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1 **CpxR/CpxA-controls *scsABCD* transcription to counteract copper and**
2 **oxidative stress in *Salmonella* Typhimurium**

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16 *Running Title:* CpxR/CpxA and Cu control *scsABCD* transcription

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19 **Abstract**

20 Periplasmic thiol/disulfide oxidoreductases participate in the formation and
21 isomerization of disulphide bonds and contribute to the virulence of pathogenic
22 microorganisms. Among the systems encoded in the *Salmonella* genome, the
23 *scsABCD* locus was shown to be required to cope with Cu and H_2O_2 stress. Here
24 we report that this locus forms an operon whose transcription is driven by a
25 promoter upstream of *scsA* and depends on CpxR/CpxA and on Cu. Furthermore,
26 genes homologues to *scsB*, *scsC* and *scsD* are always detected immediately
27 downstream *scsA* and in the same genetic arrangement in all *scsA*-harboring
28 enterobacterial species. Also, a CpxR-binding site is detected upstream *scsA* in
29 most of these species, providing evidences of an evolutionary conserved function
30 and regulation. Each individual *scs* gene shows a different role in copper and/or
31 H_2O_2 resistance, evidencing a hierarchical contribution of these factors in the
32 defense against these intoxicants. A protective effect of Cu-preincubation against
33 H_2O_2 toxicity and the increased Cu-mediated activation of *cpxP* in the Δ*scsABCD*
34 mutant suggest that the CpxR/CpxA-controlled transcription of the ScsABCD
35 system contribute to prevent Cu-toxicity and to restore redox balance at the
36 *Salmonella* envelope.

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37 **Importance**

38 Copper intoxication triggers both specific and non-specific responses in
39 *Salmonella*. The *scs* locus -coding for periplasmic thiol/disulfide
40 oxidoreductases/isomerases-like proteins- has been the focus of attention because
41 it is necessary for copper resistance, oxidative stress and virulence, and because it
42 is not present in non-pathogenic *Escherichia coli*. Still, the conditions at which the
43 *scs* locus is expressed, or the role of its individual components remain unknown. In
44 this report we examine the contribution of each Scs factors for survival under H_2O_2
45 and copper stress. We establish that the *scs* genes are forming a copper-activated
46 operon controlled by the CpxR/CpxA signal transduction system and provide
47 evidence of its conserved gene-arrangement and regulation in other bacterial
48 pathogens.

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49 **Introduction**

50 Copper (Cu) is required in trace amounts as a cofactor or as a structural
51 component of several enzymes mainly linked to aerobic metabolism, but in excess
52 is also very harmful (1, 2). Free Cu ions can displace other essential metals such a
53 Fe from Fe-S clusters on enzymes or catalyze redox cycling reactions with oxygen
54 or nitrogen species promoting the formation of reactive radicals resulting in cell
55 dead (3, 4). The envelope of Gram negative bacteria is the primary barrier against
56 external injuries and, in consequence, the first target of Cu toxicity. Cu(II)/Cu(I)
57 cycling in this compartment was proposed to increase the formation of non-specific
58 disulfide bonds on proteins affecting redox homeostasis (5-7).

59 Recent evidences indicate that eukaryotic cells use the biocidal properties of
60 Cu to defend themselves against microbial pathogens (8-10). Macrophages
61 actively deliver the metal ion to specific compartments where the pathogen resides
62 contributing to its intoxication (11).Therefore, the ability to rapidly and actively
63 handle and eliminate the incoming toxic metal or to repair Cu-induced damages
64 become crucial for the survival of intracellular pathogens such as *Salmonella*
65 *enterica*. This Gram negative species contains a dedicated copper resistance
66 system controlled at the transcriptional level by the cytoplasmic Cu(I) sensor CueR
67 (1, 12). In the presence of Cu, CueR induces the expression of the membrane
68 bound P_{1B}-type ATPase CopA, which removes Cu(I) from the cytoplasm, as well as
69 of two periplasmic proteins, the multicopper oxidase CueO and the *Salmonella*-
70 specific Cu(II)-binding protein CueP, that contribute to avoid further toxic reactions
71 as well as back diffusion of the metal ion into the cytoplasm (13-15). Recently, we

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72 demonstrated that contrary to other CueR-controlled genes, expression of CueP
73 also depends on CpxR/CpxA (16), an ancestral envelope stress-responding two
74 component system that activates gene expression in response to Cu excess (7, 17,
75 18). Unlike *Escherichia coli* and a number of Gram negative species, and with the
76 exception of new strains isolated from copper-fed cattle (19, 20), *S. enterica* does
77 not harbor in its core genome the CusCFBA efflux pump to remove Cu ions from
78 the cell envelope (14). Although CueP was found to partially restore copper-
79 resistance of an *E. coli* Δ*cus* mutant (14, 21), it is currently unknown how
80 *Salmonella* eliminates the excess of the metal ion from this compartment to
81 counteract its toxic effects.

82 Protein cysteine SH groups are likely to oxidize at the periplasmic redox
83 potential (22). A set of dedicated systems of oxidoreductases of the thioredoxin
84 superfamily are required to promote the correct S-S formation and to preserve
85 specific functional SH groups at this compartment, particularly under stress (23-
86 25). These systems are composed of periplasmic proteins that oxidize or reduce
87 thiol groups using electrons transferred from the cytoplasm by membrane-
88 integrated components. *Salmonella* harbors the widely-distributed DsbA/DsbB pair
89 responsible for *de novo* S-S formation, and two isomerase/reductase activity
90 complexes, DsbC/DsbD and DsbG/DsbD that fix improper S-S bonds or keep S-
91 groups reduced on different Cys-containing substrate (26-28). Also present in the
92 pathogen are a DsbA homolog, SrgA, a substrate specific DsbA/DsbB paralog,
93 DsbL/DsbI, essential for virulence (29), and the ScsC/ScsB pair, initially identified
94 as part of the *Salmonella* specific *scsABCD* locus that suppresses copper

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95 sensitivity of *E. coli* mutants after overexpression (30), with no identified
96 substrates. The periplasmic component, ScsC, displays structural similarities to
97 DsbA and DsbG. It forms monomers in solution like DsbA, but its catalytic domain
98 is typical of the disulfide isomerases and almost identical to DsbG (31). ScsB
99 shows similarities to *Caulobacter crescentus* or *Proteus mirabilis* ScsB proteins,
100 members of the DsbD superfamily that were shown to provide electrons to the
101 specific ScsC homologs and to an envelope peroxide reduction pathway (32, 33).
102 All Scs proteins, including ScsC and ScsB, and the other two inner-membrane
103 associated proteins with unknown function, ScsD and ScsA, contain Cys-X-X-Cys
104 motifs, a hallmark of the oxidoreductase-thioredoxin superfamily and a putative Cu-
105 binding site (10, 23). Mutants deleted in *scsC*, *scsB* or *scsD*, or in the whole *scs*
106 locus, but not in *scsA*, equally decreased Cu resistance (34). On the other hand,
107 only the Δ*scsA* strain was affected by H_2O_2 and enhanced protein carboxylation at
108 the periplasmic space in the presence of H_2O_2 was reported for the Δ*scsABCD*
109 strain (34). The *scs* locus was also found to be required for SPI1-mediated
110 secretion of SipB and for bacterial proliferation inside cortisol-activated
111 macrophages (34, 35).

112 In this work, we report that *scsABCD* transcription is induced by Cu and
113 depends on CpxR/CpxA (36, 37). The contribution of each of the Scs component
114 together with those of the DsbC-DsbG/DsbD systems to Cu tolerance is evaluated
115 as well as their role in oxidative stress resistance. Our results indicate that the
116 *scsABCD* operon is part of the Cpx regulon that increases *Salmonella* survival

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117 under severe Cu and oxidative stress, hostile conditions encountered by the
118 pathogen during its intracellular survival.

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119 **Results**

120 **Transcription of the *scs* genes is induced by Cu**

121 A genome-wide transcriptome analysis of the response of *Salmonella* after a 10
122 min-shock to Cu or Zn salts (7) revealed that the *scs* locus is specifically
123 upregulated in the presence of $CuSO_4$, both when cells were grown in minimal or
124 rich medium (Fig. S1A). Under these conditions, no activation of other
125 oxidoreductase-thioredoxin superfamily coding gene, i.e., *dsbA*, *dsbB*, *dsbC*, *dsbG*,
126 *dsbD*, *dsbL*, *dsbI* or *srgA*, was observed (Fig. S1B). Cu-mediated activation of the
127 *scs* genes was verified using real-time reverse transcription-PCR (qRT-PCR).
128 Transcription of both *scsA* and *scsB* increased with time and reached a maximum
129 at 40 min from Cu addition (Fig. 1), although with differences in the magnitude of
130 the response achieved at different times after metal addition, particularly at times
131 shorter than 20 minutes. This and the 48 pb spacing between *scsA* and the rest of
132 partially overlapping *scs* genes (see Fig. S2A) may suggest that transcription of
133 these genes could originate from two separated promoters, one located upstream
134 *scsA* and the other upstream *scsB*, as prior reports proposed (30, 34, 35). Insertion
135 of a chloramphenicol resistance cassette at 100 pb downstream of the translation
136 start site of *scsA* and in the opposite orientation (*scsA*::Cm^R-INT), leaving a 311-bp
137 region upstream *scsB*, decreased more than ten-fold the maximal Cu-promoted
138 induction of *scsB* transcription (Fig. 1), indicating that the transcription of the whole
139 *scs* locus under copper stress is driven by the *scsA* promoter.

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140 **Transcription of the *scs* locus is stimulated by the CpxR/CpxA regulatory**
141 **system**

142 The Cu-mediated induction of *scs* transcription (see Fig. 1) and the role of the Scs
143 proteins in alleviating the damage cause by Cu and oxidative stress (34), prompted
144 us to investigate whether transcription of the *scs* locus is controlled by regulatory
145 factors involved in preserving the Cu- or envelope-homeostasis, such as CueR, the
146 CueR-paralog GolS, the CpxR/CpxA and Rcs two component-systems, and the
147 extracytoplasmic sigma E factor (7, 13, 14, 16, 36). A chromosomal *lacZ* reporter
148 fusion to the promoter upstream *scsA* (P*scs*-*lacZ*) was introduced in cells deleted
149 in *cueR, golS*, *cpxR-cpxA*, *rcsB* or *rpoE*. As shown in Fig. 2A, only deletion of the
150 genes encoding the sensor kinase CpxA or its cognate response regulator CpxR
151 decreased P*scs*-*lacZ* expression. CpxR-mediated regulation was verified by qRT-
152 PCR (Fig. 1). Deletion of CpxR abrogated the Cu-induced transcription of both
153 *scsA* and *scsB*, including the remnant *scsB* transcription observed in the
154 *scsA*::Cm^{R}-INT strain. Wild-type expression of the reporter construction was
155 restored by complementing the Δ*cpxR* strain with *cpxR* expressed in *trans* under
156 the control of an inducible promoter (Fig. 2B). As expected for a CpxR/CpxA-
157 regulated gene, its expression was reduced at acidic pH (Fig. 2B), a condition at
158 which the CpxA kinase is not active (38), and increased by NlpE overexpression
159 (Fig. 2C), a condition that is known to activate the kinase (38, 39).

160 An *in silico* analysis of the promoter region upstream *scsA* revealed the
161 presence of a putative CpxR-binding site between nt –46 and –60 relative to the
162 *scsABCD* transcriptional start site at the intergenic *scsA-cbpA* region (Fig. S2A).

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163 This sequence differs in 3 bases to the consensus 5'-GTAAAN$_5$GTAAA-3' CpxR-
164 binding site (40). No putative CpxR-binding sequence was identified within the
165 *scsA* gene or in the intergenic *scsA*-*scsB* region. To confirm CpxR interaction with
166 the predicted binding site on the *scsA* promoter (P*scs*), electrophoresis mobility
167 shift (EMSA) and DNase I footprinting assays were performed using increasing
168 amounts of phosphorylated CpxR protein (CpxR-P) (Fig. 3A and Fig. S2B). The
169 regulator protected from nt -41 to nt -65 relative to the transcription start site of
170 *scsA* in the coding strand, and from nt -43 to nt -67 in the non-coding strand (Fig.
171 3A), encompassing the predicted CpxR-binding sequence (Fig. S2A).

172 We modified by site-directed mutagenesis two key bases of the consensus
173 CpxR-binding site identified at the P*scs* promoter rendering the P*scs** promoter (5'-
174 CGCCGACATAACTTcAgAGG-3'). Both the modified and the native P*scs* (5'-
175 CGCCGACATAACTTTACAGG-3') promoters were cloned upstream the
176 promoterless *lacZ* gene in the pMC1871 plasmid. As shown in Fig. 3B, the
177 mutation reduced the levels of β-galactosidase activity measured in wild-type cells,
178 and it was not affected by the *cpxR* deletion, contrary to the strains harboring the
179 native P*scs*-*lacZ* plasmid. Furthermore, CpxR-P was unable to interact with the
180 modified P*scs** promoter (Fig. 3C), confirming the role of the CpxR/CpxA system in
181 *scsABCD* transcriptional regulation.

182

183 **Primary role of the ScsC/ScsB pair and ScsD in the defense of the cell**
184 **envelope against Cu stress**

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185 It was previously reported that the absence of either *scsB*, *scsC* or *scsD*, but not
186 *scsA*, produce a moderate and identical effect in the susceptibility to Cu (34). In
187 fact, it was shown that a strain deleted of all four genes, Δ*scsABCD*, is as sensitive
188 to Cu as the individual Δ*scsB*, Δ*scsC* or Δ*scsD* strains. We reexamined the
189 contribution of each Scs protein in the defense against Cu stress by recording the
190 optical density of the culture for 15 hours at 600 nm (OD_{600}) and by the
191 development of colonies on LB agar plates containing increasing amounts of
192 $CuSO_4$, a more direct and accurate method to evidence small differences between
193 strains. With both methods we confirmed that ScsA, although co-regulated with the
194 other *scs* genes, is not involved in copper resistance. Importantly, we observed
195 that ScsB, ScsC and ScsD differentially contribute to Cu tolerance (Table 1, see
196 also Fig. S3) (34). Only the strain deleted of *scsB* was as sensitive to Cu as the
197 Δ*scsABCD* mutant, which could not form colonies at concentrations higher than
198 3.25 mM $CuSO_4$ (Fig. S3B). Identical Cu-sensitive phenotype was observed for the
199 strain with a polar *cat* cassette inserted in *scsA*, i.e., *scsA*::Cm^R-INT, or the
200 Δ*scsBCD* mutant (Fig. S3), providing further support to the presence of a major
201 Cu-induced promoter upstream *scsA* controlling *scsB*, *scsC* and *scsD* transcription.
202 In these conditions, single Δ*scsC* or Δ*scsD* mutants were less sensitive to Cu than
203 the strains delete in *scsB* or in the whole *scs* locus (Table 1). As shown in Fig.
204 S3B, the absence of ScsC or ScsD impaired colony formation at copper
205 concentrations higher than 3.5 or 3.75 mM, respectively. These results highlight a
206 hierarchical contribution of the components of the *scs* locus in copper-resistance,
207 being the membrane-associated reductase ScsB the most important factor in this
208 phenotype, followed by its putative periplasmic partner ScsC, and the inner-

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209 membrane associated protein ScsD. As expected (32, 33), the strain lacking both
210 ScsB and ScsC was as sensitive to Cu as the Δ*scsB* strain (Table 1). In contrast,
211 the Δ*scsCD* strain was more sensitive to the metal than the individual mutants in
212 *scsC* or *scsD*, resembling the Δ*scsB* phenotype (Fig. S3B), and suggesting that
213 ScsB could provide electrons not only to its periplasmic ScsC partner, but also to
214 the membrane-bound ScsD.

215 The periplasmic Cu(II)-binding protein CueP was recently reported to be a
216 substrate of DsbC (28), a disulfide isomerase that although not induced by Cu in
217 *Salmonella* (see Fig. S1B) contributes to Cu tolerance in *E. coli* (5). We analyzed
218 the sensitivity to Cu of strains deleted in *dsbC*, *dsbG* or the associated membrane-
219 bound reductase gene *dsbD* (also known as *dipZ* in *Salmonella*) in the presence or
220 absence of a functional ScsC/ScsB system. Unlike the Δ*scsB* or Δ*scsC* mutants,
221 either deletion of *dsbC*, *dsbG* or *dsbD* had little or no effect on Cu resistance
222 (Table 1). All these mutants exhibited similar minimum inhibitory concentration
223 (MIC) for $CuSO_4$ than the wild-type strain, although smaller colonies were
224 observed in the Δ*dsbC* or the Δ*dsbD* strains at 4.75 mM $CuSO_4$ (data not shown).
225 Interestingly, the simultaneous deletion of *dsbC*, *dsbG* and *scsC*, or of *dsbD* and
226 *scsB* severely affected Cu resistance, decreasing the MIC for $CuSO_4$ to 3.0 mM,
227 lower than the MIC exhibited either by the Δ*scsC* or the Δ*scsB* single mutants
228 (Table 1). These results indicate that in the absence of a functional ScsC/ScsB
229 system, DsbC-DsbG/DsbD also contributes to Cu resistance, supporting an
230 overlapping role between different oxidoreductase pairs under conditions of severe
231 redox imbalance, as previously proposed (9, 23).

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233 **Role of the Scs proteins in response to H_2O_2**

234 ScsA was reported to affect survival after 2 h incubation with 2 or 4 mM H_2O_2 (34).
235 Surprisingly, under these conditions, Anwar *et al* reported that strains deleted in
236 the other three *scs* genes or in whole *scs* locus exhibited wild-type sensitivity to the
237 oxidant, although the Δ*scsABCD* strain showed enhanced protein carboxylation at
238 the periplasmic space (34), a hallmark of oxidative damage. In view of these
239 somehow contradictory results, we first compared the tolerance to H_2O_2 of the wild-
240 type, the Δ*scsABCD* and the Δ*scsBCD* strains by recording their growth (OD_{600}) in
241 LB for 15 h. A mutant in *tpx*, coding for a periplasmic peroxiredoxin with low H_2O_2
242 tolerance (41), was used as a control. All the strains, but particularly the Δ*tpx*,
243 exhibited an extended lag phase as the concentration of H_2O_2 added to the
244 medium increased (Fig. S4). The differences in the susceptibility to H_2O_2 between
245 the wild-type strain and the *scs* mutants as well as between the Δ*scsABCD* and
246 Δ*scsBCD* strains were more evident at 6 mM H_2O_2. At this concentration, the
247 Δ*scsABCD* mutant was unable to grow (Fig. S4), while the Δ*scsBCD* mutant
248 showed a very extended lag phase, indicative of a severe damage. (To note, the
249 growth observed for the Δ*scsBCD* after 8 h incubation with 6 mM H_2O_2 could be
250 only attributed to some survival cells after H_2O_2 treatment and not to the
251 appearance of suppressor mutations, as the re-incubation with 6 mM H_2O_2 of these
252 mutant cells recovered from the 15 h peroxide-treated culture time point showed
253 the same behavior.)

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254 The above results confirm the requirement of ScsA in the defense against
255 oxidative stress, and demonstrate that the rest of the Scs proteins are also
256 required for full H_2O_2 resistance. The analysis of the single *scs* mutants supports
257 this suggestion (Fig. 4). The Δ*scsA* strain exhibited the same H_2O_2 sensitivity than
258 the Δ*scsABCD* mutant at 6 mM H_2O_2. However, deletion of either *scsC* or *scsD*
259 also increased the sensitivity to the oxidant (Fig. 4C), highlighting the importance of
260 these proteins against oxidative stress. Finally, a small but significant delay in the
261 lag-phase compared with the wild-type was observed for the Δ*scsB* strain (Fig.
262 4C). These results indicate that all Scs proteins, not just ScsA, are involved in
263 balancing oxidative stress at the *S.* Typhimurium envelope.

264 As the expression of the Scs proteins is induced by Cu, we tested the effects
265 of the simultaneous addition of 6 mM H_2O_2 and 1 mM $CuSO_4$, a metal
266 concentration that does not affect growth of the wild type or the mutant strains
267 tested in this study (Fig. 4B, see also Fig. S5). Addition of Cu partially reversed the
268 susceptibility to H_2O_2 of all *scs* mutants, with the exception of the Δ*scsB* strain
269 (compare Fig. 4C and D). The Cu-mediated protection was not caused by the Cu-
270 catalyzed elimination of the oxidant (42), since it did not improve the wild-type
271 strain resistance to H_2O_2 (see Fig. S5). Most probably, it could be caused by the
272 nonspecific Cu-catalyzed oxidation of thiol groups at the envelope of the mutant
273 cells, as previously proposed (5), or the Cu-induced expression of protecting
274 factors in these strains. To discern between these possibilities, we pre-incubated
275 both the wild-type and mutant Δ*scsABCD* for 60 min with 1 mM $CuSO_4$ and then
276 removed the metal ion prior to H_2O_2-exposure. In this condition, only minimal

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277 nonspecific thiol-oxidizing activity of the metal was expected. As shown in Fig. 5A,
278 preincubation with Cu markedly decreased the lag-phase of the mutant in the
279 presence of H_2O_2, while it had no effect on the wild-type strain, supporting the
280 hypothesis of the Cu-induced expression of factors that protect/repair the redox
281 damage in cells lacking a functional Scs systems. We tested whether these
282 putative factors were also under CpxR-control. Indeed, the Δ*cpxR* strain had an
283 increase sensitivity to H_2O_2 (Fig. 5A) supporting our observations and the
284 importance of the Cpx-response to cope with this oxidative damage. However,
285 preincubation of the Δ*cpxR* strain with copper increased its resistance to peroxide -
286 although not to the Δ*scsABCD* level- suggesting that other unidentified factors are
287 involved in the Cu-induced protection against H_2O_2 besides those controlled by
288 CpxR.

289

290 **Deletion of the *scs* locus augments the CpxR/CpxA response in the presence**
291 **of Cu**

292 The contribution of the Scs system in alleviating Cu and oxidative stress (see Fig. 4
293 and S3), its CpxR/CpxA-dependence (Fig 1, 2 and 3), and the CpxR-dependent
294 protective effect of Cu against oxidative stress observed in the *scs* mutants (Fig.
295 5A) prompted us to evaluate whether the absence of the Scs system affects the
296 envelope homeostasis and in consequence CpxR/CpxA activity. To test this, we
297 determined the expression of a chromosomal *lacZ* gene fusion to *cpxP*, the
298 archetypal Cpx regulated factor (37), in the wild-type or in the Δ*scsABCD* mutant

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299 grown in the presence or absence of 1 mM $CuSO_4$. As shown in Fig. 5B, addition
300 of Cu increased transcription from the P*cpxP* promoter in the wild type strain as
301 expected for a Cpx-regulated gene (1, 16-18). Remarkably, in the absence of a
302 functional *scsABCD* locus, Cu-mediated *cpxP* induction showed a significant
303 increase (Fig. 5B), suggesting a role for the Scs proteins in restoring the envelope
304 homeostasis after a surge of the metal ion, therefore preventing the overstimulation
305 of the Cpx-system.

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306 **Discussion**

307 The *scs* (suppressor of copper sensitivity) locus was initially identified for its ability
308 to restore copper tolerance of *E. coli* strains carrying mutations in *cutF* (*nlpE*) or in
309 *cutC*, but also in *cutA* (*dsbD*), *lnt* or *lgt*, coding for a periplasmic disulfide
310 isomerase, an inner membrane apolipoprotein N-acyltransferase and a
311 phosphatidylglycerol-prolipoprotein diacylglyceryl transferase, respectively (30, 43).
312 Because of the homology of the *scs* gene products with thioredoxin-like proteins
313 involved in oxidative disulfide folding and disulfide isomerization at the cell
314 envelope of Gram-negative bacteria, they were tested for their role in redox
315 biology, and were found to alleviate the stress caused either by copper or by H_2O_2
316 (30, 31, 34). Their presence also reduces H_2O_2-mediated protein carbonylation in
317 the periplasm (34), a common damage exerted by reactive oxygen species (ROS)
318 generating-agents like copper (44). Several reports suggested that the locus
319 contains two separate and independent transcriptional units, *scsA* and *scsBCD*,
320 with different biological functions, being the first required for oxidative stress as
321 well as for *scsBCD* regulation, and the second involved in copper-resistance (30,
322 31, 34, 35). Deletion of *scsA*, but not of the rest of the *scs* genes, decreased H_2O_2
323 tolerance (34). Also, a Δ*scsA* mutant showed an increased transcription of *scsB,*
324 *scsC and scsD*, suggesting a role of ScsA in repressing the expression of *scsBCD*
325 (35). Finally, mutants deleted in *scsB*, *scsC* or *scsD*, but not in *scsA*, were shown
326 to affect copper-resistance (31, 34).

327 In contrast to these observations, here we establish that transcription of all
328 four *scs* genes is driven by a Cu-activated promoter located upstream of *scsA* (Fig.

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329 1). We demonstrate that the insertion of a polar chloramphenicol-resistance
330 cassette downstream the *scsA* translational start site decreased more than ten
331 folds the Cu-mediated activation of *scsB* transcription (Fig. 1). We also show that
332 Cu-induction of *scsABCD* depends on the CpxR/CpxA (Fig. 1, 2 and 3), a two
333 component system essential for preserving/repairing periplasmic or inner
334 membrane proteins damaged by different physical or chemical agents, including
335 metals like Cu, Zn or Au (36, 37, 45). Our results indicate that like *scsA*,
336 transcription of *scsB*, *scsC* and *scsD* is driven from the CpxR-dependent *scsA*
337 promoter, at least during copper-stress. The identification of a single transcription
338 start site located upstream of *scsA* in a S. Typhimurium global gene expression
339 study (46, 47) give further support to our observation. In addition, we observed that
340 in all *scsA*-harboring enterobacterial species genomes, the *scsA* gene is always
341 followed by homologues to *scsB*, *scsC* and *scsD*, in this order (Fig. S6), suggesting
342 that the four *scs* products operate in an integrated biological pathway. In fact, the
343 identification of a putative Cpx-binding sequence at the P*scs* promoter in most of
344 these species (Table S3), provides also evidence of a conserved regulation.
345 Nevertheless, the presence of an alternative transcription start site upstream of
346 *scsB* promoting transcription of *scsBCD* independent of *scsA* under a yet
347 unidentified condition cannot be rule out, as a short intergenic region separating
348 *scsA* and *scsB* was observed in all the analyzed genomes (Fig. S6).

349 Anwar *et al* reported that deletion of either *scsB*, *scsC* or *scsD* had the same
350 effect on Cu-tolerance in *Salmonella* (34) suggesting that their products form part
351 of a single detoxification complex. By performing a more detailed analysis of the

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352 contribution of these Scs proteins to copper resistance, here we establish that each
353 of them plays a distinct role in alleviating the stress produced by the metal ion. We
354 show that the absence of ScsB provoked the most dramatic effect on Cu tolerance
355 under the conditions tested, followed in relevance by the mutant deleted in the
356 gene encoding its putative coupled periplasmic oxidoreductase ScsC, and the
357 strain deleted in *scsD*, coding for a still uncharacterized integral membrane protein
358 with a putative periplasmic tioredoxin-like domain (Table 1 and Fig. S3). In
359 addition, we demonstrate that other envelope thioredoxin-like proteins, such as
360 DsbC, DsbG and DsbD, are dispensable for Cu tolerance, except in the absence of
361 a functional ScsC/ScsB system (Table 1), highlighting the importance of ScsB and
362 its putative partner proteins ScsC and ScsD in Cu resistance. Our results also
363 suggest that the Scs proteins and the DsbC/DsbD and DsbG/DsbD pairs acts on
364 different substrates, and provide evidences about the functional cross-talk between
365 different Dsb-like systems to favor *Salmonella* survival under stressfull conditions.

366 ScsC and ScsD are not only important in alleviating the damage caused by
367 Cu, but also in the defense against oxidative stress. Together with the major H_2O_2-
368 detoxification factor of the system, ScsA (34), deletion of either *scsC* or *scsD*
369 severely decreased growth at high concentrations of H_2O_2 (Fig. 4 and Fig. S4). In
370 fact, we observe that Cu contributes to protect the Δ*scs* mutant strain from H_2O_2
371 damage, not only by stimulating non-specific corrections of misformed S-S bonds
372 on periplasmic proteins, as previously suggested in *E. coli* (5) but also by triggering
373 the expression of damage-correcting factors. In this sense, induction of
374 transcription of the canonical CpxR/CpxA regulated gene *cpxP* in the Δ*scsABCD*

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375 under Cu stress (Fig. 5B) suggests the existence of a feedback loop between Scs
376 and CpxR/CpxA to restore envelope homeostasis after severe Cu and/or redox
377 injury. The Cu-mediated induction of Cpx-independent oxidation-protecting factors
378 (Fig. 5A), strengthen the relevance of the Scs system in restoring envelope
379 homeostasis after severe Cu and/or redox injury.

380 The Cu-dependent transcriptional activation of the *scs* operon and the role of
381 ScsB, ScsC, and ScsD in Cu resistance (Fig. 1 and S3, and Table 1) predicts a
382 role of these proteins in folding of periplasmic Cu-resistant determinants. CueP is
383 of particular relevance, among the two periplasmic factors controlled by the copper
384 sensor CueR (1). It is the major periplasmic Cu-binding protein required for Cu-
385 tolerance under anaerobic conditions (14, 21), and it was shown to deliver Cu to
386 the periplasmic Cu, Zn-superoxide dismutase SodCII (48), linking Cu and oxidative
387 stress. Three cysteine residues involved in metal binding and intra/interchain
388 interactions in CueP are essential for its biological function (49). Like the Scs
389 system, CueP is present in *Salmonella* and in a small set of bacterial species, but
390 not in *E. coli* (14), and its transcription is also dependent on CpxR/CpxA (16).
391 However, a recent report indicates that CueP is a DsbC substrate (28). Current
392 work is undertaken at our lab to determine the Scs target factors and their role in
393 copper and H_2O_2 tolerance.

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394 **Materials and Methods**

395 **Bacterial strains and growth conditions**

396 *S. enterica* serovar Typhimurium strains and plasmids used in this study are listed
397 in Table S1. Oligonucleotides are listed in Table S2. Cells were routinely grown at
398 37 °C in Luria–Bertani (LB broth) or on LB-agar plates, except when indicated.
399 Ampicillin, tetracycline, kanamycin, and chloramphenicol were used when
400 necessary at 100, 15, 50, and 20 µg ml^{-1}, respectively. All reagents and chemicals
401 were from Sigma, except the Luria-Bertani culture media that were from Difco, and
402 oligonucleotides and enzymes that were from Life Technologies.

403

404 **Genetic and molecular biology techniques**

405 The strains carrying gene deletions, or *lacZ* reporter fusion to promoter on the
406 chromosome, all derivatives of ATCC 14028s, were generated by Lambda Red-
407 mediated recombination following by P22-mediated transduction using previously
408 described protocols (16, 50, 51) and the primers listed in Table S2. When
409 necessary, the antibiotic resistance cassette inserted at the deletion point was
410 removed using FLP-mediated recombination (52). A similar procedure was
411 employed to construct the *scsA*::Cm^R-INT strain harboring the resistance cassette
412 inserted at 100 pb from the translational start site of *scsA*. DNA fragments as well
413 as plasmids were introduced into bacterial cells by electroporation using a Bio-Rad
414 device following the manufacturer's recommendations. All constructs were verified

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415 by DNA sequencing.

416 Plasmids pPB1334 carrying the transcriptional fusion of the native *Salmonella*
417 P*scs* promoter to *lacZ* (Table S1) was constructed by cloning a 303 pb PCR-
418 amplified product into the XmaI site of pMC1871 (Amersham) using previously
419 described protocols (51). The reporter pPB1477 plasmid carrying the modified
420 CpxR-independent P*scs* promoter, P*scs** (Table S1), was constructed by PCR-
421 mediated site-directed mutagenesis. First, we amplified a 119 bp fragment using
422 oligonucleotides Pscs*-Fw and PscsA-Rv (*Xma*I) (Table S2) and pPB1334 (Table
423 S1) as template. Then this fragment was used as a primer along with PscsA-Fw
424 (*Xma*I) to generate the final product carrying the mutant promoter for cloning into
425 XmaI-digested pMC1871.

426

427 **Induction and inhibition assays**

428 β-galactosidase activity was measured on total extracts from cells cultured in LB
429 adjusted at either pH 7.0 or pH 5.5 by the addition of 100 mM MES and 1 mM
430 $CuSO_4$ for 18 h at 37°C essentially as previously described (51). When indicated,
431 100 μM isopropyl-β-thiogalactopyranoside (IPTG) was added to induce expression
432 of *cpxR* or *nlpE* from plasmids.

433 Quantitative real-time reverse transcription-PCR (qRT-PCR) assay was
434 performed basically as previously described (7). Total RNA was prepared from
435 wild-type, Δ*cpxR*, *scsA*::Cm^R-INT or *scsA*::Cm^R-INT Δ*cpxR* cells grown to mid

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436 exponential phase [optical density at 620 nm($OD_{620\ nm}$) of 0.4–0.7] after incubation
437 for 0, 10, 20, 40 and 60 min, with or without 1 mM $CuSO_4$ as indicated in the
438 figures using the RNAzol RT reagent (Molecular Research Center). After RQ1
439 DNase (Promega) treatment to improve quality, cDNA was obtained using Super
440 Script II reverse transcriptase (Invitrogen), dNTPs and the specific sets of
441 oligonucleotides (listed in Table S2) to amplify either *scsA*, *scsB* or the *rnpB* gene,
442 used as housekeeping to normalize transcription levels. Relative transcription was
443 calculated as the ratio between normalized expression levels obtained after
444 incubation in the presence or absence of Cu ions.

445 Copper-sensitivity assays in liquid media were done by recording the $OD_{600\ nm}$
446 of the cultures grown in aerobic condition in LB without or with the indicated
447 concentrations of $CuSO_4$. Metal-sensitivity assays were done in LB-agar plates
448 containing increasing concentrations of $CuSO_4$. To estimate the MIC values by
449 colony-forming unit, over-night cultures of each strain were diluted until 10^{-6} in PBS
450 prior to applying 10 μl of the indicated dilution on the top of the plate. Colonies
451 were allowed to develop for 24 h at 37°C before photographical recording. The MIC
452 values were determined as the minimal concentration of $CuSO_4$ at which no growth
453 was observed.

454 Sensitivity to H_2O_2 was tested by recording OD_{620} every 60 min in a BioTek
455 Synergy 2 multimode microplate reader for 15 h at 37°C. Overnight cultures of the
456 indicated strains were diluted 1:100 in LB and applied in duplicate into a sterile 96-
457 well microplate (Greiner Bio-one) containing fresh H_2O_2 at the indicated final

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458 concentration and/or 1 mM $CuSO_4$ (see figure legends for details). When indicated,
459 the metal salt was used to treat the cultures before sensitivity testing.
460

461 **Protein–DNA interaction assays**

462 EMSA assays and DNase I footprinting assays were done using 6 fmol of a ^{32}P-
463 labeled DNA fragment containing the *scsABCD* promoter and phosphorylated
464 CpxR (CpxR-P) basically as described (16, 45). CpxR-P was obtained by
465 incubating purified His-tagged CpxR with 25 mM of acetyl phosphate for 1 h at 30
466 °C. Protein concentration was routinely determined by Bradford assay, using
467 bovine serum albumin as standard. The DNA sequence ladder was generated in
468 parallel using the reverse primer and the Sequenase DNA Sequencing kit
469 (Affimetrix). After electrophoresis, the gels were dried and exposed to
470 autoradiography.
471

472 ***In silico* analysis**

473 The Seed tool (http://pubseed.theseed.org) was used to search for *scsA* homologs
474 in other bacterial genomes (53).
475

476 **Statistical analysis**

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477 One-way analysis of variance (ANOVA) and the Tukey-Kramer multiple
478 comparison test with an overall significance level of 0.05 were used. In the figure,
479 asterisks denote the values among the treatment groups in which a statistically
480 significant difference was determined.

481

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493

494 **Conflicts of Interest**

495 The authors declare no conflict of interest.

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677 **Table 1.** Contribution of ScsC/ScsB and DsbC-G/DsbD to copper tolerance.

Strain	**MIC (mM)***
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†
Δ*dsbG*	5.00
Δ*scsC* Δ*dsbC* Δ*dsbG*	3.00
Δ*dsbD*(*dipZ*)	5.00†
Δ*scsB* Δ*dsbD*	3.00

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679 *MIC values were determined on LB plates containing increasing amounts of
680 $CuSO_4$ under aerobic condition (see Methods for details). The data correspond to
681 mean values of three independent experiments done in duplicate. [†]Smaller
682 colonies were observed compared with the wild-type strain.

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683 **Figure Legends**

684 **FIG. 1. Transcription of the *scsABCD* is induced by Cu and depends on**
685 **CpxR/CpxA.** The *scsA* and *scsB* relative mRNA levels were determined by
686 quantitative real-time reverse-transcription PCR (qRT-PCR) using LB medium
687 cultures obtained at 0, 5, 10, 20, 40 and 60 minutes after challenging the wild-type
688 (W-t), the Δ*cpxR*, the *scsA*::Cm^R-INT or the Δ*cpxR scsA*::Cm^R-INT strains with 1
689 mM $CuSO_4$. At each time point transcription levels were first normalized to the
690 expression of *rnpB* and then relativized to the levels obtained in the absence of
691 metal. Data correspond to the mean value of three independent experiments
692 performed in triplicate. Standard deviation error bars (SD) are depicted.

693

694 **FIG. 2. CpxR/CpxA controls the expression of the *scs* locus. (A)** β-
695 galactosidase activity from a *scsA*::*lacZ* transcriptional fusion expressed on wild-
696 type (W-t), Δ*cpxR*, Δ*cpxA*, Δ*cueR*, Δ*golS*, Δ*rpoE* or Δ*rcsB* cells grown overnight in
697 LB broth. **(B)** β-galactosidase activity was determined from the W-t, the Δ*cpxR*, or
698 the Δ*cpxR* strain complemented with pCpxR (Δ*cpxR*/pCpxR), all carrying the the
699 *scsA*::*lacZ* reporter fusion. Cells were grown overnight in LB 100 mM MES buffer
700 adjusts the pH value to 7.0 or pH 5.5 without (-IPTG) or with the addition of 100 μM
701 IPTG (+IPTG), as indicated. The data shown in A and B correspond to mean
702 values of four independent experiments performed in duplicate. Error bars
703 representSD. **(C)** β-galactosidase activity from W-t cells carrying *scsA*::*lacZ* (*scsA-*
704 *lacZ*) or *cpxP*::*lacZ* (*cpxP-lacZ*) -included as a CpxR-regulated positive control-

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705 transcriptional fusions and transformed with either the empty vector pUHE21-2*lacI*^{q}
706 (vector), or with pNlpE, grown in LBwith the addition of 100 μM IPTG. (To note, the
707 *lacZ* in the *cpxP*::*lacZ* construction was introduced after the 3' end of *cpxP* in order
708 to avoid undesirable disturbance of the CpxR/CpxA signal transduction pathway
709 (36, 37, 54)). All values were normalized by the average activity obtained for cells
710 with control vector. Bars represent the average normalized values from at least
711 three separate experiments. Error bars represent SD.

712

713 **FIG. 3. CpxR interacts with the *scsA* promoter at the predicted CpxR binding**
714 **site. (A)** DNase I footprinting analysis of the promoter region of *scsABCD*
715 performed on both end-labelled coding and non-coding strands. Purified and
716 acetyl-phosphate preincubated CpxR (CpxR-P) at the final concentrations of 0.5
717 and 1 μM was added to the DNA fragments. Solid vertical lines and sequences on
718 the left indicate the CpxR-protected region. **(B)** β-galactosidase activity from the
719 wild-type (W-t) and Δ*cpxR* strains carrying a reporter plasmids in which expression
720 of the *lacZ* gene is directed by the native *scsABCD* promoter (P*scs*) or by the
721 promoter harboring the mutations at the CpxR-binding site (P*scs**). The activity
722 was determined on overnight cultures grown in LB, 100 mM MES, pH 7.0. The data
723 correspond to mean values of three independent experiments performed in
724 duplicate. Error bars correspond to the SD. **(C)** DNase I footprinting analysis of the
725 native (P*scs*) or of the mutant (P*scs**) promoter regions performed on the non-
726 coding strand. CpxR-P was added at the same final concentrations than in part A.
727 The predicted CpxR-protected region is shown with a solid vertical line.

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729 **FIG. 4. The Scs proteins are involved in balancing oxidative stress at the**
730 ***Salmonella* envelope. (A-D)** Wild-type (W-t), Δ*scsA*, Δ*scsB*, Δ*scsC*, Δ*scsD* and
731 Δ*scsABCD* cells were grown in aerobic condition in LB without or with the addition
732 of 6 mM H_2O_2 and/or 1 mM $CuSO_4$, as indicated. $OD_{600\ nm}$ of the cultures was
733 recorded every hour for 15 h. Results are the means standard deviations for four
734 independent experiments, each in duplicate.

735

736 **FIG. 5. Cu protects mutant *scs* cells from oxidative stress by activating the**
737 **expression of protecting factors. (A)** Pre-incubation with copper protects
738 *scsABCD*-deficient mutants from stress by H_2O_2. The *S*. Typhimurium wild type (W-
739 t) and mutant deleted in *scsABCD* (Δ*scsABCD*) or in *cpxR* (Δ*cpxR*) were grown to
740 the early log phase (60 minutes) in LB without or with 1 mM $CuSO_4$. The metal was
741 then removed and cultures were continued or challenged with addition of 5 or 6
742 mM H_2O_2, as indicated. The growth was monitored as above. Results are average
743 of three independent assays performed in duplicate, and error bars correspond to
744 SD. **(B)** The CpxR/CpxA response is enhanced by Cu in the absence of the *scs*
745 locus. β-galactosidase activity from a *cpxP-lacZ* transcriptional fusion expressed on
746 wild-type (W-t) or Δ*scsABCD* cells grown in LB, 100 mM MES at pH 7.0 after 180
747 min exposure to 1 mM $CuSO_4$. Data correspond to mean values of three
748 independent experiments performed in triplicate. Error bars depict SD. The Cu-

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749 induction of the P*cpxP* reporter in the Δ*scsABCD* strain differs significantly from the

750 wild-type strain (***, $P< 0.001$).

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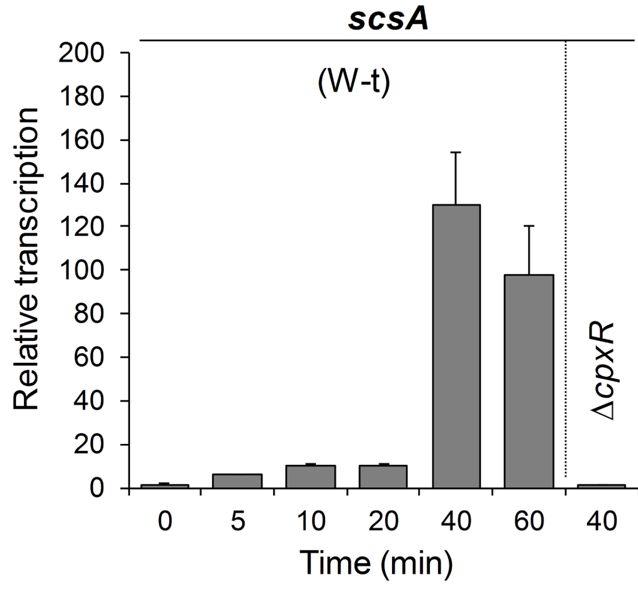

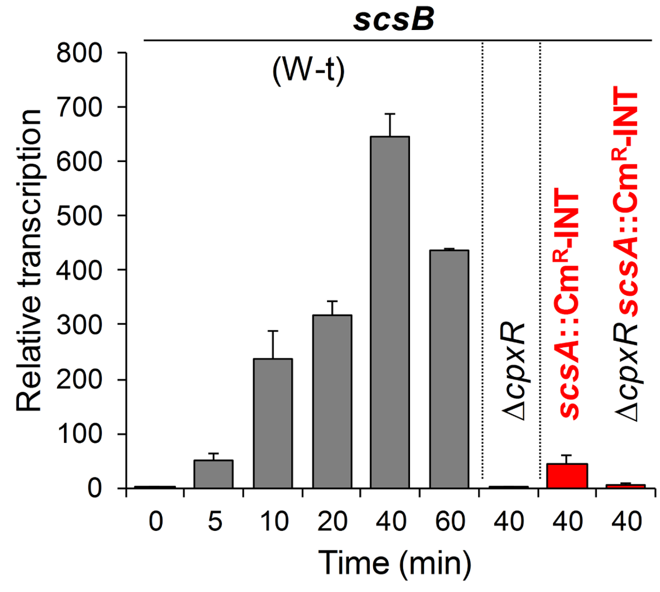

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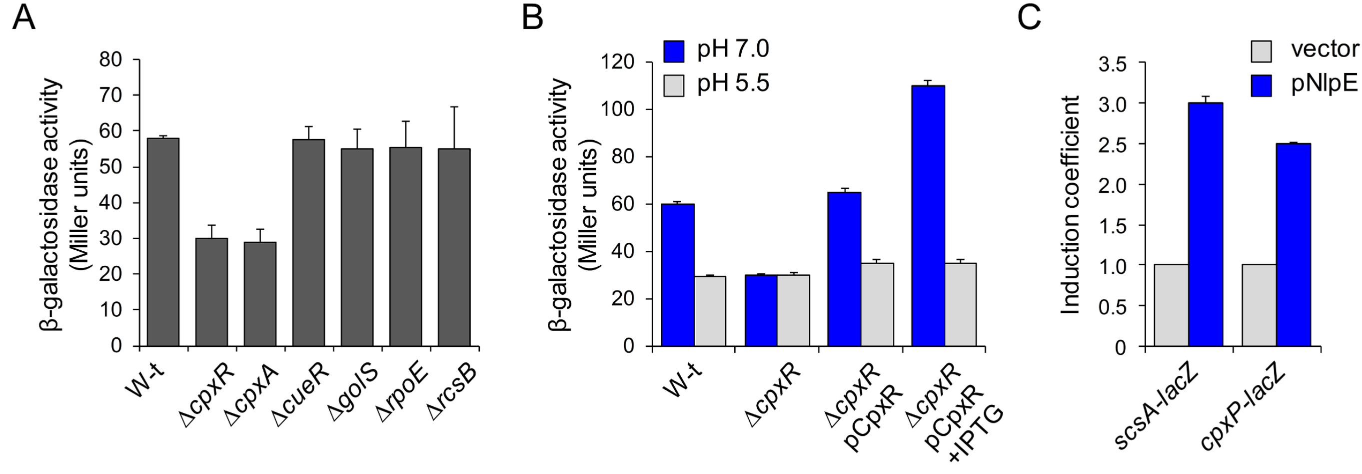

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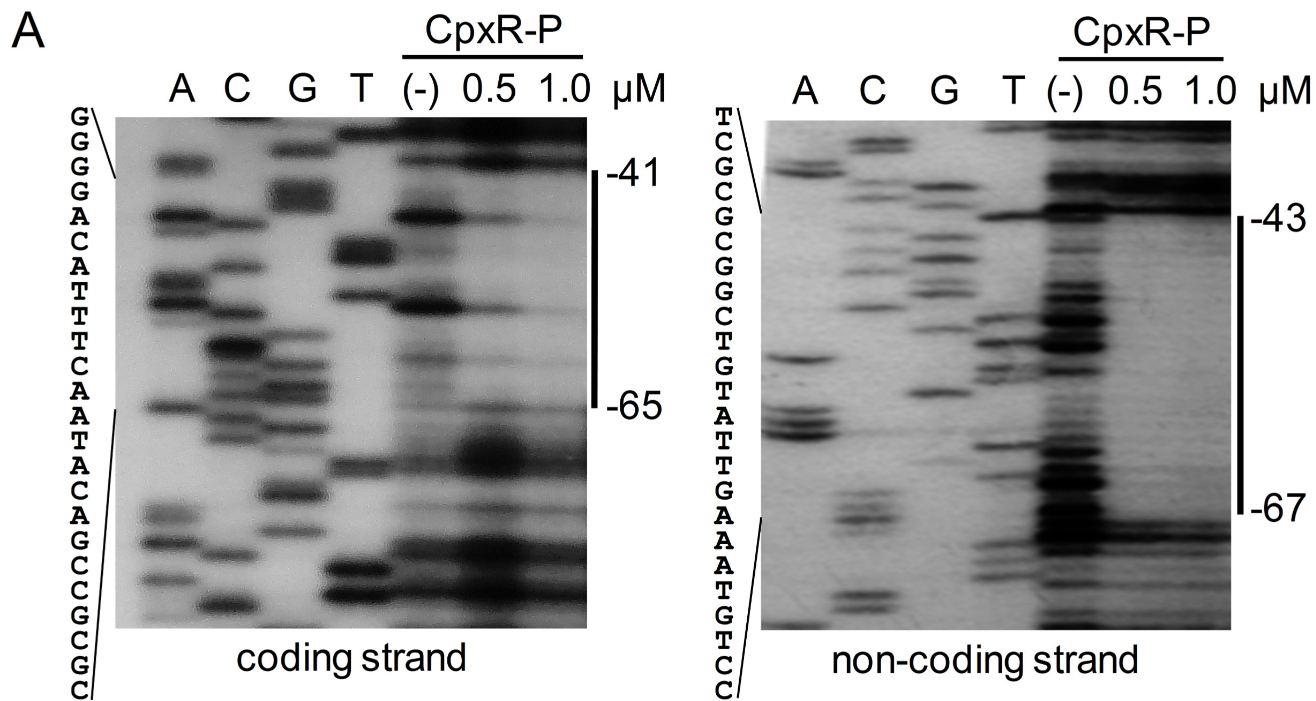

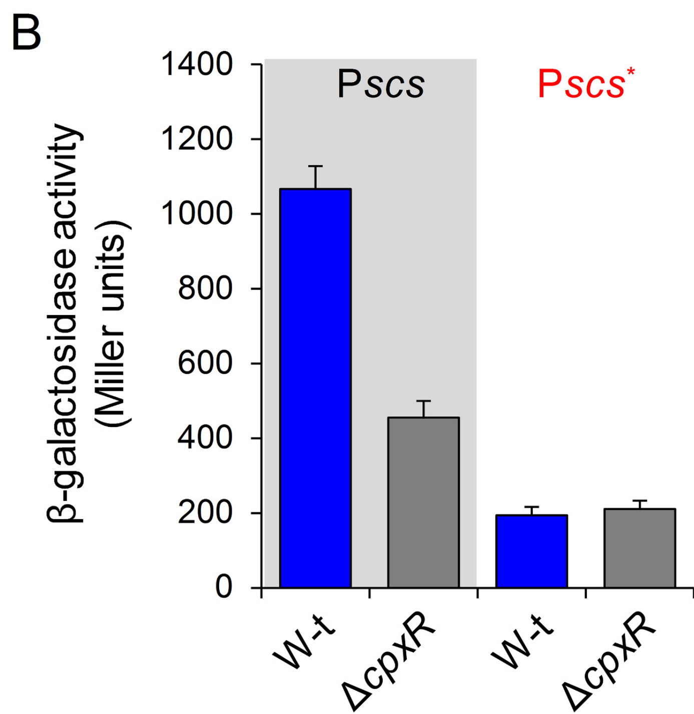

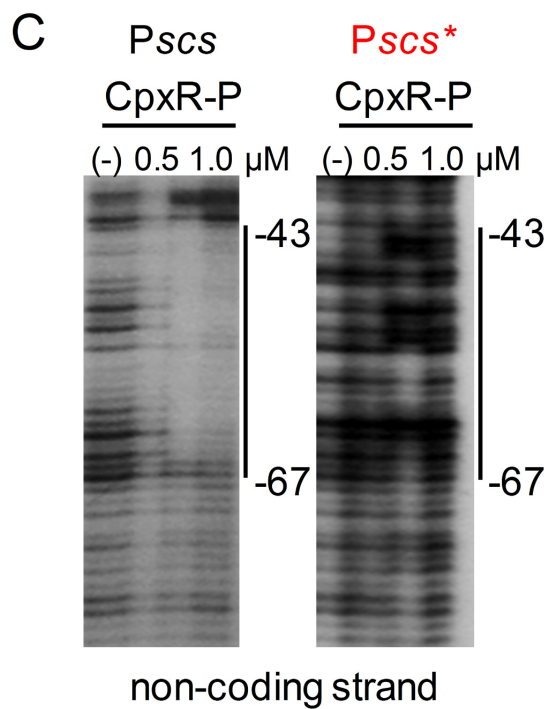

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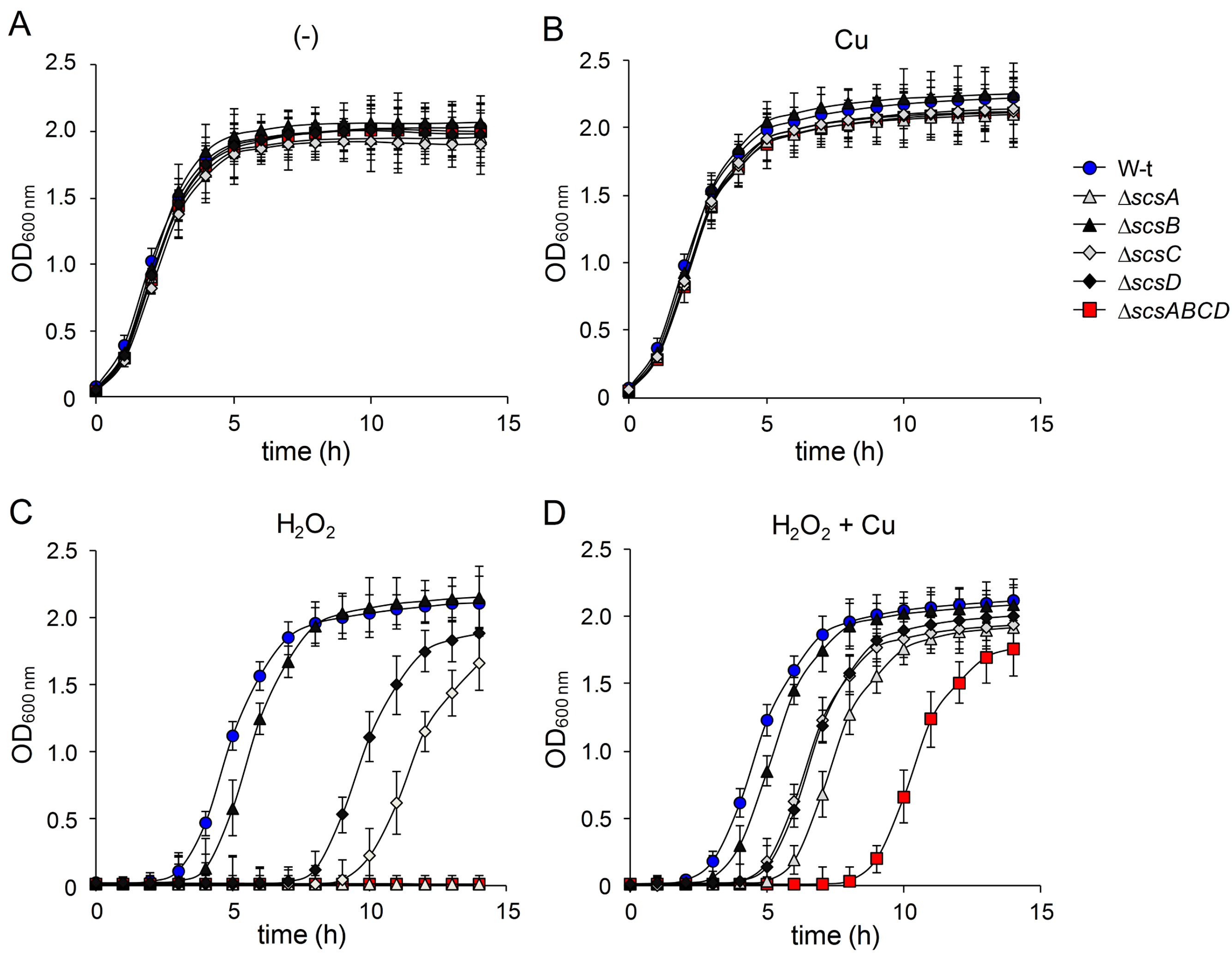

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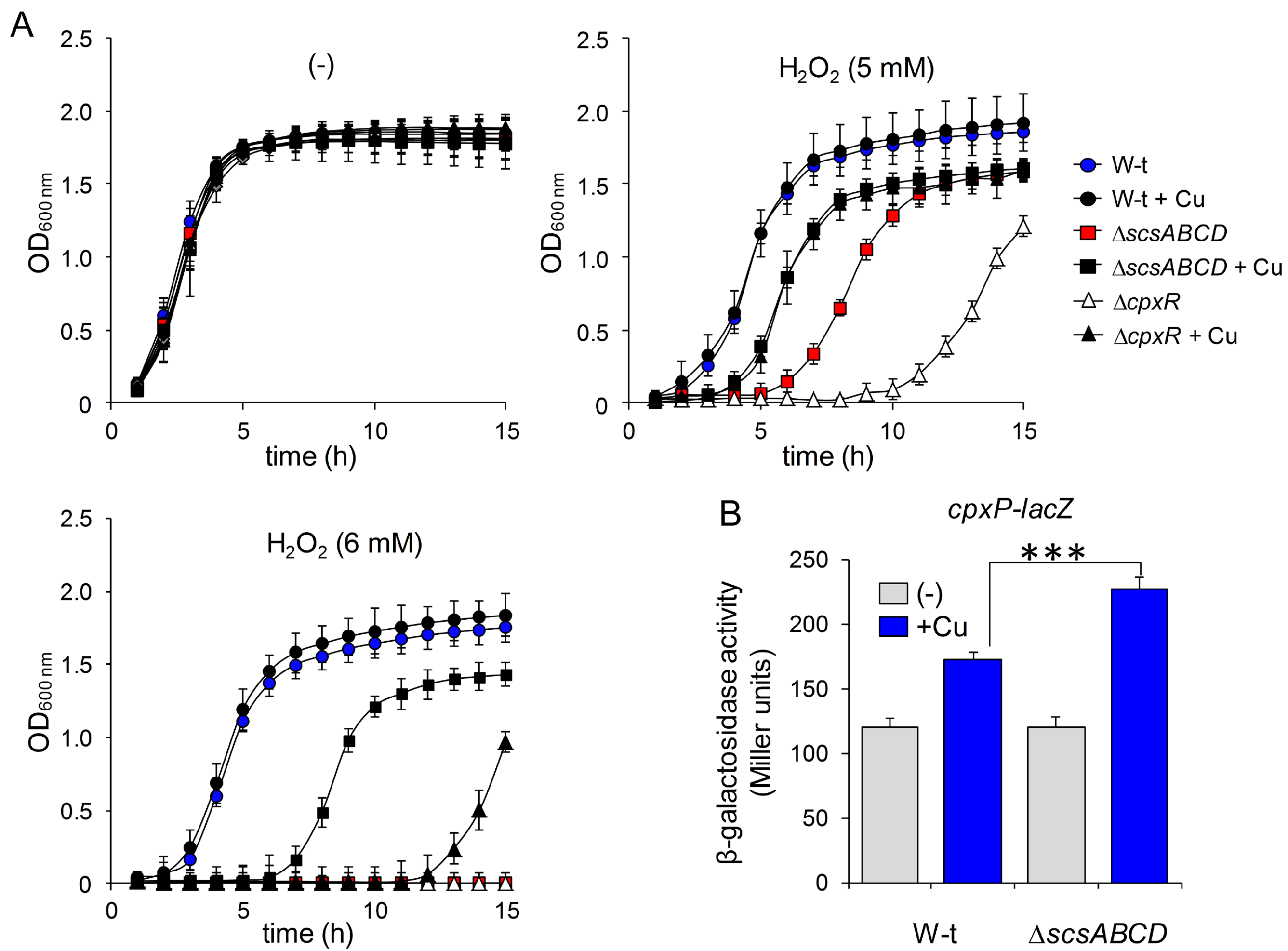

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Supplementary Information

CpxR/CpxA-controls *scsABCD* transcription to counteract copper and oxidative stress in *Salmonella* Typhimurium

Carolina López, Susana K. Checa and Fernando C. Soncini

Table S1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant properties	Reference or source
*S.*Typhimurium Strain		
14028s	Wild type	ATCC
PB10122	Δ*cpxR*	(1)
PB9618	Δ*scsA*	This study
PB9409	Δ*scsBCD*::Cm^R	This study
PB10431	Δ*scsABCD*::Cm^R	This study
PB10807	Δ*scsABCD*	This study
PB10673	Δ*scsB*	This study
PB11084	Δ*scsC*	This study
PB11020	Δ*scsD*	This study
PB13672	Δ*scsBC*	This study
PB13673	Δ*scsCD*	This study
PB9410	Δ*dsbC*::Cm^R	This study
PB10688	Δ*dsbC*	This study
PB12111	Δ*dsbG*	This study
PB12071	Δ*dsbD*	This study
PB12131	Δ*scsC* Δ*dsbC* Δ*dsbG*	This study
PB12132	Δ*scsB* Δ*dsbD*	This study
PB12110	Δ*tpx*::Cm^R	This study
PB10668	*scsA*::Cm^R-INT	This study
PB12345	*scsA*::*lacZY*$^+$	This study
PB12397	Δ*cpxR*::Cm^R*scsA*::*lacZY*$^+$	This study
PB12814	Δ*cpxA*::Cm^R*scsA*::*lacZY*$^+$	This study
PB12398	Δ*cueR*::Cm^R*scsA*::*lacZY*$^+$	This study
PB12399	Δ*golS*::Cm^R*scsA*::*lacZY*$^+$	This study
PB12815	Δ*rpoE*::Cm^R*scsA*::*lacZY*$^+$	This study
PB12400	Δ*rcsB*::Cm^R*scsA*::*lacZY*$^+$	This study
PB10864	*cpxP-lacZ*-Cm^R	(1)

PB13408	Δ*scsABCD cpxP*-*lacZ*-Cm^R	This study
Plasmids		
pUH21–2lacIq	ori_{pMB1} Ap^R *lacI*^{q}	Laboratory stock
pPB1466	pUH::cpxR (pCpxR)	(1)
pPB1474	pUH::nlpE (pNlpE)	(1)
pPB1467	pQE-32::6x-His-*cpxR*	(1)
pPB1334	pMC1871-derived P*scs*::*lacZ* reporter plasmid(pP*scs*) Tc^R	This study
pPB1477	pMC1871-derived CpxR-independent P*scs*::*lacZ* reporter plasmid (pP*scs**) Tc^R	This study

Table S2. Oligonucleotides used in this study

Primer name	Sequence (5'-3')	Purpose
scsAFw	ATGGCGAAACAACAACG	qRT-PCR
scsA Rv	GTAGACAAAGCGCAAGG	qRT-PCR
scsB Fw	CTGTTGCTGGATGTCAAACTGG	qRT-PCR
scsB Rv	TCCCTGGGTGGTGATATTCG	qRT-PCR
scsA-INTRv	GTTGCGCGGTACAAACC	qRT-PCR
rnpB Fw	TCATCTAGGCCAGCAATCG	qRT-PCR
rnpB Rv	GTGAAAGGGTGCGGTAAGAG	qRT-PCR
scsA-P1	GATAACTCGCGTTAAACAGTGAGGGCGCAGTG TAGGCTGGAGCTGCTTCG	λRed deletion of *scsA* or *scsABCD.* Construction of *scsA*::*lacZY*$^{+}$transcriptionalfu sion
scsA-P2	GTAATTAACCGTTAGCAATAACCGGTCTGCATA TGAATATCCTCCTTA	λ Red deletion of *scsA.* Construction of *scsA*::*lacZY*$^{+}$transcriptional fusion
scsB-P1	GGTTAATTACTCATTCACGGAGAAAAAATGTGT AGGCTGGAGCTGCTTCG	λ Red deletion of *scsB, scsBC* or *scsBCD*
scsB-P2	GCTTTTTTCAACGTTTGTAATACCGCATCCATA TGAA TATCCTCCTTA	λ Red deletion of *scsB*
scsC-P1	AATACATGATTGTTTTACTGCTGGCGCTGGTGT AGGCTGGAGCTGCTTCG	λ Red deletion of *scsC or scsCD*
scsC-P2	CTGCGCCCGGAATCAGCTCGTCGCCAATGCAT ATGAATATCCTCCTTA	λ Red deletion of *scsC or scsBC*
scsD-P1	ACTGCGGCGTTGGCTGCGTGAAGCCGCGGGT GTAGGCTGGAGCTGCTTCG	λ Red deletion of *scsD*
scsD-P2	GGTTAATTACTCATTCACGGAGAAAAAATGTGT AGGCTGGAGCTGCTTCG	λ Red deletion of *scsD*, *scsCD*, *scsBCD* or *scsABCD*
scsA::Cm-INT P1	TTGTACCGCGCAACGCATGGCGGGCCTGCAC GCCTTGCGTGTAGGCTGGAGCTGCTTCG	For insertion of Cm^{R} at 100bp from the start of *scsA*
scsA::Cm-INT P2	GGAGCGCTGACCACCGCAGCAGAGGCCGTCG CCTGCATCTCATATGAATATCCTCCTTA	For insertion of Cm^{R} at 100bp from the start of *scsA*
PscsA-Fw (XmaI)	GACCCGGGCATAATGGCGTAATAATC	P*scs* cloning; EMSA; DNAse I footprinting
PscsA-Rv (XmaI)	GACCCGGGGCAAAGAAACCACCAG	P*scs* cloning; EMSA; DNAse I footprinting

Pscs*-Fw	GCCGACATAACTTCAGAGGGGAAAGGTTG C	Construction of the CpxR-independent P*scs* promoter (Pscs*)
dsbC-P1	CATCCGCGGGCATGAGTGCTGACAGGAAAGG ATAGTTTGTGTAGGCTGGAGCTGCTTCG	λ Red deletion of *dsbC*
dsbC-P2	CTCGCGCCGACGAAGTTGTCTCTGTTGTTTCA CGCGTACATATGAATATCCTCCTTA	λ Red deletion of *dsbC*
dsbG-P1	GGCGTGGTACGGTTAAGCTCCAGCTTTGCTAA TTTCTGCGGTGTAGGCTGGAGCTGCTTCG	λ Red deletion of *dsbG*
dsbG-P2	ATACTCTTTTCGCTTTGATATTAAAAGGAAAAAA TAT GCATATGAATATCCTCCTTA	λ Red deletion of*dsbG*
dsbD-P1	GGTTTTACCTGTTACTCACGGAGATACTGATTA CCTCTCGTGTAGGCTGGAGCTGCTTCG	λ Red deletion of *dsbD*
dsbD-P2	CACGGTATTCTCCTCCGTCTTTGCTTTTGCAAG TGTCGCCATATGAATATCCTCCTTA	λ Red deletion of *dsbD*
tpx-P1	GGATTTCGACGTTAACTATAAGTAAATAGGAAC ATAA TTGTGTAGGCTGGAGCTGCTTCG	λ Red deletion of *tpx*
tpx-P2	CCTGTAGCACTGACAAACGTAGCGCCAGCAG GCGCTGGCCATATGAATATCCTCCTTA	λ Red deletion of *tpx*

Table S3. Predicted *scsA* promoter region in selected species from Fig S3

>fig|99287.1.peg.1079 Suppression of copper sensitivity: putative copper binding protein ScsA [*Salmonella typhimurium* LT2]
AGAAGGTTGCGCAGCGCGCCGACATAACTTTACAGGGGAAAGGTTGCCAAAACCGCGCCAGTGG
CTAAGATAACTCGC***G***TTAAACAGTGAGGGCGCA**ATG**

>fig|12149.1.peg.1066 Suppression of copper sensitivity: putative copper binding protein ScsA [*Salmonella bongori* 12149]
AGCAGGCTGGGTGGCGCGTCGACATAACTTTACAGGGGAAAGGTTGCCAAAACTGCGTCAGTGG
ATAAGATAACTCGCGTTAAACAGTGAGGGCGCA**ATG**

>fig|670484.3.peg.739 Suppression of copper sensitivity: putative copper binding protein ScsA [*Citrobacter freundii* str. ballerup 7851/39]
GTATATCCTGCTGATGACAGGACATAACTTTACAGCGCAAAGGTTGCCAAAACCTGGTGAGTGGTT
AAGATAACTCGCATCAATCAGTGAGGGTATA**ATG**

>fig|469595.3.peg.1618 Suppression of copper sensitivity: putative copper binding protein ScsA [*Citrobacter* sp. 30_2]

GTATATCCAGCAGATAGCAGGACATAACTTTACAGCGGAAAGGTTGCCAAAACCTGGCGAGTGGT
TAAGATAACTCGCATCAATGAGTGAGGGTATA**ATG**

>fig|500640.5.peg.3473 Suppression of copper sensitivity: putative copper binding protein ScsA [*Citrobacter youngae* ATCC 29220]
CCGGGGGCTTTTTCTGCGCCGACATAACTTTACAGGGTAAAGGTTGCCAAAACCTTGCCAGTGGC
TAAGATAACCCGCATCAATCAGTGAGGGTAGA**ATG**

>fig|290338.6.peg.1728 Suppression of copper sensitivity: putative copper binding protein ScsA [*Citrobacter koseri* ATCC BAA-895]
GTAATGAGCGGGGCGACGCCGACATAACTTTACAGCGCAAAGGTTGCTAAAACCTTGTCAGTGGC
TAAGATAGCTCGCGTCAATCAGTGAGGATGTC**ATG**

>fig|399741.3.peg.3356 Suppression of copper sensitivity: putative copper binding protein ScsA [*Serratia proteamaculans* 568]
GCGACCAATACCGTCCTCAGACATTTCTTTACAGGCAAAACATTGCTAAAAGCGTCTGCGCCGCTA
AGATAAGCGCAGACTTGGAATTGAGGGCGGC**ATG**

>fig|656440.3.peg.3142 Suppression of copper sensitivity: putative copper binding protein ScsA [*Escherichia coli* TA206]
TCAGTGGATTTTCAACGTCACGACGTAACTTTACAGTGTAAGAGTTGCGAAAAGTTAATCTGCAGAT
AAACTGATTTGTGTCGATCAGTGAGGTTTT**ATG**

>fig|615.1.peg.3098 Suppression of copper sensitivity: putative copper binding protein ScsA [*Serratia marcescens* Db11]
GCCATCAATACGTCGCTCAGACATTTCTTTACCGACAAAACGTTGCTAATCGCGGCCGCGCCGCTA
AGATAACCCCAGATCTAAACATGAGGATGGC**ATG**

>fig|527004.3.peg.2045 Suppression of copper sensitivity: putative copper binding protein ScsA [*Yersinia rohdei* ATCC 43380]
TAACCTTGACCAAATGTTTACGCACCAAACATTGATAATGATAATTATATGGTTAAGATGAAGCCCT
ATCACTGCATGGGATTATTAACTTACCGTC**ATG**

>fig|349968.3.peg.3654 Suppression of copper sensitivity: putative copper binding protein ScsA [*Yersinia bercovieri* ATCC 43970]
AATCTTGATTATGCCGCTAAGATGAGCGCTCATCCTGTATGGGACTGACTCAATAATTTTTGAGATG
AATCGCCCGGATAATGGTTAAACAAGGATA**ATG**

>fig|349965.3.peg.1057 Suppression of copper sensitivity: putative copper binding protein ScsA [*Yersinia intermedia* ATCC 29909]

ATCTTAATTATGCCGTTAAGATGAGCGCTCAGTCTTGAATGAGACTAATTTATCAATTTTACTGATGA
ATTATACGGATCAGGATTAAACACGGTAA**ATG**

>fig|349966.5.peg.2167 Suppression of copper sensitivity: putative copper binding protein ScsA [*Yersinia frederiksenii* ATCC 33641]
CCTAAATTGACCTTTGTTTACCAACCAAGCATTGATAATGATAATTATATCGTTAAGATGAAGCGCT
ATAACTGAACGGAATTATTAAGTAACAAGA**ATG**

>fig|527002.3.peg.2301 Suppression of copper sensitivity: putative copper binding protein ScsA [*Yersinia aldovae* ATCC 35236]
AGGTACAAACGTTGATAATCCTGATTATGCCGCTAAGATGGGCGTTCAGGATTGAATGGAAATGAT
TTAACAATTTTAAGATGATTAAACAACGATA**ATG**

>fig|888063.3.peg.3601 Suppression of copper sensitivity: putative copper binding protein ScsA [*Enterobacter hormaechei* ATCC 49162]
GGCAAACCGCTTATCTTCCAGACACTTCTTTACAGCTTTTGCGTTGCCAAAATATGTTCGCCCGATA
ATATAGGCGTCCTGAATATTTGAGGCAAAC**ATG**

>fig|630.2.peg.1275 Suppression of copper sensitivity: putative copper binding protein ScsA [*Yersinia enterocolitica* 8081]
CCTTGAATGGAATTGATTTAACAATTCCAAGGATAATTTATTCGATACAAGGTTGATCATGGATATCG
TTGAGTACGGATAGGGTTAAATAGGGATA**ATG**

>fig|349967.3.peg.3661 Suppression of copper sensitivity: putative copper binding protein ScsA [*Yersinia mollaretii* ATCC 43969]
TAGGTTCAGACATTGATAATCTTGATTATGCCGCTAAGATGGGCGCTCATCCTGAATGGGACTGATT
CAATAATTTTTGAGACGAATAGCACGGAAC**ATG**

>fig|640131.3.peg.739 Suppression of copper sensitivity: putative copper binding protein ScsA [*Klebsiella variicola* At-22]
GGCAAATCACTTATCCGCCAGACACTTCTTTACAGCTTTACCATTGCCAAAACGCGTTCGCCCGAT
AAGATAGGCCGCGTGAATATTTGAGGCGATC**ATG**

>fig|527012.3.peg.2960 Suppression of copper sensitivity: putative copper binding protein ScsA [*Yersinia kristensenii* ATCC 33638]
CTGATTTAACCATTTAAGGGATAACTCATTCAGCTCGTGGCTGATAATAGTTAAGCATGGATAATGG
TCAAATACGGATAATGTTTAAATACAGATA**ATG**

>fig|469608.3.peg.3262 Suppression of copper sensitivity: putative copper binding protein ScsA [*Klebsiella* sp. 1_1_55]
GGCAAATCACTTATCCGCCAGACACTTCTTTACAGCTTTACCATTGCTAAAACGCGTTCGCCCGAT
AAGATAGGCCTCGTGAATATTTGAGGCGACC**ATG**

>fig|1028307.3.peg.567 Suppression of copper sensitivity: putative copper binding protein ScsA [*Enterobacter aerogenes* KCTC 2190]
GGCAAATCACTTATCCGCCAGACACTTCTTTACAGCTTTACCATTGCTAAAACGCGTTCGCCCGAT
AAGATAGGCCTCGTGAATATTTGAGGCGACC**ATG**

>fig|701347.4.peg.667 Suppression of copper sensitivity: putative copper binding protein ScsA [*Enterobacter cloacae* SCF1]
GAGGGCGCGTTCCGCTGAAGACACTTCTTTACAGCATTACCATTGCCAAAACGCGCTCACCCTATA
AGATAAGCCTCGTGAATAATTGAGGCCGATC**ATG**

NOTE: ScsA start codons are in bolt. Where it was detected, predicted -10 regions are underlined and the transcription start site determined in *Salmonella* is underlined, in italic, and bold. Putative CpxR-binding sequences are shown in boxes. The “The Seed” genes identifications were conserved here as well as in Fig. S3, to easily find them on-line.

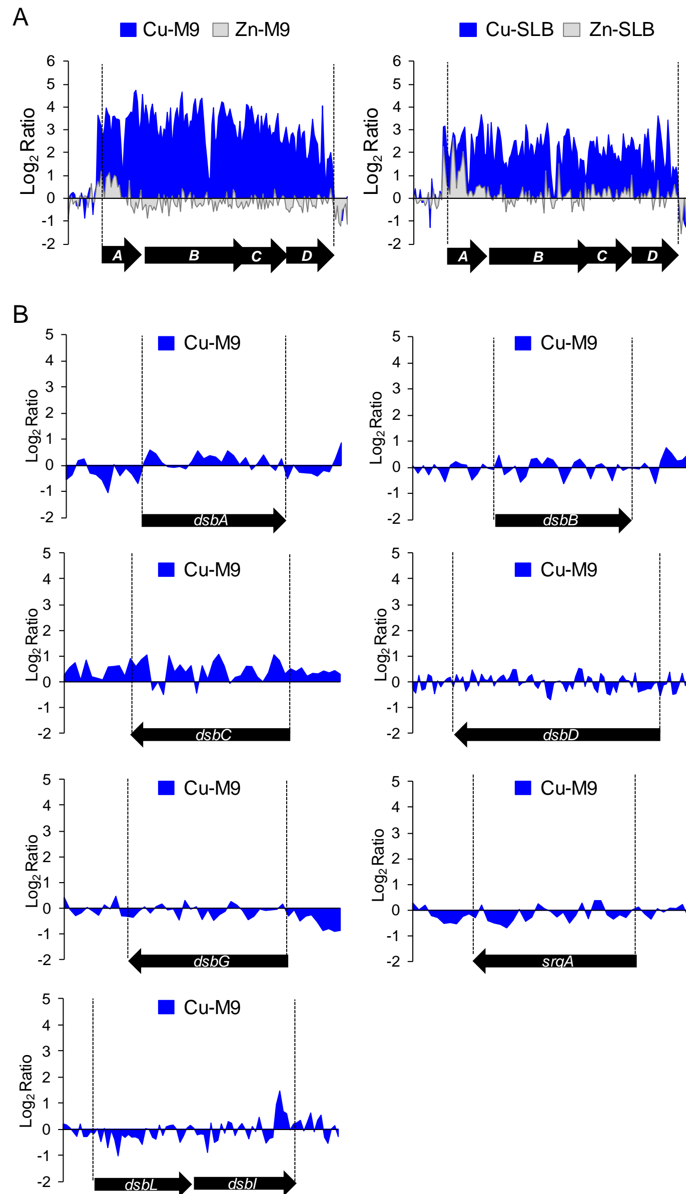
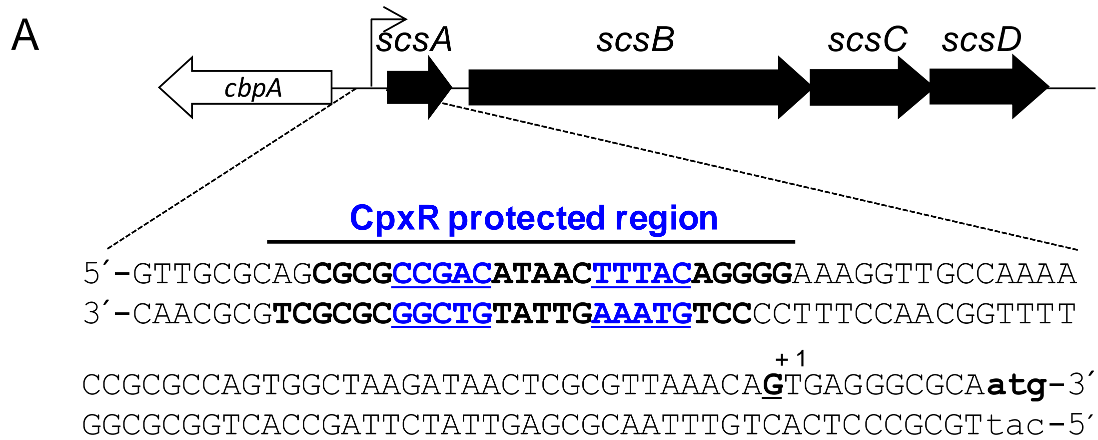


FIG. S1. Transcription of the *scs* locus is upregulated in the presence of Cu. WebArrayDB analysis for *scs* locus **(A)** or the indicated thioredoxin-like genes **(B)** performed in M9 medium after a 10 min exposure to 10 μM $CuSO_4$ or 50 μM $ZnSO_4$ or after exposure to 1 mM $CuSO_4$ or 250 μM $ZnSO_4$ in SLB as indicated. The mean change in intensity of two biological replicates, $\log_2$ ratio, for each probe within the ORF is plotted against the genome location. For each probe Log_2 Ratio was calculated, using the mean intensity of the probe obtained for the sample subjected to metal treatment against the mean intensity of the probe obtained for the control sample, without metal added, as previously described (2).



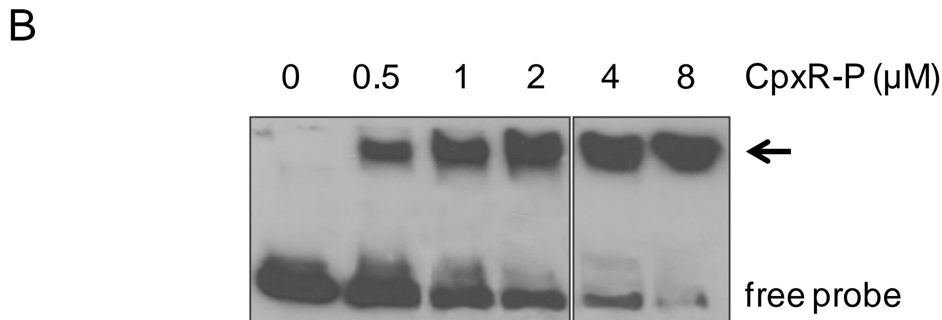
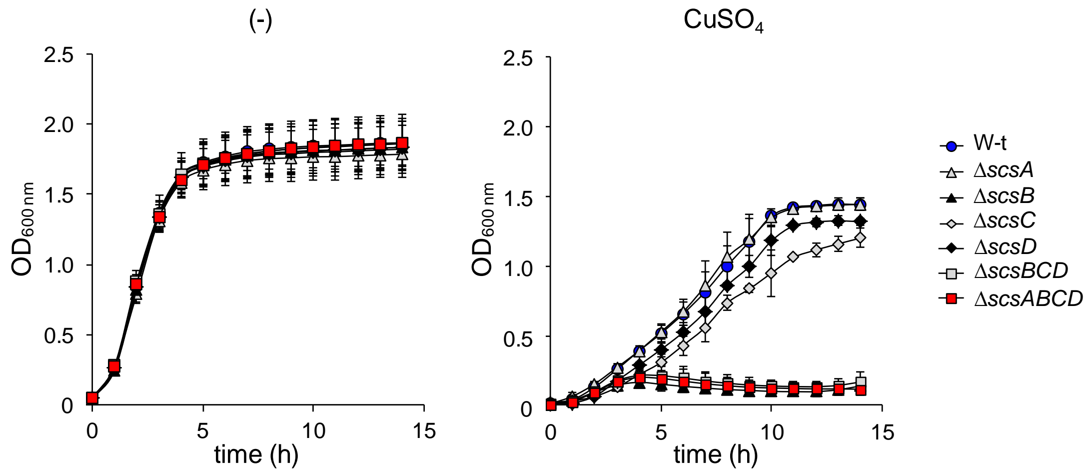


FIG. S2. CpxR binds to the *scsA* promoter. (A) Schematic representation of the *S.* Typhimurium *cbpA*-*scsABCD* region and DNA sequence of the *scsA* promoter region. The position of *scsA* transcription start site (+1) is indicated by an arrow in the scheme. Based upon the *E. coli* consensus sequence of *5′-GTAAA(N)$_{4–8}$GTAAA-3′*, a predicted CpxR-direct repeat is in blue and underlined in the sequence. The DNaseI protected sequence in each strand is shown in bold. **(B)** EMSA was performed using the ^{32}P 3′-end-labelled PCR fragment of the promoter region of *scsABCD* incubated with purified and acetyl-phosphate preincubated CpxR at the indicated final concentrations. The arrow shows the CpxR-DNA complex.



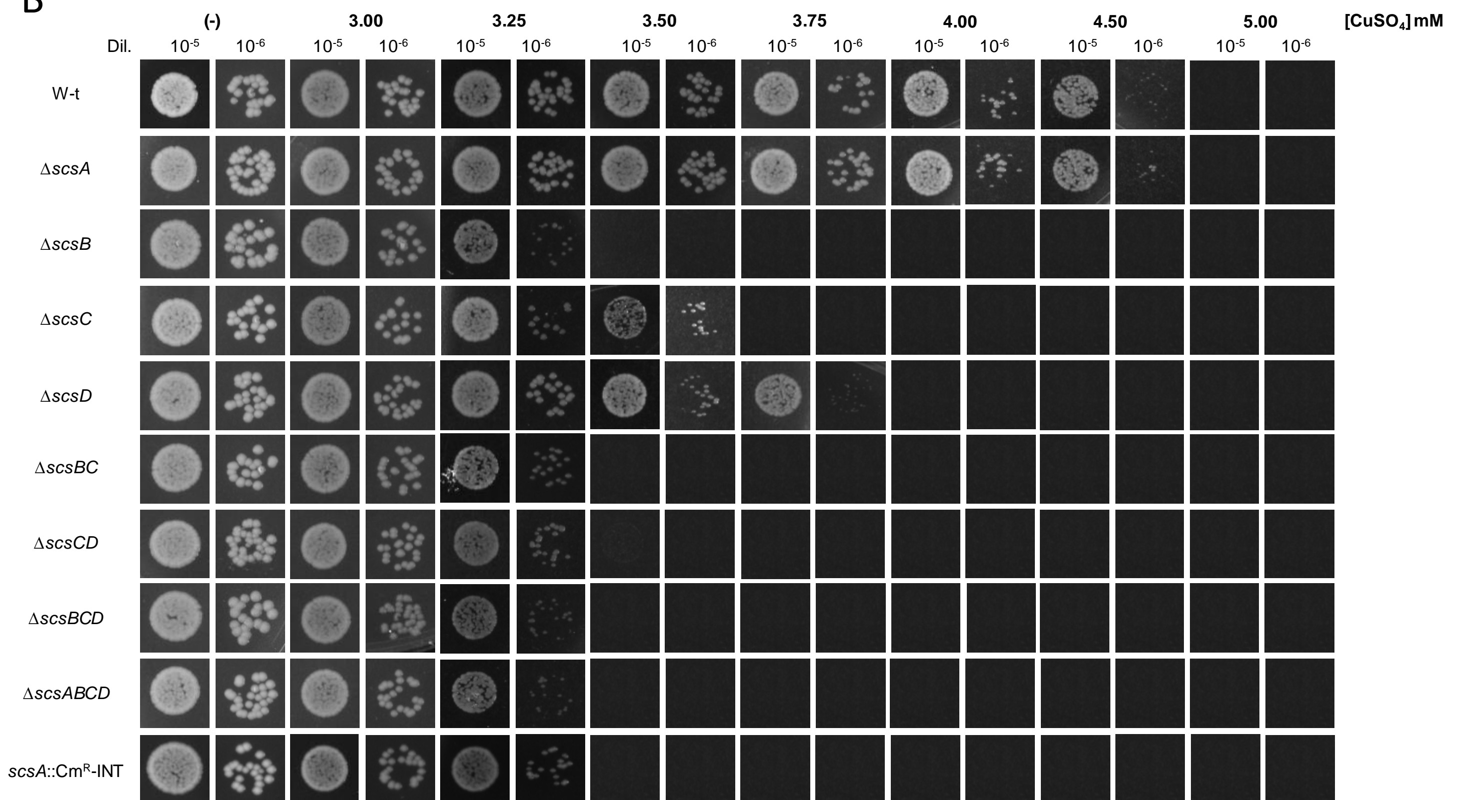


FIG. S3. Contribution of each individual Scs proteins to copper resistance. Comparative copper-sensitivity assays of the wild-type (W-t) or the indicated deletion mutant or the strain carrying the resistance cassette at 100 pb from the translational start site of *scsA* (*scsA*::Cm^{R}-INT) were done in LB **(A)** or in LB-agar plates **(B)**

supplemented with the addition of $CuSO_4$. (-) indicate no metal addition. In **(A)** $CuSO_4$ was added to a final concentration of 3.25 mM. $OD_{600\ nm}$ was recorded every hour for 15 h. Results are the means and standard deviations for four independent experiments, each in duplicate. In **(B)** 10 µl-aliquots from a 10^{-5} or 10^{-6} dilution in PBS of overnight cultures were applied on top of LB agar plates supplemented with the indicated $CuSO_4$ concentrations. After incubation at 37°C for 24 h the plates were photographed. The data correspond to representative images of at least three independent experiments done in duplicate.

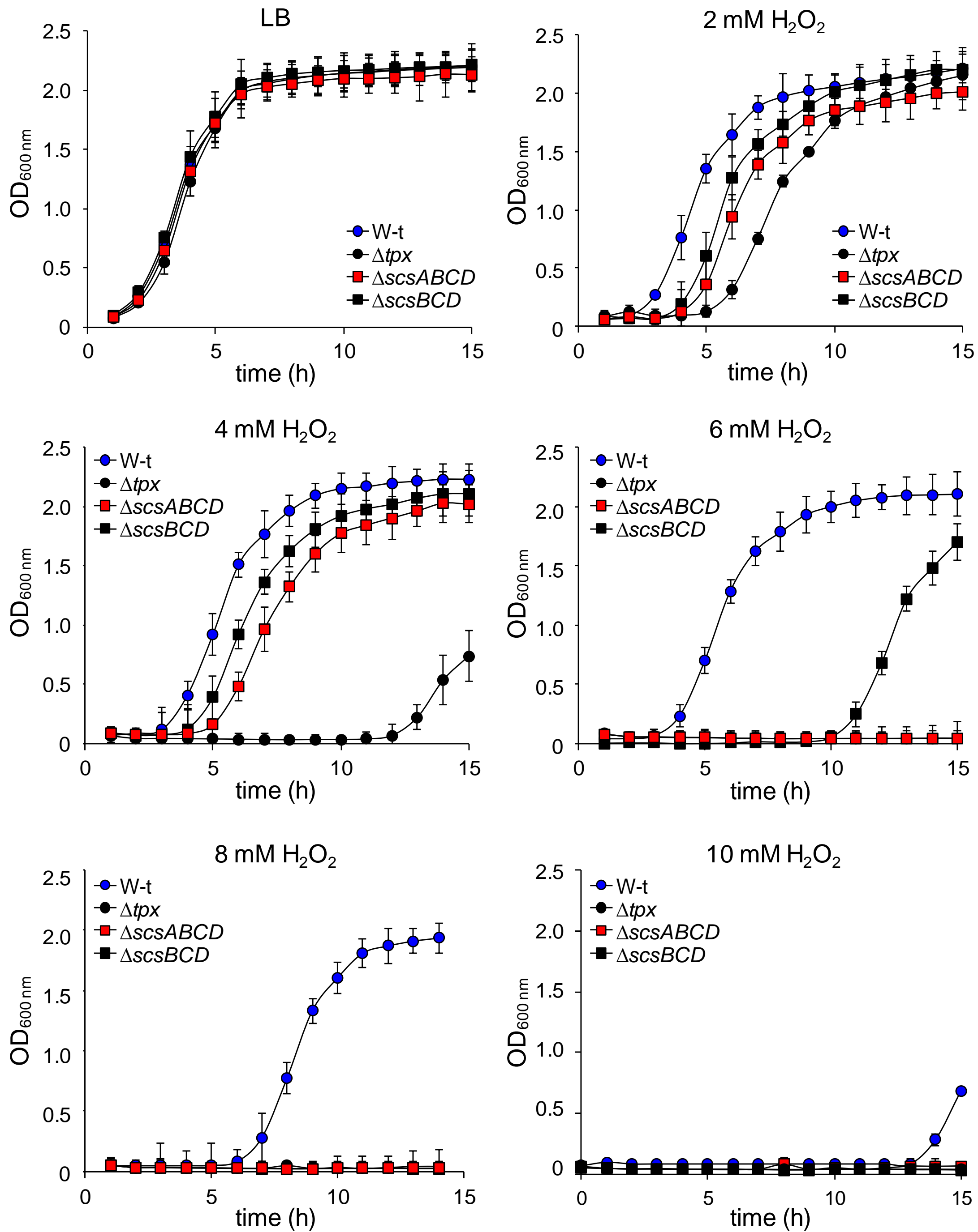


FIG. S4. **Role of the Scs factors in H_2O_2 tolerance.** Wild-type, Δ*tpx* (positive control), Δ*scsABCD* or Δ*scsBCD* *Salmonella* strains were grown in LB without (-) or with addition of the indicated concentration of H_2O_2. The $OD_{600\ nm}$ was recorded every hour for 15 h. Results are the average of three independent assays performed in duplicate, and error bars correspond to SD.

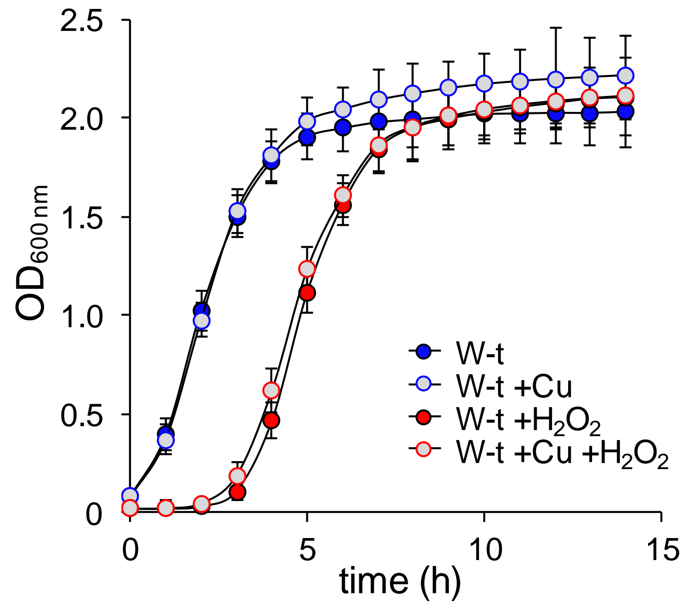


FIG. S5. Copper does not affect the wild-type strain growth in the presence of H_2O_2. Wild-type cell (W-t) were grown in LB supplemented with 1 mM $CuSO_4$ (Cu) and/or 6 mM H_2O_2, as indicated. $OD_{600\ nm}$ was recorded every hour for 15 h. The results (taken form Fig. 4) are the average of three independent assays performed in duplicate, and error bars correspond to SD.

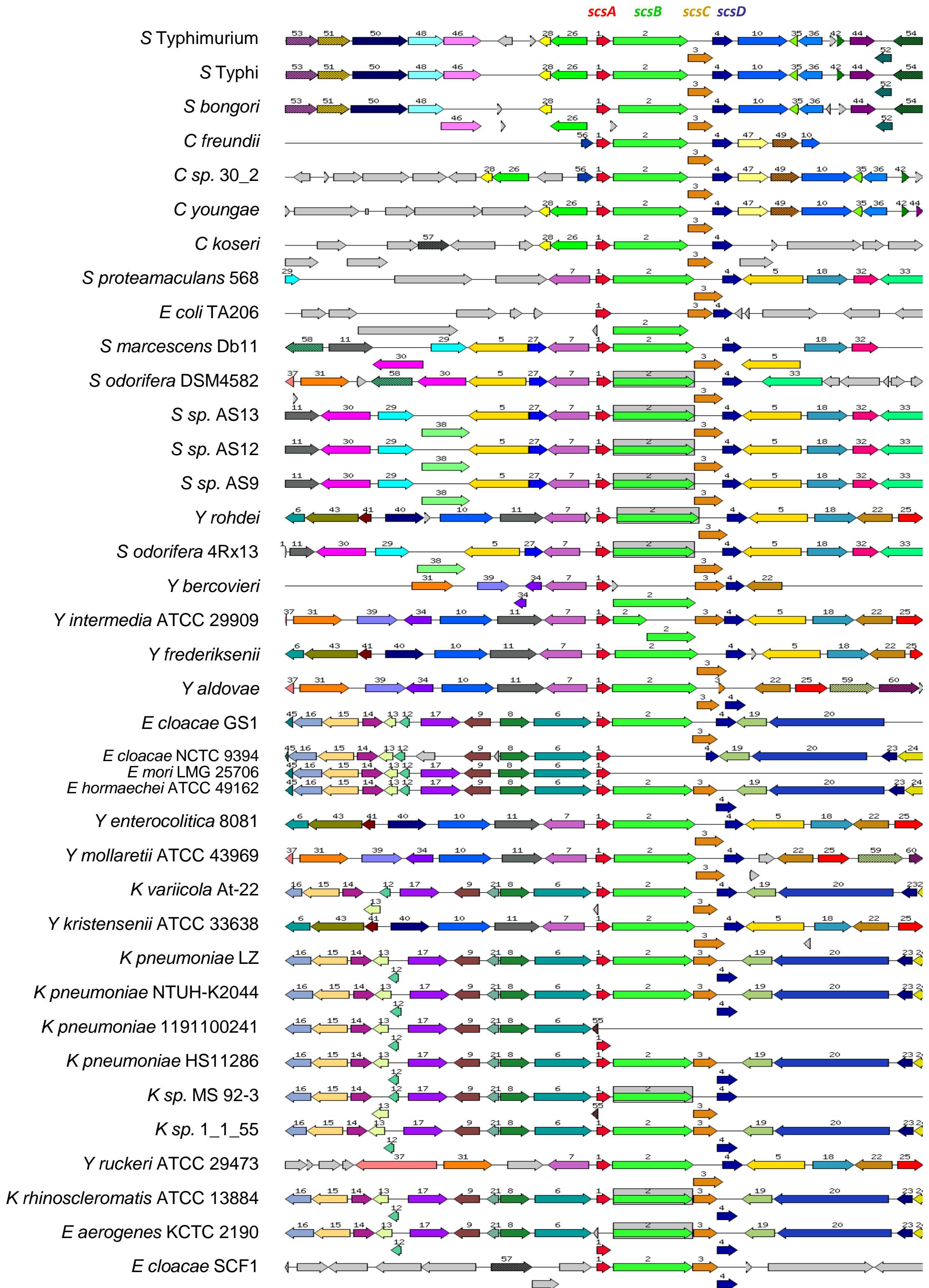

scsA
scsB
scsC
scsD
S Typhimurium
S Typhi
S bongori
C freundii
C sp. 30_2
C youngae
C koseri
S proteamaculans 568
E coli TA206
S marcescens Db11
S odorifera DSM4582
S sp. AS13
S sp. AS12
S sp. AS9
Y rohdei
S odorifera 4Rx13
Y bercovieri
Y intermedia ATCC 29909
Y frederiksenii
Y aldovae
E cloacae GS1
E cloacae NCTC 9394
E mori LMG 25706
E hormaechei ATCC 49162
Y enterocolitica 8081
Y mollaretii ATCC 43969
K variicola At-22
Y kristensenii ATCC 33638
K pneumoniae LZ
K pneumoniae NTUH-K2044
K pneumoniae 1191100241
K pneumoniae HS11286
K sp. MS 92-3
K sp. 1_1_55
Y ruckeri ATCC 29473
K rhinoscleromatis ATCC 13884
E aerogenes KCTC 2190
E cloacae SCF1

FIG. S6. The *scsA* gene is always upstream *scsB*, *scsC* and *scsD* homologues in *scsA*-harboring species. The figure shows the chromosomal region of organisms harboring *scsA* homologues by "The Seed Viewer" tool (http://pubseed.theseed.org). In the figure *scsA*, *scsB*, *scsC* and *scsD* are marked as 1, 2, 3, and 4, respectively because of their conservation among these genomes. *scsB*, *scsC* and *scsD* are present in all but *Enterobacter mori* LMG 25706 and *Klebsiella pneumoniae* 1191100241 genomes which lack sequence information beyond *scsA*. (Only *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2, *Salmonella enterica* subsp. *enterica* serovar Typhi str. CT18, and *Salmonella bongori* 12149 were included in the figure, but all *Salmonella* serovars harbor the *scsABCD* locus). **Species:** *Salmonella enterica* subsp. *enterica* serovar Typhimurium str. LT2; *Salmonella enterica* subsp. *enterica* serovar Typhi str. CT18; *Salmonella bongori* 12149; *Citrobacter freundii* str. ballerup 7851/39; *Citrobacter sp.* 30_2; *Citrobacter youngae* ATCC 29220; *Citrobacter koseri* ATCC BAA-895; *Serratia proteamaculans* 568; *Escherichia coli* TA206; *Serratia marcescens* Db11; *Serratia odorifera* DSM 4582; *Serratia sp.* AS13; *Serratia sp.* AS12; *Serratia sp.* AS9; *Yersinia rohdei* ATCC 43380; *Serratia odorifera* 4Rx13; *Yersinia bercovieri* ATCC 43970; *Yersinia intermedia* ATCC 29909; *Yersinia frederiksenii* ATCC 33641; *Yersinia aldovae* ATCC 35236; *Enterobacter cloacae* subsp. *cloacae* GS1; *Enterobacter cloacae* subsp. *cloacae* NCTC 9394; *Enterobacter mori* LMG 25706; *Enterobacter hormaechei* ATCC 49162; *Yersinia enterocolitica* 8081; *Yersinia mollaretii* ATCC 43969; *Klebsiella variicola* At-22; *Yersinia kristensenii* ATCC 33638; *Klebsiella pneumoniae* subsp. *pneumoniae* LZ; *Klebsiella pneumoniae* NTUH-K2044; *Klebsiella pneumoniae* 1191100241; *Klebsiella pneumoniae* subsp. *pneumoniae* HS11286; *Klebsiella sp.* MS 92-3; *Klebsiella sp.* 1_1_55; *Yersinia ruckeri* ATCC 29473; *Klebsiella pneumoniae* subsp. *rhinoscleromatis* ATCC 13884; *Enterobacter aerogenes* KCTC 2190; and *Enterobacter cloacae* SCF1.

References

1. **Pezza A, Pontel LB, Lopez C, Soncini FC.** 2016. Compartment and signal-specific codependence in the transcriptional control of *Salmonella* periplasmic copper homeostasis. Proc Natl Acad Sci U S A **113:**11573-11578.

2. **Pontel LB, Scampoli NL, Porwollik S, Checa SK, McClelland M, Soncini FC.** 2014. Identification of a *Salmonella* ancillary copper detoxification mechanism by a comparative analysis of the genome-wide transcriptional response to copper and zinc excess. Microbiology **160:**1659-1669.