The copper-responsive ScsC protein of *Salmonella* promotes intramacrophage survival and interacts with the arginine sensor ArtI

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Running title

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Abbreviations

AMS, 4-Acetamido-4'-Maleimidylstilbene-2,2'-Disulfonic Acid; CcScsB, ScsB proteins of *Caulobacter crescentus*; CcScsC, ScsC proteins of *Caulobacter crescentus*; DTNB, 5,5′-Dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; FBS, Fetal-bovine serum; GSSG, oxidised glutathione; INF-γ, interferon gamma; IPTG, isopropyl β-D-1-thiogalactopyranoside; LPS, Lipopolysaccharides; PmScsB, ScsB protein of *Proteus mirabilis*; PmScsC, ScsC protein of *Proteus mirabilis*; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; StScsA, ScsA protein of *S.* Typhimurium; StScsB, ScsB protein of *S.* Typhimurium; StScsC, ScsC protein of *S.* Typhimurium; StScsD, ScsD protein of *S.* Typhimurium; TEV protease, Tobacco Etch Virus protease; TRX, thioredoxin.

Abstract

The *scsABCD* (suppressor of copper sensitivity) locus of *Salmonella* encodes four proteins that resemble the disulphide-folding machinery of other bacteria. Previous work has shown that *Salmonella* encounters toxic levels of copper during infection and the Scs system provides protection against this copper-mediated toxicity. The current work reports that expression of the soluble periplasmic protein StScsC is induced by copper, and that intramacrophage survival in the presence of copper is diminished by the loss of StScsC. Using a combination of genetic and proteomics approaches, the abundance of various cysteine-containing periplasmic proteins were found to be elevated by StScsC in the *Salmonella* periplasm, implicating StScsC in the disulphide folding of superoxide dismutases and proteins involved in amino acid sensing and import. Co-purification and mass spectrometry approaches confirmed that the arginine-sensing periplasmic protein ArtI associates with StScsC via a disulphide interaction, and purified ArtI was shown to alter the thiol redox state of purified StScsC. This work reports the first demonstration of a redox partner for the Scs system of *Salmonella*, and provides insights into how this bacterial pathogen responds to copper stress during infection.

Introduction

Disulphide folding is of particular importance in the periplasm of pathogenic bacteria, where motility, type III secretion, and the assembly of a variety of secreted enzymes and toxins all rely upon disulphide folding machinery [1]. The correct formation of disulphides in γ-proteobacteria proceeds via two routes: (i) oxidation of cysteine residues; (ii) disulphide isomerisation. The archetypal DsbAB system catalyses thiol oxidation via the periplasmic DsbA protein, and the electrons are then shuttled to the respiratory chain via the membrane protein DsbB [2]. Non-native disulphides are then corrected by the periplasmic disulphide isomerase DsbC, a process that is driven by electrons from the membrane protein DsbD (previously reviewed [3]). In addition to the DsbAB and DsbCD systems, *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) encodes DsbLI similar to that of uropathogenic *E. coli* [4-6], as well as a plasmid-encoded SrgA that is similar to DsbA [7]. In addition, *S.* Typhimurium encodes a further four thioredoxin (TRX)-like proteins at the *scs* locus (**s**uppressor of **c**opper **s**ensitivity), named after the first functional role to be assigned: the expression of the entire *scsABCD* operon was shown to restore tolerance to copper in copper-sensitive mutants of *E. coli* [8].

ScsA from *S.* Typhimurium (StScsA) has previously been predicted to be a copper-binding protein, and ScsB from *S.* Typhimurium (StScsB) was suggested to be a DsbD-like membrane spanning protein [8]. ScsC from *S.* Typhimurium (StScsC) is a soluble periplasmic protein and StScsD is a membrane-anchored periplasmic protein [9]. Scs proteins are expressed in many other Gram-negative bacteria, including *Proteus mirabilis* [10], *Caulobacter crescentus* [11], and species of *Citrobacter*, *Klebsiella* and *Yersinia* [12]. The ScsB protein of *C. crescentus* (CcScsB) has been shown to maintain the ScsC protein (CcScsC) in a reduced state, which subsequently delivers electrons for peroxide reduction via redox interactions with peroxiredoxins [11]. Similarly, the ScsC protein of *P. mirabilis* (PmScsC) was found to be in the reduced state in the presence of copper [10], and the ScsB protein (PmScsB) was found to provide reducing power for PmScsC-mediated disulphide isomerase activity [13]. The PmScsC protein was found to be important for swarming activity in the presence of copper, although unlike StScsC it did not affect growth in the presence of copper in rich media [10]. Furthermore, CcScsC and PmScsC are dimeric (7) and trimeric [10], respectively, and this oligomerization is mediated via N-terminal extensions that are not present in the monomeric StScsC from *S.* Typhimurium [9, 10, 13]. The presence and absence of the N-terminal extension of the ScsC homologues from different species is easily illustrated using sequence alignments (Fig. 1), and is likely to have implications for the *in vivo* role. Given that the well-known disulphide isomerase of *E. coli* DsbC is a dimer, this suggested that oligomerization of PmScsC was necessary for isomerase activity: indeed, deletion of the N-terminal oligomerization domain converted this protein into a monomeric dithiol oxidase [10]. Hence, these studies are consistent with two functionally distinct classes of ScsC protein, with the *in vivo* role for StScsC remaining elusive prior to the current work.

Copper is an important micronutrient for both bacterial pathogens and hosts cells. However, excess copper can be toxic, disrupting Fe-S clusters [10] and catalysing the production of reactive oxygen species via Fenton chemistry [14]. During infection, ATP7A-dependent copper transport into phagosomes promotes bacterial killing [15], and *Salmonella* responds by exporting copper via the GoI and Cus copper export systems [16]. As mentioned above, the *scsABCD* operon (suppressor of copper sensitivity) of *Salmonella* has previously been shown to be important for copper tolerance [8, 9], and subsequent work has demonstrated that in *Salmonella*-infected macrophages the *scs* locus is required for bacterial proliferation [17]. Indeed, there is a precedent for the involvement of thioredoxin-like proteins in copper tolerance, as the membrane-spanning DsbD (or DipZ) protein has previously been shown to confer tolerance to copper exposure [18]. A subsequent study demonstrated that the conserved cysteine residues Cys^{163} and Cys^{285} of the ScsB homologue DsbD were required for copper tolerance in *E. coli* [19]. The observation that redox-active copper (II) can directly promote the oxidation of disulphide bonds [20] is consistent with these numerous reports that link copper exposure to disulphide folding. Furthermore, given the potential impact of redox stress upon correct disulphide formation, it is unsurprising that the Scs system has also been reported to protect proteins from carbonylation resulting from hydrogen peroxide exposure [12, 21].

The *scs* operon was first described over 20 years ago, when it was suggested that the *scs* locus had two promoter regions that regulate two transcripts encoding StScsA and StScsBCD [8]. However, until recently the transcriptional regulation at this locus has remained understudied. A

recent study showed that deletion of the *scsA* gene results in the upregulation of the *scsBCD* operon, and that the *scsA* gene has a role in the proliferation of *S.* Typhimurium in cortisol-stressed macrophages [17]. Subsequent work has identified a CpxR-binding site upstream of *scsA* genes from a number of enterobacteriaceae, and transcription of the *scs* locus was confirmed to be stimulated by the CpxR/A regulatory system [12], a well-known system that can respond to copper and other envelope stressors previously characterised in *E. coli* [22].

Prior to the current study, several intriguing questions remained for the Scs system of *S.* Typhimurium related to Scs protein expression in response to metal ions, the impact of ScsABCD upon the folding of disulphide-containing proteins, and the potential redox partners for the periplasmic StScsC. Herein, these questions are addressed using a combination of genetic, proteomic and biochemical approaches.

Results

StScsC expression is induced by copper

Given that the all four *scsABCD* mRNA transcripts had previously been shown to be regulated by copper [12], it was of interest to confirm that protein synthesis was also elevated by copper. Hence, wild type *S.* Typhimurium was exposed to 2 mM copper (II) and native StScsC was quantified in the *Salmonella* periplasm via mass spectrometry, which resulted in a 10-fold increase in the abundance of StScsC (Fig. 2A). To confirm that the expression of StScsC was modulated by copper and not by other divalent metals, the culture medium was also supplemented with $ZnSO_4$, $FeSO_4$, and the expression of StScsC was measured by Western blotting with an anti-StScsC antibodies [9]. Only the presence of $CuSO_4$ (Fig. 2B) was shown to increase the expression of the StScsC protein.

Salmonella survival in murine macrophages is significantly impaired by loss of StScsC

Given that membrane-bound disulphide oxidoreductases are generally quite promiscuous and the unique roles of the *scs* operon are therefore likely to be mediated through the periplasmic chaperones StScsC and StScsD, it was of interest to investigate whether loss of the *scsC* gene affected bacterial loads during infection. Activated macrophages were infected with WT and Δ*scsC* strains in the presence of copper (i.e. to stimulate StScsC expression in the wild type), and survival after 3 h and 24 h was expressed as a % of bacterial load following uptake (20 min post-infection). 100 µM copper was used as this concentration has previously been shown to have no effect on the survival of WT *Salmonella* [29]. At 20 min post-infection, there was no significant difference between bacterial loads of the wild-type and the mutant strains (Fig. 3A), confirming that loss of *scsC* did not influence uptake into macrophages. At 3 h post-infection, survival of the wild-type strain was 8-fold higher than that of the Δ*scsC* mutant (Fig. 3B). At 24 h post infection, this trend continued with the wild-type strain displaying approximately 5-fold higher survival compared to the Δ*scsC* mutant.

StScsC elevates the abundance of disulphide-containing proteins involved in peptide uptake

Given the likely role of StScsC in disulphide chemistry in the periplasm of *S.* Typhimurium, it was of interest to investigate the impact of this protein upon the abundance of cysteine- and disulphide-containing secreted and periplasmic proteins. Several studies have reported that DsbA target proteins are degraded in the absence of DsbA (and exhibit lower abundance) [23-25], so a similar approach was employed to identify potential StScsC targets. It was hypothesised that redox active copper may catalyse the introduction of unwanted thiol modifications, and that StScsC is up-regulated under these conditions to promote the correct disulphide folding of specific periplasmic/secreted proteins. It was therefore anticipated that in the absence of StScsC, copper exposure will more readily promote misfolding and degradation of these the target proteins (compared to a wild type strain), resulting in lower abundance. Hence, wild type and Δ*scsC S.* Typhimurium strains were grown in the presence of copper (to induce disulphide stress and to ensure that StScsC is expressed in the WT strain), and protein abundance in periplasmic preparations was analysed via mass spectrometry. From the intial dataset of periplasmic/secreted proteins (Table S1), cysteine- and disulphide-containing proteins were identified and were analysed further (Fig. 4A). All

proteins from Fig. 4A were mapped onto a diagram to illustrate *in vivo* functions for these candidate target proteins (Fig. 4B), and those fitting the hypothesised abundance changes for a StScsC target were again highlighted in red. Notably, proteins involved in amino acid and peptide uptake displayed greater abundance when copper and StScsC were present suggesting that folding of these disulphide-containing periplasmic proteins may be facilitated by StScsC.

StScsC binds to ArtI in the E. coli periplasm

While measuring protein abundance is a useful approach to predict potential interaction partners for StScsC (Fig. 4), further investigation was undertaken using a biochemical technique to trap StScsC bound to redox partners. A recombinant $StScsC_{CXXA}$ variant was engineered where the second cysteine of the CXXC catalytic motif was replaced by an alanine, with the idea that this recombinant $StScsC_{CXXA}$ would form a mixed disulphide *in vivo* with a redox partner (Fig. 5A). The absence of the second thiol group in $StScsC_{CXXA}$ would then limit the ability of $StScsC_{CXXA}$ to dissociate from the redox partner. There is a precedent for using this approach with the TRX-like chaperone DsbG in *E. coli* [26]. The work herein involved expression of his_6-tagged recombinant $StScsC_{CXXA}$ in the *E.coli* periplasm, followed by affinity chromatography and analysis of mixed disulphides via SDS-PAGE in the presence and absence of DTT (Fig. 5B). With the exception of DsbL and SopA, the vast majority of the cysteine-containining potential redox partners highlighted in Fig. 4B have orthologues in both *Salmonella* and *E. coli.* Futhermore, *E. coli* is more amenable to protein expression studies, so was therefore selected as the test system for this work. Copper was omitted from this experiment as it was not required for expression of recombinant $StScsC_{CXXA}$ and was found to cause self-dimerisation problems for the $StScsC_{CXXA}$ protein that did not occur with the wild type protein (data not shown). The 23 kDa band (in both lanes 1 and 2) is the monomeric StScsC protein, and it was hypothesised that the the slower-migrating bands (highlighted by the box in lane 1) that disappear in the presence of DTT could be mixed disulphide complexes containing StScsC. DTT-mediated dissociation of the putative disulphide-bonded complexes was also confirmed by Western blotting using anti-his_6 antibodies (Fig. 5C). The upper bands from lane 1 (Fig. 5B) were then excised from the silver-stained SDS-PAGE gel and analysed via mass spectrometry. The band highlighted by the arrow on Fig. 5B was identified as StScsC bound to ArtI (Arginine ABC transporter substrate binding protein) via identification of peptide fragments using the Mascot search engine [27], N.B. *E. coli* ArtI has a closely-related homologue in *S.* Typhimurium (sequence identity = 95.5 %). Other bands contained StScsC but no other partner protein could be detected, indicating that $StScsC_{CXXA}$ may form an intermolecular disulphide with itself (other bands are likely to be StScsC dimers at various stages of degradation).

Oxidised StScsC is reduced by S. Typhimurium ArtI

To further investigate the role of StScsC in modulating the thiol redox state of ArtI, the redox state of oxidised and reduced StScsC was measured in the presence and absence of ArtI. The *artI* gene from *S.* Typhimurium was cloned and his_6-tagged ArtI was purified from the E. coli cytoplasm via affinity chromatography. His_6-tagged StScsC was also purified in the same way. Both proteins were reated with 5 mM DTT and 20 mM GSSG, exogenous reductant/oxidant was removed using a PD-10 column, and the anticipated redox state of StScsC was confirmed using DTNB as previously described [28]. An example of StScsC-mediated thiol quantitation is shown in Fig. 6A. However, the cysteines of ArtI could not be modified with AMS following addition of DTT even when 1% SDS was present (Fig. 6B), and reduction and alkylation with chloroacetamide was also unsuccessful in modifying ArtI (Fig. 6C). Hence, the interaction of 'as purified' ArtI with oxidised and reduced StScsC was investigated using AMS and Western blotting with anti-StScsC antibodies (Fig. 7). These data show that addition of 'as purified' ArtI to reduced StScsC had no effect (lanes 1-4), whereas incremental addition of 'as purified' ArtI to oxidised StScsC (lanes 5-8) results in a corresponding reduction of StScsC. Hence, these data indicate that ArtI is purified in the reduced state and can undergo disulphide exchange with StScsC. Together with the co-purification data in Fig. 5, these data support a *bona fide* physical interaction between StScsC and ArtI.

Discussion

Disulphide folding of proteins is important for their activity and stability. Copper has previously been shown to accumulate in the *Salmonella* containing vacuoles of macrophages [29], and copper has also been shown to promote the oxidation of native and non-native disulphide bonds [20]. The *scs* operon of *Salmonella* encodes TRX-like proteins that have been shown to confer tolerance to copper [8] that are also controlled by copper at the transcriptional level [12]. Hence, it was hypothesised that the Scs system and copper play a combined role in disulphide folding during infection. Herein, our initial goal was to demonstrate that copper could induce the expression of the periplasmic StScsC protein beyond the mRNA level tested in previous studies [12]. Expression of the soluble periplasmic StScsC protein was confirmed to be induced by copper using Western blotting (and not by zinc or iron). Given the presence of a copper-responsive CpxR-binding site in the *scsA* promoter region [12] it is logical that copper is an activator.

To gain insights into the importance of the periplasmic StScsC protein during infection, macrophage survival assays were carried out for wild type and Δ*scsC* strains in the presence of exogenous copper (i.e. to ensure that StScsC was expressed in the wild type). These experiments gave a clear result that showed a significant decrease in survival when StScsC was lost, indicating an important role for StScsC targets for intramacrophage survival. This observation is consistent with previous work that demonstrated that loss of the *scsA* gene gave a similar result [17], especially in light of subsequent reports that the entire operon is under the control of the *scsA* promoter [12] and that polar effects of the *scsA* mutation might be expected to diminish the expression of StScsC.

A powerful approach to investigate the functional role for TRX-like proteins is to measure the abundance of candidate target proteins under conditions where the chaperone of interest is differentially expressed: the hypothesis being that in the absence of the chaperone the target proteins will undergo misfolding and degradation, resulting in lower abundance. To this end, the proteomics study described herein was undertaken to identify protein targets that displayed elevated abundance in the presence of copper (which stimulates StScsC production) and were less abundant when copper-mediated expression of StScsC could not take place (i.e. in the *scsC* mutant). This approach identified the Zn/Cu superoxide dismutases SodC/SodCII and a range of peptide/amino acid transporters (ArgT, GltI, and HisJ) as potential target proteins for StScsC (Fig. 4B, highlighted in red). The abundances of these proteins are clearly elevated by the presence of StScsC, indicating that StScsC may facilitate disulphide folding in these proteins during copper exposure. Since StScsC is only expressed in the presence of copper, we presume that disulphide bonds are introduced into these target proteins by the Dsb system when copper is absent, but additional disulphide folding chaperones (i.e. the Scs system) are required to facilitate this process when copper is present. While the current work has identified a number of proteins involved in amino acid uptake as potential interaction partners for StScsC, the lower abundances of the superoxide dismutases in the *scsC* mutant may render this strain susceptible to an oxidative burst and may partially explain the macrophage survival data (Fig. 3).

To provide an independent approach to identify target proteins for StScsC, a his-tagged $ScsC_{CXXA}$ mutant protein was expressed in *E. coli* with the aim of trapping StScsC as a mixed disulphide with a redox partner (Fig. 5A). This method has been used previously to identify DsbG targets [26]. Using a combination of affinity chromatography and mass spectrometry, the arginine-binding periplasmic protein (ArtI) was found to form a disulphide bridge with $ScsC_{CXXA}$ (Fig. 5B). In addition, loss of *scsC* did decrease ArtI abundance, although *t*-tests did not support this as a significant difference (Table S1). Interestingly, ArtI is part of a family of disulphide-containing peptide/amino acid binding proteins, including HisJ, ArgT, GltI, ArtJ, and ProX (Fig. 4B). HisJ, ArgT, and GltI were measured at significantly lower abundance in the Δ*scsC* strain compared to wild type, suggesting a potential interaction of these periplasmic chaperones with StScsC. Given that the relative abundance of ArtI compared to these other chaperones is much lower (Fig. 4A), it is surprising that ArtI is the only protein that was identified in the $StScsC_{CXXA}$ trapping experiment, although this might reflect a preferential binding to ArtI.

To further invesigate the interaction between StScsC and ArtI, the 'as purified' ArtI was added to oxidised and reduced StScsC and was shown to reduce the oxidised StScsC in a dose-dependent manner (Fig. 7). This clearly supports the idea that a disulphide bond can form between StScsC and ArtI, and that the heterodisulphide complex that was purified (Fig. 5) is not an artefact of the CXXA mutation described above. At this stage it is not possible to conclude whether *in vivo* reduced ArtI interacts with oxidised StScsC, or whether the reverse reaction takes place (i.e. oxidised ArtI interacts with reduced StScsC). However, given that StScsC has previously been shown to exist

predominantly in the reduced state in the *S.* Typhimurium periplasm [9], the working hypothesis is that reduced StScsC may act to repair an oxidised cysteine species in ArtI. Furthermore, the ArtI protein has only two cysteine residues so non-native internal disulphides are not possible, as is the case for all potential targets identified in Fig. 4. It is therefore hypothesised that StScsC may act to repair low molecular weight modifications of cysteine sidechains on periplasmic proteins, as depicted for ArtI on the model shown in Fig. 8. Clearly, it is a little premature to speculate on the exact mechanism for StScsC, but since copper has been shown to catalyse disulphide formation [20] and *Salmonella* is exposed to peroxide stress within the macrophage (via superoxide dismutase activity), two potential thiol modifications would be glutathionylation or sulfenylation (reversible addition of S-OH in the presence of peroxides). In addition, the Scs system has previously been shown to be important for tolerance to peroxide stress [12], which adds weight to the hypothesis that StScsC may play a role in the repair of sulfenylated cysteines. However, this modification is depicted as S-X in the working hypothesis shown in Fig. 8 until future studies can determine the exact nature of the *in vivo* interaction between StScsC and ArtI. Given the difficulties encountered with modifying the cysteine residues in ArtI (Fig. 6B-C), future work may include a range of thiol modification experiments under a variety of denaturing conditions.

To further investigate potential structural interactions between StScsC and ArtI, a structural model for ArtI was generated with the RaptorX server [30] using a homologous template (PDBid = 2Y7I) from the PDB (Fig. 9). This revealed a structural role for the intramolecular disulphide in ArtI that is remote from the arginine binding cleft. In addition, while the disulphides in both StScsC and the ArtI model are largely buried in their oxidised state, it is conceivable that sulphur atoms on ArtI may become exposed if modified with low-molecular weight adducts and become available for disulphide exchange with reduced StScsC.

During infection, the utilisation of amino acids has previously been shown to be important for intracellular proliferation of *S. Typhimurium* [31]. The *artI* gene is encoded by the *artMQIP* operon in *E. coli* and *Salmonella* [32], and is regulated by the ArgR repressor in response to arginine. While *in vitro* amino acid binding experiments with purified ArtI were unsuccessful [32], a functional role in L-arginine sensing has been reported where ArtI is required for the L-arginine-mediated modulation of c-di-GMP levels in *S.* Typhimurium [33]. At this point, it is also worth noting that the L-arginine uptake protein ArgT [32], a predicted target for StScsC (Fig. 4), has previously been shown to deplete the L-arginine pool in macrophages to use as a carbon source. Furthermore, preliminary investigations demonstrate a modest growth stimulation for wild type *Salmonella* following the addition of L-arginine, which is not seen in a *scsC* mutant strain (Fig. 10). Hence, it is hypothesised that during copper exposure (e.g. within the macrophage) StScsC may also protect ArgT from deleterious thiol modifications and promote arginine acquisition from host cells. This work provides novel insights into how copper and StScsC impacts directly upon arginine metabolism in *S.* Typhimurium, which has broader implications for growth and survival within the macrophage.

Materials and methods

Bacterial strains, plasmids, and culture conditions

S. Typhimurium SL1344 strain (wild-type and deletion mutant Δ*scsC*) and *E. coli* strains (BL21[DE3], DH5α and JM109) were cultured at 37°C on solid Luria Bertani (LB) agar or in liquid LB broth supplemented with the appropriate antibiotics; streptomycin (40 µg/ml), ampicillin (125 µg/ ml), chloramphenicol (34 µg/ ml), kanamycin (50 µg/ ml). Growth medium was supplemented with 1- 2 mM $CuSO_4$, $FeSO_4$, and $ZnSO_4$. *E. coli* culture media were supplemented with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) to induce expression of StScsC, ArtI and $ScsC_{CXXA}$ from plasmids shown in Table S2. Cloning of $scsC_{CXXA}$ and *artI* was performed using the primers shown in Table S3. Genomic DNA of SL1344 was isolated with GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich) according to manufacturer's instructions. The *artI* gene was then amplified by PCR from SL1344 genomic DNA with primers shown in Table S3. Amplified PCR product was cloned into the pTrcHis plasmid by Gibson assembly according to the manufacturer's instructions (NEB). The mutant $scsC_{CXXA}$ gene was designed to encode an OmpA signal peptide and a C-terminal His_6-tag, and was synthesised using the Invitrogen GeneArt Gene synthesis service. (Thermo-fisher). This ORF was then amplified and sub-cloned into the BamHI/FspI sites of the pSU2718 plasmid. His-

tagged StScsC protein was overexpressed in *E. coli* BL21 (DE3) cells using the pET21a_*scsC*_LIC vector plasmid as previosuly described [9].

Macrophage survival assays

RAW-Blue macrophage (derived from the murine RAW 264.7 macrophages) (raw-sp, Invivogen) stocks (~1.5 ml) were maintained in DMEM (41966029, Gibco) containing 10 % (v/v) heat-inactivated FBS (Sigma-Aldrich) and 200 U/ml of penicillin-streptomycin (Sigma-Aldrich) at 37°C in humidified air containing 5 % CO_2. The day before infection, macrophages were harvested (500 xg for 5 min) and 1.5 x 10^5 RAW-Blue macrophages were plated in a flat bottom 96-well plate. In order to prime and activate the macrophages, 1ng/ ml of INF-γ Recombinant Human Protein (PHC4033, Gibco) and 10 ng/ ml LPS (Sigma-Aldrich) were added to the culture and the total volume of the suspension was adjusted to 100 µl by the addition of DMEM without Penicillin-Streptomycin. Cells were then incubated for 18 h (37°C in humidified air containing 5% CO_2). Wild-type and ***ΔscsC S.*** Typhimurium overnight cultures were harvested and the pellets were washed in 1x PBS. Bacterial pellets were resuspended in DMEM media and bacterial counts were obtained by measuring the optical density of the cultures at 600 nm. Pre-primed Raw-Blue macrophages were infected with *S.* Typhimurium at a multiplicity of infection (MOI) of 10 (10 bacteria: 1 macrophage). To ensure that StScsC was expressed in *S.* Typhimurium, infections were performed in the presence of 100 µM $CuSO_4$, a concentration previously used to avoid toxicity to macrophages [29]. After 20 min of infection, medium was removed and 100 µl of media containing 200 µg/ ml gentamicin was added to the wells for elimination of the extracellular bacteria. After 10 min of incubation, spent medium was removed and macrophages were washed three times with 100 µl PBS then the macrophages were lysed with 100 µl of 0.01% Triton X-100 in PBS to enumerate bacterial uptake. Identical samples were also not lysed, and 35 µg/ ml gentamicin was added for continuous infections for 3 h and 24 h prior to macrophage lysis. Lysates were serially 10-fold diluted and plated on agar plates with antibiotics (Streptomycin (40 µg/ ml) for wild-type *S.* Typhimurium and Kanamycin (50 µg/ ml) for ***ΔscsC S.*** Typhimurium). Plates were incubated at 37°C for 16 h and intracellular bacterial counts were determined by enumerating colony forming units (CFU).

Periplasmic protein fractionation

Periplasmic protein fractions were prepared by cold osmotic shock. 1 L bacterial cultures were grown to an OD_{600} of 1.5 at 37°C and were mixed with 14.25 mL of 1 M NaCl and 14.25 mL of 1 M Tris/HCl pH 7.3. The suspension was then centrifuged at 3,000 rcf for 20 min at 20°C. The supernatant was then collected and the pellet was resuspended in 3.5 mL of supernatant and 3.5 mL of TSE buffer (40 % sucrose, 33 mM Tris/HCl pH 7.3 and 2 mM EDTA), incubated at room temperature for 20 min and then centrifuged at 5,000 rcf for 15 min at 20°C. The pellet was then resuspended in 10 mL of ice cold dH_2O and mixed on ice for 45 s. 10 µl of 1 M $MgCl_2$ was then added and the suspension was mixed on ice for 45 s and the incubated statically on ice for 20 min. The suspension was centrifuged at 5,000 rcf for 15 min at 4°C, and the supernatant was collected (periplasmic fraction). The concentrations of protein samples were measured using the Markwell assay [34].

Mass spectrometry

For native ESI-MS analysis, 10 µg of periplasmic protein samples were dissolved in solubilisation buffer (8 M urea, 100 mM ammonium carbonate, 20 mM DTT, 0.2 % octyl-beta glucoside) for 1 h then alkylated with 100 mM iodoacetamide for 15 min at room temperature. Subsequent trypsin digestion was performed where appropriate. BSA standard was used as an internal standard to allow label free quantitation where known concentration of BSA was added to the protein samples. Protein samples were placed into an ACQUITY UPLC M-Class System (Waters) for sample separation. 4 µl of each sample was injected to the column. Peptides were trapped on a 180 µM X 20 mm Acquity Symmetry C18 (5µM) column for 3 min and then separated on a 75 µM X 150 mm Acquity UPLC HSS T3 column with 1.8 µM particles (Waters). All peptides were separated with a 40 min gradient and data were collected for 60 min. The trapping flow rate was 15 µl/min with 97 % A and 3 % B (A = water with 0.1 % formic acid, B = acetonitrile with 0.1 % formic acid). The flow rate was then

changed to 0.3 µl/min and the mobile phase was replaced with 3 % A and 40 % B over a 40 min linear gradient. Peptides were analysed by Synapt G2-Si Mass spectrometer (Waters) fitted with a Zspray nanospray source. The fragment ion data was collected via the HDMSE approach. Peptides were then analysed using Progenesis QI software against the TrEMBL *Salmonella* database.

For identification of the peptides from SDS-PAGE, the bands of interest were excised from the gel, reduced, alkylated and digested with trypsin as previously described [35]. Extracted peptides were spotted onto a MTP Anchorchip MALDI-TOF plate (Bruker). Samples were allowed to dry then 1 µl of matrix solution was added on top (0.7 mg/ml alpha-Cyano-4-hydroxycinnamic acid dissolved in solvent mixture containing 85% acetonitrile, 15% water, 0.1% TFA and 1 mM $NH_4H_2PO_4$). The plate was placed into a MALDI-ToF mass spectrometer (Ultraflextreme, Bruker). The instrument was calibrated with commercially-available Bruker Peptide Calibration Standard II (Bruker, part number 222570). The spectra from the extracted peptides were collected using the settings as described; Polarity: +ve, Laser frequency: 2 kHz, ion sources: 25 kV and 22.35 kV, Lens: 7.5 kV, pulsed ion extraction: 80 nS, range: 700-3500 Da, data sampling rate: 4 Gs/s. For each sample, 3500 shots were summed and saved. The mass spectrometry data files were exported from the Flexicontrol software from Bruker Matrix Science. An automatic search against the TrEMBL *E. coli* database (downloaded from Uniprot) was performed using the Mascot search engine.

Electrospray LC-MS of Intact Proteins

Purified ArtI was alkylated with chloroacetamide or reduced with DTT and alkylated with chloroacetamide. Electrospray mass spectra were recorded on a Bruker micrOTOF-Q II mass spectrometer. An aliquot of each sample, corresponding to approximately 20 picomoles of protein was desalted on-line by reverse-phase HPLC on a Phenomenex Jupiter C4 column (5 µm, 300Å, 2.0 mm x 50 mm) running on an Agilent 1100 HPLC system at a flow rate of 0.2 ml/min using a short water, acetonitrile, 0.05% trifluoroacetic acid gradient. The eluent was monitored at 280 nm and then directed into the electrospray source, operating in positive ion mode, at 4.5 kV and mass spectra recorded from 500-3000 m/z. Data was analysed and deconvoluted to give uncharged protein masses with Bruker's Compass Data Analysis software.

Protein purification

StScsC, and ArtI were overexpressed in the *E. coli* cytoplasm as N-terminally his_6-tagged proteins, and $StScsC_{CXXA}$ was overexpressed in the *E. coli* periplasm as a C-terminally his_6-tagged protein. Cells were harvested (9,000 rpm, 4 °C, 30 min), sonicated on ice, and cell debris was removed via centrifugation (15,000 rpm, 4 °C, 30 min). The soluble cell extract was purified by metal affinity chromatography as previously described [9, 36]. Resin was washed with 10 column volumes of buffer (25 mM Tris pH7, 150 mM NaCl and 0.5 % Triton X-100) containing 10 mM imidazole and the protein was eluted with buffer containing 300 mM imidazole. Proteins were exchanged into 150 mM HEPES or 50 mM NaCl pH7 (pH 8 for ArtI) using a PD-10 column. To cleave the his-tag on purified StScsC, 1 µg TEV protease was incubated with purified StScsC overnight at room temperature, and uncleaved protein was removed the following day via affinity chromatography.

Isolation of StScsC heterodisulphide complexes from the periplasm

His-tagged $StScsC_{CXXA}$ was overexpressed in *E. coli* JM109 cells grown in LB broth (180 rpm, 37 °C). When the OD_{600} reached 0.5, 1 mM IPTG was added and the culture was grown for a further 4 h. Cells were harvested and his-tagged $StScsC_{CXXA}$ was purified as described above. Purified protein samples were mixed with loading dye (50 mM Tris, 2 % SDS, 0.5 % Bromophenol Blue, 10 % Glycerol, +/- 100 mM DTT) (1:1), boiled for 5 min at 95 °C and resolved on a 12 % SDS-PAGE gel. Proteins were then transferred to a PVDF membrane for Western blotting. An anti-his_6 antibody coupled with alkaline phosphatase (ab49746, Abcam) (1:5000) was used for the detection of his-tagged $StScsC_{CXXA}$ protein. Blots were stained with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (B5655, Sigma-Aldrich).

Redox exchange reactions with ArtI and StScsC

Purified non-tagged StScsC and his_6-tagged ArtI were quantified using the Markwell assay [34]. StScsC was reduced with 5 mM DTT and oxidised with 20 mM GSSG for 1 h at room temperature, and the thiol redox state was assessed using DTNB as previously described [28]. StScsC was incubated with 'as purified' ArtI for 1 h at room temperature and was then exposed to the thiol modification reagent AMS as previously described [37]. Briefly, protein samples were precipitated by mixing with excess cold acetone in a 4:1 ratio (v/v). The samples were then kept at -70 °C for 10 min then incubated at - 20 °C for 4 h. The samples were centrifuged at 5,000 x g for 15 min at 4 °C. Supernatant was removed and the tubes were dried using a vacuum centrifuge for 15 min to eliminate any acetone residue. Precipitated protein samples were then resuspended in 8 µL of 20 mM AMS in 50 mM Tris/ HCl, 1% SDS buffer and incubated at room temperature for 10 min. Samples were then resolved via SDS-PAGE for analysis of the mass shift [37]. Western blots for the detection of StScsC protein were performed using an anti-StScsC antibody [9] (1:5000) and an anti-rabbit IgG coupled with alkaline phosphatase (Sigma-Aldrich) (1:5000) as the secondary antibody. Blots were stained with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (Sigma-Aldrich).

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Author contributions

BY GKR and MS planned experiments; BY and MS performed the experiments; BY, GKR and MS analysed the data; BY, GKR and MS wrote the paper.

Conflicts of interest

There are no conflicts of interest that could inappropriately influence, or be perceived to influence, this work.

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Supporting Information

Table S1. Effects of StScsC upon protein abundance in the *S.* Typhimurium periplasm.
Table S2. List of bacterial strains and plasmids
Table S3. List of primers

Figure legends

Fig. 1. Sequence alignment of ScsC proteins. The amino acid sequences of ScsC from *Salmonella* Typhimurium *SL1344* (Uniprot accession number: A0A0H3NBX5), *Klebsiella pneumoniae* (Uniprot accession number: A0A377VNR4), *Yersinia frederiksenii* (Uniprot accession number: A0A380PSJ7), *Citrobacter youngae ATCC 29220* (Uniprot accession number: D4BBN6), *Caulobacter crescentus* (Uniprot accession number: Q9A747) and *Proteus mirabilis* (Uniprot accession number: A0A1Z1SYD5). Fully conserved residues are shaded in gray and residues showing 50 % conservation are shaded in purple. The CXXC motif is highlighted by the red box. The top three sequences (*Salmonella*, *Klebsiella* and *Citrobacter)* are shown to lack the N-terminal oligomerisation domain. The alignment was generated using the Clustal tool in Bioedit.

Fig. 2. StScsC expression is induced by copper in *S.* Typhimurium. (A) The abundance of StScsC in the *Salmonella* periplasm was measured using electrospray ionisation mass spectrometry (ESI-MS). In the presence of 2 mM copper, the abundance of StScsC was found to be elevated by ~10-fold (red bar). Error bars showing SEM of 4 repeats. *P*-value < 0.05 for unpaired *t-test* is indicated by an asterisk. (B) Wild-type *Salmonella* was grown in the presence of 2 mM $ZnSO_4$, $FeSO_4$ and $CuSO_4$, and StScsC expression was detected via Western blotting with anti-StScsC antibodies. The black box highlights that the 23 kDa StScsC is only expressed in the presence of copper. Equal amounts of protein was loaded for each periplasmic sample measured by Markwell assay [34]. This experiment was performed twice and data shown are representative of reproducible trends.

Fig. 3. StScsC promotes *S . Typhimurium* survival within macrophages during copper stress. RAW-Blue macrophages were infected with wild-type (black bars) and Δ*scsC* (red bars) *S .* Typhimurium strains using culture medium supplemented with 100 μM $CuSO_4$. (A) Bacterial uptake into macrophages where intracellular bacterial survival is expressed as % survival at 20 min post-infection (i.e. following bacterial uptake). (B) Intracellular bacterial survival is expressed as % survival at 3 h and 24 h post infection compared to 20 min post-infection (i.e. following bacterial uptake). Datapoints represent averages of five repeats. Asterisks denote *P* < 0.01 for two-way ANOVA, and error bars show standard deviation.

Fig. 4. StScsC influences the abundance of disulphide- and cysteine-containing periplasmic secreted in *S.* Typhimurium. (A) Proteomic quantitation of disulphide-containing periplasmic/secreted proteins. The protein abundance (fmol) in 0.27 μg of total periplasmic protein of wild-type and ***ΔscsC*** *S.* Typhimurium strains grown in the presence of 2 mM copper (II). Data points are averages of 6 repeats and error bars show SD. P-values for unpaired *t*-tests are indicated (* p<0.1, ** p<0.05 and *** p<0.001). (B) Diagram to show the function of proteins in Fig. 4A. The abundances of the proteins highlighted in red are predicted StScsC targets where protein abundance is diminished by loss of StScsC.

Fig. 5. StScsC forms a mixed disulphide with ArtI. (A) Model for $StScsC_{CXXA}$ forming a mixed disulphide with target proteins (T) in the presence of low molecular weight thiols (LMTs). (B) SDS-PAGE analysis of purified $ScsC_{CXXA}$ in the absence (lane 1) and the presence (lane 2) of DTT. Black boxes indicate a higher molecular weight protein hypothesised to be StScsC disulphide-bonded to a target protein, which dissociates when DTT is present. The protein band indicated by the arrow was analysed using MALDI-TOF mass spectrometry and determined to contain peptides from both StScsC and ArtI. This experiment was performed four times and data shown are representative of

reproducible trends. (C) Western blot detection of StScsC partner. Western blot analysis of purified recombinant $ScsC_{CXXA}$ in the absence (lane 1) and the presence (lane 2) of DTT. $ScsC_{CXXA}$ was detected with anti-his_6 antibodies. Boxes indicate higher molecular weight proteins that disappear when DTT is added. This experiment was performed twice and data shown are representative of reproducible trends.

Fig. 6. Measurement of thiol redox state for StScsC and inaccessibility of ArtI cysteine residues. (A) Measurement of thiol redox state in StScsC**.** The redox state of StScsC was determined using DTNB as previously described [28], and an example dataset is shown above for 'as purified' StScsC. The inset shows the change in A_{412} upon the addition of increasing StScsC. Fully oxidised StScsC displayed no change in A_{412}. To calculate the concentration of reduce thiols, ε_{412} (TNB) = 13.8 $mM^{-1}cm^{-1}$ was used. (B) SDS-PAGE analysis of thiol redox status of ArtI. Purified ArtI was treated with 5 mM DTT and 20 mM GSSG, and ArtI protein was isolated by acetone precipitation and resuspended in buffer containing 20 mM AMS, 50 mM Tris/ HCl, 1% SDS. Samples were resolved via SDS-PAGE, and both reduced (R) and oxidised (O) ArtI migrated at the same rate, suggesting that AMS cannot modify cysteine residues of ArtI. This experiment was performed twice and data shown are representative of reproducible trends. (C) As-purified ArtI (black trace) was analysed via mass spectometry and was found to be 27630 Da, corresponsing to the expected molecular weight for his_6-tagged ArtI (27761 Da) minus the initiation methionine (loss of 131 Da). Exposure to the alkylating agent chloroacetamide (red trace) or 'reduction with DTT + chloroacetamide' (blue trace) did not alter the molecular weight of ArtI.

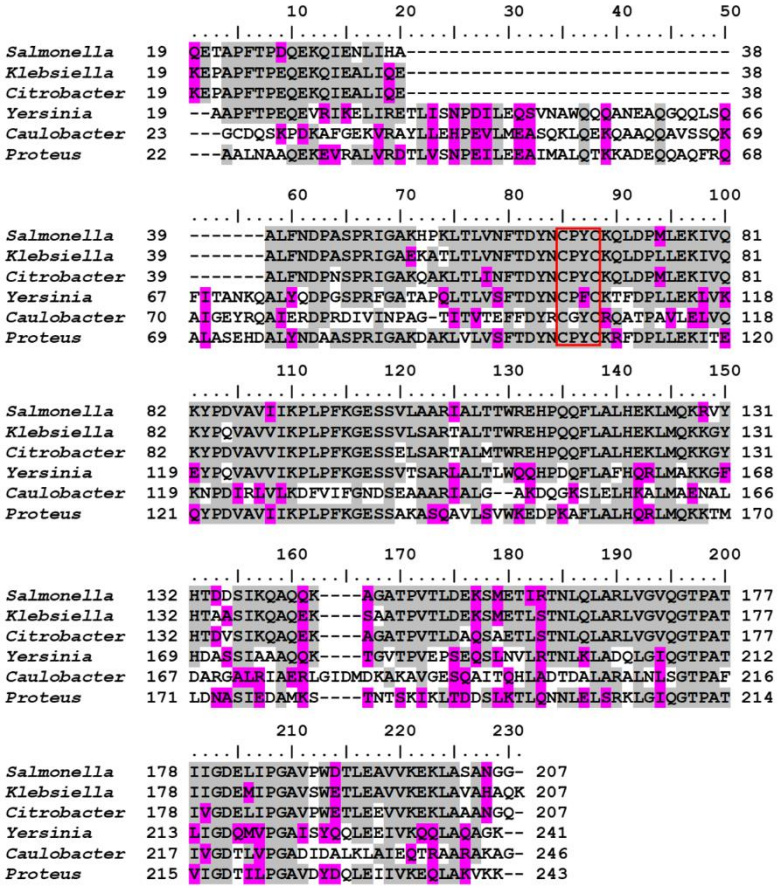
Fig. 7. ArtI alters the redox status of oxidised StScsC. 'As-purified' ArtI and oxidised (O)/reduced (R) StScsC were incubated together under various conditions and Western blot analysis with anti-StScsC antibodies was used to probe the redox status of StScsC. The decrease in migration rate of oxidized StScsC with increasing concentrations of ArtI, following treatment with AMS, demonstrates that ArtI promotes the reduction of StScsC. ArtI does not have any effect on reduced StScsC. Dual colour protein standards (Bio-Rad) were used (M).

Fig. 8. Model for copper and StScsC in arginine sensing/uptake during intramacrophage survival. During infection, *Salmonella* is engulfed by macrophages and copper ions are targetted to the *Salmonella* containing vacuoles (SCVs). (1) Copper ions present in the bacterial periplasm may promote deleterious thiol modifications (S-X) in periplasmic proteins, including ArtI, leading to misfolding and degradation. (2) StScsC repairs modified thiols on ArtI, and the Dsb system introduces a disulphide. (3) Disulphide-folded ArtI senses L-arginine (R).

Fig. 9. Crystal structure of *Salmonella* ScsC and structural model of ArtI. (A) Ribbon structure of StScsC. Coordinates were obtained from PDB file 4GXZ (39). Yellow spheres indicate sulphur atoms of cysteine residues at positions Cys48 and Cys51. (B) Electrostatic surface structure of StScsC. Surface is omitted from the exposed active site cysteine residues to show the sulphur atoms (yellow spheres). White surface indicates hydrophobic surface adjacent to the cysteine residues. (C) The predicted structure of SL1344 ArtI was obtained using the RaptorX server based on a homologous template from the PDB (PDBid = 2Y7I). Arginine that was bound to the protein is indicated. Sulphur atoms are shown in yellow spheres. (D) Electrostatic surface of the protein was shown. Surface is omitted from the exposed active site cysteine residues to show the sulphur atoms (yellow spheres).

Fig. 10. Loss of *scsC* abolishes L-arginine-mediated stimulation of growth. (A) Wild-type *S.* Typhimurium was grown in M9 medium (inc. 0.2% glucose, 1 mM copper) in the presence and absence of 0.5 mM L-arginine. After 8 h, cell density was higher in the presence of L-arginine. (B) *S.* Typhimurium Δ*scsC* strain was grown as described for panel A in the presence and absence of 0.5 mM L-arginine. (C) *S.* Typhimurium Δ*scsC* strain complemented with plasmid pScs (the *scs* genes were reintroduced) [9] was grown as described for panel A in the presence and absence of 0.5 mM L-arginine. After 8 h, cell density was higher in the presence of L-arginine. Datapoints represent averages of a minimum of three repeats. Error bars show standard error of the mean.

FIGURE 1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Klebsiella 19 KEPAPFTPEQEKQIEALIQE---------- 38
Citrobacter 19 KEPAPFTPEQEKQIEALIQE---------- 38
Yersinia 19 --AAPFTPEQEVRIKELIRETLISNPDILEQSVNAWQQQANEAQGQQLSQ 66
Caulobacter 23 ---GCDQSKPDKAFGEKVRAYLLEHPEVLMEASQKLQEKQAAQQAVSSQK 69
Proteus 22 ---AALNAAQEKEVRALVRDTLVSNPEILEEAIMALQTKKADEQQAQFRQ 68
60 70 80 90 100
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Klebsiella 39 -------ALFNDPASPRIGAEKATLTLVNFTDYNCPYCKQLDPLLEKIVQ 81
Citrobacter 39 -------ALFNDPNSPRIGAKQAKLTLINFTDYNCPYCKQLDPMLEKIVQ 81
Yersinia 67 FITANKQALYQDPGSPRFGATAPQLTLVSFTDYNCPFCKTFDPLLEKLVK 118
Caulobacter 70 AIGEYRQAIERDPRDIVINPAG-TITVTEFFDYRCGYCRQATPAVLELVQ 118
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110 120 130 140 150
Salmonella 82 KYPDVAVIIKPLPFKGESSVLAARIALTTWREHPQQFLALHEKLMQKHVY 131
Klebsiella 82 KYPQVAVVIKPLPFKGESSVLSARTALTTWREHPQQFLALHEKLMQKKGY 131
Citrobacter 82 KYPDVAVVIKPLPFKGESSELSARTALMTWREHPQQFLALHEKLMQKKGY 131
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Caulobacter 119 KNPDIRLVLKDFVIFGNDSEAAARIALG--AKDQGKSLELHKALMAENAL 166
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Yersinia 169 HDASSIAAAQQK----TGVTPVEPSEQSLNVLRTNLKLADQLGIQGTPAT 212
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Yersinia 213 LIGDQMVPGAISYQQLEEIVKQQLAQAGK-- 241
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FIGURE 2

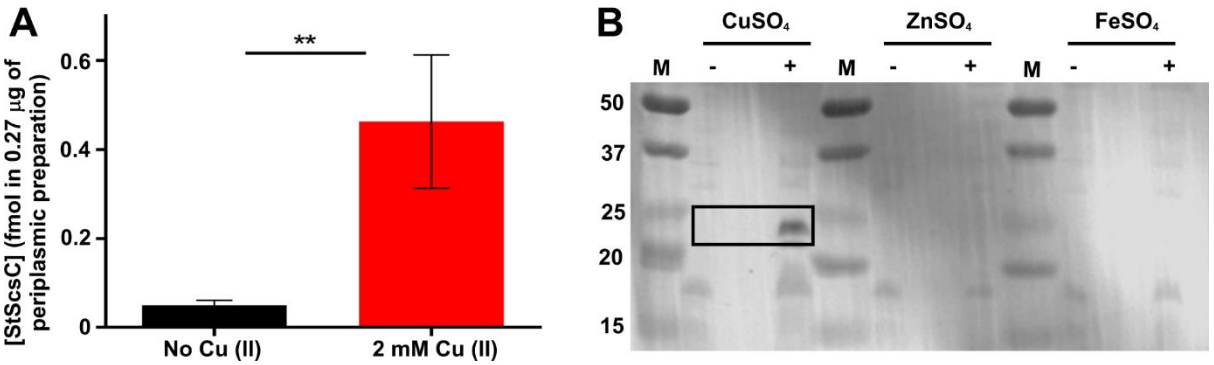
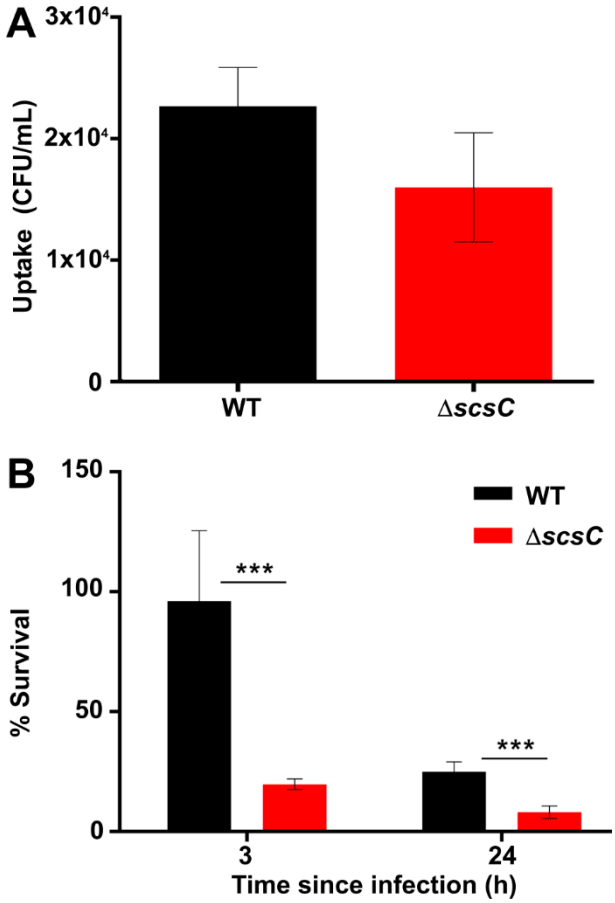

A
[StScsC] (fmol in 0.27 μg of periplasmic preparation)
0.6
0.4
0.2
0
**
No Cu (II)
2 mM Cu (II)
B
CuSO4
ZnSO4
FeSO4
M
-
+
M
-
+
M
-
+
50
37
25
20
15

FIGURE 3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3x10⁴
2x10⁴
1x10⁴
0
Uptake (CFU/mL)
WT
ΔscsC
B
150
100
50
0
% Survival
WT
ΔscsC
***
***
3
24
Time since infection (h)

FIGURE 4

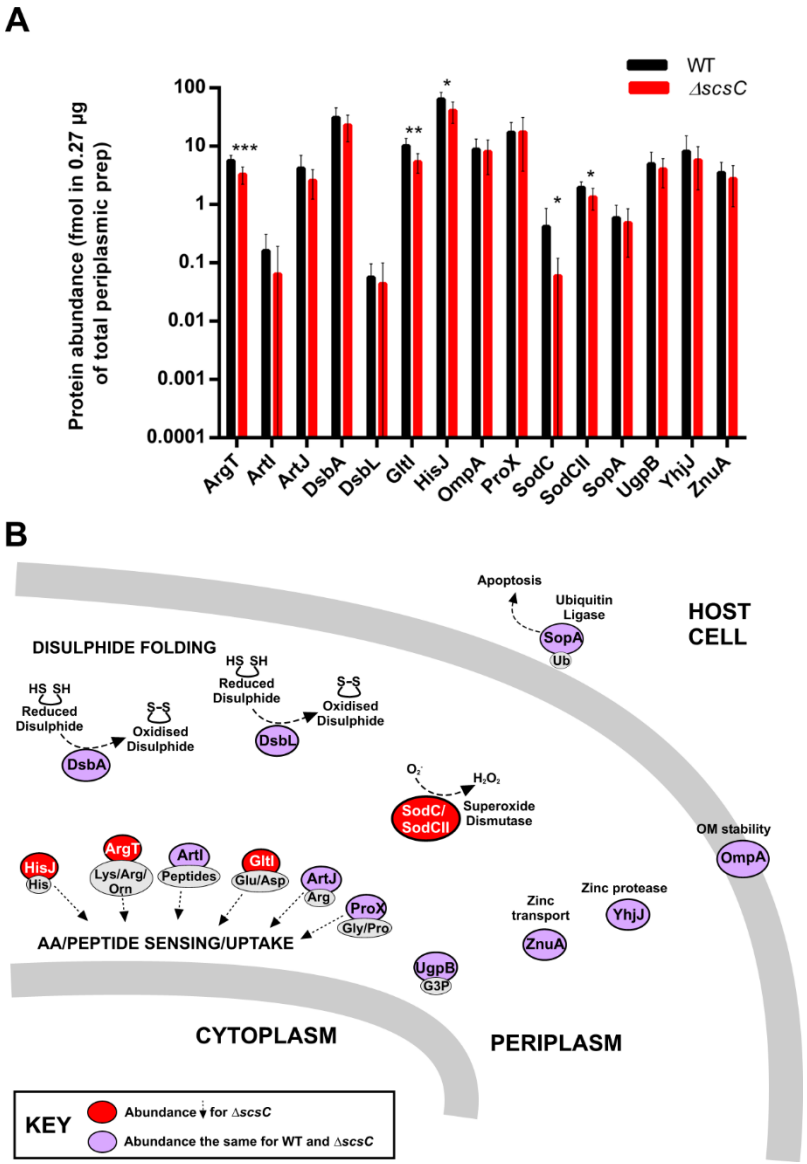

A
WT
ΔscsC
Protein abundance (fmol in 0.27 µg of total periplasmic prep)
100
10
1
0.1
0.01
0.001
0.0001
***
**
*
*
*
ArgT
ArtI
ArtJ
DsbA
DsbL
GltI
HisJ
OmpA
ProX
SodC
SodCII
SopA
UgpB
YhjJ
ZnuA
B
HOST CELL
Apoptosis
Ubiquitin Ligase
SopA
Ub
DISULPHIDE FOLDING
HS SH
Reduced Disulphide
S-S
Oxidised Disulphide
DsbA
DsbL
O2-
H2O2
SodC/ SodCII
Superoxide Dismutase
OM stability
OmpA
HisJ
His
ArgT
Lys/Arg/ Orn
ArtI
Peptides
GltI
Glu/Asp
ArtJ
Arg
ProX
Gly/Pro
AA/PEPTIDE SENSING/UPTAKE
Zinc transport
ZnuA
Zinc protease
YhjJ
UgpB
G3P
CYTOPLASM
PERIPLASM
KEY
Abundance ↓ for ΔscsC
Abundance the same for WT and ΔscsC

FIGURE 5

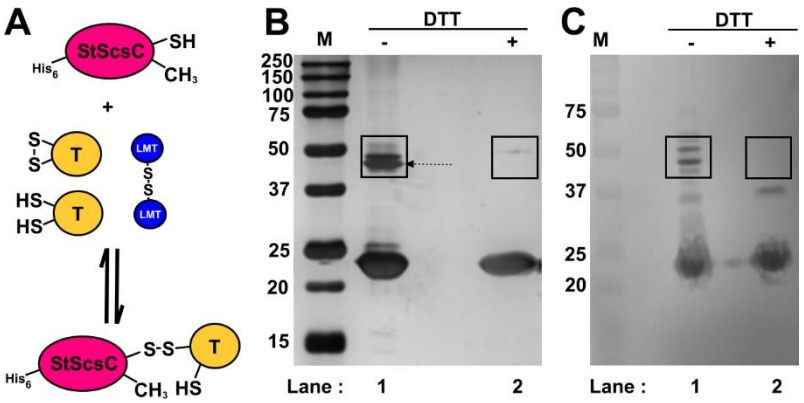

A
StScsC
SH
His6
CH3
+
S
S
T
LMT
HS
HS
T
LMT
StScsC
S-S
T
His6
CH3
HS
B
M
DTT
-
+
250
150
100
75
50
37
25
20
15
Lane :
1
2
C
M
DTT
-
+
75
50
37
25
20
Lane :
1
2

FIGURE 6

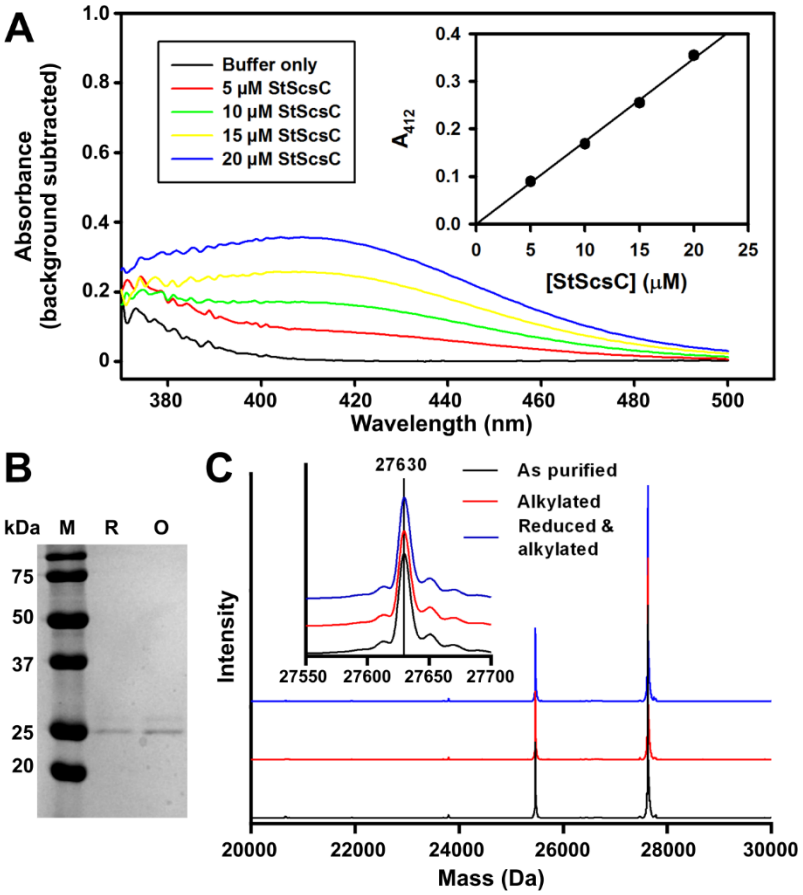

A
Absorbance (background subtracted)
Wavelength (nm)
Buffer only
5 µM StScsC
10 µM StScsC
15 µM StScsC
20 µM StScsC
A_{412}
[StScsC] (µM)
B
kDa M R O
75
50
37
25
20
C
Intensity
27630
As purified
Alkylated
Reduced & alkylated
Mass (Da)

FIGURE 7

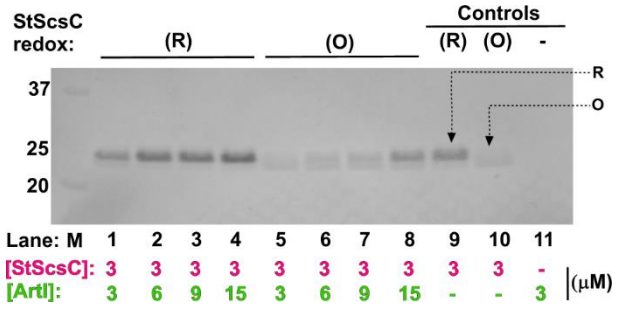

StScsC redox:
(R)
(O)
Controls
(R) (O) -
37
25
20
R
O
Lane: M 1 2 3 4 5 6 7 8 9 10 11
[StScsC]: 3 3 3 3 3 3 3 3 3 3 -
[ArtI]: 3 6 9 15 3 6 9 15 - - 3
(μM)

FIGURE 8

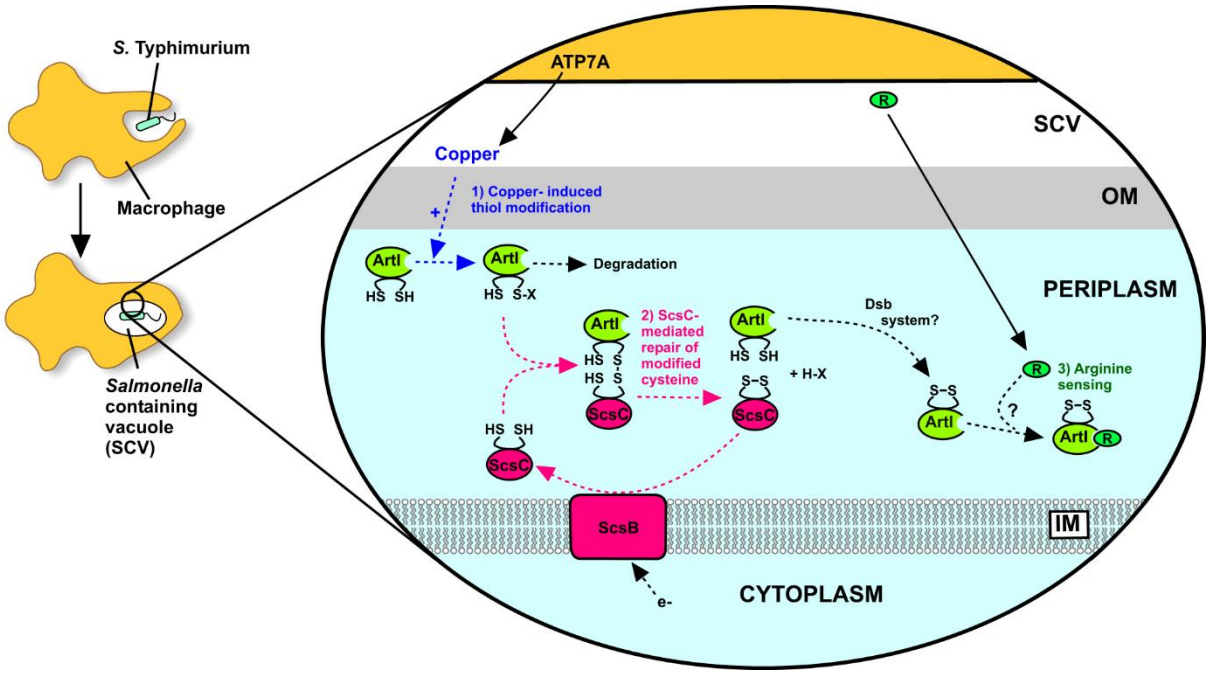

S. Typhimurium
Macrophage
Salmonella containing vacuole (SCV)
ATP7A
Copper
SCV
OM
1) Copper- induced thiol modification
PERIPLASM
ArtI
HS SH
HS S-X
Degradation
2) ScsC-mediated repair of modified cysteine
ScsC
Dsb system?
+ H-X
S-S
3) Arginine sensing
R
?
ScsB
IM
e-
CYTOPLASM

FIGURE 9

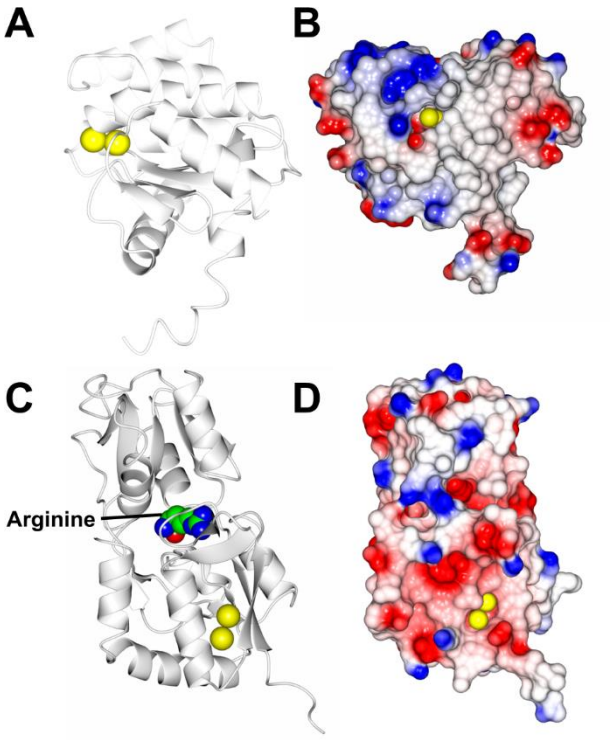
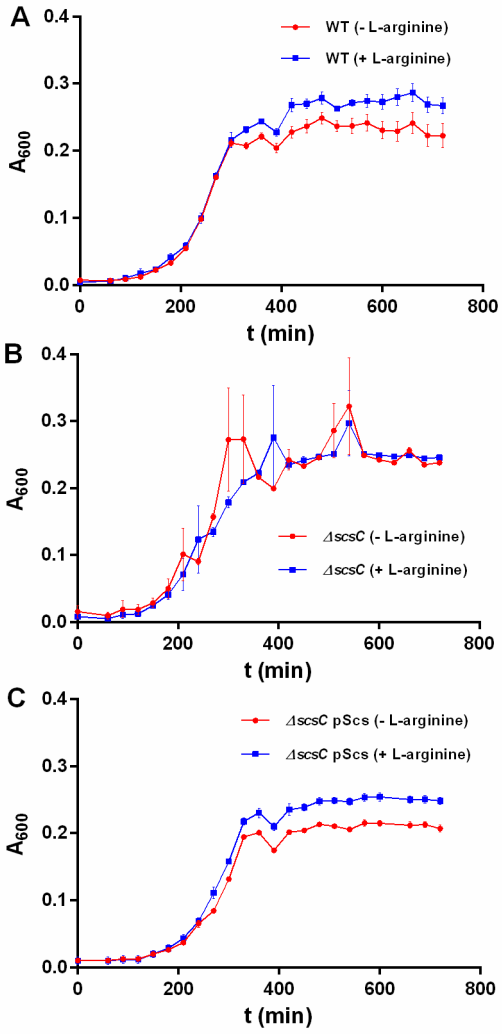


FIGURE 10


A
B
C
WT (- L-arginine)
WT (+ L-arginine)
ΔscsC (- L-arginine)
ΔscsC (+ L-arginine)
ΔscsC pScs (- L-arginine)
ΔscsC pScs (+ L-arginine)
A600
t (min)
0.0
0.1
0.2
0.3
0.4
0
200
400
600
800