ScsCox (Figure 5D, Table 2). Intriguingly, equivalent experiments with ScsCred estimated an indistinguishable $K_D = 10^{-9.6}$ M with either DP1 or DP2 probe (Figure 5C, Figure 5D). This revealed that the Cys-xx-Cys motif is not involved in Cu(II) binding, although they are the key ligands for Cu(I). Although an equal molar mixture of Cu(II) and ScsCred detected partial oxidation of the two Cys thiol groups (Figure 5A), such redox chemistry is less likely under the experimental conditions where Cu(II) is constrained stably by the DP probes. However, the nature of the Cu(I) binding site in ScsC has yet to be determined.

An equivalent experiment with the DP1 probe and n-ScsBox estimated a Cu(II) binding affinity of $K_D = 10^{-8.4}$ M, which is weaker than that of ScsCox but is slightly stronger than that of DP1 (Figure S3). Furthermore, with the higher affinity probe DP2, we were not able to detect the weak Cu(II) binding to the n-ScsBox protein. However, the low levels of Cu(II) reduction by n-ScsBred prevented a meaningful estimation of its Cu(II) binding affinity.

**ScsB and ScsC transfer Cu(I) to CueP**

To further explore the biological role of Scs proteins in copper resistance (12, 24), we investigated the ability of Scs proteins to transfer bound copper to CueP using a well-established protocol for detection of copper transfer (29, 31, 32). The periplasmic Cu chaperone CueP binds copper in its reduced state (22). The Cu(I) bound forms of n-ScsB and an MBP fused SscC, which was prepared to increase the molecular weight of SscC for separation from CueP, were incubated for half an hour with reduced apo-CueP in a 1:1 molar ratio. The lack of Cu(I) in reduced apo-CueP was confirmed prior to mixing with the Cu(I) loaded Ssc proteins (Figure S4). The protein mixture was then loaded onto a size exclusion column and elution fractions were analysed by SDS-PAGE as well as spectrophotometrically after the addition of the Cu(I) probe BCS. These experiments showed that Cu(I) is primarily present in the CueP-containing elution fractions but not in those corresponding to n-ScsB and SscC (Figure 6A, Figure 6B) indicating that both n-ScsB and SscC transferred Cu(I) to CueP in vitro. The similar Cu(I) binding affinities of SscB and CueP are consistent with an incomplete transfer of Cu(I) between the two proteins (Figure 6B). Conversely, the Cu(I) binding affinity of SscC is ~100 times weaker than that of CueP (Table 2), allowing a complete transfer of Cu(I) from SscC to CueP (Figure 6A).

Given that the reduced form of CueP is the functional form for copper binding (33), we also investigated whether CueP is maintained reduced by the Ssc reducing system. AMS free thiol trapping assays did not show any productive redox interactions between SscBred and SscCred with CuePox (Figure S5A, Figure S5B). Similarly, under our experimental conditions, we did not observe reduction of CuePox by DsbCred (Figure S5C). These results seem to contradict previous work that described DsbC as a CueP reductase (33).

**DISCUSSION**

*S. Typhimurium* contains an extended collection of periplasmic oxidoreductases including the Dsb proteins, which contribute to *Salmonella* fitness and virulence (2, 34, 35) and the less studied *scs* gene cluster, which plays a role in tolerance to high levels of copper (12, 24). The work presented here provides the first mechanistic evidence of the dual function of the Ssc machinery, which acts as a Dsb-like disulfide reductase system with an additional function in copper homeostasis.

The limited number of detailed studies examining Ssc proteins are beginning to uncover an intriguing diversity among individual Ssc proteins across different organisms, particularly SscC. For example, *Caulobacter crescentus* and *Proteus mirabilis* SscC are dimeric and trimeric isomerases, respectively that form redox relays with the membrane protein SscB (13, 36). Conversely, *S. Typhimurium* SscC is a monomeric protein which structurally resembles DsbA (14). In this study we aimed at characterizing the *S. Typhimurium* SscC/SscB pair and defining the molecular basis for SscC/SscB mediated copper resistance trait.

We first tested the oxidase activity of *S. Typhimurium* SscC and showed that despite the remarkable structural similarity with the DsbA
thiol oxidase (Figure 1B) and their very close reduction potentials (~132 mV vs ~126 mV), ScsC is not able to catalyze disulfide formation in the standard peptide substrate used to assay DsbA disulfide transfer rates (9,14). This suggests that ScsC may have a different substrate specificity or act as a reductase of substrates with higher reduction potentials. We have shown that ScsC is maintained in the reduced state in the periplasm of Salmonella suggesting that this is the functional form of the protein (14). The next question to address was to identify its cognate reductase. The \textit{scsC} gene is often located directly downstream of \textit{scsB}, a predicted DsbD-like membrane disulfide reductase (12,13). Using AMS-gel-shift assays we showed that oxidized \textit{S. Typhimurium} ScsC is rapidly reduced upon addition of reduced n-ScsB, which demonstrates that the two proteins form a redox relay. Previous work showed functional redox interactions between SesB and oligomeric SesC in \textit{P. mirabilis} and \textit{C. crescentus} (13,36) and our new data demonstrates that monomeric ScsC is also efficiently reduced by n-ScsB.

SPR analysis of the interactions between n-ScsB and ScsC in different redox states revealed relatively low binding affinities (~300 µM; Table 1), which are consistent with \(K_D\) values previously reported for transient electron transfer interactions between Dsb proteins (27,37). Remarkably, these affinities were independent of the redox-state of the interacting proteins and did not provide a mechanism for the unidirectional flow of electrons between n-ScsB and ScsC. Determination of the reduction potential of the n-ScsB active site disulfide by equilibration with DTT showed a more reducing reduction potential of n-ScsB (-256 mV) compared to ScsC (-132 mV) (14), specifying that the unidirectional electron transfer between these two proteins is thermodynamically driven.

In the \textit{Salmonella} periplasm the ScsC/ScsB pair co-exists with Dsb oxidative and reductive pathways and therefore there is a possibility of an interplay between these thiol/disulfide exchange systems. As shown by SPR and AMS-gel shift assays, n-ScsB does not efficiently interact with and reduce DsbA under the conditions tested. This is remarkable given the structural similarity between DsbA and ScsB’s cognate substrate ScsC. Similarly, we did not detect any functional interaction between ScsC and DsbA, indicating that these pathways are maintained separate with little or no cross talk. This is consistent with previous work that showed large kinetic barriers between \textit{E. coli} oxidative DsbA/B and reductive DsbC/D pathways (38). Additionally, we measured the interplay between the ScsC/ScsB and DsbC/DsbD reducing systems and showed that while reduced n-DsbD efficiently transfers electrons to oxidized ScsC, n-ScsB reduction of DsbC is inefficient. The apparent redundancy in the catalytic activity of ScsB and the common disulfide reductase DsbD questions the need for a specific reductase for ScsC. We postulate that the presence of a dedicated reductase in the \textit{scs} gene cluster may be necessary to facilitate the transfer of a fully functional gene cluster across bacteria and to ensure co-transcription and functional coupling of all \textit{scs} genes. Further, under copper stress co-transcription of ScsC with its reductase ScsB supports the importance of maintaining reduced ScsC to remove toxic Cu(I) from the cell.

\textit{Salmonella} Scs proteins have been shown to play a role in protection against copper toxicity (14,24,39). Although all Scs proteins contribute to \textit{Salmonella} copper tolerance, ScsB is the most important Scs factor followed by ScsC (24). In this work we aimed at deciphering the molecular basis of ScsB/ScsC mediated copper resistance. Our data showed that the two main Scs players in \textit{Salmonella} copper homeostasis bind Cu(I) with high affinities (\(K_D\) of \(10^{-14.8}\) M and \(10^{-13.1}\) M for ScsB and ScsC, respectively). The stoichiometry was 1:1 and only the proteins in the reduced form were able to bind Cu(I), suggesting that the catalytic cysteines play a dominant role in Cu(I) binding, which is consistent with the role played by cysteines in other comparable systems (40-44). Under aerobic conditions periplasmic copper may cycle between Cu(I) and Cu(II) oxidation states (45). Similar to other metallochaperones (46) ScsC is also able to bind Cu(II) but the determined affinity is much weaker than that for Cu(I).

The transcription of \textit{scs} and \textit{cueP} genes is co-regulated by the CpxR/CpxA signal transduction system (24). Although the CueP metallochaperone has been shown to contribute to copper tolerance in the absence of oxygen, CueP has also been shown to express and to contribute to copper resistance under aerobic conditions (16,17,47). We therefore
investigated a possible interplay between these two machineries. Our studies showed that both ScsB and ScsC can efficiently bind Cu(I) and transfer it to the periplasmic chaperone CueP. As such, our data allowed us to propose a mechanism where the Scs and CueP systems work in concert in Salmonella copper resistance. Under copper stress conditions, the envelope stress response system CpxR/CpxA drives the production of Scs proteins and the metallochaperone CueP. These periplasmic proteins can bind Cu(I) with high-affinity, combating copper toxicity in the periplasm and limiting its movement to the cytosol. The different Cu(I) binding affinities displayed by ScsC, ScsB and CueP may play a crucial role in copper homeostasis by allowing a dynamic transfer of toxic copper ions from Scs proteins to CueP, which in turn can deliver it to the stress response enzyme Cu, Zn-superoxide dismutase (Cu/Zn-SOD) for its functional activation (Figure 7). Copper loaded CueP has been found to be essential for the activation of Cu/Zn-SOD in Salmonella (22). The inner membrane copper transporters CopA and GotT are known to transfer copper from the cytoplasm to CueP (22). Similarly, we show that ScsB/ScsC fulfill a similar role, being able to bind copper and pass it to CueP independent of ATP hydrolysis. Additionally, ScsC also binds Cu(II) with a nanomolar affinity and this may help to scavenge periplasmic Cu(II).

In light of previous work, our findings reveal the ability of the n-ScsB tandem immunoglobulin structural fold to efficiently interact with and reduce a diverse set of ScsC targets including monomers as shown here (Salmonella ScsC), dimers (Caulobacter ScsC) (13) and trimers (Proteus ScsC) (36,48), while discriminating against closely related Dsb proteins such as monomeric DsbA and dimeric DsbC. Future studies will be required to determine the molecular basis for the intriguing specificity of ScsB for diverse ScsC proteins. The Scs proteins may also transfer reducing power to other periplasmic proteins yet to be identified that could play a role in defence mechanisms against oxidative stress.

In summary, our findings show that ScsB and ScsC form a reducing system in the Salmonella cell envelope. We have provided the first biochemical insight into the molecular mechanism underlying Scs mediated copper tolerance in Salmonella. In this machinery, Scs reducing proteins provide protection against copper toxicity by sequestering Cu(I) with sub-picomolar affinities and transferring it to a major periplasmic copper chaperone CueP that binds Cu(I) at femtomolar affinity. Given the distribution of the scs operon across bacteria, similar Scs mediated copper resistance traits may also be present in other organisms including Citrobacter koseri, Klebsiella pneumoniae, and Proteus mirabilis, which encode the important Scs proteins are required for copper homeostasis.

MATERIALS AND METHODS

Production of purified proteins

The coding sequence for the n-terminal domain of Salmonella ScsB (n-ScsB) lacking the signal peptide (residues 26-231) was cloned into a modified version of the expression vector pMCSG7 which encodes the targeted gene as a fusion protein containing a n-terminal (6xHis)-tag, thioredoxin (TRX) and a TEV protease cleavage site. The coding sequences of the mature form (lacking the signal peptide) of Salmonella n-DsbD (residues 23-128) and DsbC (residues 22-237) were cloned into a pET28a-His-TEV vector which encodes the targeted gene as a fusion protein containing a n-terminal (6xHis)-tag followed by a TEV protease cleavage site. The recombinant proteins were produced using a method similar to the one described previously for other S. Typhimurium DsbA-like proteins (14,49). Briefly, all proteins were overexpressed using an autoinduction method (50) and then purified by Ni⁺⁺ affinity chromatography, TEV protease was used to cleave the 6xHis-TRX tag and the cleaved proteins were further purified by reverse Ni⁺⁺ affinity chromatography followed by size exclusion chromatography. Similarly, S. Typhimurium CueP (residues 22–180, accession locus tag STM3650) was purified using a similar method to the one described for n-DsbD and DsbC (49,51). For copper transfer experiments, an MBP fusion construct of ScsC was prepared in order to increase the size of ScsC relative to CueP. The coding DNA sequence for ScsC lacking the signal peptide (residues 19–189) was cloned between EcoRI and HindIII sites in pMAL-c2x vector using the Gibson assembly reagent kit (NEB) (see Supplementary Table S3, for cloning primers) (52). The recombinant fusion
protein was overexpressed using autoinduction and purified using amylose resin (New England Biolabs) with 10 mM maltose.

**Peptide oxidation assay**

The ability of ScsC to catalyze disulfide bond formation in a synthetic peptide substrate was assessed as previously described (26). Briefly, the peptide substrate CQQGFDGTQNSCK with a 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) chelate attached to the N-terminus and a methylcoumarin amide (MCA) group coupled to the ε-amino group of the lysine was purchased from AnaSpec (Fremont, CA). The lyophilized peptide was reconstituted at 2 mM in 100 mM imidazole, pH 6.0. Europium trifluoromethansulfonate (Sigma Aldrich) was added to the peptide solution at a molar ratio of 1:2. Assays were performed in a 96-well Optitrap, on an Envision Multilabel Plate Reader (Perkin Almer) using excitation at 340 nm and emission at 615 nm. Total reaction volume in each well was 50 µl, containing 80 nM of ScsC or DsbA and 2 mM oxidized glutathione (GSSG) in a buffer of 50 mM MES, pH 5.5, 50 mM NaCl, 2 mM EDTA. The reactions were initiated by the addition of 8 µM peptide substrate. The disulfide bond formation was measured using time-resolved fluorescence with a 100 ms delay before reading and a 400 ms reading time. Test was carried out in triplicates per plate and tested on two occasions. The data was analyzed using GraphPad Prism version 5.0 (GraphPad Software, Inc.).

**Electron transfer experiments**

All proteins (n-ScsB, ScsC, DsbA, n-DsbD and DsbC) were prepared at 50 µM and were oxidized and reduced by incubating at 4°C for 1 hour using 10 mM GSSG (Sigma Aldrich, Australia) and DTT<sub>red</sub> (Astral Scientific, Australia), respectively. Excess glutathione and DTT in the reaction mixture were removed by size exclusion chromatography (Superdex 200 10/300 GL GE Healthcare, USA) equilibrated in 10 mM NaPO₄, pH 7.0, 50 mM NaCl and 1 mM EDTA. The final redox state was confirmed by AMS gel shift analysis.

Electron transfer reactions were performed as described previously (53) with some modifications. Briefly, 100 µl of 20 µM reduced catalysts (N-ScsB, N-DsbD) were mixed with 100 µl 20 µM oxidized substrate proteins (ScsC, DsbA, DsbC) (1:1 ratio). Immediately after mixing, 50 µl samples were taken at 15, 120 and 300 second timepoints and quenched with 10% (w/v) trichloroacetic acid (TCA). The samples were centrifuged (16,100 x g for 10 minutes at 4°C) and the pellets were washed with acetone (100% (v/v) and resuspended in 50 mM Tris, pH 7.0, 1% SDS containing 2 mM AMS (4-acetoamido-4'-maleimido stilbene-2,2'-disulfonic acid) (Life Technologies, Australia) to alkylate free thiols (-SH). The alkylation reaction was stopped by adding non-reducing loading dye to each sample and the separation of oxidized and reduced proteins was carried out by SDS-PAGE. Experiments were carried out on three independent occasions.

**Surface Plasmon Resonance (SPR) binding analysis**

A Biacore T200 biosensor instrument (GE healthcare) was used to measure the affinity of the protein-protein interactions. All experiments were performed at 25°C in 20 mM HEPES, pH 7.4, 150 mM NaCl, 0.001% Tween 20. Ligands were immobilized using a standard amine coupling method. Briefly, following activation of the CM5 sensor chip (GE healthcare) with 0.1 M 1-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and 0.25 M n-hydroxysulfosuccinimide (NHS), 20 µg/ml ligand proteins in 10 mM acetate buffer, pH (4.0-4.5) was injected at 10 µl/min until a immobilization level of 500–1000 RU was reached. Residual active NHS esters were blocked by injecting 1 M ethanolamine–HCl (pH 8.5) for 7 min. All analytes were exchanged into the running buffer and injected over the surface at a constant flow of 50 µl/min. To restore the redox state of the immobilized protein after each run, 10 mM reduced DTT was injected to the relevant flow cell at 10 µl/min for 2 min twice. A steady-state affinity model was used to determine the affinity of the protein-protein interactions using the BIAcore evaluation 4.1 software. Experiments were performed in triplicate using different immobilization levels (500-1000 RU).
**Measurement of reduction potential**

The reduction potential of n-ScsB was measured using AMS gel shift analysis as described previously (37). Recombinant n-ScsB (22 µM) was incubated in degassed nitrogen purged buffer containing 100 mM sodium phosphate, pH 7.0, 1 mM EDTA supplemented with 100 mM oxidized DTT (DTT<sub>ox</sub>) (Sigma Aldrich, Australia) and varying concentrations (8 µM-120 mM) of reduced DTT (DTT<sub>red</sub>) (Astral Scientific, Australia). After overnight equilibration at room temperature, the reactions were quenched with 10% (w/v) TCA, sedimented (21,600 x g for 10 minutes at 4°C) and processed as described previously (37). Separation of oxidized and reduced proteins was carried out by SDS-PAGE and the quantities of reduced vs oxidized n-ScsB was determined using density analysis software (ImageJ 1.45)(54). The amount of reduced protein (R) in each reaction was plotted against DTT<sub>red</sub>/DTT<sub>ox</sub> to obtain the equilibrium constant (K<sub>eq</sub>) of the redox reaction using equation 1.

\[
R = \frac{[\text{DTT}_{\text{red}}]}{[\text{DTT}_{\text{ox}}]} = \frac{K_{\text{eq}}}{1 + K_{\text{eq}}}
\]

The corresponding reduction potential values were calculated by substituting the K<sub>eq</sub> value in the Nernst equation (Equation 2).

\[
E^0' = E_{\text{DTT}_{\text{red}}/\text{DTT}_{\text{ox}}}^0 - \frac{RT}{nF} \ln K_{\text{eq}}
\]

Where \(E^0'_{\text{DTT}_{\text{red}}/\text{DTT}_{\text{ox}}} = -312\) mV, F denotes Faraday constant (96485.3 C mol<sup>-1</sup>), n = 2 (number of electron transferred) and RT is the product of the gas constant (8.314 J K<sup>-1</sup> mol<sup>-1</sup>) and the absolute temperature (298.15 K) (55).

**Quantification of Cu(I) binding**

It was conducted based on equations 3 and 4 employing Cu(I) chromophoric complex \([\text{Cu}^{\text{II}}\text{Bca}]^3-\) as a detection probe (Bca = bicinchoninic anion; \(\log \beta_2 = 17.3\); \(\varepsilon_{562} = 7,900\) M<sup>-1</sup> cm<sup>-1</sup>) (31,56):

\[
[\text{Cu}^{\text{II}}\text{L}_2]^3- + P \rightleftharpoons \text{Cu}^{\text{II}}P + 2\text{L}^2- (L = \text{Bca})
\]

\[
\frac{[P]_{\text{tot}}}{[\text{Cu}^{\text{II}}]_{\text{tot}}} = 1 - \frac{[\text{Cu}^{\text{II}}\text{L}_2]}{[\text{Cu}^{\text{II}}]_{\text{tot}}} + K_{\beta_2} \beta_2 \left(\frac{[P]_{\text{tot}}}{[\text{Cu}^{\text{II}}]_{\text{tot}}} - 2\right)^2 \left(1 - \frac{[\text{Cu}^{\text{II}}\text{L}_2]}{[\text{Cu}^{\text{II}}]_{\text{tot}}}\right) (4)
\]

Under the condition of an effective competition for Cu(I) between the probe ligand Bca and the target protein P of equation 3, the Cu(I) speciation is quantitatively described by equation 4. Consequently, the dissociation constant \(K_D\) for the complex \([\text{Cu}^{\text{II}}\text{P}]\) can be estimated via curve-fitting of the experimental equilibrium concentration of the probe complex \([\text{Cu}^{\text{II}}\text{Bca}]^3-\) to equation 4, together with the known \(\beta_2\) for \([\text{Cu}^{\text{II}}\text{Bca}]^3-\) and the known total concentrations of related components (i.e., Cu(I), P and Bca). The equilibrium concentration of \([\text{Cu}^{\text{II}}\text{Bca}]^3-\) may be determined directly from its characteristic absorbance at 562 nm. Decreasing the probe ligand Bca concentration in equation 3 may lead to a non-competitive Cu(I) transfer from \([\text{Cu}^{\text{II}}\text{Bca}]^3-\) to protein P and this allows estimation of the Cu(I) binding stoichiometry of the protein P. The experiments were performed under anaerobic condition in deoxygenated buffers according to the reported protocols (31,56).

**Quantification of Cu(II) binding**

Cu(II) binding to protein was analyzed with two fluorescence dansyl peptide (DP) probes DP1 and DP2 according to a recent report (57). The quantification was based on the competition reaction (5) and the two associated equations 6 and 7:

\[
\text{Cu}^{\text{II}}\text{P} + \text{P} \rightleftharpoons \text{Cu}^{\text{II}}\text{P} + \text{DP}
\]

\[
K_{\text{ex}} = \frac{[\text{Cu}^{\text{II}}\text{P}][\text{P}]}{[\text{Cu}^{\text{II}}\text{P}][\text{P}]} = \frac{K_{\beta_2}\beta_2}{K_D}
\]

\[
\frac{[\text{Cu}^{\text{II}}\text{P}]}{[\text{DP}]} = \frac{F_0 - F_1}{F_0 - F_1} = \frac{\Delta F}{\Delta F_1}
\]

The Cu(II) affinity of the target protein P (expressed as \(K_D(\text{Cu}^{\text{II}}\text{P})\)) may be estimated from equation 6 with the known \(K_D(\text{Cu}^{\text{II}}\text{DP})\) of the DP probe. Since the fluorescence quenching responds to the Cu(II) binding to the DP probe only, the equilibrium concentration \([\text{Cu}^{\text{II}}\text{DP}]\) in equations 5 and 6 can be estimated via equation 7 where \(F_0\) and \(F_1\) are the fluorescence intensity of the DP probe upon binding 0, <1.0 and 1.0 equivalent of Cu(II), respectively. Other terms in equation 6 were determined via mass balance relationships. The experimental protocols followed those reported (57).
**Cu(I) transfer experiments**

All proteins used in the copper transfer experiments were fully reduced prior to each experiment and redox state confirmed by the Ellman assay (58). For the copper transfer experiments an MBP fused form of ScsC (ScsC-MBP) was prepared, to increase the molecular weight of ScsC relative to CueP (MBP has been previously shown not to bind copper (59, 60)). Cu(I)-loaded forms of n-ScsB and ScsC-MBP were prepared by incubation of each metal-free apo-proteins with CuSO₄ and NH₂OH at a 1:5:10 molar ratio in 20 mM MOPS, 50 mM NaCl pH 7.0 for 30 min. To remove free (excess) copper, the reaction mixture (~1 mL) was applied onto a size exclusion chromatography column (Superdex 75 10/300 GL, (GE Healthcare, USA)) equilibrated in 20 mM MOPS, 50 mM NaCl pH 7.0. Peak fractions were analyzed by SDS-PAGE and the Cu(I) chromophoric ligand BCS was added to detect copper content at 483 nm (31).

To assess the copper transfer ability of these proteins, Cu(I) bound forms of n-ScsB and ScsC-MBP, were incubated for half an hour with reduced apo-CueP in a 1:1 molar ratio. The protein mixture was loaded onto a size exclusion column (Superdex 75 10/300 GL, (GE Healthcare, USA)) and elution fractions were analysed by SDS-PAGE for protein and by BCS for Cu(I).
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Conflict of Interest
The authors declare that they have no conflicts of interest with the contents of this article.
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This article contains Figure S1-S5, Table S1-S3

Abbreviations

Dsb, disulfide bond; *S. Typhimurium*, *Salmonella enterica* serovar Typhimurium; AMS, 4-acetoamido-4′-maleimidylstilbene-2,2′-disulfonic acid; BCA, bicinchoninic acid; BCS, bathocuproinedisulfonic acid; r.m.s.d., root mean square deviation
Table 1: Equilibrium dissociation constants (K_D). K_D values for selected complexes between *Salmonella* Scs and Dsb proteins in the different redox states.

| Immobilized ligand | Injected analyte | K_D (μM) |
|--------------------|-----------------|----------|
| n-ScsB_red         | ScsC_ox         | 310 ± 46 |
| n-ScsB_ox          | ScsC_red        | 336 ± 38 |
| n-ScsB_red         | ScsC_red        | 233 ± 56 |
| n-ScsB_ox          | ScsC_ox         | 361 ± 12 |
| n-ScsB_red         | DsbA_ox         | No interaction |
| n-ScsB_ox          | DsbC_ox         | 334 ± 26 |
| n-DsbD_red         | ScsC_ox         | 422 ± 26 |
| n-DsbD_ox          | DsbC_ox         | 300 ± 51 |
| n-DsbD_ox          | DsbA_ox         | No interaction |

Table 2. Summary of Cu(I) and Cu(II) binding properties

| Protein      | Cu(I) binding at pH 7.3 | Cu(II) log K_D at pH 7.4 | log K_D^a | with DP1 | with DP2 |
|--------------|-------------------------|--------------------------|-----------|---------|---------|
|              | Stoichiometry           |                          |           |         |         |
| n-ScsB(SH)_2 | 1                       | -14.8(1)                 | nd^b      | nd^b    |         |
| n-ScsB(SS)   | 0                       | -                        | -8.4(2)   |         | -       |
| ScsC(SH)_2   | 1                       | -13.1(1)                 | -9.5(1)   | -9.6(1) |         |
| ScsC(SS)     | 0                       | -                        | -9.5(1)   | -9.6(1) |         |
| CueP(SH)_3   | 2                       | -15.0(1)                 | nd^b      | nd^b    |         |

^a The bracketed values are the errors in the last digits estimated from two sets of experiments with total about 10 individual experimental data points.

^b not determined due to the redox sensitivity between Cu^{2+} and the reduced protein form.
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FIGURES

Figure 1. Salmonella Dsb-like systems. A, Schematic representation of Dsb-like systems in Salmonella. Salmonella has evolved complex machineries to support disulfide bond formation in the periplasm. This includes the classic Dsb oxidases, Dsb reductases and a less characterized Scs proteins. Figure was created with BioRender. B, Structural comparison of S. Typhimurium ScsC and DsbA. Ribbon representation of the secondary structure elements of DsbA (left; PDB entry 3L9S) and ScsC (right; PDB entry 4GXZ). The thioredoxin (TRX)-fold and inserted helical domain of both proteins are shown in dark blue and dark pink, respectively. Inset shows a close-up view of the CxxC active sites with sulfur atoms shown as yellow sticks. DsbA and ScsC superposition gave a r.m.s.d. value of 3.3 Å for 128 of the 189 Cα aligned. C, In vitro disulfide oxidase activity. Top panel: Schematic representation of the peptide oxidation assay. A europium ion (Eu(III)) in a 1,4,7,10-tetraazaacyclododecane-1,4,7,10-tetra acetic acid (DOTA) chelate fluoresces when in close proximity to the coumarin chromophore (aminoacyl 7-amino-4-methyl-coumarin amide; MCA) (i.e. when the two cysteines form a disulfide bond). Bottom panel: Representative fluorescence curves of peptide cysteine oxidation by Salmonella DsbA (closed squares, ■), ScsC (closed circles, ●) and buffer alone (open square, □). DsbA efficiently oxidizes a fluorescently labelled peptide substrate in the presence of 2 mM GSSG. ScsC shows no oxidizing activity as compared with the buffer control. FRET: Fluorescence Resonance Energy Transfer. Error bars correspond to the standard deviation calculated from three independent experiments.
Figure 2. Characterization of the SscC – SscB redox relay pair. A, Electron transfer from reduced n-SscB to oxidized SscC. Stoichiometric amounts (20 µM) of both proteins were mixed and the reaction mixtures were quenched with 10% TCA after 15, 120 and 300 seconds, followed by AMS alkylation and separation by SDS-PAGE. Control samples: (1) reduced SscC; (2) oxidized SscC; (6) reduced n-SscB and (7) oxidized n-SscB. Electron transfer from reduced n-SscB to oxidized SscC was confirmed by the change of redox state judged by the shift of the bands corresponding oxidized SscC and reduced n-SscB by 1 kDa up and down, respectively (lanes 3 to 5). B, Electron transfer from reduced SscC to oxidized n-SscB. Control samples: (1) reduced n-SscB; (2) oxidized n-SscB; (6) reduced SscC and (7) oxidized SscC. The lack of an upward band-shift for oxidized n-SscB (lanes 3 to 5) indicates no reduction of this protein upon mixing with an equimolar amount of reduced SscC. C, SPR analysis of SscC binding to immobilized n-SscB. The representative sensogram of n-SscBred binding to ScsCox was double referenced and shown together with equilibrium binding analysis. A series of concentrations (0-650 µM of oxidized SscC), were injected over the reduced n-SscB immobilized surface. The apparent equilibrium dissociation constants \( K_D \) was determined using a steady-state affinity model. The data are expressed as mean ± standard error of the means (SEM). All experiments were conducted on at least three independent occasions with fresh immobilization. D, Reduction potential determination of n-SscB. Redox equilibrium of n-SscB. n-SscB was equilibrated with redox buffers containing various concentrations of reduced and oxidized DTT. After 24 h, all the samples were treated with 10% TCA, followed by AMS alkylation and separation using SDS-PAGE (bottom panel). Fraction of reduced n-SscB plotted against the \([\text{DTT}_\text{red}]/[\text{DTT}_\text{ox}]\) ratio (top panel). The plots were fitted to equation 1 to determine \( K_{eq} \) as described in the materials and methods. Error bars correspond to the standard deviation calculated from three independent experiments.
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Figure 3. Interaction between the Scs system and Dsb system in Salmonella. A, Electron transfer from reduced n-ScsB to oxidized DsbA. Control samples: (1) reduced DsbA; (2) oxidized DsbA; (6) reduced n-ScsB and (7) oxidized n-ScsB. Partial electron transfer from reduced n-ScsB to oxidized DsbA was confirmed by the change of redox state for some of the proteins as judged by the shift of the band corresponding oxidized DsbA and reduced n-ScsB by 1 kDa up and down, respectively (lanes 3 to 5). B, Electron transfer from ScsC to DsbA. Control samples: (1) reduced DsbA; (2) Oxidized DsbA; (6) reduced ScsC and (7) oxidized ScsC. The lack of a band-shift for oxidized DsbA and reduced ScsC (lanes 3 to 5) suggests an absence of electron transfer from reduced ScsC to oxidized DsbA. C, Electron transfer from n-DsbD to ScsC. Control sample lane (1) reduced ScsC; (2) oxidized ScsC; (6) reduced n-DsbD and (7) oxidized n-DsbD. The band-shift for ScsC and n-DsbD by 1 kDa up and down, respectively (lanes 3 to 5) suggests an efficient electron transfer from reduced n-DsbD to oxidized ScsC. D, Electron transfer from n-ScsB to DsbC. Control samples: (1) reduced DsbC; (2) oxidized DsbC; (6) reduced n-ScsB and (7) oxidized n-ScsB. Only a small amount of oxidized DsbC (lane 2) and reduced n-ScsB (lane 6) shift by 1 kDa up and down, respectively (lanes 3 to 5) suggesting a less efficient electron transfer from reduced n-ScsB to oxidized DsbC. Red; Reduced, Ox; Oxidized.
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Figure 4. Quantification of Cu(I) binding with chromophoric probe \([\text{Cu(I}(\text{Bca})_2]^{3-}\). Change in \([\text{Cu}(\text{Bca})_2]^{3-}\) concentration for a series of solutions containing \([\text{Cu(I)}]_{\text{tot}} = 32-33\ \mu M\) and \([\text{Bca}]_{\text{tot}} = 80\ \mu M\) for (i) or 300 \(\mu M\) for (ii) as a function of increasing concentrations of fully reduced protein n-ScsB (A), ScsC (B) or CueP (C). The red empty circles are the experimental data for each fully oxidized protein form under the condition (i) and the red cross are the data upon further addition of reductant dithionite (1.0 mM) into the solution. The dotted lines in (A) and (C) are simple interpolations of the experimental data for non-competitive Cu(I) transfer that defined Cu(I) binding stoichiometry of one and two for n-ScsB and CueP, respectively. The solid traces show fitting of the experimental data to binding models of one Cu(I) site for n-ScsB and ScsC and of two indistinguishable Cu(I) sites for CueP. The derived affinity data (expressed as dissociation constant \(K_D\)) are listed in Table 2. All experiments were conducted under anaerobic condition in MOPS buffer (50 mM, pH 7.3) containing 200 \(\mu M\) \(\text{NH}_2\text{OH}\) and 100 mM NaCl.
Figure 5. Analysis of interaction of Cu(II) with ScsC and CueP. A, B, ESI-MS analysis of fully reduced ScsC (A) and fully reduced CueP (B) upon alkylation of free Cys thiols with iodoacetamide (IAA) in the presence of EDTA and BCS: (i) fully reduced apo-protein control; (ii) an equal molar mixture of fully reduced apo-protein and CuSO₄ after incubation for 30 min. The identity of each protein component was labelled, and their molar masses are given in supplementary Table S2. P(SA) refers to the alkylated protein thiol P(S-CH₂CONH₂) with a net increase in molar mass by 57 kDa. C, D, Quantification of Cu(II) binding to ScsC via fluorescence quenching of probe DP1 (C) or DP2 (D) upon Cu²⁺ titration: (i) Cu(II) binding curve for each probe only (2.0 µM); (ii) binding curves for equal molar mixtures of DP probe and ScsC (each 2.0 µM; green, fully reduced form; red, fully oxidized form). The solid traces are the simple interpolations of the experimental data. All experiments were conducted in MOPS buffer (5 mM, pH 7.4).
Figure 6. Copper transfer from n-ScsB and ScsC to CueP. SEC chromatograms corresponding to: A, top panel: Cu(I)-ScsC only; bottom panel: Cu(I)-ScsC after mixing with one molar equivalent of apo-CueP. B, top panel: Cu(I)-n-ScsB only; bottom: Cu(I)-n-ScsB after mixing with one molar equivalent of apo-CueP. Fractions of the eluted protein under each peak were analysed by SDS-PAGE (bottom panels). The copper content of the eluted protein containing fractions was measured by adding 25 mM ascorbic acid and 0.2 mM BCS and then measuring absorbance at 483 nm (dashed blue line). Asterisk indicates the protein mixtures before the SEC analysis. Fractions under the peak labelled as ‘excess copper’ were also analysed by BCS assay and SDS-PAGE, which in all cases showed a high abundance of copper but no protein content (data not shown).
**Figure 7. Overview of the proposed model for ScsC/ScsB mediated copper protection of *Salmonella*.** Under copper stress conditions, the envelope stress response system CpxR/CpxA drives the production of Scs proteins and the metallochaperone CueP. ScsB and ScsC form a functional redox relay that efficiently transfers electrons from the cytoplasm via ScsB to ScsC through a cascade of thiol–disulfide exchange reactions. Reduced ScsC and ScsB can then bind Cu(I) with sub-picomolar affinity and transfer it to the metallochaperone CueP, which in turn delivers it to the stress response Cu, Zn-superoxide dismutase (Cu/Zn-SOD). Collectively, these systems contribute to combating copper toxicity in the *Salmonella* periplasm and limit copper movement to the cytosol as well as contributing to defence mechanisms against reactive oxygen species. Figure was created with BioRender.
The Ses disulfide reductase system cooperates with the metallochaperone CueP in Salmonella copper resistance

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