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RESEARCH ARTICLE

CpxR/CpxA Controls *scsABCD* Transcription To Counteract Copper and Oxidative Stress in *Salmonella enterica* Serovar Typhimurium

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ABSTRACT Periplasmic thiol/disulfide oxidoreductases participate in the formation and isomerization of disulfide bonds and contribute to the virulence of pathogenic microorganisms. Among the systems encoded in the *Salmonella* genome, the system encoded by the *scsABCD* locus was shown to be required to cope with Cu and H_2O_2 stress. Here we report that this locus forms an operon whose transcription is driven by a promoter upstream of *scsA* and depends on CpxR/CpxA and on Cu. Furthermore, genes homologous to *scsB*, *scsC*, and *scsD* are always detected immediately downstream of *scsA* and in the same genetic arrangement in all *scsA*-harboring enterobacterial species. Also, a CpxR-binding site is detected upstream of *scsA* in most of those species, providing evidence of evolutionarily conserved function and regulation. Each individual *scs* gene shows a different role in copper and/or H_2O_2 resistance, indicating hierarchical contributions of these factors in the defense against these intoxicants. A protective effect of Cu preincubation against H_2O_2 toxicity and the increased Cu-mediated activation of *cpxP* in the Δ*scsABCD* mutant suggest that the CpxR/CpxA-controlled transcription of the ScsABCD system contributes to prevent Cu toxicity and to restore the redox balance at the *Salmonella* envelope.

IMPORTANCE Copper intoxication triggers both specific and nonspecific responses in *Salmonella*. The *scs* locus, which codes for periplasmic thiol/disulfide-oxidoreductase/isomerase-like proteins, has been the focus of attention because it is necessary for copper resistance, oxidative stress responses, and virulence and because it is not present in nonpathogenic *Escherichia coli*. Still, the conditions under which the *scs* locus is expressed and the roles of its individual components remain unknown. In this report, we examine the contribution of each Scs factor to survival under H_2O_2 and copper stress. We establish that the *scs* genes form a copper-activated operon controlled by the CpxR/CpxA signal transduction system, and we provide evidence of its conserved gene arrangement and regulation in other bacterial pathogens.

KEYWORDS copper resistance, CpxRA, envelope homeostasis, oxidative stress, ScsABCD, thioredoxin-like proteins

Copper is required in trace amounts as a cofactor or structural component of several enzymes, mainly linked to aerobic metabolism, but is very harmful in excess (1, 2). Free Cu ions can displace other essential metals, such a Fe from Fe-S clusters on enzymes, or catalyze redox cycling reactions with oxygen or nitrogen species, promoting the formation of reactive radicals and resulting in cell death (3, 4). The envelope of Gram-negative bacteria is the primary barrier against external injuries and, in consequence, is the first target of Cu toxicity. Cu(II)/Cu(I) cycling in this compartment was

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proposed to increase the formation of nonspecific disulfide bonds on proteins, affecting redox homeostasis (5–7).

Recent evidence indicates that eukaryotic cells use the biocidal properties of Cu to defend themselves against microbial pathogens (8–10). Macrophages actively deliver the metal ion to specific compartments where the pathogen resides, contributing to its intoxication (11). Therefore, the ability to handle and to eliminate the incoming toxic metal rapidly and actively or to repair Cu-induced damage is crucial for the survival of intracellular pathogens such as *Salmonella enterica*. This Gram-negative species contains a dedicated copper resistance system controlled at the transcriptional level by the cytoplasmic Cu(I) sensor CueR (1, 12). In the presence of Cu, CueR induces the expression of the membrane-bound P_{1B}-type ATPase CopA, which removes Cu(I) from the cytoplasm, and of two periplasmic proteins, namely, the multicopper oxidase CueO and the *Salmonella*-specific Cu(II)-binding protein CueP, which contribute to avoidance of further toxic reactions and back-diffusion of the metal ion into the cytoplasm (13–15). Recently, we demonstrated that, in contrast to other CueR-controlled genes, the expression of CueP also depends on CpxR/CpxA (16), an ancestral envelope stress-responding two-component system that activates gene expression in response to Cu excess (7, 17, 18). Unlike *Escherichia coli* and a number of Gram-negative species, and with the exception of new strains isolated from copper-fed cattle (19, 20), *S. enterica* does not harbor in its core genome genes that encode the CusCFBA efflux pump to remove Cu ions from the cell envelope (14). Although CueP was found to partially restore the copper resistance of an *E. coli* Δ*cus* mutant (14, 21), it is currently not known how *Salmonella* eliminates the excess metal ion from this compartment to counteract its toxic effects.

Protein cysteine SH groups are likely to oxidize at the periplasmic redox potential (22). A set of dedicated systems of oxidoreductases of the thioredoxin superfamily are required to promote the correct S-S formation and to preserve specific functional SH groups in this compartment, particularly under stress (23–25). These systems are composed of periplasmic proteins that oxidize or reduce thiol groups using electrons transferred from the cytoplasm by membrane-integrated components. *Salmonella* harbors the widely distributed DsbA/DsbB pair, which is responsible for *de novo* S-S formation, and two isomerase/reductase activity complexes, DsbC/DsbD and DsbG/DsbD, which fix improper S-S bonds or keep S groups reduced on different Cys-containing substrates (26–28). Also present in the pathogen are a DsbA homologue, SrgA, a substrate-specific DsbA/DsbB paralogue, DsbL/DsbI, which is essential for virulence (29), and the ScsC/ScsB pair, which was initially identified as part of the *Salmonella*-specific *scsABCD* locus, which suppresses the copper sensitivity of *E. coli* mutants after overexpression (30), with no identified substrates. The periplasmic component ScsC displays structural similarities to DsbA and DsbG; it forms monomers in solution like DsbA, but its catalytic domain is typical of the disulfide isomerases and is almost identical to that of DsbG (31). ScsB shows similarities to *Caulobacter crescentus* and *Proteus mirabilis* ScsB proteins, members of the DsbD superfamily that were shown to provide electrons to the specific ScsC homologues and to an envelope peroxide reduction pathway (32, 33). All Scs proteins, including ScsC, ScsB, and the other two inner-membrane-associated proteins with unknown function, ScsD and ScsA, contain Cys-X-X-Cys motifs (a hallmark of the oxidoreductase-thioredoxin superfamily) and a putative Cu-binding site (10, 23). Mutants with deletions of *scsC*, *scsB*, or *scsD* or the whole *scs* locus but not *scsA* showed equally decreased Cu resistance (34). Only the Δ*scsA* strain was affected by H_2O_2, however, and enhanced protein carboxylation in the periplasmic space in the presence of H_2O_2 was reported for the Δ*scsABCD* strain (34). The *scs* locus was also found to be required for SPI1-mediated secretion of SipB and for bacterial proliferation inside cortisol-activated macrophages (34, 35).

In this work, we report that *scsABCD* transcription is induced by Cu and depends on CpxR/CpxA (36, 37). The contributions of the Scs components, together with those of the DsbC-DsbG/DsbD systems, to Cu tolerance, as well as their roles in oxidative stress resistance, are evaluated. Our results indicate that the *scsABCD* operon is part of the Cpx

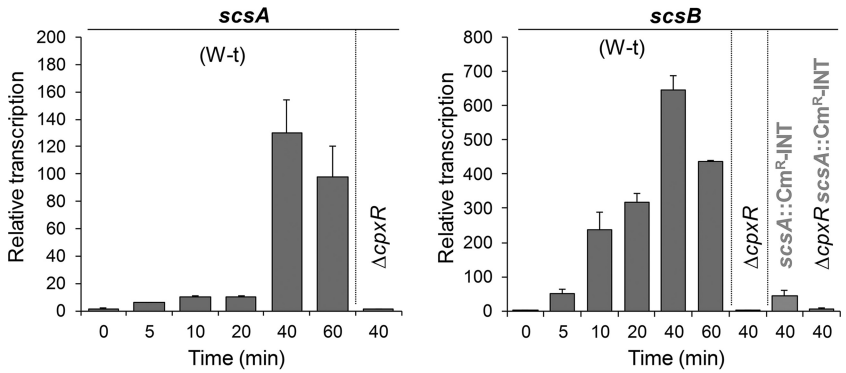

FIG 1 Transcription of *scsABCD* is induced by Cu and depends on CpxR/CpxA. The relative *scsA* and *scsB* mRNA levels were determined by real-time qRT-PCR using LB medium cultures obtained 0, 5, 10, 20, 40, and 60 min after challenge of the wild-type (W-t), Δ*cpxR*, *scsA*::Cm^r-INT, or Δ*cpxR scsA*::Cm^r-INT strain with 1 mM $CuSO_4$. At each time point, transcription levels were first normalized to the expression of *rnpB* and then relativized to the levels obtained in the absence of metal. Data correspond to the mean values from three independent experiments performed in triplicate. Error bars depict standard deviations (SDs).

regulon, which increases *Salmonella* survival under severe Cu and oxidative stress, hostile conditions encountered by the pathogen during its intracellular survival.

RESULTS

Transcription of the *scs* genes is induced by Cu. A genome-wide transcriptome analysis of the response of *Salmonella* after a 10-min shock with Cu or Zn salts (7) revealed that the *scs* locus was specifically upregulated in the presence of $CuSO_4$ when cells were grown in either minimal or rich medium (see Fig. S1A at http://www.ibr-conicet.gov.ar/investigacion/publicaciones). Under these conditions, no activation of other genes encoding proteins of the oxidoreductase-thioredoxin superfamily, i.e., *dsbA*, *dsbB*, *dsbC*, *dsbG*, *dsbD*, *dsbL*, *dsbI*, or *srgA*, was observed (see Fig. S1B at http://www.ibr-conicet.gov.ar/investigacion/publicaciones). Cu-mediated activation of the *scs* genes was verified using real-time quantitative reverse transcription-PCR (qRT-PCR). Transcription of both *scsA* and *scsB* increased with time and reached a maximum 40 min after Cu addition (Fig. 1), although with differences in the magnitude of the response achieved at different times after metal addition, particularly at times shorter than 20 min. This and the 48-bp spacing between *scsA* and the rest of the partially overlapping *scs* genes (see Fig. S2A at http://www.ibr-conicet.gov.ar/investigacion/publicaciones) might suggest that transcription of these genes could originate from two separate promoters, one located upstream of *scsA* and the other upstream of *scsB*, as prior reports proposed (30, 34, 35). Insertion of a chloramphenicol resistance (Cm^r) cassette 100 bp downstream of the translation start site of *scsA* and in the opposite orientation (*scsA*::Cm^r-INT), leaving a 311-bp region upstream of *scsB*, decreased by >10-fold the maximal Cu-promoted induction of *scsB* transcription (Fig. 1), indicating that transcription of the whole *scs* locus under copper stress is driven by the *scsA* promoter.

Transcription of the *scs* locus is stimulated by the CpxR/CpxA regulatory system. The Cu-mediated induction of *scs* transcription (Fig. 1) and the role of the Scs proteins in alleviating the damage caused by Cu and oxidative stress (34) prompted us to investigate whether transcription of the *scs* locus is controlled by regulatory factors involved in preserving the Cu or envelope homeostasis, such as CueR, the CueR paralogue GolS, the CpxR/CpxA and Rcs two-component systems, and the extracytoplasmic sigma E factor (7, 13, 14, 16, 36). A chromosomal *lacZ* reporter fusion to the promoter upstream of *scsA* (P*scs-lacZ*) was introduced into cells with deletions of *cueR*, *golS*, *cpxR-cpxA*, *rcsB*, or *rpoE*. As shown in Fig. 2A, only deletion of the genes encoding the sensor kinase CpxA or its cognate response regulator CpxR decreased P*scs-lacZ* expression. CpxR-mediated regulation was verified by qRT-PCR (Fig. 1). Deletion of

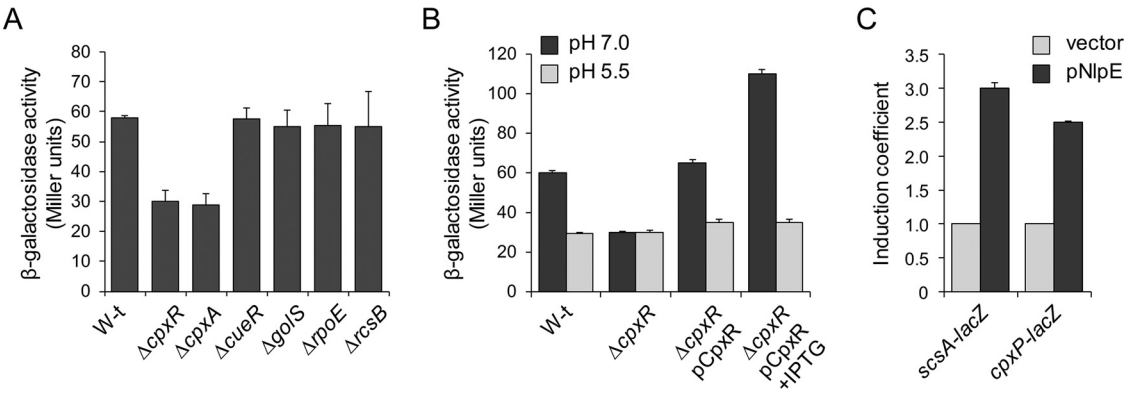

FIG 2 CpxR/CpxA controls the expression of the *scs* locus. (A) β-Galactosidase activity from a *scsA*::*lacZ* transcriptional fusion expressed in wild-type (W-t), Δ*cpxR*, Δ*cpxA*, Δ*cueR*, Δ*golS*, Δ*rpoE*, and Δ*rcsB* cells grown overnight in LB broth. (B) β-Galactosidase activity determined for the wild-type strain, the Δ*cpxR* strain, and the Δ*cpxR* strain complemented with pCpxR (Δ*cpxR*/pCpxR), all carrying the *scsA*::*lacZ* reporter fusion. Cells were grown overnight in LB medium with 100 mM MES buffer to adjust the pH value to 7.0 or 5.5, without (−IPTG) or with (+IPTG) the addition of 100 μM IPTG as indicated. The data shown in panels A and B correspond to mean values from four independent experiments performed in duplicate. Error bars represent SDs. (C) β-Galactosidase activity determined for wild-type cells carrying *scsA*::*lacZ* (*scsA-lacZ*) or *cpxP*::*lacZ* (*cpxP-lacZ*) (included as a CpxR-regulated positive control) transcriptional fusions and transformed either with the empty vector pUHE21-2*lacI*^{q} (vector) or with pNlpE and then grown in LB medium with the addition of 100 μM IPTG. (It should be noted that the *lacZ* in the *cpxP*::*lacZ* construction was introduced after the 3′ end of *cpxP*, in order to avoid undesirable disturbance of the CpxR/CpxA signal transduction pathway [36, 37, 54].) All values were normalized to the average activity obtained for cells with the control vector. Bars represent the average normalized values from at least three separate experiments. Error bars represent SDs.

CpxR abrogated the Cu-induced transcription of both *scsA* and *scsB*, including the remnant *scsB* transcription observed in the *scsA*::Cm^r-INT strain. Wild-type expression of the reporter construct was restored by complementing the Δ*cpxR* strain with *cpxR* expressed in *trans* under the control of an inducible promoter (Fig. 2B). As expected for a CpxR/CpxA-regulated gene, expression was reduced at acidic pH (Fig. 2B), a condition under which the CpxA kinase is not active (38), and was increased by NlpE overexpression (Fig. 2C), a condition that is known to activate the kinase (38, 39).

An *in silico* analysis of the promoter region upstream of *scsA* revealed the presence of a putative CpxR-binding site between position −46 and position −60, relative to the *scsABCD* transcriptional start site in the intergenic *scsA-cbpA* region (see Fig. S2A at http://www.ibr-conicet.gov.ar/investigacion/publicaciones). This sequence differs in 3 bases from the consensus 5′-GTAAAN$_5$GTAAA-3′ CpxR-binding site (40). No putative CpxR-binding sequence was identified within the *scsA* gene or in the intergenic *scsA-scsB* region. To confirm CpxR interaction with the predicted binding site on the *scsA* promoter (P*scs*), electrophoretic mobility shift assays (EMSAs) and DNase I footprinting assays were performed using increasing amounts of phosphorylated CpxR protein (CpxR-P) (Fig. 3A; also see Fig. S2B at http://www.ibr-conicet.gov.ar/investigacion/publicaciones). The regulator provided protection from position −41 to position −65, relative to the transcription start site of *scsA* in the coding strand, and from position −43 to position −67 in the noncoding strand (Fig. 3A), encompassing the predicted CpxR-binding sequence (see Fig. S2A at http://www.ibr-conicet.gov.ar/investigacion/publicaciones).

We modified, by site-directed mutagenesis, 2 key bases of the consensus CpxR-binding site identified at the P*scs* promoter, yielding the P*scs** promoter (5′-CGCCGA CATAACTTcAgAGG-3′, in which the modified bases are underlined and lowercase). Both modified and native (5′-CGCCGACATAACTTTACAGG-3′) P*scs* promoters were cloned upstream of the promoterless *lacZ* gene in the pMC1871 plasmid. As shown in Fig. 3B, the mutation reduced the levels of β-galactosidase activity measured in wild-type cells, and it was not affected by the *cpxR* deletion, in contrast to the strains harboring the native P*scs-lacZ* plasmid. Furthermore, CpxR-P was unable to interact with the modified P*scs** promoter (Fig. 3C), confirming the role of the CpxR/CpxA system in *scsABCD* transcriptional regulation.

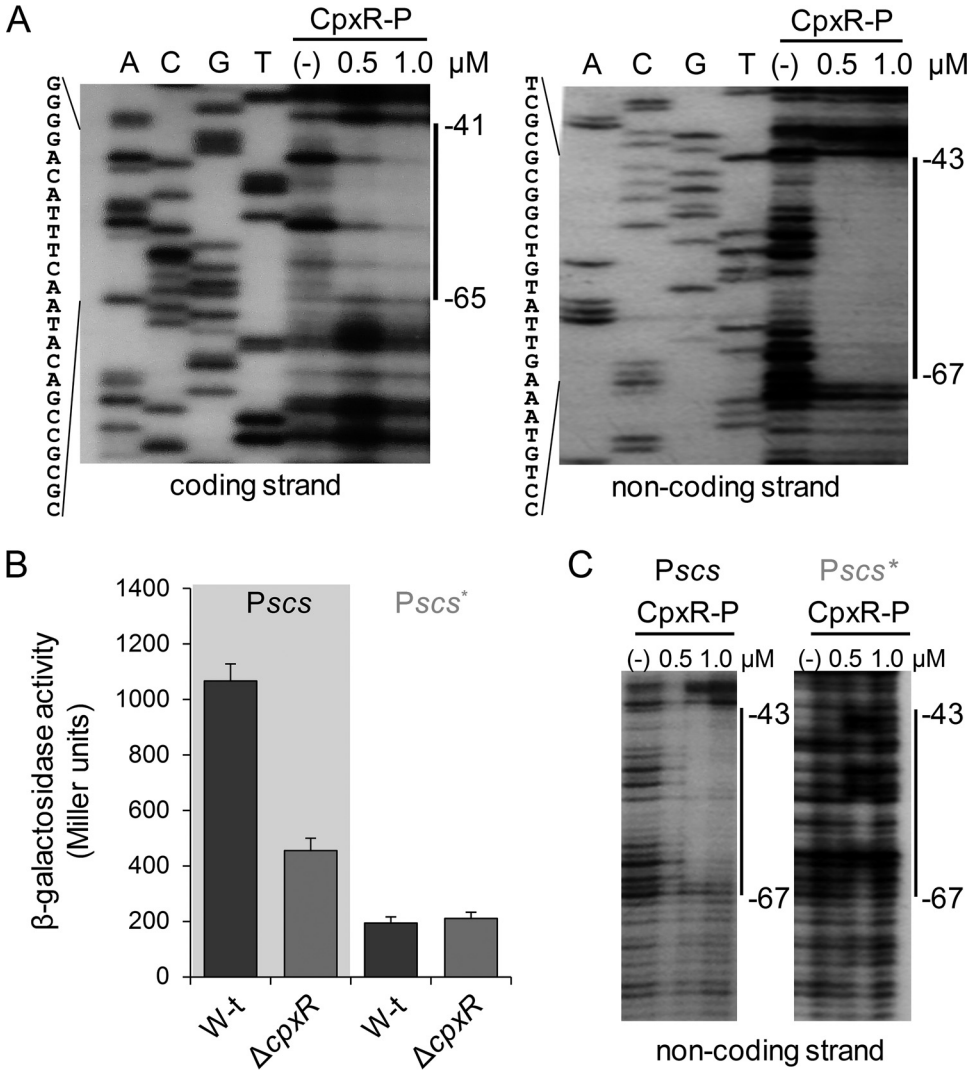

FIG 3 CpxR interacts with the *scsA* promoter at the predicted CpxR binding site. (A) DNase I footprinting analysis of the promoter region of *scsABCD*, performed on both end-labeled coding and noncoding strands. Purified and acetyl-phosphate-preincubated CpxR (CpxR-P), at the final concentrations of 0.5 and 1 μM, was added to the DNA fragments. Solid vertical lines on the right and sequences on the left indicate the CpxR-protected region. (B) β-Galactosidase activity of wild-type (W-t) and Δ*cpxR* strains carrying reporter plasmids in which expression of the *lacZ* gene was directed by the native *scsABCD* promoter (P*scs*) or by the promoter harboring mutations at the CpxR-binding site (P*scs**). The activity was determined on overnight cultures grown in LB medium with 100 mM MES (pH 7.0). The data correspond to mean values from three independent experiments performed in duplicate. Error bars correspond to SDs. (C) DNase I footprinting analysis of the native (P*scs*) or mutant (P*scs**) promoter regions performed on the noncoding strand. CpxR-P was added at the same final concentrations as in panel A. The predicted CpxR-protected region is shown with solid vertical lines.

Primary role of the ScsC/ScsB pair and ScsD in the defense of the cell envelope against Cu stress. It was reported previously that the absence of *scsB*, *scsC*, or *scsD*, but not *scsA*, produced moderate and identical effects on the susceptibility to Cu (34). In fact, it was shown that a strain with deletions of all four genes, Δ*scsABCD*, was as sensitive to Cu as the individual Δ*scsB*, Δ*scsC*, and Δ*scsD* strains. We reexamined the contribution of each Scs protein in the defense against Cu stress by recording the optical density at 600 nm (OD_{600}) of the cultures for 15 h and by assessing the development of colonies on Luria-Bertani (LB) agar plates containing increasing amounts of $CuSO_4$, a more direct and accurate method to detect small differences between strains. With both methods, we confirmed that ScsA, although coregulated with the other *scs* genes, was not involved in copper resistance. Importantly, we observed that ScsB, ScsC, and ScsD contrib-

TABLE 1 Contributions of ScsC/ScsB and DsbC-DsbG/DsbD to copper tolerance

Strain description	MIC (mM)[a]
Wild type	5.00
Δ*scsA*	5.00
Δ*scsB*	3.50
Δ*scsC*	3.75
Δ*scsD*	3.75
Δ*scsBC*	3.50
Δ*scsCD*	3.50
Δ*scsBCD*	3.50
Δ*scsABCD*	3.50
scsA::Cm^r-INT	3.50
Δ*dsbC*	5.00[b]
Δ*dsbG*	5.00
Δ*scsC* Δ*dsbC* Δ*dsbG*	3.00
Δ*dsbD* (*dipZ*)	5.00[b]
Δ*scsB* Δ*dsbD*	3.00

[a]MIC values were determined on LB plates containing increasing amounts of $CuSO_4$, under aerobic conditions (see Materials and Methods for details). The data correspond to mean values from three independent experiments performed in duplicate.
[b]Smaller colonies were observed, compared with the wild-type strain.

uted differently to Cu tolerance (Table 1; also see Fig. S3 at http://www.ibr-conicet.gov.ar/investigacion/publicaciones) (34). Only the strain with the deletion of *scsB* was as sensitive to Cu as the Δ*scsABCD* mutant, which could not form colonies at concentrations higher than 3.25 mM $CuSO_4$ (see Fig. S3B at http://www.ibr-conicet.gov.ar/investigacion/publicaciones). Identical Cu-sensitive phenotypes were observed for the strain with a polar *cat* cassette inserted in *scsA*, i.e., *scsA*::Cm^r-INT, and the Δ*scsBCD* mutant (see Fig. S3 at http://www.ibr-conicet.gov.ar/investigacion/publicaciones), providing further support for the presence of a major Cu-induced promoter upstream of *scsA* controlling *scsB*, *scsC*, and *scsD* transcription. Under these conditions, single Δ*scsC* or Δ*scsD* mutants were less sensitive to Cu than were strains with deletions of *scsB* or the whole *scs* locus (Table 1). As shown in Fig. S3B at http://www.ibr-conicet.gov.ar/investigacion/publicaciones, the absence of ScsC or ScsD impaired colony formation at copper concentrations higher than 3.5 or 3.75 mM, respectively. These results highlight hierarchical contributions of the components of the *scs* locus in copper resistance, with the membrane-associated reductase ScsB being the most important factor in this phenotype, followed by its putative periplasmic partner ScsC and the inner-membrane-associated protein ScsD. As expected (32, 33), the strain lacking both ScsB and ScsC was as sensitive to Cu as the Δ*scsB* strain (Table 1). In contrast, the Δ*scsCD* strain was more sensitive to the metal than were the mutants with individual deletions of *scsC* or *scsD*, resembling the Δ*scsB* phenotype (see Fig. S3B at http://www.ibr-conicet.gov.ar/investigacion/publicaciones), which suggests that ScsB could provide electrons not only to its periplasmic ScsC partner but also to the membrane-bound ScsD.

The periplasmic Cu(II)-binding protein CueP was recently reported to be a substrate of DsbC (28), a disulfide isomerase that, although not induced by Cu in *Salmonella* (see Fig. S1B at http://www.ibr-conicet.gov.ar/investigacion/publicaciones), contributes to Cu tolerance in *E. coli* (5). We analyzed the sensitivity to Cu of strains with deletions of *dsbC*, *dsbG*, or the associated membrane-bound reductase gene *dsbD* (also known as *dipZ* in *Salmonella*), in the presence or absence of a functional ScsC/ScsB system. Unlike the Δ*scsB* or Δ*scsC* mutants, deletion of *dsbC*, *dsbG*, or *dsbD* had little or no effect on Cu resistance (Table 1). All of those mutants exhibited similar MICs for $CuSO_4$, compared with the wild-type strain, although smaller colonies were observed for the Δ*dsbC* and Δ*dsbD* strains at 4.75 mM $CuSO_4$ (data not shown). Interestingly, the simultaneous deletion of *dsbC*, *dsbG*, and *scsC* or of *dsbD* and *scsB* severely affected Cu resistance, decreasing the MIC for $CuSO_4$ to 3.0 mM, lower than the MICs exhibited by the Δ*scsC* and Δ*scsB* single mutants (Table 1). These results indicate that, in the absence of a functional ScsC/ScsB system, DsbC-DsbG/DsbD also contributes to Cu resistance, which

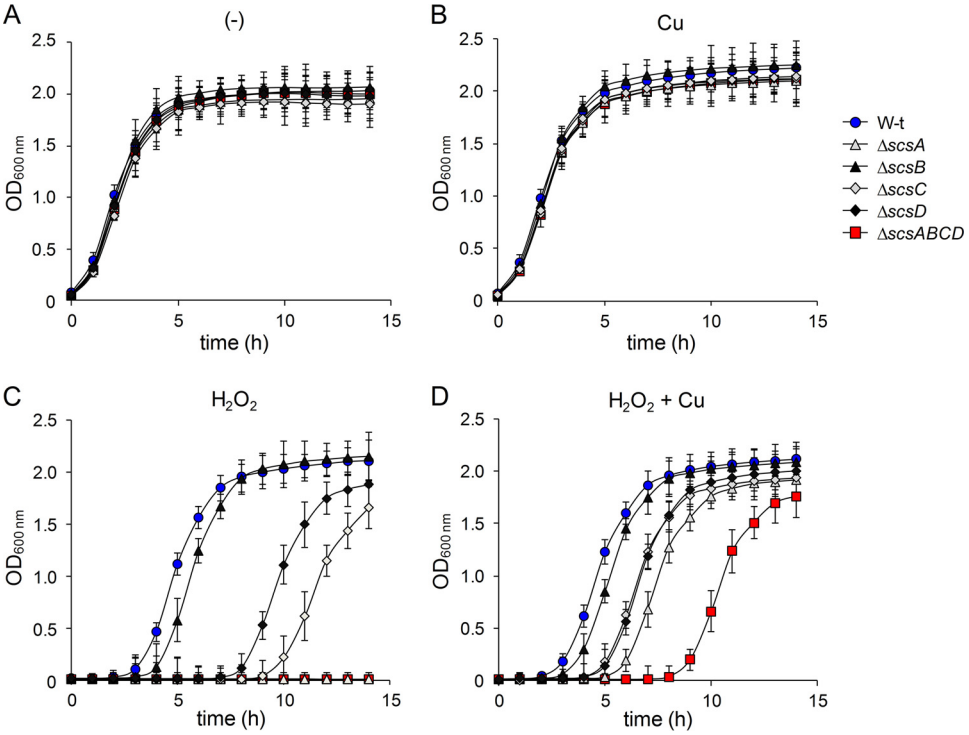

FIG 4 The Scs proteins are involved in balancing oxidative stress at the *Salmonella* envelope. Wild-type (W-t), Δ*scsA*, Δ*scsB*, Δ*scsC*, Δ*scsD*, and Δ*scsABCD* cells were grown under aerobic conditions in LB medium, without (−) or with the addition of 6 mM H_2O_2 and/or 1 mM $CuSO_4$, as indicated. The OD_{600} of the cultures was recorded every 1 h for 15 h. Results are the means and SDs from four independent experiments, each performed in duplicate.

supports overlapping roles for different oxidoreductase pairs under conditions of severe redox imbalance, as proposed previously (9, 23).

Role of the Scs proteins in response to H_2O_2. ScsA was reported to affect survival after 2 h of incubation with 2 or 4 mM H_2O_2 (34). Surprisingly, under those conditions, Anwar et al. reported that strains with deletions of the other three *scs* genes or the whole *scs* locus exhibited wild-type sensitivity to the oxidant, although the Δ*scsABCD* strain showed enhanced protein carboxylation in the periplasmic space (34), a hallmark of oxidative damage. In view of these somewhat contradictory results, we first compared the tolerance to H_2O_2 of the wild-type, Δ*scsABCD*, and Δ*scsBCD* strains, by recording their growth (OD_{600}) in LB broth for 15 h. A mutant in *tpx*, which codes for a periplasmic peroxiredoxin with low H_2O_2 tolerance (41), was used as a control. All of the strains, but particularly the Δ*tpx* strain, exhibited an extended lag phase as the concentration of H_2O_2 added to the medium increased (see Fig. S4 at http://www.ibr-conicet.gov.ar/investigacion/publicaciones). The differences in susceptibility to H_2O_2 between the wild-type strain and the *scs* mutants, as well as between the Δ*scsABCD* and Δ*scsBCD* strains, were more evident at 6 mM H_2O_2. At that concentration, the Δ*scsABCD* mutant was unable to grow (see Fig. S4 at http://www.ibr-conicet.gov.ar/investigacion/publicaciones), while the Δ*scsBCD* mutant showed a very extended lag phase, indicative of severe damage. (It should be noted that the growth observed for the Δ*scsBCD* strain after 8 h of incubation with 6 mM H_2O_2 could be attributed only to some surviving cells after H_2O_2 treatment and not to the appearance of suppressor mutations, because reincubation with 6 mM H_2O_2 of mutant cells recovered from the culture treated with peroxide for 15 h showed the same behavior.)

The results describe above confirm the requirement for ScsA in the defense against oxidative stress and demonstrate that the rest of the Scs proteins are also required for full H_2O_2 resistance. The analysis of the single *scs* mutants supports this suggestion (Fig. 4). The Δ*scsA* strain exhibited the same H_2O_2 sensitivity as the Δ*scsABCD* mutant at 6

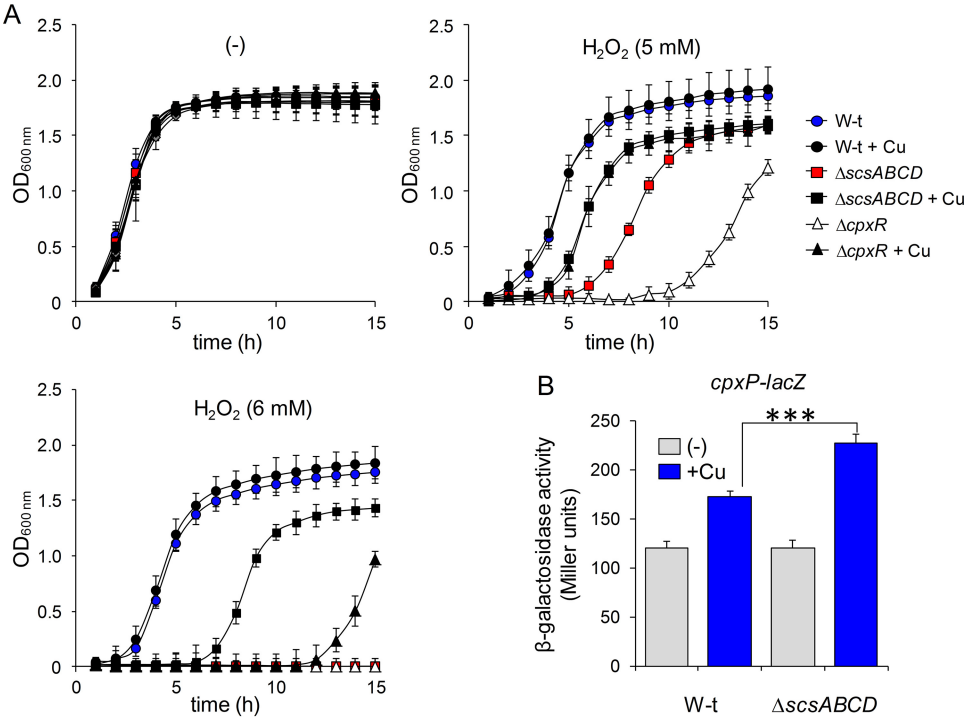

FIG 5 Cu protects mutant *scs* cells from oxidative stress by activating the expression of protecting factors. (A) Preincubation with copper protects *scsABCD*-deficient mutants from stress caused by H_2O_2. The *S.* Typhimurium wild-type (W-t) strain and mutant strains with deletion of *scsABCD* (Δ*scsABCD*) or *cpxR* (Δ*cpxR*) were grown to the early log phase (60 min) in LB medium without (−) or with 1 mM $CuSO_4$. The metal was then removed, and cultures were continued or challenged with the addition of 5 or 6 mM H_2O_2, as indicated. The growth was monitored as described above. Results are averages from three independent assays performed in duplicate, and error bars correspond to SDs. (B) The CpxR/CpxA response is enhanced by Cu in the absence of the *scs* locus. β-Galactosidase activity from a *cpxP-lacZ* transcriptional fusion expressed in wild-type or Δ*scsABCD* cells grown in LB medium with 100 mM MES (pH 7.0) was determined after 180 min of exposure to 1 mM $CuSO_4$. Data correspond to mean values from three independent experiments performed in triplicate. Error bars depict SDs. The Cu induction of the P*cpxP* reporter in the Δ*scsABCD* strain differed significantly from that in the wild-type strain. ***, $P < 0.001$.

mM H_2O_2. However, deletion of either *scsC* or *scsD* also increased the sensitivity to the oxidant (Fig. 4C), highlighting the importance of these proteins against oxidative stress. Finally, a small but significant increase in the lag phase, compared with the wild-type strain, was observed for the Δ*scsB* strain (Fig. 4C). These results indicate that all Scs proteins, not just ScsA, are involved in balancing oxidative stress at the *Salmonella enterica* serovar Typhimurium envelope.

Because expression of the Scs proteins is induced by Cu, we tested the effects of the simultaneous addition of 6 mM H_2O_2 and 1 mM $CuSO_4$, a metal concentration that does not affect the growth of the wild-type strain or the mutant strains tested in this study (Fig. 4B; also see Fig. S5 at http://www.ibr-conicet.gov.ar/investigacion/publicaciones). The addition of Cu partially reversed the susceptibility to H_2O_2 of all *scs* mutants, with the exception of the Δ*scsB* strain (compare Fig. 4C and D). The Cu-mediated protection was not caused by Cu-catalyzed elimination of the oxidant (42), since it did not improve the resistance to H_2O_2 of the wild-type strain (see Fig. S5 at http://www.ibr-conicet.gov.ar/investigacion/publicaciones). Most probably, it could be caused by nonspecific Cu-catalyzed oxidation of thiol groups at the envelope of the mutant cells, as proposed previously (5), or Cu-induced expression of protecting factors in these strains. To distinguish between these possibilities, we preincubated both the wild-type strain and the Δ*scsABCD* mutant for 60 min with 1 mM $CuSO_4$ and then removed the metal ion prior to H_2O_2 exposure. Under these conditions, only minimal nonspecific thiol-oxidizing activity of the metal was expected. As shown in Fig. 5A, preincubation with Cu markedly decreased the lag phase of the mutant in the presence of H_2O_2, while it

had no effect on the wild-type strain, supporting the hypothesis of Cu-induced expression of factors that protect against and/or repair redox damage in cells lacking a functional Scs system. We tested whether these putative factors were also under CpxR control. Indeed, the Δ*cpxR* strain had increased sensitivity to H_2O_2 (Fig. 5A), supporting our observations and confirming the importance of the Cpx response in coping with this oxidative damage. However, preincubation of the Δ*cpxR* strain with copper increased its resistance to peroxide (although not to the Δ*scsABCD* level), suggesting that other unidentified factors, besides those controlled by CpxR, are involved in the Cu-induced protection against H_2O_2.

Deletion of the *scs* locus augments the CpxR/CpxA response in the presence of Cu. The contribution of the Scs system in alleviating Cu and oxidative stress (Fig. 4; also see Fig. S3 at http://www.ibr-conicet.gov.ar/investigacion/publicaciones), its CpxR/CpxA dependence (Fig. 1, 2, and 3), and the CpxR-dependent protective effect of Cu against oxidative stress observed in the *scs* mutants (Fig. 5A) prompted us to evaluate whether the absence of the Scs system affected envelope homeostasis and consequently CpxR/CpxA activity. To test this, we determined the expression of a chromosomal *lacZ* gene fusion to *cpxP*, the archetypal Cpx-regulated factor (37), in the wild-type strain and the Δ*scsABCD* mutant, grown in the presence or absence of 1 mM $CuSO_4$. As shown in Fig. 5B, addition of Cu increased transcription from the P*cpxP* promoter in the wild-type strain, as expected for a Cpx-regulated gene (1, 16–18). Remarkably, in the absence of a functional *scsABCD* locus, Cu-mediated *cpxP* induction showed a significant increase (Fig. 5B), suggesting a role for the Scs proteins in restoring envelope homeostasis after a surge of the metal ion, thus preventing overstimulation of the Cpx system.

DISCUSSION

The *scs* (*s*uppressor of *c*opper *s*ensitivity) locus was initially identified for its ability to restore the copper tolerance of *E. coli* strains carrying mutations in *cutF* (*nlpE*) or *cutC*, as well as in *cutA* (*dsbD*), *lnt*, or *lgt* (which code for a periplasmic disulfide isomerase, an inner membrane apolipoprotein *N*-acyltransferase, and a phosphatidylglycerol-prolipoprotein diacylglyceryl transferase, respectively) (30, 43). Because of the homology of the *scs* gene products with thioredoxin-like proteins involved in oxidative disulfide folding and disulfide isomerization at the cell envelope of Gram-negative bacteria, the gene products were tested for their roles in redox biology and were found to alleviate the stress caused by copper or H_2O_2 (30, 31, 34). Their presence also reduces H_2O_2-mediated protein carbonylation in the periplasm (34), a common type of damage produced by reactive oxygen species (ROS)-generating agents such as copper (44). Several reports suggested that the locus contains two separate and independent transcriptional units, i.e., *scsA* and *scsBCD*, with different biological functions, with the first being required for oxidative stress responses and for *scsBCD* regulation and the second being involved in copper resistance (30, 31, 34, 35). Deletion of *scsA* but not the rest of the *scs* genes decreased H_2O_2 tolerance (34). In addition, a Δ*scsA* mutant showed increased transcription of *scsB*, *scsC*, and *scsD*, suggesting a role for ScsA in repressing the expression of *scsBCD* (35). Finally, deletions of *scsB*, *scsC*, or *scsD* but not *scsA* were shown to affect copper resistance (31, 34).

In contrast to these observations, here we establish that transcription of all four *scs* genes is driven by a Cu-activated promoter located upstream of *scsA* (Fig. 1). We demonstrated that the insertion of a polar chloramphenicol resistance cassette downstream from the *scsA* translational start site decreased the Cu-mediated activation of *scsB* transcription >10-fold (Fig. 1). We also showed that Cu induction of *scsABCD* depended on CpxR/CpxA (Fig. 1, 2, and 3), a two-component system essential for preserving and/or repairing periplasmic or inner membrane proteins damaged by different physical or chemical agents, including metals such as Cu, Zn, and Au (36, 37, 45). Our results indicate that, like *scsA* transcription, transcription of *scsB*, *scsC*, and *scsD* is driven by the CpxR-dependent *scsA* promoter, at least during copper stress. The identification of a single transcription start site located upstream of *scsA* in a

S. Typhimurium global gene expression study (46, 47) gives further support to our observation. In addition, we observed that, in all *scsA*-harboring enterobacterial species genomes, the *scsA* gene is always followed by homologues of *scsB*, *scsC*, and *scsD*, in that order (see Fig. S6 at http://www.ibr-conicet.gov.ar/investigacion/publicaciones), suggesting that the four *scs* products operate in an integrated biological pathway. In fact, the identification of a putative Cpx-binding sequence at the P*scs* promoter in most of those species (see Table S3 at http://www.ibr-conicet.gov.ar/investigacion/publicaciones) also provides evidence of conserved regulation. However, the presence of an alternative transcription start site upstream of *scsB*, promoting transcription of *scsBCD* independent of *scsA* under currently unidentified conditions, cannot be ruled out, as a short intergenic region separating *scsA* and *scsB* was observed in all of the analyzed genomes (see Fig. S6 at http://www.ibr-conicet.gov.ar/investigacion/publicaciones).

Anwar et al. reported that deletion of either *scsB*, *scsC*, or *scsD* had the same effect on Cu tolerance in *Salmonella* (34), suggesting that their products form part of a single detoxification complex. By performing a more detailed analysis of the contributions of these Scs proteins to copper resistance, here we establish that each of them plays a distinct role in alleviating the stress produced by the metal ion. We showed that the absence of ScsB produced the most dramatic effect on Cu tolerance under the conditions tested, followed in relevance by the mutant with deletion of the gene encoding its putative coupled periplasmic oxidoreductase, ScsC, and the strain with deletion of *scsD*, coding for a still uncharacterized integral membrane protein with a putative periplasmic thioredoxin-like domain (Table 1; also see Fig. S3 at http://www.ibr-conicet.gov.ar/investigacion/publicaciones). In addition, we demonstrated that other envelope thioredoxin-like proteins, such as DsbC, DsbG, and DsbD, are dispensable for Cu tolerance, except in the absence of a functional ScsC/ScsB system (Table 1), highlighting the importance of ScsB and its putative partner proteins ScsC and ScsD in Cu resistance. Our results also suggest that the Scs proteins and the DsbC/DsbD and DsbG/DsbD pairs act on different substrates, and we provide evidence regarding the functional cross talk between different Dsb-like systems to favor *Salmonella* survival under stressful conditions.

ScsC and ScsD are important not only in alleviating the damage caused by Cu but also in the defense against oxidative stress. Together with the major H_2O_2 detoxification factor of the system, ScsA (34), deletion of either *scsC* or *scsD* severely decreased growth at high concentrations of H_2O_2 (Fig. 4; also see Fig. S4 at http://www.ibr-conicet.gov.ar/investigacion/publicaciones). In fact, we observed that Cu contributed to protect the Δ*scs* mutant strain from H_2O_2 damage not only by stimulating nonspecific corrections of misformed S-S bonds on periplasmic proteins, as suggested previously for *E. coli* (5), but also by triggering the expression of damage-correcting factors. In this sense, the induction of transcription of the canonical CpxR/CpxA-regulated gene *cpxP* in the Δ*scsABCD* strain under Cu stress (Fig. 5B) suggests the existence of a feedback loop between Scs and CpxR/CpxA to restore envelope homeostasis after severe Cu and/or redox injury. The Cu-mediated induction of Cpx-independent oxidation-protecting factors (Fig. 5A) strengthens the relevance of the Scs system in restoring envelope homeostasis after severe Cu and/or redox injury.

The Cu-dependent transcriptional activation of the *scs* operon and the role of ScsB, ScsC, and ScsD in Cu resistance (Fig. 1 and Table 1; also see Fig. S3 at http://www.ibr-conicet.gov.ar/investigacion/publicaciones) predict a role for these proteins in the folding of periplasmic Cu-resistant determinants. CueP is of particular relevance, among the two periplasmic factors controlled by the copper sensor CueR (1); it is the major periplasmic Cu-binding protein required for Cu tolerance under anaerobic conditions (14, 21), and it was shown to deliver Cu to the periplasmic Cu,Zn-superoxide dismutase SodCII (48), linking Cu and oxidative stress. Three cysteine residues involved in metal binding and intrachain/interchain interactions in CueP are essential for its biological function (49). Like the Scs system, CueP is present in *Salmonella* and in a small set of bacterial species but not in *E. coli* (14), and its gene's transcription is also dependent on

CpxR/CpxA (16). However, a recent report indicates that CueP is a DsbC substrate (28). Current work is under way in our laboratory to determine the Scs target factors and their roles in copper and H_2O_2 tolerance.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *S. enterica* serovar Typhimurium strains and plasmids used in this study are listed in Table S1 at http://www.ibr-conicet.gov.ar/investigacion/publicaciones. Oligonucleotides are listed in Table S2 at http://www.ibr-conicet.gov.ar/investigacion/publicaciones. Cells were routinely grown at 37°C in LB broth or on LB agar plates, except when indicated. Ampicillin, tetracycline, kanamycin, and chloramphenicol were used, when necessary, at 100, 15, 50, and 20 μg ml^{-1}, respectively. All reagents and chemicals were from Sigma, except for the LB culture media, which were from Difco, and oligonucleotides and enzymes that were from Life Technologies.

Genetic and molecular biological techniques. The strains carrying gene deletions or a *lacZ* reporter fusion to a promoter on the chromosome, all derivatives of strain ATCC 14028s, were generated by Lambda Red-mediated recombination followed by P22-mediated transduction, using previously described protocols (16, 50, 51) and the primers listed in Table S2 at http://www.ibr-conicet.gov.ar/investigacion/publicaciones. When necessary, the antibiotic resistance cassette inserted at the deletion point was removed using FLP-mediated recombination (52). A similar procedure was employed to construct the *scsA*::Cm^r-INT strain, harboring the resistance cassette inserted 100 bp from the translational start site of *scsA*. DNA fragments as well as plasmids were introduced into bacterial cells by electroporation using a Bio-Rad device, following the manufacturer's recommendations. All constructs were verified by DNA sequencing.

The pPB1334 plasmid carrying the transcriptional fusion of the native *Salmonella* P*scs* promoter to *lacZ* (see Table S1 at http://www.ibr-conicet.gov.ar/investigacion/publicaciones) was constructed by cloning a 303-bp PCR-amplified product into the XmaI site of pMC1871 (Amersham), using previously described protocols (51). The reporter pPB1477 plasmid carrying the modified, CpxR-independent P*scs* promoter, P*scs** (see Table S1 at http://www.ibr-conicet.gov.ar/investigacion/publicaciones), was constructed by PCR-mediated site-directed mutagenesis. First, we amplified a 119-bp fragment using oligonucleotides Pscs*-Fw and PscsA-Rv (XmaI) (see Table S2 at http://www.ibr-conicet.gov.ar/investigacion/publicaciones), with pPB1334 (see Table S1 at http://www.ibr-conicet.gov.ar/investigacion/publicaciones) as the template. This fragment was then used as a primer along with PscsA-Fw (XmaI) to generate the final product carrying the mutant promoter for cloning into XmaI-digested pMC1871.

Induction and inhibition assays. β-Galactosidase activity was measured in total extracts from cells cultured for 18 h at 37°C in LB broth adjusted to either pH 7.0 or pH 5.5 by the addition of 100 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) and 1 mM $CuSO_4$, essentially as described previously (51). When indicated, 100 μM isopropyl-β-thiogalactopyranoside (IPTG) was added to induce expression of *cpxR* or *nlpE* from plasmids.

Real-time qRT-PCR assays were performed basically as described previously (7). Total RNA was prepared from wild-type, Δ*cpxR*, *scsA*::Cm^r-INT, or *scsA*::Cm^r-INT Δ*cpxR* cells grown to mid-exponential phase (OD_{620} of 0.4 to 0.7) after incubation for 0, 10, 20, 40, and 60 min with or without 1 mM $CuSO_4$, as indicated in the figures, using the RNAzol RT reagent (Molecular Research Center). After RQ1 DNase (Promega) treatment to improve quality, cDNA was obtained using Super Script II reverse transcriptase (Invitrogen), deoxynucleoside triphosphates (dNTPs), and specific sets of oligonucleotides (listed in Table S2 at http://www.ibr-conicet.gov.ar/investigacion/publicaciones) to amplify *scsA*, *scsB*, or the *rnpB* gene, which was used as a housekeeping gene to normalize transcription levels. Relative transcription levels were calculated as the ratio of normalized expression levels obtained after incubation in the presence or absence of Cu ions.

Copper sensitivity assays in liquid media were performed by recording the OD_{600} of cultures grown under aerobic conditions in LB broth without or with the indicated concentrations of $CuSO_4$. Metal sensitivity assays were performed on LB agar plates containing increasing concentrations of $CuSO_4$. To estimate the MIC values by CFU determination, overnight cultures of each strain were diluted to 10^{-6} in phosphate-buffered saline (PBS) and 10 μl of the indicated dilution was applied to the top of the plate. Colonies were allowed to develop for 24 h at 37°C before photographic recording. The MIC values were determined as the minimal concentration of $CuSO_4$ at which no growth was observed.

Sensitivity to H_2O_2 was tested by recording OD_{620} values, with a BioTek Synergy 2 multimode microplate reader, every 60 min for 15 h at 37°C. Overnight cultures of the indicated strains were diluted 1:100 in LB broth and applied in duplicate to a sterile 96-well microplate (Greiner Bio-One) containing fresh H_2O_2, at the indicated final concentration, and/or 1 mM $CuSO_4$ (see figure legends for details). When indicated, the metal salt was used to treat the cultures before sensitivity testing.

Protein-DNA interaction assays. EMSAs and DNase I footprinting assays were performed using 6 fmol of a ^{32}P-labeled DNA fragment containing the *scsABCD* promoter and CpxR-P, basically as described previously (16, 45). CpxR-P was obtained by incubating purified His-tagged CpxR with 25 mM acetyl phosphate for 1 h at 30°C. Protein concentrations were routinely determined with the Bradford assay, using bovine serum albumin as the standard. A DNA sequence ladder was generated in parallel by using the reverse primer and a Sequenase DNA sequencing kit (Affymetrix). After electrophoresis, the gels were dried and exposed for autoradiography.

***In silico* analysis.** The Seed tool (http://pubseed.theseed.org) was used to search for *scsA* homologues in other bacterial genomes (53).

Statistical analysis. One-way analysis of variance and the Tukey-Kramer multiple-comparison test, with an overall significance level of 0.05, were used.

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C.L. made major contributions to the design of the study and to the acquisition, analysis, and interpretation of the data. S.K.C. and F.C.S. made major contributions to the conception of the study, the interpretation of the data, and the writing of the manuscript.

We declare no conflicts of interest.

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