

Oxidoreductases that Act as Conditional Virulence Suppressors in *Salmonella enterica* Serovar Typhimurium

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Abstract

In *Salmonella enterica* serovar Typhimurium, oxidoreductases of the thioredoxin superfamily contribute to bacterial invasiveness, intracellular replication and to the virulence in BALB/c mice as well as in the soil nematode *Caenorhabditis elegans*. The *scsABCD* gene cluster, present in many but not all enteric bacteria, codes for four putative oxidoreductases of the thioredoxin superfamily. Here we have analyzed the potential role of the *scs* genes in oxidative stress tolerance and virulence in *S. Typhimurium*. An *scsABCD* deletion mutant showed moderate sensitization to the redox-active transition metal ion copper and increased protein carbonylation upon exposure to hydrogen peroxide. Still, the *scsABCD* mutant was not significantly affected for invasiveness or intracellular replication in respectively cultured epithelial or macrophage-like cells. However, we noted a significant copper chloride sensitivity of SPI1 T3SS mediated invasiveness that strongly depended on the presence of the *scs* genes. The *scsABCD* deletion mutant was not attenuated in animal infection models. In contrast, the mutant showed a moderate increase in its competitive index upon intraperitoneal challenge and enhanced invasiveness in small intestinal ileal loops of BALB/c mice. Moreover, deletion of the *scsABCD* genes restored the invasiveness of a *trxA* mutant in epithelial cells and its virulence in *C. elegans*. Our findings thus demonstrate that the *scs* gene cluster conditionally affects virulence and underscore the complex interactions between oxidoreductases of the thioredoxin superfamily in maintaining host adaptation of *S. Typhimurium*.

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Introduction

Salmonella enterica serovar Typhimurium (*S. Typhimurium*) is a facultative intracellular enteric pathogen that traditionally has been used as a model organism for studying typhoid fever caused by the human adapted serovar Typhi [1,2]. In the murine model for typhoid fever, *S. Typhimurium* invades the intestinal epithelium after *per oral* challenge. Invasion is followed by dissemination and intracellular bacterial replication in phagocytic cells of the liver and spleen.

Virulence of *S. Typhimurium* in mammalian cell culture and in mice, as well as in alternative host infection models, strongly relies on horizontally acquired DNA regions termed as *Salmonella* pathogenicity islands (SPI:s) [3]. Two of the SPI:s, SPI1 and SPI2, code for protein type III secretion systems (T3SS) that translocate bacterial effector proteins into host cells. The effector proteins act to manipulate central host cell functions, such as actin polymerization, vesicular trafficking, and signal transduction [4,5,6]. In mice, SPI1 and SPI2 enable the bacteria to transcytose the intestinal epithelial barrier, and disseminate to replicate in macrophages of the liver and spleen respectively [7,8]. Moreover, SPI1 and SPI2 T3SS also modulate pro-inflammatory responses of the host during *Salmonella* infection [9,10,11].

Reactive oxygen species (ROS) generated by *Salmonella*-induced inflammatory responses most evidently contribute to the clearance

and pathogenesis of salmonellosis. Mice (*phox -/-*) lacking a functional phagocyte oxidase rapidly succumb upon infection doses well controlled by wild type littermates [12]. Furthermore, individuals suffering from chronic granulomatous disease, a condition marked by a defective oxidative phagocyte response, show an increased prevalence of extraintestinal infections caused by non-typhoidal serovariants of *Salmonella* [13].

The genome sequence of *S. enterica* reveals a number of enzymes that potentially provide protection against ROS, such as H₂O₂. These enzymes include super oxide mutases, catalases, thiol peroxidases and methionine sulphoxide reductases [14,15,16,17]. In addition, oxidoreductases of the thioredoxin superfamily contribute to the assembly or activities of many bacterial virulence factors. SPI2 activity in part relies on the periplasmic DsbA and SrgA proteins, which both belong to the thioredoxin superfamily of oxidoreductases [18]. SrgA is in fact coded for by a plasmid-carried fimbrial operon *pef*, and moreover is required for the assembly of Pef fimbriae [19]. The activity of SPI2 also depends on the cytoplasmic thioredoxin 1 (TrxA, *trxA*), whereby *trxA* mutants are severely attenuated for replication in macrophage-like cells and in mice [20,21]. TrxA additionally mediates a redox-associated lethality caused by *S. Typhimurium* in an infection model based on the soil nematode *C. elegans* [22].

Many of the oxidoreductases such as TrxA, DsbA and thiol peroxidase Tpx implicated in virulence of *S. Typhimurium* and in

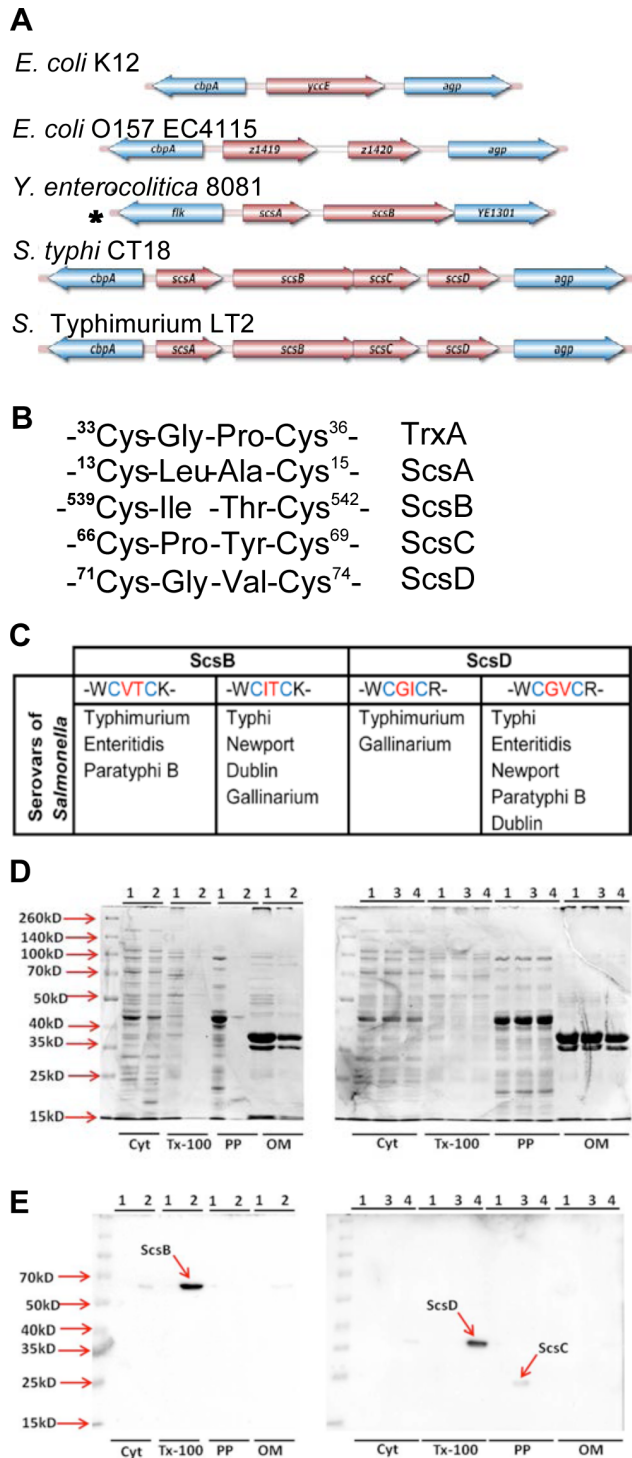


Figure 1. In silico analyses and localization of the ScsABCD proteins. A) Illustrates the *cbpA*-*agg* genomic region in selected strains of *Escherichia coli* (*E. coli*), *Yersinia enterocolitica* (*Y. enterocolitica*) and *Salmonella enterica* (*S. enterica*); * No *cbpA* or *agg* present. B) and C) detail and compare the Cys-X-X-Cys motives of Scs proteins with the prototype TrxA and their variability in predicted ScsB and ScsD proteins. D) shows Coomassie blue stained 12%SDS-polyacrylamide gels presenting sub-cellular fractionation of the proteins E) presents immunoblot with anti-His to show the localization of Scs proteins in different subcellular fractions. In (D) and (E), cyt = cytoplasmic fraction; Tx-100 = Triton X-100 soluble fraction; PP = periplasmic fraction and OM = Tx-100 insoluble integral outer membrane fraction. 1 = WT/

pET32a; 2 = *scsB* clone (pNA15), 3 = *scsC* clone (pNA16) and 4 = *scsD* clone (pNA17).

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redox tolerance of *E. coli*, are highly conserved within enterobacteria [15,18,23]. In contrast to laboratory *E. coli* strains, *S. Typhimurium* includes the *scsABCD* gene cluster that encodes four proteins each with a Cys-X-X-Cys motif characteristic for the thioredoxin superfamily [24] (Figs. 1A and 1B). When cloned into *E. coli*, the *scs* genes restore copper tolerance of selected copper-sensitive mutants [24]. However, the actual role of the *scs* genes for redox tolerance in *S. Typhimurium* and their contribution to virulence, if any, has remained enigmatic. Here we demonstrate that a *S. Typhimurium scsABCD* deletion mutant shows moderate sensitization to copper chloride and, surprisingly, a conditional enhanced invasiveness in epithelial cells and virulence in *C. elegans*.

Results

The *scsABCD* Gene Cluster

The predicted primary sequences of the *S. Typhimurium* ScsABCD proteins were presented in 1997 [24]. Since, a number of new genome sequences and protein prediction algorithms have been annotated, whereby homologues of Scs proteins have been identified in a number of diverse bacterial species [25]. Hence, we revisited the *S. enterica scsABCD* genes and protein predictions for the ScsABCD protein sequences in terms of *in silico* analyses using more recent public databases.

In the genome sequence of *S. Typhimurium* LT2, the *scsABCD* genes position between *cbpA* and *agg* genes [26]. The *scs* genes are contained in two transcriptional units read from the same DNA strand [24]; one consisting of *scsA* followed by a second including the *scsBCD* genes. In contrast, *E. coli* K-12 and many other members of *Enterobacteriaceae* and related organisms lack *scs* gene sequences, or contain only selected *scs* genes positioned between *cbpA* and *agg* genes (Fig. 1A).

Each of the *scs* genes codes for a predicted protein with a single Cys-X-X-Cys motif and an enriched containment of hydrophobic amino acids between the Cys residues (Fig. 1B). Such a motif fits the hallmark of the catalytic site of the thioredoxin/glutaredoxin family of oxidoreductases. The Cys-X-X-Cys motifs for ScsA and ScsC are conserved in all annotated *S. enterica* genome sequences, whereas for ScsB and ScsD either one of the two residues between the cysteines is variable (Fig. 1C) potentially leading to alterations in the redox potential of the proteins [27,28].

According to signal sequence predictions for the *S. Typhimurium* Scs proteins, only ScsC would contain a classical signal sequence. This would also fit with its suggested role as a periplasmic disulphide isomerase in *Caulobacter crescentus* [25]. ScsA and ScsB both contain a putative lipobox at their N-termini that predict the proteins to be outer membrane lipoproteins. However, for ScsA any processing at the predicted lipobox sequence would delete the Cys-X-X-Cys motif, while for ScsB the putative lipobox sequence positions at only 13 residues from the initiation methionine. Still, ScsB as well as ScsD were predicted to be integral membrane proteins.

Expression and Localization of Recombinant Scs Proteins

To get indications for the actual localization of the Scs proteins we first cloned them individually in the pBAD30 vector that allows for L-arabinose assisted inductions of recombinant proteins in *S. Typhimurium*. Each *scs* gene was amplified individually including its putative ribosome binding site at the 5'-end, and coding for C-terminal 6xHis tag. Attempts to express the proteins in *S.*

Typhimurium only revealed signal for ScsC upon immunoblotting for the His-tag at the expected mass position (data not shown). However, detection of plain ScsA, ScsB or ScsD proteins in *S. Typhimurium* appeared difficult.

One possibility could be extreme sensitivity to proteolytic degradation, or suboptimal induction of ScsA, ScsB or ScsD in *S. Typhimurium* from our recombinant plasmid constructs. In a next attempt we tried to express all Scs proteins individually in *E. coli* BL21 strain, deficient in the cytoplasmic Lon protease and expressing the phage T7 RNA polymerase, using the T7 promoter-based expression vector pET32a. The recombinant BL21 *E. coli* strain was next fractionated into a detergent insoluble cell wall fraction (representing integral outer membrane proteins), detergent soluble cell wall fraction, periplasmic protein fraction and cytosolic fraction. The fractions were separated on SDS-PAGE gels (Fig. 1D) and used for immunoblotting of the His-tag. In this scenario, a band corresponding to calculated molecular mass of ScsC-6xHis clearly appeared enriched in the periplasmic fraction at 23-kDa as was detected in *S. Typhimurium* (Fig. 1E). This would be consistent with the predicted periplasmic localization of the protein.

Similarly, cloning of *scsB* with His-tag coding region into pET32a allowed for detection of a 67-kDa band in the detergent soluble fraction (Fig. 1E). This would be consistent with the predicted localization of ScsB within the inner membrane, the localization predictions recently presented for the ScsB superfamily of redox transporters [25] and with the predicted apparent mass of the fusion protein.

In selected cases, introduction of an N-terminal thioredoxin 1 tag results in stabilization of recombinant proteins. Such a strategy was considered inappropriate for ScsA as a concomitant fusion protein would be severely affected for any subsequent signal peptide processing. However, predicted to be devoid of the signal sequence, for ScsD we generated a translational thioredoxin-ScsD-His-tag fusion protein as coded for by pET32a. Upon induction in *E. coli* BL21, we noted an additional protein band of 36-kDa molecular mass in the detergent soluble fraction in Coomassie stained protein gel (data not shown). This matched the predicted size of a TrxA-ScsD-His fusion protein. A band at the same position was detected upon immunoblotting for the His-tag (Fig. 1E). When excised from the gel, and subjected to mass-spectrometric analyses, it revealed both thioredoxin and ScsD peptides. Admittedly, this fusion protein clearly is artificial, but the analyses would be in line with an assumed cytoplasmic membrane association of ScsD, and assuming that ScsD contains a strong enough internal topological membrane insertion domain(s).

Construction of *scs* Deletion Mutants

In order to study any functional aspect of the *scs* genes in *S. Typhimurium*, we set out to delete each reading frame using recombinase-assisted site-directed mutagenesis [29]. For *scsA*, the deletion comprised the entire reading frame, but not extending into the intergenic region between *scsA* and *scsB* (for primer details, see Table S2). The open reading frames (ORF) of *scsB* and *scsC* partially overlap [24]. Therefore the deletions of the individual *scsB*, *scsC* and *scsD* genes were designed not to interfere with the ORFs of the rest. We also created a strain construct, a $\Delta scsABCD$ quadruple deletion mutant, lacking all the four *scs* genes from the 5'-end of *scsA* to the 3'-end of *scsD* ORFs. Finally, to exclude any polar effects caused by the resistance cassette used for tagging the mutations, the cassettes were removed from all deletion mutants.

The *scsBCD* Genes Equally Contribute to Copper Chloride Tolerance in *S. Typhimurium*

Copper chloride (CuCl_2) sensitivity in *E. coli* mutants is reversed when provided with the cloned *scsABCD* genes from *S. Typhimurium* [24]. Therefore we started to probe for the contribution of *scs* genes to the intrinsic copper tolerance in *S. Typhimurium* using the deletion mutants defined above.

The $\Delta scsA$ mutant did not reveal CuCl_2 sensitivity while deleting either the *scsB*, *scsC* or *scsD* gene resulted in a moderate but equal and highly reproducible sensitization to CuCl_2 (Fig. 2A). Similarly, the $\Delta scsABCD$ quadruple deletion mutant showed the same sensitization as the individual $\Delta scsB$, $\Delta scsC$ and $\Delta scsD$ mutants (Fig. 2A).

Attempts to complement individual Δscs mutants with a cloned corresponding gene failed (data not shown). However, all copper sensitive single Δscs mutants as well the quadruple deletion mutant, were fully complemented for CuCl_2 tolerance by the *scsABCD* genes cloned in cloning vector pSU41 (pNA10) (Fig. 2A and data not shown). None of the mutants revealed increased sensitization to another antibacterial transition metal in the form of zinc chloride (Fig. 2B).

The *scsABCD* Genes Protect against Protein Carbonylation

As CuCl_2 mediates cysteine disulphide bond formation and potentially promotes generation of ROS, we next tested for any sensitization of the *scs* mutants to hydrogen peroxide (H_2O_2). In doing this we neither observed H_2O_2 sensitization for the $\Delta scsB$, $\Delta scsC$ and $\Delta scsD$ single mutants nor for the $\Delta scsABCD$ quadruple mutant (Fig. 2D). Notwithstanding the fact, the $\Delta scsA$ single mutant showed increased sensitivity to H_2O_2 (Fig. 2D).

Apart from mediating disulphide bond formation, ROS also results in protein carbonylation in *S. Typhimurium* [17]. Thus, we assayed protein carbonylation in cultures of *S. Typhimurium* exposed to 0.75 mM H_2O_2 to detect any discernible differences. To exclude any confounding effects by the $\Delta scsA$ single mutant, we continued using the $\Delta scsABCD$ mutant only. The rationale for this was that this mutant lacked all the *scs* genes yet did not reveal sensitization to H_2O_2 . In this experiment we detected a more pronounced accumulation of carbonylated proteins in the $\Delta scsABCD$ mutant (Fig. 3A), notably in the periplasmic fraction (Fig. 3C). Expressing the cloned *scsABCD* genes from pNA10 in the quadruple mutant reduced the H_2O_2 -mediated protein carbonylation (Fig. 3B). That rather few proteins became increasingly carbonylated in the $\Delta scsABCD$ mutant could explain why this *scs* mutant did not display increased loss of viability upon exposure to H_2O_2 . Still, these series of experiments strongly implicated that the Scs proteins are involved in balancing periplasmic oxidative stress in *S. Typhimurium*.

S. Typhimurium Still Relies on *trxA* for Copper Chloride Tolerance

TrxA also contributes to copper tolerance in *E. coli* [30]. We thereby set out to test whether the presence of *scsABCD* genes made *trxA* dispensable for CuCl_2 tolerance in *S. Typhimurium*. We noted a decreased CuCl_2 tolerance of a *S. Typhimurium* $\Delta trxA$ mutant that in magnitude equaled to that of the $\Delta scsABCD$ mutant (Fig. 4A). *S. Typhimurium* lacking *trxA* as well as the *scsABCD* genes did not show any further sensitization to CuCl_2 (Fig. 4A).

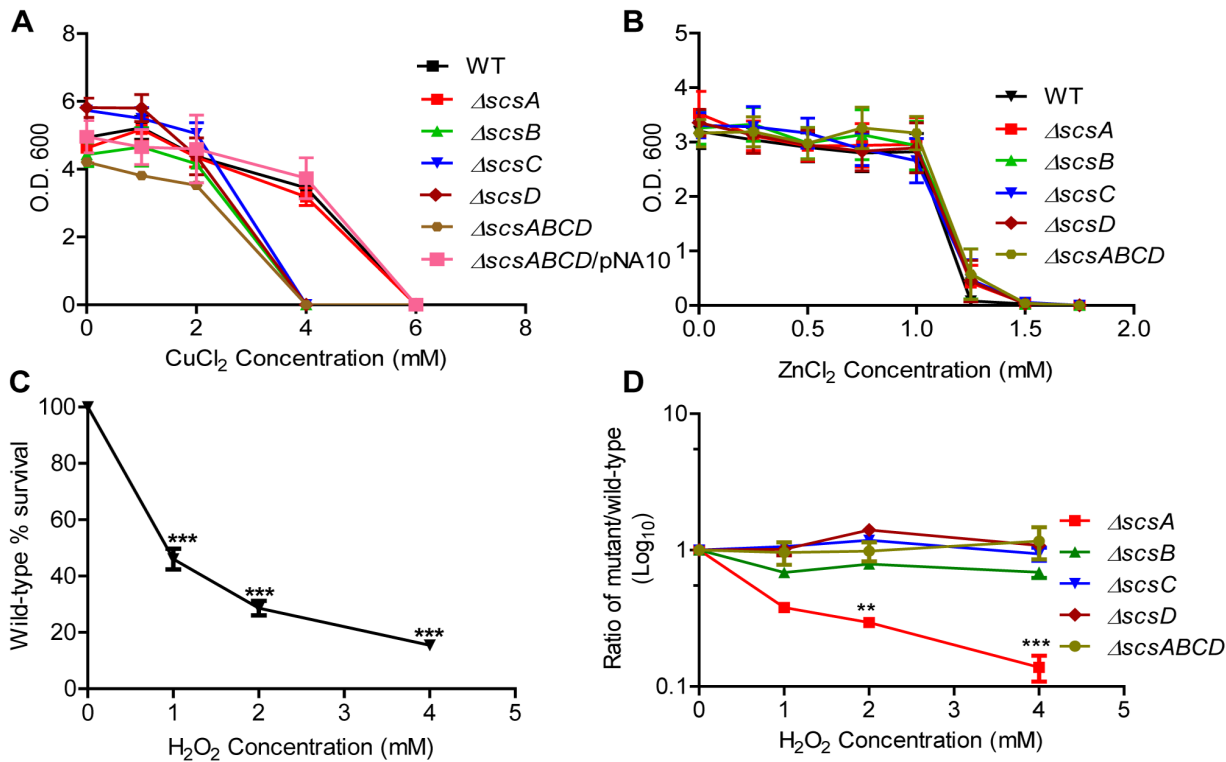


Figure 2. Sensitivity to copper chloride, zinc chloride and hydrogen peroxide. A) Optical densities of overnight wild type *S. Typhimurium* and different *scs* mutants grown in the presence of CuCl₂. This sensitivity is reversed by trans-complementation using the cloned *scsABCD* genes on plasmid pNA10 for Δ*scsABCD* mutant. B) shows equal sensitization to ZnCl₂ for wild type *S. Typhimurium* and its Δ*scs* mutants. C) Loss in viable count of the wild type *S. Typhimurium* strain after exposure to H₂O₂. Values are given as percentage relative to a hydrogen peroxide deficient control. *** = $p \leq 0.001$ D) Relative indices of viable counts of different *scs* knockouts compared to wild type reveals sensitization only for the Δ*scsA* mutant. ** = $p \leq 0.01$, *** = $p \leq 0.001$. Error bars indicate standard error of the mean. doi:10.1371/journal.pone.0064948.g002

Redox Sensitivity and Dependency on *scsABCD* Genes for *in vitro* Invasiveness of *S. Typhimurium*

Full invasiveness of *S. Typhimurium* in mammalian cell cultures relies on *trxA* [20]. As *trxA* and the *scsABCD* mutants revealed an equal sensitization to CuCl₂, we set testing that to what extent the invasiveness was affected in our mutants, and whether invasiveness would be influenced by CuCl₂. For this, the cultures were grown to induce SPI1 expression in the absence or presence of CuCl₂ for 4 hours, and subsequently exposed to MDCK epithelial cells without CuCl₂ for one hour.

When cultures were propagated in the absence of CuCl₂, we corroborated the decreased invasion reported for a Δ*trxA* mutant [20] (Fig. 4B). This decrease in invasiveness was retained when the mutant was provided with the cloning vector pBAD33 or when provided with a pBAD33-derivative coding for a catalytically inactive TrxA. When complemented with the wild type *trxA* in pBAD33, invasiveness was enhanced above wild type levels (Fig. 4B). These observations clearly implicate a role for TrxA in invasiveness of *S. Typhimurium*.

The Δ*scsABCD* quadruple mutant invaded as efficiently as the parental wild type strain (Fig. 4B). Surprisingly though, deleting the *scsABCD* genes from the Δ*trxA* mutant resulted in increased invasiveness (Fig. 4C).

When the cultures were grown to induce SPI1-mediated invasiveness at 3 mM of CuCl₂ we noted a moderate decrease in colony forming units in relation to the optical density of the cultures for the Δ*scsABCD* and Δ*trxA* mutants (Fig. 4D). Still, while not reduced for viability, the invasiveness of the wild type *S.*

Typhimurium strain was virtually lost upon growth under CuCl₂ stress (Fig. 4E and 4F). In contrast, even though the Δ*trxA* mutant showed decreased invasion under ordinary growth conditions, the invasion index for the mutant became increased by pre-exposure to CuCl₂ (Fig. 4E and 4F). Also, the relative invasiveness of both the Δ*scsABCD* and Δ*scsABCD*/Δ*trxA* mutant was much retained upon growth in the presence of CuCl₂ (Fig. 4E and 4F). These results showed that invasiveness of *S. Typhimurium* is strongly reduced by CuCl₂, yet that this chemical attenuation depended on the Scs and TrxA oxidoreductases.

As the invasiveness of *S. Typhimurium* in cell cultures strongly relies on SPI1 T3SS and the fact that *scsABCD* mutant retained invasiveness at CuCl₂ stress evoked the question whether this enhanced invasion of the *scsABCD* relied on SPI1 T3SS. Therefore, we inserted a polar Tn5::lacZ'Y' mutation in the *scsABCD* mutant inactivating the main SPI1 transcriptional activator gene *hilA*, and another in the *prgH* gene coding for a central component of the SPI1 T3SS apparatus. When exposed to CuCl₂, the accompanying *hilA* and *prgH* mutants failed to reveal invasiveness in the MDCK cell based assay, to an extent that approached our detection limit (data not shown). Thus, the retained invasiveness of the *scsABCD* mutant at CuCl₂ exposure continued to be SPI1-dependent.

The *scsABCD* and SPI1 Gene Expression

That the *scsABCD* mutant retained invasiveness upon CuCl₂ stress in a SPI1-mediated manner led us to test whether the *scs* gene cluster affected SPI1 gene expression. For this we measured

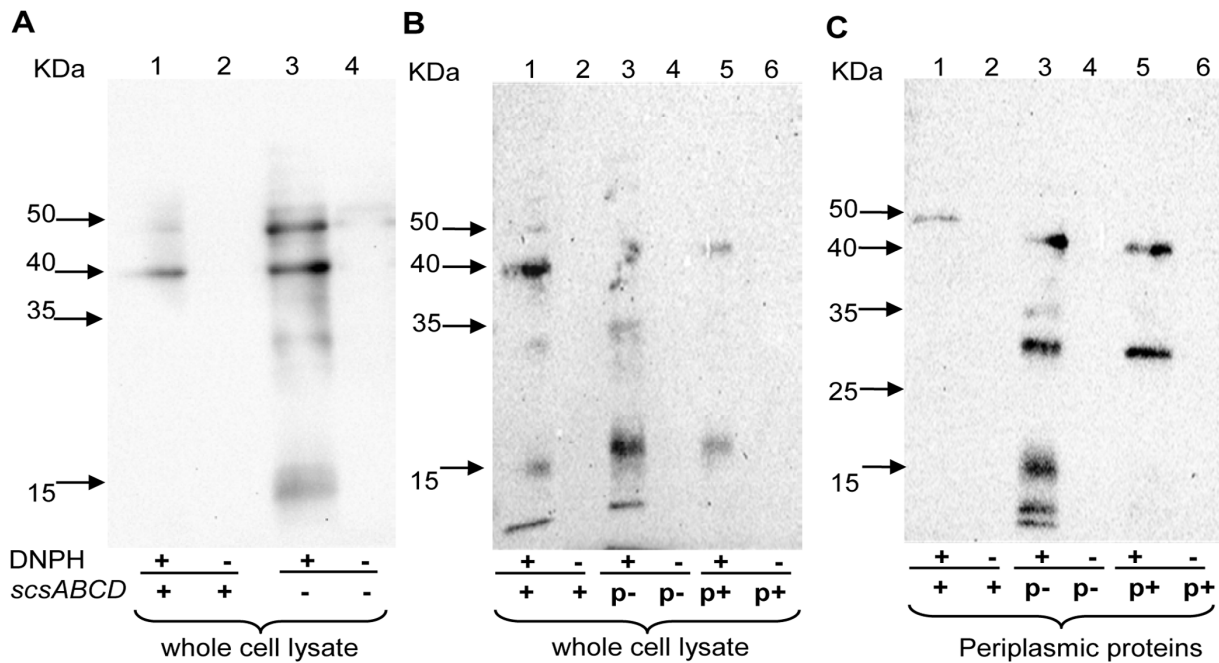


Figure 3. Carbonylated protein profiles after exposure to hydrogen peroxide. Protein carbonylation was detected by immunoblotting after coupling to dinitrophenol and protein separation on 12% SDS-PAGE gels. A) Whole cell lysate obtained from the $\Delta scsABCD$ mutant reveals a higher concentration of carbonylated proteins after exposure to 0.75 mM hydrogen peroxide (B). Trans-complementation with cloned *scsABCD* (pNA10) reduces the level of protein carbonylation in the whole cell fraction, as well as in the periplasmic fraction (C). DNPH=Dinitrophenyl hydrazine (+)=presence (-)=absence. p+ indicates the trans-complementing *scsABCD* genes in pNA10. p- indicates the vector control pSU41. doi:10.1371/journal.pone.0064948.g003

SPI1 gene expression using the two forth mentioned SPI1 Tn5::lacZ constructs. When grown over night in LB we noted clear reading of the *hilA::lacZ* and *prgH::lacZ* transcriptional fusions by assaying beta-galactosidase activities (Fig. 5A and B). Addition of CuCl₂ to the culture prior to the inoculation resulted in an overall decreased reporter activity with a slight relative decrease in both fusions for the *scsABCD* mutant (Fig. 5A and B). However, this difference disappeared when bacteria were grown for invasiveness in cull culture medium supplemented with CuCl₂ (Fig 5C and D).

The *scsABCD* Genes and SPI1 Activity

To address whether *scsABCD* genes affected SPI1 activity, we followed the expression and secretion of a SPI1 effector fusion protein. For this we used plasmid pAUN1 that carries the 5'-end of the effector protein gene *sipB* translationally fused to TEM beta-lactamase gene devoid of its region coding for the signal sequence [31]. We chose to use cultures propagated in cell culture medium as this is the medium used to generate invasion competent bacteria, and as the *lacZ* reporter activities did not differ between the wild type and mutant in this medium (Fig. 5C and D). When grown to generate invasiveness, we noted a clear presence of the SipB-Bla fusion protein in the culture supernatant and cell lysates from the wild type bacteria carrying pAUN1 as indicated by the enzymatic activity of the SipB-Bla fusion protein (Fig. 5E). For the *scsABCD* mutant we could likewise detect enzymatic activity from both fractions. However, as compared to the activities expressed by the wild type, the proportion of the extracellular activity was reduced and the cell-bound activity increased in the *scsABCD* mutant (Fig. 5E). The enzymatic activity became much reduced when the medium was supplemented with CuCl₂ at 2 mM (Fig. 5F), and decreased below detection limit at 3 mM (data not shown). Interestingly though, at 2 mM CuCl₂, the proportions of

enzymatic activities for cell-bound and secreted fusion protein appeared the same for the wild type and *scsABCD* mutant (Fig. 5F).

To exclude any potential distractions emerging from alternations in specific enzyme activity generated by CuCl₂, we also assayed for the SipB-Bla fusion protein by immunoblotting. In the absence of CuCl₂, the amounts of the fusion protein in the supernatant appeared much higher in the wild type, and with a substantial accumulation in cell lysate for the $\Delta scsABCD$ mutant (Fig. 5G). At 2 mM CuCl₂ the signals for the fusion protein decreased, but remained detectable upon longer exposure. Still, the proportions of the fusion protein in the extracellular versus intracellular fraction of the wild type appeared more pronounced compared to the $\Delta scsABCD$ mutant (Fig. 5G).

Combined, these observations indicated that while SPI1 expression was redox sensitive, the *scs* gene cluster did affect *prgH* expression in LB. Under conditions where the *scsABCD* deletion mutant did not reveal difference in *prgH* expression, the deletion still affected secretion of a SipB fusion protein.

The *scsABCD* Genes, Intracellular Replication and *in vivo* Virulence of *S. Typhimurium*

Apart from affecting invasion, TrxA strongly affects bacterial intracellular replication in RAW264.7 macrophage-like cells, as well as virulence in BALB/c mice [20]. Regarding intracellular replication in RAW264.7 cells we did not note any significant deviation for the $\Delta scsABCD$ mutant compared to the wild type (Fig. 6A).

Upon intraperitoneal challenge the $\Delta scsABCD$ mutant showed a moderate relative increase in splenic loads three days post challenge (Fig. 6B). When comparing the ratios of wild type and mutant bacterial ratios by a nonparametric test, the difference did not breach statistical significance. However, we also extracted the

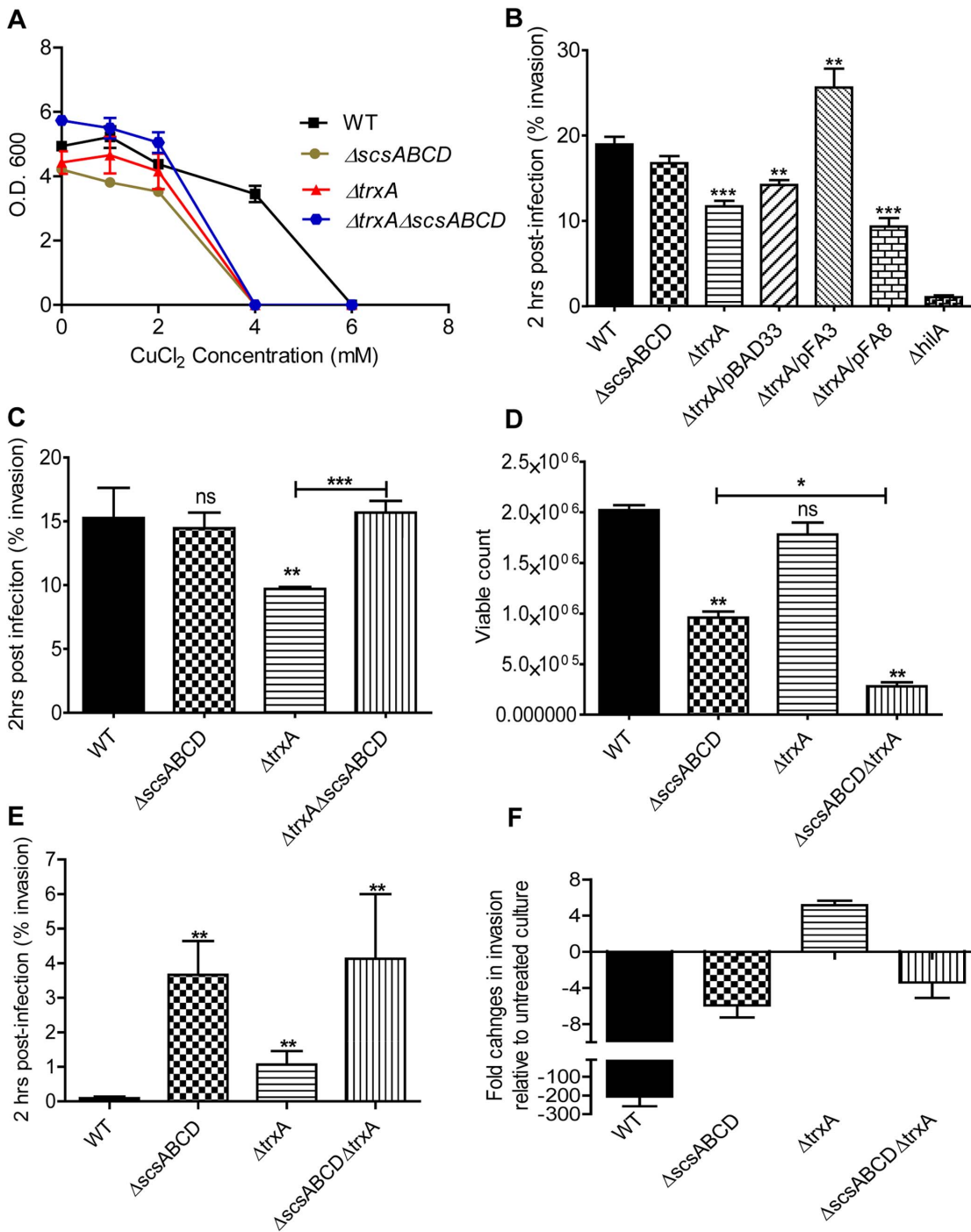


Figure 4. Sensitivity to copper chloride and invasion in MDCK epithelial cell line, with or without copper chloride stress. A) Optical density of overnight wild type *S. Typhimurium*, $\Delta scsABCD$ and different $\Delta trxA$ mutants grown in the presence of $CuCl_2$. B) The compromised invasion of a $\Delta trxA$ deletion mutant can be trans-complemented with cloned *trxA* (pFA3) but not with a plasmid coding for catalytically inactive TrxA (pFA8). C) The *scsABCD* deletion acts as a suppressor mutation for the decreased invasion of the $\Delta trxA$ mutant. D) and E) The viability of $\Delta scsABCD$ and $\Delta trxA$ under $CuCl_2$ stress (3 mM) is compromised, yet the invasiveness is highly enhanced at this concentration. F) shows fold changes in invasion of 3 mM $CuCl_2$ treated culture relative to the untreated culture. ns = non-significant; * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$. The error bars indicate standard error of the mean.
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actual viable counts of individual strains from the data to allow the use of a parametric test. In this, the differences in viable counts became statistically significant ($P < 0.05$). Despite this enhancement in the replication of *scsABCD* mutant, the $\Delta scsABCD$ deletion

neither restored the strong attenuation exhibited by the $\Delta trxA$ mutant background in RAW264.7 nor in mice (Figs. 6A and 6B). In other words, in contrast to the decreased invasiveness, the

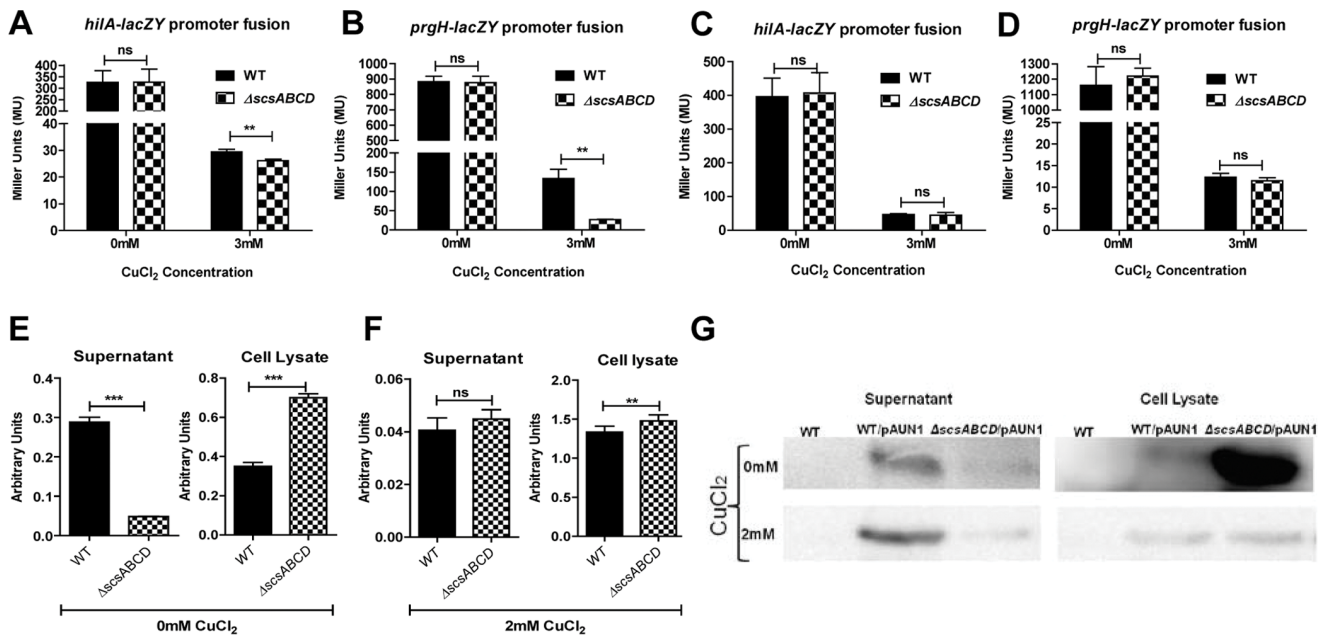


Figure 5. β -galactosidase and β -lactamase activities and expression measurements. A) and B) β -galactosidase activities for *hilA* and *prgH* promoter fusions from overnight LB culture are slightly reduced for *scsABCD* mutant in the presence of CuCl₂. C) and D) β -galactosidase activities for *hilA* and *prgH* promoter fusions from 4 hrs DMEM-based invasive culture are unaffected irrespective of *scsABCD* mutation. β -lactamase activities from supernatants and cell lysates of the invasive cultures of wild type and in $\Delta scsABCD$ mutant bacteria in the absence (E) and presence (F) of CuCl₂. G) β -lactamase as assayed from the same cultures by immunoblotting for β -lactamase. ns = non-significant; ** = $p \leq 0.01$; *** = $p \leq 0.001$. Error bars indicate standard error of the mean.

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attenuation of the $\Delta trxA$ mutant in RAW264.7 cells and BALB/c mice evidently did not depend on the *scs* genes.

SPI1 is important in mediating invasion of *S. Typhimurium* from the intestine of mice. However, the redox conditions in the intestine are likely to deviate from the ones prevailing in cell culture settings. Hence we continued by comparing invasiveness of wild type and $\Delta scsABCD$ mutant of *S. Typhimurium* in ileal loops of BALB/c mice. In these experiments, the $\Delta scsABCD$ mutant indeed revealed an increase in numbers of invading bacteria (Fig. 6C).

The *scsABCD* Gene Cluster Conditionally Suppresses Virulence in *C. elegans*

Feeding *C. elegans* with *S. Typhimurium* results in a significant shortening of the nematode life span, which in part relies on a massive hypodermal oxidative stress response in the nematode [22]. This stress response is initiated at the intestine some 24 hours post infection, and at 36 to 48 hours post infection fills the nematode hypodermal space, as revealed by staining with the fluorescent ROS indicator 2',7'-dichlorodihydrofluorescein diacetate (H₂DCFDA) [22]. Nematodes fed with a $\Delta trxA$ mutant of *S. Typhimurium* lack this oxidative response and gain a significant restoration of their life span [22]. Therefore, we set out to test whether *scsABCD* genes would bear any role in the pathogenesis of *S. Typhimurium* in *C. elegans*.

C. elegans infected with the $\Delta scsABCD$ mutant of *S. Typhimurium* behaved as nematodes infected with the wild type regarding life span (Fig. 7A). Still, transfer of the *scsABCD* deletion into the $\Delta trxA$ mutant resulted in retrieved virulence as evidenced by a shortening of the nematode life span (Fig. 7A).

Nematodes infected with the wild type or $\Delta scsABCD$ mutant evoked an apparently equal hypodermal ROS staining with H₂DCFDA (wild type, Figs 7B and C; $\Delta scsABCD$ mutant, Figs 7F

and G). This observation was corroborated by quantification of oxidative stress induced fluorescence of H₂DCFDA (Fig. 7J). To corroborate our previous finding [22], the oxidative stress response was much reduced upon infection with the $\Delta trxA$ mutant (Figs. 7D and E, Fig. 7J). Surprisingly, infection with the $\Delta scsABCD/\Delta trxA$ mutant accompanied with a substantial restoration in the hypodermal oxidative stress such that the H₂DCFDA staining reached wild type levels at 72 h post infection (Figs. 7H and I, Fig. 7J).

Thereby, the *scsABCD* genes affected the $\Delta trxA$ mutant phenotype not only regarding invasiveness of epithelial cells, but also regarding the virulence and pathogenesis of the $\Delta trxA$ mutant in *C. elegans*.

Discussion

Reactive oxygen species play an important role in the pathogenesis of many bacterial species both in cell culture setting and for virulence in man, mice and nematodes [12,13,14,15,16,17]. In *E. coli* selected oxidoreductases of the thioredoxin superfamily, including thioredoxin 1 (TrxA) itself, are not only needed for tolerance to H₂O₂ but also for resistance to the redox active transition metal copper [30,32,33]. Copper ions mediate non-enzymatic catalysis of protein disulphide formation [34,35], which mimics a facet of oxidative stress that interferes with periplasmic disulphide bond formation. *S. enterica* carries a gene cluster, the *scsABCD* genes which, when expressed in copper sensitive laboratory strains of *E. coli*, enhances their copper tolerance [24]. Recently, copper was coupled to the ability of phagocytes to control infection with *S. Typhimurium* [36]. Furthermore, when *S. Typhimurium* replicates in macrophages, the *scs* genes are significantly induced for their expression [37]. Given the coupling between tolerance to oxidative stress and

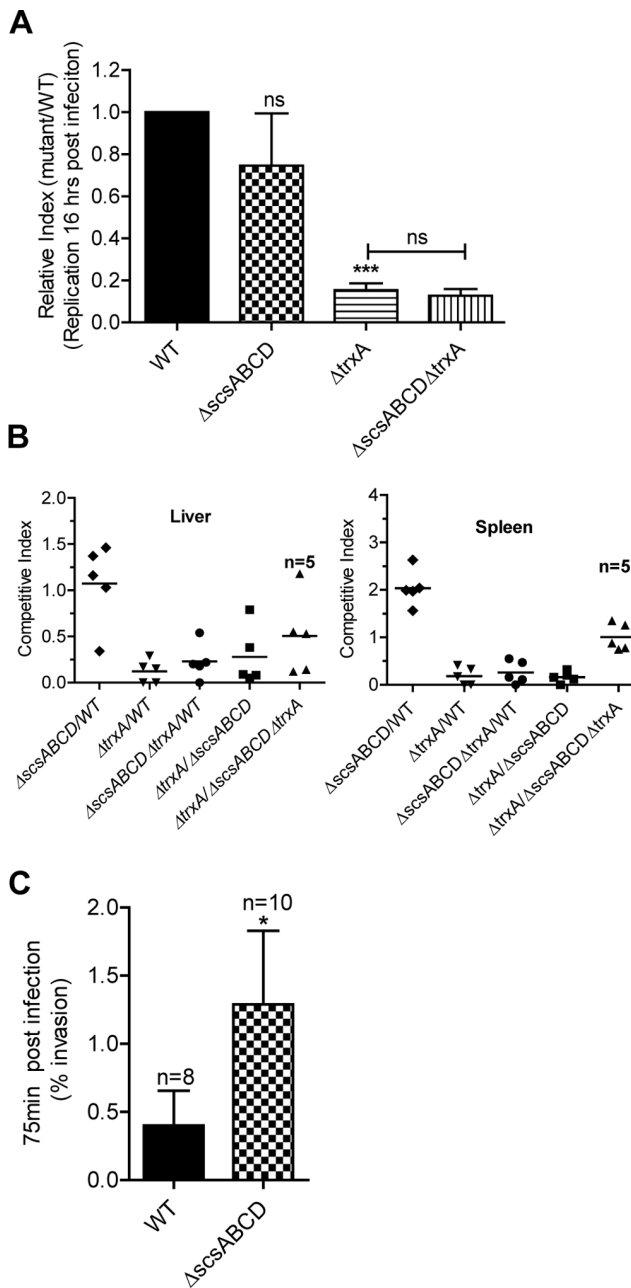


Figure 6. Replication in RAW264.7 macrophages and mice, and invasion in ileal loops of mice. A) The $\Delta scsABCD$ does not restore replication of $\Delta trxA$ in macrophage cell line RAW 264.7 B) The $\Delta scsABCD$ mutation shows a moderate increase in splenic load in BALB/c mice on intraperitoneal challenge; n = number of mice C) The $\Delta scsABCD$ invades more efficiently in ileal loop ligation experiment. n = number of loops. ns = non-significant; * = $p \leq 0.05$; *** = $p \leq 0.001$. Error bars indicate standard error of the mean. doi:10.1371/journal.pone.0064948.g006

copper [30,32,33], we have searched for a role of the ScsABCD proteins, all putative members of the thioredoxin superfamily of oxidoreductases, for oxidative stress tolerance and virulence in *S. Typhimurium*.

Individual $\Delta scsB$, $\Delta scsC$, $\Delta scsD$ and $\Delta trxA$ mutants showed equal sensitization to copper chloride, and that deleting the $\Delta scsABCD$ genes in a $\Delta trxA$ mutant did not cause further sensitization. This deduced that the $scsABCD$ and $trxA$ genes contributed to copper

tolerance through a common pathway. This conclusion would fit well with recent analyses in *C. crescentus* where ScsB represented a DsbD-like cytoplasmic membrane electron transporter and ScsC, a putative periplasmic disulphide isomerase and a reduction substrate for ScsB [25]. TrxA is also known to provide DsbD with electrons [38], which could adopt a connection between the ScsABCD system and TrxA in mediating copper tolerance. Exposure to H_2O_2 resulted in protein carbonylation both in the wild type *S. Typhimurium* and in the strain lacking the $scsABCD$ genes. Still, the level of protein carbonylation was higher in the $\Delta scsABCD$ mutant as compared to wild type, notably in the periplasmic sub-cellular fraction. Such results further implicate a role of the Scs proteins in balancing redox stress in *S. Typhimurium*.

ScsA did not add to $CuCl_2$ tolerance despite the presence of a *Cys-X-X-Cys* motif. However, this motif resides within its predicted signal sequence. Hence a processed ScsA would be missing a thioredoxin-like catalytic motif. Such a scenario invites for speculating that processing of ScsA could rely on disulfide formation in the putative signal sequence, and hence depend on periplasmic redox status for processing and transport. Still the $\Delta scsA$ mutant showed increased sensitization to H_2O_2 , while no such sensitization was noted for any of the other scs deletion mutants or for the $\Delta scsABCD$ mutant. That is, an imbalance in the Scs protein content rather than lack of ScsA might explain the selective sensitization to H_2O_2 . Such reports have already been published with periplasmic protein DsbA, where overexpression of DsbA protein suppresses the motility contrary to the requirement of DsbA for proper motility [39]. This said, we could not at present functionally distinguish ScsA from the rest of the Scs proteins or TrxA. The strong contribution of thiol peroxidase and methionine sulfoxide reductases for protection from H_2O_2 mediated killing in *S. Typhimurium* [17] could further explain the redundancy of ScsBCD proteins in mediating any H_2O_2 stress tolerance *in vitro*.

When the $\Delta scsABCD$ mutant was tested in cell culture based virulence assays we did not note any alterations in its ability to invade epithelial cells or to replicate in macrophage-like cells. We did however observe that $CuCl_2$ blocked invasion with extreme efficacy. Furthermore, this decrease in invasion strongly relied on the $scsABCD$ and $trxA$ genes. When given intraperitoneally to BALB/c mice, the $\Delta scsABCD$ mutant showed a moderate enhancement in fitness as compared to the wild type. Also, when assaying invasion in ileal loops of mice, we noted an enhanced invasion efficacy for the $\Delta scsABCD$ mutant.

Apart from exhibiting decreased invasiveness *in vitro*, a $\Delta trxA$ mutant of *S. Typhimurium* also reveals strong virulence attenuation in the soil nematode *C. elegans* [22]. Here we demonstrate that both these attenuations depended on the scs genes. That is, selected attenuations caused by $CuCl_2$ or TrxA-deficiency are “conditionally” dependent on the scs genes.

Conditional phenotypes for oxidoreductase mutants of the thioredoxin superfamily have previously been described in *E. coli*. For example, H_2O_2 tolerance of *E. coli* relies on TrxA [30]. Surprisingly though a thioredoxin reductase (TrxB) deficient mutant while sensitive to H_2O_2 in stationary phase, shows even an increased tolerance to H_2O_2 in logarithmic phase of growth. This conditionality with H_2O_2 sensitivity comes from the fact that TrxB-deficiency associates with increased catalase expression evidently induced by accumulated oxidized TrxA [33]. A second example comes from the conditional contribution of oxidoreductases to copper chloride. In *E. coli*, lack of the periplasmic disulfide isomerase DsbC results in copper chloride sensitization, while deleting the disulfide oxidase DsbA does not [40]. Still, $CuCl_2$

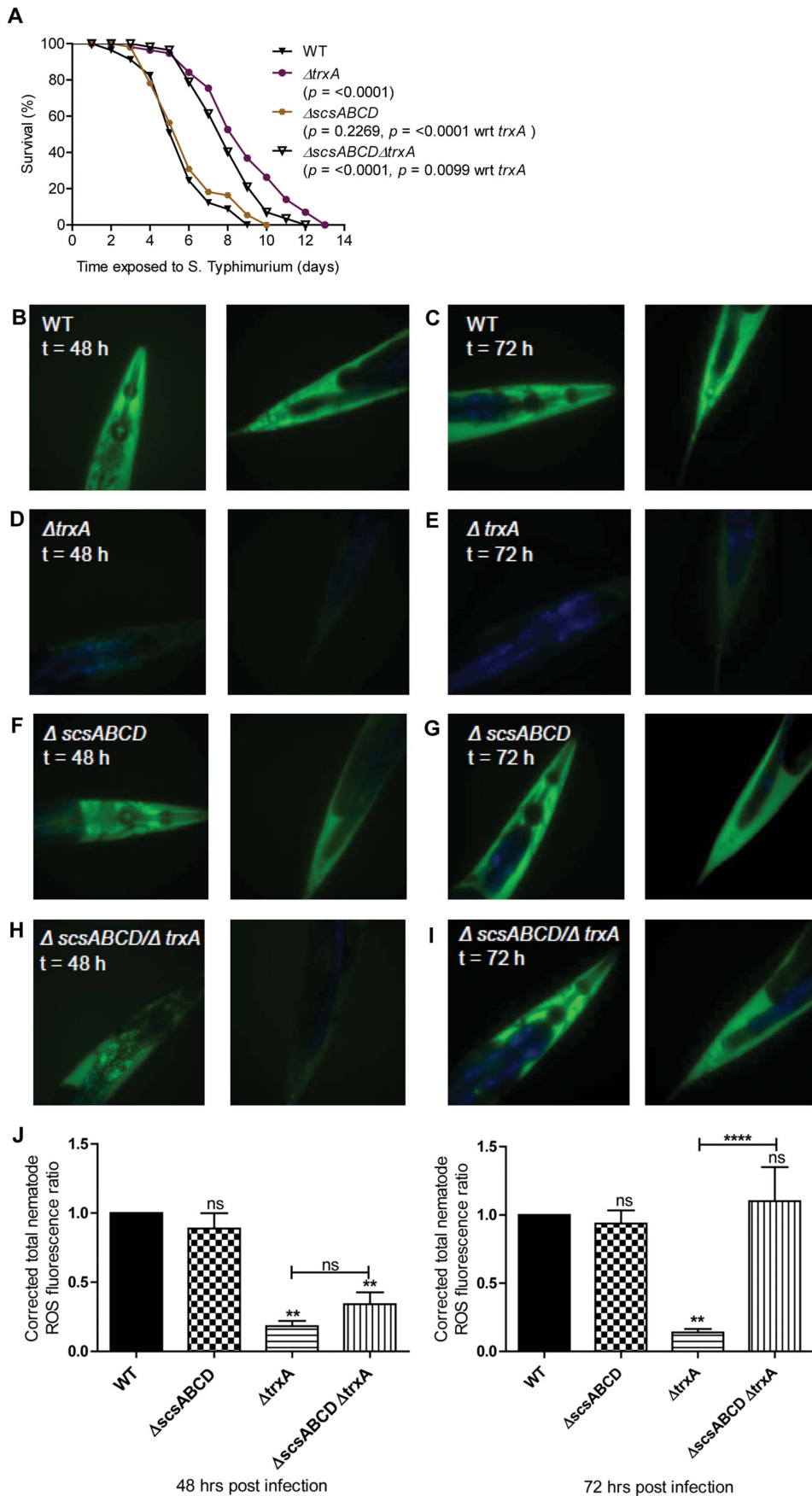


Figure 7. Virulence and ROS dissemination are restored in *C. elegans* by removing $\Delta scsABCD$ in the $\Delta trxA$ mutant. A) Survival of wild-type N2 nematodes was compared when infected with *S. Typhimurium* 14028 wild-type, $\Delta trxA$, $\Delta scsABCD$ or the $\Delta scsABCD/\Delta trxA$ mutant. B to I). Nematodes were harvested 48 or 72 hours post infection and stained with H_2DCFDA to detect intracellular ROS. In these images, ROS is shown in green and intestinal autofluorescence in blue. Images are shown at 40x magnification and are representative of at least 20 nematodes from 2 independent assays. J) Florescence intensities are compared with wild type and between different mutants. ns = non-significant, ** = $p \leq 0.01$, **** = $p \leq 0.0001$. Error bars indicate the standard error of the mean.
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sensitivity is enhanced in strains lacking both DsbA and DsbC. The explanation to this apparent contradiction may come from $CuCl_2$ itself; $CuCl_2$ is an efficient but evidently a non-specific oxidant that introduces non-native disulfide bonds in periplasmic proteins such as alkaline phosphatase [40]. Thus in the absence of a proteins catalyzing and proof-reading disulphide bond formation, $CuCl_2$ might cause accumulation of wrongly oxidized proteins to the extent of compromising bacterial fitness.

Assuming the Scs system participates in periplasmic redox shuffling, its conditional effects on virulence could relate to mechanisms similar to that proposed for the TrxA-TrxB and DsbA-DsbC interactors; presence of $CuCl_2$ or TrxA-deficiency could cause differential Scs-mediated accumulation of oxidized periplasmic proteins. Such accumulating proteins, being the Scs proteins themselves or their substrates, could subsequently relate to altered T3SS activity, and thus explain the containment of the $scsABCD$ genes in *S. enterica*. Indeed, the DsbA activity of *S. Typhimurium* has been connected to both SPII gene expression and in the SPII T3SS apparatus functionality [41]. Such connections led us to probe for the impact of the $scsABCD$ gene cluster for SPII gene expression and secretion potential. When applying $CuCl_2$ stress in cell culture medium, the transcriptional activity of the *hilA* and *prgH* promoters were reduced, as was the expression of the SipB-Bla fusion protein. Still, whether applying copper stress or not, the $\Delta scsABCD$ mutant appeared defective in secreting the SipB-Bla fusion protein. Clearly, the fusion protein does not represent an original T3SS effector protein, but the results still implicate a role for the Scs system in balancing SPII T3SS activity.

The above findings also provide a plausible mechanistic explanation for the invasiveness of the mutant. Under ordinary conditions the expression of SPII T3SS mediated invasion genes in wild type is highly active but leaky allowing secretion of effector proteins even in the absence of host cell contact. In the $\Delta scsABCD$ mutant the apparatus is less active, or stricter. Effector proteins accumulating inside mutant bacteria create a secretion competent pool applicable even after copper induced down-regulation of SPII gene expression, and notably, only used for translocation. Effectors secreted by SPII activate host JNK and p38 kinases, as well as nuclear localization of transcription factor NF- κ B [9]. These kinases and transcription factors are described to potentiate expression of the inducible nitric oxide synthase [42,43] as well as the phagocyte oxidase subunit gp91 [44]. Thereby a rationale for *S. enterica* maintaining the *scs* genes could relate to the ability of regulating SPII secretion activity.

The redox-dependency of T3SS is not restricted to *S. Typhimurium*. The activity of the virulence-associated T3SS of *Pseudomonas aeruginosa* and *Shigella flexneri* relies on their DsbA homologues [45,46]. Furthermore, expression of fimbrial adhesins in uropathogenic and enterotoxigenic *E. coli* [47,48] as well as secretion of cholera toxin in *Vibrio cholerae* [49] rely on corresponding DsbA homologues. Such reports in combination with our data on the *scs* genes implicate dependence and a highly balanced interaction of virulence factors with evolutionary conserved bacterial oxidoreductases belonging to the thioredoxin superfamily. Thereby, phenotypes caused by mutations in genes

for oxidoreductases could be conditional in more general terms, and not restricted to *scs* genes in *S. Typhimurium*. If indeed so, then one must consider that mutating oxidoreductases would not only cause alterations in virulence but also act as suppressor mutations.

Materials and Methods

Ethical Statement

The work did not involve human subjects or non-human primates. However, the mice were used in this study and the ethical permits were obtained from Stockholm Norra djurförsöksetiska Nämnd with N491/11 for intraperitoneal challenge and N172/08 for ileal loop infections. The animals were housed and monitored according to the national and international guidelines.

In silico Analyses

The *S. Typhimurium* strain LT2 and 14028s genome sequences annotated in 2001 [26] and 2010 [50] respectively, were used as reference for all the *in silico* analyses and primer design. Sequences from additional genome annotations were searched and compared in NCBI protein cluster for *Cys-X-X-Cys* motives. Search for Lipobox, classical signal peptide sequences and cleavage sites were conducted using the LipP 1.0 and SignalP servers [51], while protein localization and topologies were predicted using PSORTb vs3.0.2 and the Phyre2 server respectively [52].

Bacterial Strains, Plasmids, Phages and Nematodes

S. Typhimurium 14028 (ATCC, Manassas, VA, USA) was used as the wild type *S. Typhimurium* strain throughout the study. The plasmids used for site-directed mutagenesis [29] were pDK3, pDK4 and pDK46. For cloning purposes, the pBAD derivative pBAD33, the pACYC-184 derivative pSU41 and T7 promoter based expression vector pET32a (Novagen) were used [53,54,55]. The primers for cloning are given in Table S1. Cultures were propagated in Luria broth or on Luria agar plates with 10g sodium chloride per liter (Duchefa Biochemie, The Netherlands). For generating invasive bacterial cultures, bacteria were propagated in defined cell culture medium as given below. When necessary, growth media were supplemented with ampicillin (100 μ g/ml), chloramphenicol (10 μ g/ml), kanamycin sulfate (50 μ g/ml) or tetracycline (10 μ g/ml). To activate the arabinose promoter in pBAD33, cultures were supplemented with L-arabinose to a final concentration of 1% (w/v). All antibiotics and L-arabinose were from Sigma-Aldrich (St. Louis, MO, USA). Phage P22 *int* transduction was used to transfer mutations between strains [56].

The *C. elegans* strain used in this study was wild-type variant Bristol N2. Nematodes were cultured and maintained at 20°C on modified nematode growth media (NGM, 0.35% peptone) agar plates and fed with *E. coli* strain OP50, as described [57]. Both strains were obtained from the *Caenorhabditis* Genetics Center (Minneapolis, USA).

Bacterial strains and plasmids used and generated in this study are summarized in Table 1.

Table 1. Strains and plasmid.

| Strains | Genotype/Property | Reference |
|-----------------|---|------------|
| MC5 | Wild Type | ATCC 14028 |
| Fia-1280 | Wildtype::Tet ^r | [63] |
| NA48 | 14028(Δ <i>scsA</i>) | This study |
| NA49 | 14028(Δ <i>scsB</i>) | This study |
| NA50 | 14028(Δ <i>scsC</i>) | This study |
| NA51 | 14028(Δ <i>scsD</i>) | This study |
| NA91 | 14028(Δ <i>scsABCD</i>) | This study |
| NA198 | 14028(Δ <i>scsABCD</i> / Δ <i>trxA</i>) | This study |
| Fia-1195 | 14028(Δ <i>trxA</i>) | [20] |
| Fia-1381 | 14028(Δ <i>prgH</i> ::Tn5 <i>lacZY</i>) | [63] |
| Fia-1199 | 14028(Δ <i>hila</i> ::Tn5 <i>lacZY</i>) | [63] |
| Plasmids | | |
| pSU41 | pACYC-184 derivative vector control, Kan ^r | [54] |
| pNA10 | The <i>scsABCD</i> gene cluster cloned in pSU41 with 209 bp upstream region of <i>scsA</i> , Kan ^r | This study |
| pAUN1 | <i>sipB</i> - β -lactamase fusion cloned in pSU41, Kan ^r | [64] |
| pET32a | T7 promoter based expression vector | [55] |
| pNA14 | The <i>scsA</i> cloned in BamHI and XhoI site of pET32a, Amp ^r | This study |
| pNA15 | The <i>scsB</i> cloned in EcoRI and XhoI site of pET32a, Amp ^r | This study |
| pNA16 | The <i>scsC</i> cloned in BamHI and XhoI site of pET32a, Amp ^r | This study |
| pNA17 | The <i>scsD</i> cloned in BamHI and XhoI site of pET32a, Amp ^r | This study |
| pBAD33 | pBAD series vector control, Cm ^r | [53] |
| pFA3 | Full length <i>trxA</i> cloned in pBAD33, Cm ^r | [20] |
| pFA8 | Catalytically inactive <i>trxA</i> cloned in pBAD33, Cm ^r | [20] |

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Generation of Knockout Mutants

Individual *scs* genes, or the entire *scsABCD* locus, were deleted in the wild type *S. Typhimurium* using phage recombinase-assisted homologous recombination [29]. Briefly, the resistance genes from pDK3 or pDK4 were PCR-amplified with primer extensions homologous to individual *scs* genes, as defined in Table S2. PCR products were electroporated into the recipients containing pDK46 and recombinants selected using proper antibiotic selection. To exclude possible secondary mutations, the inserted cassettes were transduced by the phage P22 *int* into a fresh *S. Typhimurium* 14028 background. Finally, the antibiotic resistance cassettes were removed with the aid of plasmid pCP20 coding for recombinase [29].

All mutants were verified by PCR amplification of inserted resistance cassette with primers designed 100 bp up- and downstream of the target genes respectively, as listed in Table S3.

Protein Expression and Cell Fractionation

Individual *scs* genes with C-terminal His-tags were cloned into the cloning site of pET32a expression vector. The clones were transformed to *E. coli* BL21 strain. The cultures were grown overnight in 2 ml LB and were subcultured as 1:100 in 20 ml of LB with aeration at 37°C. The cultures were either induced with 2 mM IPTG or left uninduced and samples were collected after 3 hrs. Harvested cultures were fractionated into cytoplasmic, Triton X-100 (Tx-100) soluble, Tx-100 insoluble and periplasmic proteins as described in [58,59] and [60] respectively.

For generation of Tx-100 soluble, Tx-100 insoluble fractions, the bacteria were pelleted and the pellets were resuspended in PBS on

ice. Chilled suspensions were sonicated in 4 ml volume using the Soniprep 150 SANYO at 12 micron amplitude for 2×1.5 min on ice. Whole Cells were removed by centrifugation at 5000 rpm for 10 min. Supernatants were collected and centrifuged for 15 min at 14000 rpm and 4°C. The supernatant was collected as cytoplasmic soluble fraction. The pellet was solubilized in cytoplasmic membrane solution; 50 mM Tris-HCl, 10 mM MgCl₂, 1% (v/v) Triton X-100 buffer (pH 8.0), and centrifuged aforesaid. The supernatant containing cytoplasmic membranes was collected as the TX-100 soluble fraction. The pellet containing integral outer membrane proteins was solubilized in reducing SDS sample buffer.

For periplasmic proteins, the pellet from 5 ml culture was resuspended in 50 μ l of chloroform, vortexed and kept at room temperature (22°C) for 15 minutes. Five hundred microlitre of 0.01 M Tris hydrochloride (pH = 8.0) was added and mixed. After centrifugation at 6000xg for 20 minutes, the aqueous supernatant containing periplasmic proteins was collected [60].

Fractions were separated using SDS-polyacrylamide gel electrophoresis [61] and transferred to PVDF membrane by using iblot system (Invitrogen). The proteins were detected by using HRP conjugated monoclonal anti-His antibody and ECL substrate (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific). Biorad Gel doc machine was used for signal capture.

For mass-spectrometry, protein bands were cut from the gel, washed with water and reduced by DTE and alkylated with iodoacetamide. Proteins were digested with trypsin (Promega) in 50 mM NH₄HCO₃ at 37°C overnight. Extracted peptides were analyzed with MALDI-ToF analysis using a Bruker Ultraflex-ToF/

Tof instrument. Identification was done by using the peptide maps for search in the NCBIInr database using the Mascot search engine.

Copper Chloride, Zinc Chloride and Hydrogen Peroxide Tolerance

Bacteria were grown overnight on Luria agar (LA) plates and resuspended in phosphate buffered saline (PBS, pH = 7.4). For testing sensitivity to either CuCl₂ or ZnCl₂ (Sigma Aldrich), bacteria were inoculated from PBS into 2 ml of LB to contain 10³ bacteria per ml. Cultures were supplemented with CuCl₂ to a final concentration of 0 mM, 2 mM, 4 mM or 6 mM. For ZnCl₂, the final concentration was adjusted to 0 mM, 0.25 mM, 0.5 mM, 0.75 mM, 1.0 mM, 1.25 mM, 1.5 mM, 1.75 mM and 2.0 mM respectively. Tubes were incubated at 37°C overnight on Brunswick roller. Overnight cultures were diluted 1:10 in PBS and OD₆₀₀ were recorded, taking respective reaction mixture as blank. For H₂O₂ test, 10³ bacteria in 100 µl of PBS were mixed with 100 µl of H₂O₂ (Sigma) solution in 96-well microtitre plate, in triplicate, giving final concentration of H₂O₂ as 0 mM, 1 mM, 2 mM and 4 mM in PBS respectively. Plates were incubated at 37°C for 2 hours and 100 µl from each well was spread on LA plate. The seeded plates were incubated overnight at 37°C. Viable counts were determined by counting CFU on plates and percent viability as related to wells devoid of H₂O₂ were calculated.

Detection of Protein Carbonylation

The protocol detailed by [17] was followed with slight modifications. Overnight 2 ml LB cultures of *S. Typhimurium* were diluted 1:100 in fresh 25 ml LB and grown for 3.5 hrs at 37°C at 200 rpm. Cultures were exposed to H₂O₂ at a final concentration of 0.75 mM and incubated for further 3 hours at 37°C without shaking. Cells were then centrifuged at 5000 rpm and pellet was resuspended in 1 ml of 10 mM Tris-HCl, 25 mM NaCl and 50 mM DTT (pH 7.5) and transferred to 2 ml Eppendorf tube. Cells were mechanically lysed using the Soniprep 150 SANYO at 20 micron amplitude for 3 min on ice. The cell debris was removed by centrifugation at 14000 rpm for 10 minutes. After another centrifugation step, the clear supernatant was collected.

The periplasmic proteins were isolated according to [60] and as described above.

Oxidized proteins content was detected by following the manual provided with OxyBlot™ protein oxidation detection kit (Millipore). Briefly, 5 µl from each culture supernatant was mixed with 5 µl of 12% SDS. After 5 min 10 µl of the derivatizing reagent 1 x DNPH (dinitrophenyl hydrazine) was added and incubated at room temperature (22°C) for 15 min. Reaction was stopped by adding 7.5 µl neutralization solution provided in the kit. The samples were analyzed on a 12% SDS-PAGE.

The proteins were blotted onto a PVDF membrane (Amersham Hybond™-P, GE Healthcare), and subsequently were blocked overnight in blocking buffer (PBS with 1% BSA and 0.05% Tween 20). The membrane was incubated in rabbit anti-DNP-antibody (1:150), diluted in blocking buffer, for 1 hr at room temperature with shaking. After subsequent washing with PBS-T (0.05% goat anti-rabbit IgG horseradish peroxidase conjugate (1:300), diluted in blocking buffer, was applied for 1h. Oxidized proteins were detected by using the ECL substrate (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific) and Biorad Gel doc machine.

In vitro Invasion Assays

To generate invasive cultures of *S. Typhimurium* 14028, overnight cultures propagated in Luria broth were diluted (1:10) in 4 ml D-MEM (Gibco) supplemented with 10 mM HEPES and 10 mM L-Glutamine as described in [20]. The cultures were grown in 15 ml polystyrene plastic tubes under shaking for 4 hours lacking or being exposed to 3 mM CuCl₂. MDCK epithelial cell line was maintained and infected as described previously [20]. Briefly, the confluent cells in 24 well plates were exposed to 1 × 10⁶ bacteria (MOI 10:1) diluted from the invasion competent culture in 1 ml buffered D-MEM devoid of serum and gentamicin. Invasion was synchronized by briefly centrifuging the plates, where after plates were incubated for 1 hour at 37°C. After washing the monolayers twice with PBS, cells were covered with 10%FBS supplemented D-MEM containing 50 µg/ml of gentamicin for 45 minutes. Subsequently, the cells were washed twice with PBS and bacteria were released by lysing cells in 0.5% sodium deoxycholate in PBS (Merck, Darmstadt, Germany). Invasion efficacy was defined as the ratio of recovered CFU in relation to the bacteria enumerated from the inoculum.

Promotor-fusions and β-galactosidase Measurements

The transcriptional activities for *hilA::lacZ* and *prgH::lacZ* promotor fusions were determined as described previously [62] from overnight cultures in LB, or from bacteria grown to induce invasiveness in cell culture medium. The *hilA::lacZ* and *prgH::lacZ* constructs, received from Catherine A. Lee [63], were transduced in *scsABCD* mutant and wild type 14028 background.

SipB-β-lactamase Fusion Proteins Determination

Activity of the fusion protein was assayed either enzymatically or by immunoblotting. For determining the β-lactamase activities, pAUN1 plasmid carrying sipB-β-lactamase fusions were transformed in respective strains and enzymatic activities from the supernatants and whole cell lysates were determined as described by Negrea *et al.* [64]. Briefly, the invasive cultures, optically normalized, were centrifuged at 5000 rpm to collect the clear supernatant and the cells pelleted. Pellets were resuspended in PBS and were sonicated as described above. The proteins from the supernatant as well as from cell lysate were precipitated with 10% final concentration of trichloro acetic acid and resuspended in reducing SDS sample buffer. Equal volumes of protein preparations were run on SDS-polyacrylamide gel and blotted to PVDF membrane with iblot-system (Invitrogen). The proteins were detected by using Rabbit-anti-β-lactamase antibody (Abcam) in 1:3000 dilutions. After treating with anti-rabbit HRP-conjugated secondary antibody, the bands were detected as described earlier.

Intracellular Replication in RAW264.7 Cells

Murine macrophage-like RAW264.7 cells were grown in RPMI (Gibco) supplemented with FBS, HEPES, L-glutamine and gentamicin as described by [20,65]. To assay for possible alterations in intracellular replication, bacteria were diluted from overnight plate cultures in PBS, opsonized with mouse serum (10% v/v final concentration) for 30 minutes, and diluted in supplemented RPMI (Gibco) devoid of serum and gentamicin to represent 1 × 10⁶ bacteria per ml. The macrophages were exposed to 1 ml of inoculum in 24-well plates. To synchronize phagocytosis, plates were briefly centrifuged and incubated at 37°C for 1 hr. Cells were after washed twice with PBS and exposed to serum supplemented RPMI containing 50 µg/ml of gentamicin for 45 minutes. At this point, a 1 hr sample was collected after washing the cells in PBS and releasing bacteria by hypotonic lysis

of cells. The second sample was collected at 16 hrs post infection. Growth yields were defined as the ratio in viable counts from the 16 and one-hour samples.

Infection Experiments in Mice

Female BALB/c J mice at the age of 6 to 8 weeks were purchased from Taconic Europe (Denmark) and housed at Microbiology Tumor and Cell biology (MTC) animal facility, Karolinska Institutet, Stockholm, Sweden, under normal conditions in accordance with both institutional and national guidelines. For competition experiment, each group of mice ($n=5$) was infected intra-peritoneal by 1×10^4 bacteria/100 μ l of infection dose consisting of wild-type, tagged with a tetracycline resistance marker inserted at a neutral genomic position, and mutant mixture in 1:1 ratio [66]. The mice were sacrificed on third day post infection and livers and spleens were excised, homogenized in PBS and appropriate dilutions were placed on L-agar plates. Both members in each infection mixture were separated by appropriate resistance conferred by each of them [67]. Competitive indices (CI) were calculated as described previously [68].

The ileal loop infection experiment was carried out as described earlier [69]. The mice were deprived of water 4 hrs prior to the experiment. Mice were anaesthetized with isoflurane and were operated to expose the bowel. The ileum was located and near cecum, minimum 1.5 cm long piece was ligated at both ends taking care not to cut the ileum. 10^8 bacteria in a dose of 100 μ l were injected into the ligated part. The bowel of the mouse was stitched aseptically and mice were allowed to wake up. The mice were closely observed for 75 min and then sacrificed by cervical dislocation. The bowel was re-opened and ligated loop was cut apart and taken out. The loops were cut open, washed in PBS and extracellular bacteria were killed with the application of gentamicin at 100 μ g/ml in PBS for 1 hr. The washed ileum tissue was smashed in PBS and appropriate dilutions were plated to get the viable counts.

Infection Experiments in *C. elegans*

Bacterial strains were grown overnight in LB broth at 37°C and lawns were prepared by spreading 200 μ l of overnight culture on modified NGM agar. 20 L4-staged wild-type N2 *C. elegans* were subsequently infected as described [70]. Briefly, nematodes were set down on unseeded agar before transferring to bacterial lawns to reduce, as much as possible, the transfer of *E. coli*. Nematode survival was scored at 24°C and nematodes were considered dead when not responding to gentle touch by a platinum wire. Nematodes were also transferred to fresh bacterial lawns every day. Results are representative of triplicates from 3 independent assays.

Detection of Reactive Oxygen Species in *C. elegans*

2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA ; Sigma-Aldrich) was used to visualize intracellular ROS in nematodes. Stock aliquots of H_2DCFDA (2 mM) were prepared in dimethyl sulfoxide and stored in the dark at $-80^\circ C$. L4-staged N2 nematodes were infected as described above. At each time point,

infected nematodes were harvested into tubes and washed twice with M9 buffer [57]. Nematodes were subsequently incubated with 25 μ M H_2DCFDA in 250 μ l M9 buffer, in the dark for 30 min in a 20°C water bath. Nematodes were subsequently washed thrice with M9 buffer and mounted for microscopy in PBS with 25 mM sodium azide (NaN_3 , Merck). Slides were visualized on a LEICA DMRE microscope and images were analyzed by GNU Image Manipulation Program. Total nematode ROS fluorescence intensities were quantified using ImageJ software and expressed as a ratio relative to wild-type ATCC 14028 as described previously [71]. Images are representative of at least 20 nematodes from 2 independent assays.

Statistical Analyses

All the experiments were repeated at least three times and data were analyzed using the PRISM (version 5.0) software. The data for Figs. 2A to 2C were analyzed by linear regression and Fig. 2D further analyzed by t-test on the raw percent survival values. The data for Fig. 4, Fig. 5, Fig. 6A and Fig. 6C were analyzed by t-test. Data for Fig. 6B were analyzed by Wilcoxin signed-rank test. Fig. 7A data were analyzed by Kaplan–Meier estimation and Fig. 7J data were analyzed by Wilcoxin signed-rank test.

Supporting Information

Table S1 Primer sequences for cloning of *scs* genes. *S. Typhimurium* LT2 genome sequence was used as reference for designing of all the primers for cloning. (DOC)

Table S2 Primers used for mutagenesis of *scs* genes. Homologous overhangs in the mutagenesis primers are designed by taking *S. Typhimurium* LT2 genome sequence as reference. (DOC)

Table S3 PCR verification primers for mutants. The primers were designed 100 bp up- and downstream of ORFs of *scs* genes to amplify the inserted antibiotic cassette. The reference genome sequence for primer designing was of *S. Typhimurium* LT2 strain. (DOC)

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Author Contributions

Conceived and designed the experiments: NA XS MR. Performed the experiments: NA XS. Analyzed the data: NA XS MR. Contributed reagents/materials/analysis tools: MR. Wrote the paper: NA XS MR. Obtained ethical permits: MR.

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