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Dissecting the *Salmonella* response to copper

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Intracellular copper homeostasis in bacteria is maintained as the result of a complex ensemble of cellular processes that in *Escherichia coli* involve the coordinated action of two systems, *cue* and *cus*. In contrast, the pathogenic bacterium *Salmonella* harbours only the *cue* regulon, including *copA,* which is shown here to be transcriptionally controlled by CueR. Mutant strains in the CueR-regulated genes were constructed to characterize the response of *Salmonella enterica* serovar Typhimurium to high concentrations of extracellular copper under both aerobic and anaerobic conditions. Unlike its counterpart in *E. coli*, inactivation of *cuiD* displays the most severe phenotype and is also required for copper tolerance under anaerobic conditions. Deletion of *copA* has a mild effect in aerobiosis, but strongly impairs survival in the absence of oxygen. In a Δ*copA* strain, a second S*almonella*-specific P-type ATPase, GolT, can substitute the copper transporter, diminishing the effect of its deletion. The overall results highlight the importance of the *cue* system for controlling intracellular copper stress. The observed differences between *Salmonella* and *E. coli* in handling copper excess may contribute to our understanding of the distinct capability of these related pathogenic bacteria to survive outside the host.

INTRODUCTION

Copper is required in trace amounts for bacterial growth as it is an essential component of proteins required for a variety of cellular processes such as hydrolytic pathways, iron transport, respiration and defence against oxidative stress. It is also a well-known bactericide, although the mechanisms involved in copper-mediated injury have not yet been resolved (Borkow & Gabbay, 2005; Macomber *et al.*, 2007). In order to prevent copper damage, the cytoplasmic concentration of free copper must be negligible. The strategies used to eliminate copper excess are diverse, including different regulatory systems (MerR-like regulators, repressors and two-component systems) that modulate the expression of factors involved in active extrusion and sequestration, as well as oxidation of the metal ion in the periplasmic space (Magnani & Solioz, 2005; Moore & Helmann, 2005; Rensing & Grass, 2003). Among these factors, there is a broad conservation of CPx/P1-type ATPases, in addition to small copper-binding proteins (copper chaperones) and the sporadic presence in certain species of an RND ancillary copper-efflux system. In *Escherichia coli*, for instance, copper homeostasis is maintained by the coordinated action of two regulatory systems, CueR, a MerR-like protein, and the two-component system CusR/CusS (Outten *et al.*, 2001; Rensing & Grass, 2003). CueR directly stimulates the transcription of *copA* and *cueO*, coding for a P-type ATPase and a multicopper oxidase, respectively. CopA is predicted to translocate Cu(I) from the cytoplasm to the periplasmic space, where it is converted to the less toxic form Cu(II) by CueO (Outten *et al.*, 2000; Petersen & Moller, 2000; Rensing & Grass, 2003).

The *E. coli cus* regulon was found to play a role in maintaining copper homeostasis under anaerobic conditions, when the oxidase CueO is inactive (Outten *et al.*, 2001). The CusR/CusS system is predicted to monitor the periplasmic concentration of the metal ion, to modulate the expression of an RND-type copper efflux pump, encoded by the *cusCFBA* operon (Franke *et al.*, 2003; Munson *et al.*, 2000). Recently, it was shown that transcription of a second, uncharacterized, two-component system, encoded by the *yedWV* operon, is activated by copper ions in a CusR-dependent manner (Yamamoto & Ishihama, 2005), although its role in copper tolerance remains unclear.

Other stress-response regulatory systems, such as CpxR/CpxA and SoxR/SoxS, have been found to be induced by the addition of copper (Kershaw *et al.*, 2005; Yamamoto & Ishihama, 2005), probably as a result of cellular damage caused by the metal ion (Macomber, 2007)

In this work, we have characterized the *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) response to

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Abbreviation: EMSA, electrophoretic gel mobility shift assay.

copper. Previous reports revealed the importance of the multicopper oxidase (named CuiD in *Salmonella*) and the transcriptional regulator CueR/SctR for copper tolerance (Kim *et al.*, 2002; Lim *et al.*, 2002). Here, we demonstrate that expression of CopA in *Salmonella* depends on CueR, and that this transporter and the multicopper oxidase CuiD are essential for full copper tolerance under both aerobic and anaerobic conditions. We also provide evidence that, in the absence of a functional CopA, the *Salmonella*-specific P-type ATPase GolT compensates for the deficiency directing active efflux of copper.

METHODS

Bacterial strains and growth conditions. Bacterial strains (all derivatives of *S.* Typhimurium 14028s) used in this study are listed in Table 1. Bacterial strains were grown overnight at 37 °C in Luria broth (LB) or LB-agar plates (Checa *et al.*, 2007). Ampicillin, kanamycin and chloramphenicol were used at 100, 25 and 10 μg ml^{-1}, respectively. When necessary, $CuSO_4$ was added to the cultures or plates at the indicated concentration. Cell culture medium reagents and chemicals were from Sigma. Oligonucleotides were purchased from Bio-Synthesis. Primer sequences are available on request.

Table 1. Bacterial strains

Strain	Relevant genotype	Reference or source
14028s	Wild-type	ATCC
PB5447	*cueR*::Km	This study
PB5449	Δ*cueR*	This study
PB3993	*copA*::Cm	Checa *et al.* (2007)
PB5062	Δ*copA*	Checa *et al.* (2007)
PB3167	*cuiD*::Mud*J*	Checa *et al.* (2007)
PB6149	*cuiD*::Mud*J* Δ*copA*	This study
PB3164	*golS*::Cm	Checa *et al.* (2007)
PB3162	*golT*::Cm	Checa *et al.* (2007)
PB3174	*golB*::Cm	Checa *et al.* (2007)
PB5257	Δ*golS*	Checa *et al.* (2007)
PB3298	Δ*golT*	Checa *et al.* (2007)
PB3987	Δ*golTSB*	Checa *et al.* (2007)
PB4110	Δ*golT golB*::Cm	Checa *et al.* (2007)
PB4104	*cuiD*::Mud*J* Δ*golT*	This study
PB4107	*cuiD*::Mud*J* Δ*golTSB*	This study
PB5826	*cuiD*::Mud*J* Δ*golT* Δ*copA*	This study
PB5557	Δ*copA* Δ*golT*	This study
PB5738	Δ*copA* Δ*golB*	This study
PB5275	Δ*copA* Δ*golT golB*::Cm	This study
PB5559	Δ*copA* Δ*golTSB*	Checa *et al.* (2007)
PB5737	Δ*copA* Δ*golS*	This study
PB5900	Δ*golS cueR*::Km	This study
PB5430	*yedWV*::Km	This study
PB5899	*yedWV*::Km Δ*cueR*	This study
PB5964	*yedWV* Δ*cueR golS*::Cm	This study
PB5292	*copA*::*lacZY*$^+$	Checa *et al.* (2007)
PB5368	*copA*::*lacZY*$^+$ *cueR*::Km	This study
PB5367	*copA*::*lacZY*$^+$ Δ*golS*	This study
PB5452	*cuiD*::Mud*J* Δ*cueR*	This study
PB5453	*cuiD*::Mud*J* Δ*golS*	This study
PB3140	*golB*::*lacZY*$^+$	Checa *et al.* (2007)
PB5898	*golB*::*lacZY*$^+$ Δ*cueR*	This study
PB5259	*golB*::*lacZY*$^+$ Δ*golS*	Checa *et al.* (2007)
PB5143	*golB*::*lacZY*$^+$ Δ*copA*	This study
PB6072	*golB*::*lacZY*$^+$ Δ*golS* Δ*copA*	This study

Genetic and molecular biology techniques. Gene disruptions or *lacZ* reporter fusions to promoters were carried out as described previously (Datsenko & Wanner, 2000; Ellermeier *et al.*, 2002) in strain LB5010 (Bullas & Ryu, 1983). All constructions were transferred to the wild-type strain 14028s by P22 transduction (Davis *et al.*, 1980). When necessary, the antibiotic-resistance cassette inserted at the deletion point was removed using the temperature-sensitive plasmid pCP20 carrying the FLP recombinase (Cherepanov & Wackernagel, 1995). All mutated DNA fragments were sequenced to confirm the required mutation and to screen against undesired mutations.

The *cueR* locus was PCR-amplified from the *Salmonella* chromosome using the primers cueR-ORF-F (5′-GAGGATCCATATGAATA-TTAGCG-3′) and cueR-ORF-R (5′-ACCCAAGCTTCAACGTGG-CTTTTGC-3′). The amplified fragment was cloned into pUH21-2 *laqI*^{q} to generate the *cueR*-expression plasmid pCUER (pPB1205). Plasmid DNA was introduced into bacterial strains by electroporation using a Bio-Rad apparatus, following the manufacturer's recommendations.

Copper induction and inhibition assays. β-Galactosidase assays were carried out essentially as described by Miller (1972). For metal-sensitivity assays, a 5×10^{-7} dilution from overnight culture of the wild-type or each mutant strain was done in PBS. A 30 μl aliquot was applied on LB plates containing increasing concentrations of $CuSO_4$. Plates were incubated at 37 °C for 40 h under aerobic conditions or 64 h under anaerobic conditions. Anaerobic environments were generated in a Gaspak jar system using AnaeroGen sachets (Oxoid). Anaerobic indicators (Oxoid) were employed to verify oxygen consumption, following the manufacturer's recommendations. After incubation, c.f.u. per ml were calculated and the percentage survival was estimated based on the count of the corresponding strain grown in the absence of metal added (Checa *et al.*, 2007).

CueR purification. *Salmonella* CueR was overproduced and purified from the wild-type strain carrying plasmid pCUER grown in the presence of 1 mM IPTG essentially as described previously (Outten *et al.*, 2000). All procedures were carried out at 4 °C. The protein profile of the purified proteins was determined by SDS-PAGE. Protein concentration was determined by Bradford assay, using BSA as standard.

RNA isolation and primer extension. Total RNA was extracted from mid-exponential-phase cultures (OD_{600} 0.4–0.6) of wild-type *S.* Typhimurium and its isogenic Δ*cueR* mutant strain grown in LB medium with or without the addition of 1 mM $CuSO_4$ as previously described (Aguirre *et al.*, 2000). cDNA synthesis was performed using 2 pmol of the ^{32}P-end-labelled primer PROM-*copA*-R (5′-CCCAAGCTTCGCCAGCTCAACATC-3′), with 100 μg total RNA and 1 U SuperScript II RNaseH2 reverse transcriptase (Life Technologies). The extension products were analysed by electrophoresis on a 6 % polyacrylamide-8 M urea gels and compared with sequence ladders initiated with the same ^{32}P-labelled primer that was used for primer extension.

Protein–DNA interaction analysis. Electrophoretic gel mobility shift assays (EMSAs) were performed essentially as previously described (Lejona *et al.*, 2003). DNA fragments (343 bp for the

copA promoter region) were PCR-amplified using the primers PROM-*copA*-F (5′-CCGGAATTCGGTGCGATAACCATT-3′) and PROM-*copA*-R (5′-CCCAAGCTTCGCCAGCTCAACATC-3′). Labelled DNA was incubated at room temperature for 20 min with purified CueR in the amounts indicated in the figure. The binding buffer used for protein–DNA interactions contained 25 mM Tris/HCl (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, 5 mM DTT and 10 % glycerol. Samples were run on an 8 % non-denaturing Tris/glycine polyacrylamide gel at room temperature. After electrophoresis, the gel was dried and autoradiographed.

DNase I footprinting assay. DNase I protection assays were done for both DNA strands essentially as previously described (Aguirre *et al.*, 2000; Lejona *et al.*, 2003). Binding reactions with different amounts of purified CueR protein and 6 fmol labelled DNA were performed as described for the EMSAs. Then 0.05 U DNase I (Life Technologies) was added in a final volume of 100 µl. After incubation for 1 min at room temperature, the reaction was stopped by adding 90 µl of 20 mM EDTA (pH 8), 200 mM NaCl and 100 µg tRNA ml^{-1}. DNA fragments were purified by phenol/chloroform extraction and resuspended in 5 µl H_2O. Samples (5 µl) were analysed by denaturing polyacrylamide (6 %) gel electrophoresis by comparison with a DNA sequence ladder generated with the appropriate primer.

RESULTS

Comparative *in silico* analysis between *E. coli*, *S.* Typhi and *S.* Typhimurium copper-response regulatory networks

We performed a BLAST search analysis in the *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) and *Salmonella enterica* serovar Typhi (*S.* Typhi) genomes, searching for gene product homologues to the components of the *E. coli* copper regulons *cue* and *cus* (Table 2). This screening revealed some important differences between these related enterobacteria. Both *S. enterica* serovars harbour orthologues to all components of the *cue* regulon, including the previously identified MerR-like regulator CueR/SctR, the periplasmic copper oxidase CuiD/CueO, as well as a close homologue to the inner-membrane P-type transporter CopA (Table 2). Interestingly, no orthologues to the components of the *cus* system were detected, except for the YedW/YedV two-component system that in *E. coli* was recently shown to be under transcriptional control of CusR (Yamamoto & Ishihama, 2005). In addition, *S.* Typhimurium harbours a second *cue*-like regulon, *gol*. We have recently shown that this regulon, which includes a P-type ATPase (GolT), a putative metal-binding protein (GolB) and a transcriptional regulator (GolS), endows *S.* Typhimurium with resistance to gold salts (Checa *et al.*, 2007). The *golTS* operon is present in most *Salmonella* subspecies and in *Salmonella bongori* (http://www.sanger.ac.uk/Projects/Salmonella/), but absent in *S.* Typhi (Table 2) and *S. enterica* serovar Paratyphi A. On the other hand, *golB* is present in all sequenced salmonellas.

Expression of *copA* in *Salmonella* is directly controlled by CueR

Unlike in *E. coli*, in *Salmonella cueR* and *copA* are located adjacent in the genome (separated by only 110 nt), and transcribed divergently from each other (Fig. 1a). To probe if expression of *copA* depends on CueR, we first constructed a strain carrying a chromosomal *lacZ* reporter fusion to the promoter of the transporter gene (see

Table 2. *E. coli* copper-homeostasis genes present in *S.* Typhimurium and *S.* Typhi

***E. coli* K-12 gene**	**Annotation**	***S.* Typhimurium LT2**		***S.* Typhi Ty2**	
		Gene	**Identity (%)***	**Gene**	**Identity (%)**
CueR regulon					
cueO	Multicopper oxidase	STM0168, *cuiD*	(80 %)	t0173, *yacK*	(80 %)
copA	P-type ATPase	STM0498, *copA*	(92 %)	t2362, *yrbA*	(92 %)
cueR	MerR-type regulator	STM0499, *sctR*	(91 %)	t2361, *cueR*	(91 %)
CusR regulon					
cusR	Response regulator (TCS)	–		–	
cusS	Sensor kinase (TCS)	–		–	
cusC	Outer-membrane efflux protein	–		–	
cusF	Periplasmic metal resistance protein	–		–	
cusB	Inner-membrane efflux protein	–		–	
cusA	Periplasmic, RND-family efflux protein	–		–	
yedW	Response regulator (TCS)	STM1096, *copR*	(75 %)	t1823, *copR*	(74 %)
yedV	Sensor kinase (TCS)	STM1095, *copS*	(54 %)	t1824, *copS*	(54 %)
Not present in *E. coli*					
	P-type ATPase	STM0353, *golT*	(42 %, *E.c.* CopA)	–	
	MerR-type regulator	STM0354, *golS*	(42 %, *E.c.* CueR)	–	
	Metal chaperone	STM0355, *golB*		t2511	(98 %, STM0355)

*The value represents % of amino acid identity with the *E. coli* K-12 homologue protein, except when stated.

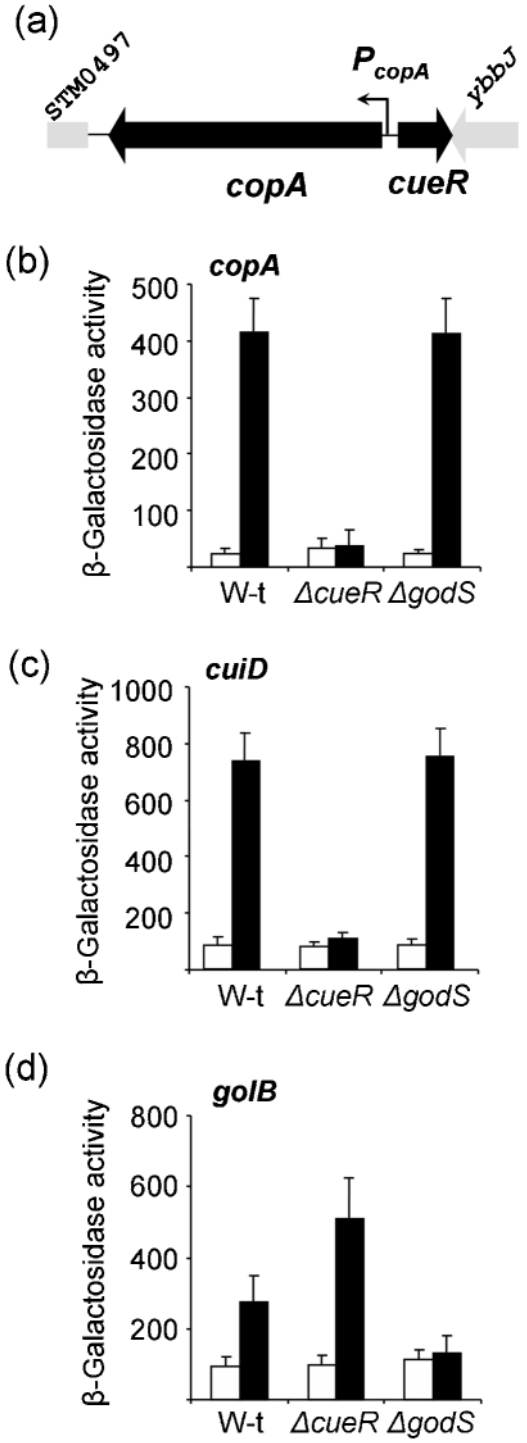

Fig. 1. Expression of *copA* is induced by copper ions, in a CueR-dependent manner. (a) Genetic organization of *copA* and *sctR* genes in the *S.* Typhimurium LT2 genome. (b–d) β-Galactosidase activity (Miller units) from *copA*::*lacZ*, *cuiD*::Mud*J* or *golB*::*lacZ* transcriptional fusions, respectively, expressed by wild-type (W-t), Δ*cueR or* Δ*golS* cells after overnight growth in LB broth without (white bars), or with the addition of 1 mM $CuSO_4$ (black bars). The data correspond to means ± SD of three independent experiments done in duplicate.

Methods for details) and assayed its expression in the presence or absence of copper ions. The *lacZ* insertion was generated as a *copA* operon fusion, without disrupting the transporter gene. This will ensure proper copper efflux. Addition of 1 mM $CuSO_4$ to the culture medium induced *copA* expression by 13-fold in LB (Fig. 1b) and by eightfold in SM9 minimal medium (Checa *et al.*, 2007). This copper-dependent activation of *copA* was eliminated in a *cueR* null mutant, but not affected in a Δ*golS* strain, confirming that CueR controls the expression of the Cu(I) transporter CopA in *Salmonella.* A similar result was obtained using a *cuiD-lacZ* reporter whose expression also depends on CueR (Fig. 1c). Interestingly, deletion of *cueR* increased rather than decreased copper-induced expression of the GolS-controlled gene *golB* (Fig. 1d). This increase probably reflects accumulation of cytoplasmic copper ions, because of low-level expression of CopA in the Δ*cueR* strain (Fig. 1b, see also below).

To confirm that *copA* transcription in *Salmonella* is controlled directly by the transcriptional regulator CueR, we first mapped the transcription start site of the gene by primer extension analysis, using RNA isolated from wild-type or Δ*cueR* mutant cells grown in the presence or absence of $CuSO_4$. A single primer extension product, corresponding to a G residue located 26 nt upstream of the *copA* start codon, was observed only in samples obtained from the wild-type strain grown in the presence of copper ions (Fig. 2a). The *Salmonella copA* transcription start site differs in one base from the one determined previously in *E. coli* by Outten *et al.*, (2000), Petersen & Moller (2000) and Stoyanov *et al.* (2001).

We performed EMSA and DNase I footprinting using purified *Salmonella* CueR to confirm direct binding (Fig. 2b, c). Both the extension of the protected region (from nt −39 to −13 relative to the transcription start site in the coding strand and from nt −15 to −40 in the non-coding strand) and the presence of hypersensitive bands (at nt −31 and −19 and at −33 and −21 in the coding and non-coding strands, respectively) are common features of the protein–DNA interaction described for the MerR family (Ansari *et al.*, 1995; O'Halloran *et al.*, 1989; Outten *et al.*, 1999). The protected sequence of the *copA* promoter encompasses the sequence 5′-TTGACCTTA-ACCTTGCTGGAAGGTTTA-3′, which includes an imperfect ACCTTCC inverted repeat sequence located between the predicted −35 and −10 elements in the *copA* promoter region, is similar to the predicted *E. coli* CueR-binding site (Fig. 2c, d; Outten *et al.*, 2000; Stoyanov *et al.*, 2001; Yamamoto & Ishihama, 2005).

CopA and CuiD are essential for copper tolerance under both aerobic and anaerobic conditions

We performed copper-sensitivity assays under both aerobic and anaerobic conditions using different mutant strains in the CueR-regulated genes. In the presence of oxygen, copper tolerance decreased in strains carrying mutations in

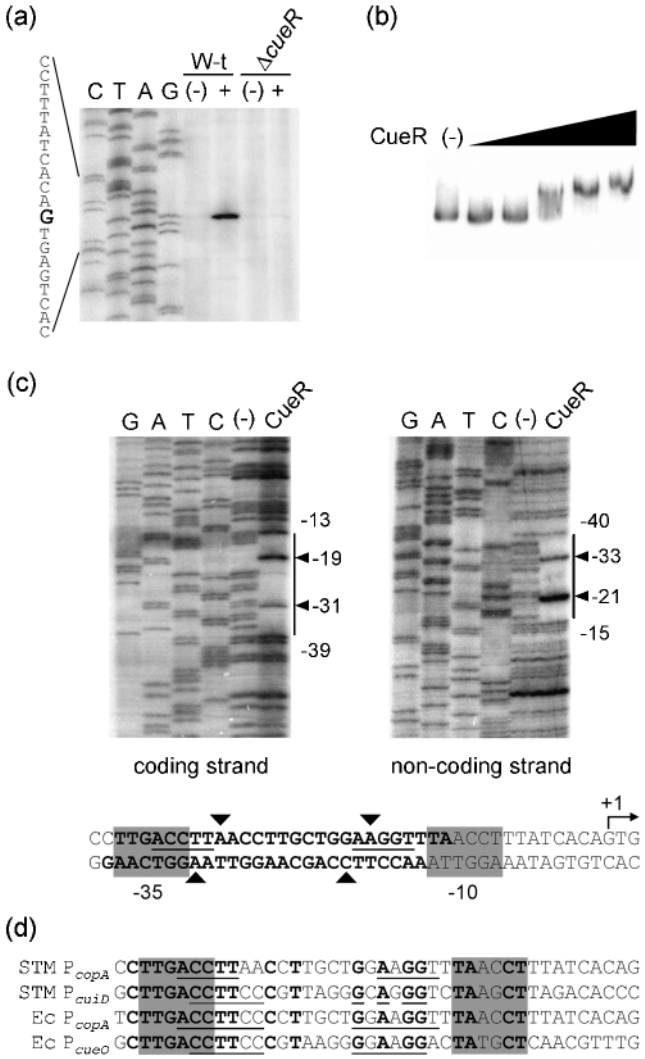

Fig. 2. CueR binds to the promoter region of *copA*. (a) Primer extension analysis of *copA* using RNA isolated from mid-exponential-phase wild-type or Δ*cueR* cells grown in LB with or without the addition of 1 mM $CuSO_4$. The sequence spanning the transcription start site (bold) is shown. (b) EMSA was performed using the ^{32}P 3′-end-labelled PCR fragment of the promoter region of *copA* incubated with purified CueR at final concentrations of 0, 0.2, 0.4, 0.8, 1.6 and 3.2 μM. (c) DNA footprinting analysis of the promoter region of *copA* was performed on both end-labelled coding and non-coding strands. Purified CueR protein (CueR, 6 μM) was added to the DNA fragments. Solid lines and arrows indicate the CueR-protected region and hypersensitive sites, respectively. The sequence at the bottom shows the *copA* promoter region. The CueR-protected region is indicated in boldface, and the inverted repeat CueR box is underlined. The DNase-hypersensitive sites are indicated by arrows. The transcription start site as well as the −10 and −35 elements (grey boxes) are also indicated. (d) Alignment of the promoter regions of *copA* and *cueO* homologues from *S.* Typhimurium and *E. coli* showing the predicted −35 and −10 regions (grey boxes), and the putative CueR operator (underlined).

either *cueR*, *copA* or *cuiD*, although the latter strain showed the most severe phenotype (Table 3). Copper susceptibility increased even more in the *cuiD copA* double mutant strain, supporting the relevant role of both proteins in maintaining copper homeostasis. The marked copper susceptibility of the single *cuiD* mutant compared with the Δ*cueR* or the Δ*copA* strains suggests that even basal levels of CuiD are enough to guarantee survival in copper-rich medium, and supports the crucial role assigned to this enzyme for copper tolerance under aerobic conditions in *Salmonella* (Lim *et al.*, 2002).

The role of CopA in copper tolerance acquired more relevance in the absence of oxygen (Table 3), when the multicopper oxidase CueO is predicted to be inactive (Outten *et al.*, 2001). Unexpectedly, we observed that survival of a *cuiD* mutant strain in the presence of copper was affected even in cells grown under anaerobic conditions (Table 3), suggesting that the encoded enzyme could play an additional role in copper homeostasis in *Salmonella*. Periplasmic multicopper oxidases are involved

Table 3. Copper tolerance phenotype of the *S.* Typhimurium mutant strains analysed in this work

Strain	MIC (mM)*	
	$+O_2$	$-O_2$
CueR regulon		
Wild-type	5.50	0.70
cueR	4.50	0.45
cuiD	1.25	0.50
copA	4.50	0.45
cuiD copA	1.00	ND
YedW/V system		
yedWV	5.50	0.70
cueR yedWV	4.50	0.45
cueR golS yedWV	4.50†	0.45
GolS regulon		
golS	5.50	0.70
golT	5.50	ND
golTSB	5.50	0.70
cuiD golT	1.25	ND
cuiD golTSB	1.25	ND
cuiD copA golT	0.75	0.23
copA golT	2.75	ND
copA golB	4.50	ND
copA golTB	2.50	ND
copA golTSB	2.50	0.23
copA golS	3.00	ND
cueR golS	4.50†	0.45

*MIC values were determined on LB plates containing increasing amounts of $CuSO_4$ under both aerobic ($+O_2$) and anaerobic ($-O_2$) conditions (see Methods for details). The data correspond to mean values of three independent experiments done in triplicate; ND, not determined.

†Smaller colonies were observed compared with the single *cueR* mutant or with the Δ*cueR* Δ*yedWV* strain.

in the conversion of the harmful Cu(I) to Cu(II) in the presence of oxygen (Singh *et al.*, 2004; Tree *et al.*, 2005).

We observed that under anaerobic conditions copper inhibited the bacterial growth even more strongly than under aerobic conditions (Table 3), as was previously observed in *E. coli* (Beswick *et al.*, 1976; Outten *et al.*, 2001; Rensing & Grass, 2003). This supports the notion that copper injury to bacterial cells cannot be mediated exclusively by oxidative DNA damage (Macomber *et al.*, 2007).

The *Salmonella* YedW/YedV system is not involved in copper homeostasis

Salmonella lacks the ancillary copper-detoxification *cus* system, but conserves genes homologous to the *E. coli yedWV* operon, STM1096 and STM1095 (Table 1). The *yedWV* operon encodes a two-component system, transcription of which in *E. coli* is activated by copper ions (Yamamoto & Ishihama, 2005).

We analysed whether YedW/YedV contributed to maintaining copper homeostasis in *Salmonella*, testing survival of the Δ*yedWV* mutant strain in the presence of $CuSO_4$ (Table 3). *yedWV* expression is not induced by addition of up to 2 mM $CuSO_4$ (data not shown). Moreover, deletion of *yedWV* does not affect copper tolerance of the wild-type strain or of the Δ*cueR* or the Δ*cueR* Δ*golS* mutants (see below), under either aerobic or anaerobic conditions, arguing against a role of this operon in copper homeostasis in *Salmonella*.

The gold transporter GolT can contribute to copper tolerance in the absence of CopA

We have recently shown that *Salmonella* has a second CueR homologue highly sensitive to gold ions, GolS, which induces the expression of a CopA-homologous protein, GolT, and a putative metal-binding protein, GolB (Checa *et al.*, 2007). We found that this *Salmonella*-specific regulon is required for gold resistance, but not for copper tolerance, except in a strain in which the main copper transporter CopA has been deleted (Checa *et al.*, 2007; see also Table 3). These results prompted us to investigate whether some of the GolS-controlled genes, including *golS*, would acquire relevance in copper homeostasis when the ancestral copper-detoxification system *cue* is inactive or absent. We constructed a series of mutant strains in which *copA* and the different genes coding for components of the *gol* regulon were deleted. As seen in Table 3, only the deletion of the P-type metal transporter gene *golT* rendered a marked reduction in copper tolerance in a Δ*copA* strain. Moreover, the simultaneous deletion of the two transporter genes, *copA* and *golT*, further increased the susceptibility of a *copA cuiD* mutant strain. The contribution of GolT to copper detoxification in the absence of a functional CopA was also observed in cells grown under anaerobic conditions (Table 3). Unlike *golT*, deletion of *golB* did not affect metal tolerance of a Δ*copA* strain, but slightly reduced survival of a strain with both CopA and GolT transporters deleted (Table 3). Neither single mutants in GolS-regulated genes, nor a mutant with the whole *gol* locus deleted, altered *Salmonella* copper susceptibility of a *cuiD* or a wild-type background (Table 3). In accordance, deletion of the gold-sensor gene *golS* had only a minor effect on copper tolerance in a Δ*cueR* mutant strain (Table 3), which was only evident in liquid media and under aerobic conditions (Fig. 3a).

From the above results, we asked how proper GolT levels could be acquired in a *copA* mutant to cope with toxic copper levels, its expression being controlled by GolS, which is a poor copper sensor (Checa *et al.*, 2007). We analysed the copper-induced expression of *golB* as a GolS-dependent gene, in wild-type and Δ*copA* backgrounds (Fig. 3b). Copper-induced *golB* expression increased up to 24-fold in the Δ*copA* strain compared with the levels obtained in the wild-type strain. This induction was dependent on the intactness of *golS* (Fig. 3b), suggesting that, in the absence of CopA, there is a rise of intracellular copper concentration that can induce the expression of the *gol* regulon. In this condition, GolT will contribute to transporting the excess copper ions out of the cytoplasm, reducing the toxicity of the cation. In support of these observations, we found an increase in copper susceptibility of the double Δ*copA* Δ*golS* mutant when compared with the single mutants (Table 3; see also Fig. 3c). Altogether, these results indicate that, in the absence of the main copper transporter, at least some of the factors controlled by GolS can assist in copper tolerance.

DISCUSSION

Copper homeostasis in bacteria is guaranteed mainly by the action of active efflux systems that remove the metal ion from the cell (Magnani & Solioz, 2005; Moore & Helmann, 2005; Nies, 2003; Rensing & Grass, 2003). Among these systems, transporters of the P-type ATPases family (Arguello *et al.*, 2007; Kuhlbrandt, 2004) are usually involved. We have demonstrated here that expression of the *Salmonella* P-type ATPase CopA is induced by copper ions in a CueR-dependent manner (Figs 1 and 2) and that deletion of the *copA* gene affects copper tolerance under both aerobic and anaerobic conditions (Table 3). These observations, in addition to the previously reported role of CueR and CuiD in copper homeostasis (Kim *et al.*, 2002; Lim *et al.*, 2002), indicate that this pathogenic enterobacterium possesses a complete and functional *cue* system, similar to the *E. coli* counterpart (Outten *et al.*, 2000; Rensing & Grass, 2003). Nevertheless, the contribution of CopA and CuiD to copper tolerance in *Salmonella* differs in some aspects from *E. coli*. First, the main mechanism apparently used by *Salmonella* to eliminate excess of copper under aerobic conditions is the conversion of Cu(I) to Cu(II) by CuiD (Table 3). Second, CuiD is also required for copper tolerance under anaerobic conditions (Table 3),

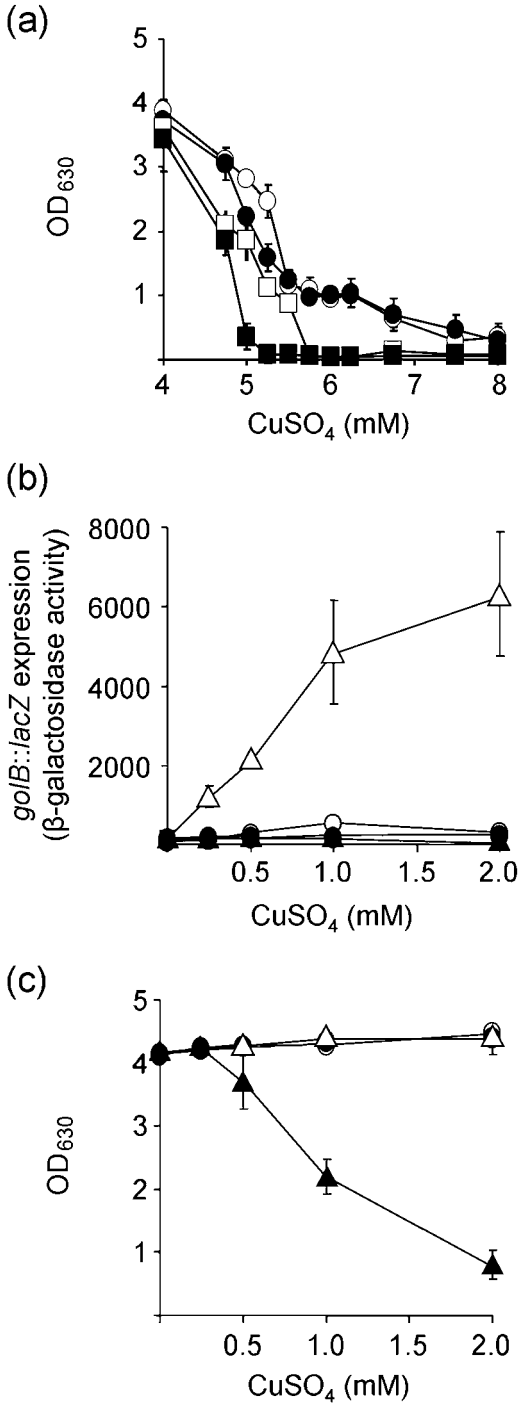

Fig. 3. GolS-controlled genes are required for copper tolerance when the *cue* system does not function. (a) Final OD_{630} reached by the cultures of wild-type (○), Δ*cueR* (□), Δ*golS* (●) or Δ*cueR* Δ*golS* (■) *Salmonella* strains grown on LB broth containing $CuSO_4$, at the specified concentrations. (b) β-Galactosidase activity (Miller units) from a *golB*::*lacZ* transcriptional fusion expressed by wild-type (○), Δ*golS* (●), Δ*copA* (△) or Δ*golS* Δ*copA* (▲) mutant cells, grown overnight in LB broth with or without the addition of the indicated amounts of $CuSO_4$. (c) Final OD_{630} reached by the cultures used in (b). The data correspond to means ± SD of three independent experiments done in duplicate.

differing from *E. coli* (Outten *et al.*, 2001). A similar effect was reported previously for the *Rhodobacter capsulatus* multicopper oxidase (Wiethaus *et al.*, 2006). It has been proposed that CueO from *E. coli* can also contribute to copper tolerance by loading of the folded protein with copper ions in the cytoplasm prior to its subsequent transport into the periplasmic space by the Tat system (Rensing & Grass, 2003). Our results suggest that this detoxification mechanism would acquire relevance in *Salmonella*, which lacks the ancillary *cus* system.

The *E. coli yedWV* orthologous genes, although present in *Salmonella* (Table 2), are neither required for copper tolerance (Table 3) nor induced under excess of copper (data not shown). The above observations, in addition to the absence of *E. coli cus* homologues in all sequenced *Salmonella* serovars (Table 2 and http://www.sanger.ac.uk/Projects/Salmonella/), highlight differences between these closely related enterobacteria in the approach used to control copper excess, in particular, in conditions where the *cue* system is overloaded.

In a previous report, we characterized the *gol* regulon that confers resistance to gold ions and demonstrated that, in the absence of the native copper transporter CopA, survival of a strain with the whole *gol* locus deleted is impaired in the presence of $CuSO_4$ (Checa *et al.*, 2007). Nevertheless, deletion of the *gol* locus did not affect copper tolerance of a wild-type or a Δ*cuiD* mutant strain. In this work, we characterized the role of the *gol* regulon in copper detoxification in more detail. We found that among the GolS-regulated factors, the P-type ATPase GolT is mainly responsible for alleviating copper toxicity in a Δ*copA* mutant strain under both aerobic and anaerobic conditions (Table 3), probably by directing active efflux of the metal ions from the cytoplasm. Our results suggest that in the absence of the main copper transporter CopA, the intracellular copper concentration increases, as was previously suggested to occur in *E. coli* (Stoyanov *et al.*, 2003). This leads to the copper-mediated activation of GolS, enhancing the expression of its target genes (Fig. 3), including *golT*, which would extrude the excess of copper, mimicking the action of CopA. A number of observations indicate, however, that the contribution of the *gol* system to copper homeostasis in nature would be incidental and lacks physiological relevance. Copper-dependent activation of the *gol* regulon was only observed when the major copper transporter CopA was deleted (Fig. 3b). In addition, *golT* and *golS* are absent in *S.* Typhi (Table 2) and *S.* Paratyphi A. The lack of part of the *gol* regulon in these two serovars of *S. enterica* subspecies I, which are well-known human-adapted pathogens (Parkhill & Thomson, 2003), supports the notion that this regulon allows *Salmonella* to gain access to different environmental niches. On the other hand, the two metal transporters CopA and GolT are structural and functional homologues: they share 42 % identity at protein level (Table 2) and both are able to mediate either copper or gold resistance under certain conditions (Table 3; Checa *et al.*, 2007). Therefore, it is

highly unlikely that GolT could physiologically replace the absent *cus* system in *Salmonella*.

The periplasmic space of Gram-negative bacteria has been proposed to be an important target for copper toxicity, because two of the three copper-resistance systems from *E. coli*, CueO and CusCFBA, work by removing Cu(I) from this compartment (Franke *et al.*, 2003; Outten & O'Halloran, 2001; Rensing & Grass, 2003). Therefore, it will be interesting to know how *Salmonella*, which lacks the *cus* system, can avoid periplasmic copper stress under anaerobic conditions when the multicopper oxidase is inactive. The complete elucidation of the mechanisms employed by *Salmonella* to eliminate the excess of copper and the differences from those previously described in *E. coli* will contribute to better understanding of the distinct lifestyles of these related bacteria.

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REFERENCES

Aguirre, A., Lejona, S., García Véscovi, E. & Soncini, F. C. (2000). Phosphorylated PmrA interacts with the promoter region of *ugd* in *Salmonella enterica* serovar Typhimurium. *J Bacteriol* **182**, 3874–3876.

Ansari, A. Z., Bradner, J. E. & O'Halloran, T. V. (1995). DNA-bend modulation in a repressor-to-activator switching mechanism. *Nature* **374**, 371–375.

Arguello, J. M., Eren, E. & Gonzalez-Guerrero, M. (2007). The structure and function of heavy metal transport P(1B)-ATPases. *Biometals* **20**, 233–248.

Beswick, P. H., Hall, G. H., Hook, A. J., Little, K., McBrien, D. C. & Lott, K. A. (1976). Copper toxicity: evidence for the conversion of cupric to cuprous copper in vivo under anaerobic conditions. *Chem Biol Interact* **14**, 347–356.

Borkow, G. & Gabbay, J. (2005). Copper as a biocidal tool. *Curr Med Chem* **12**, 2163–2175.

Bullas, L. R. & Ryu, J. I. (1983). *Salmonella typhimurium* LT2 strains which are r^- m^+ for all three chromosomally located systems of DNA restriction and modification. *J Bacteriol* **156**, 471–474.

Checa, S. K., Espariz, M., Pérez Audero, M. E., Botta, P. E., Spinelli, S. V. & Soncini, F. C. (2007). Bacterial sensing of and resistance to gold salts. *Mol Microbiol* **63**, 1307–1318.

Cherepanov, P. P. & Wackernagel, W. (1995). Gene disruption in *Escherichia coli*: Tc^R and Km^R cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. *Gene* **158**, 9–14.

Datsenko, K. A. & Wanner, B. L. (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* **97**, 6640–6645.

Davis, R. W., Bolstein, D. & Roth, J. R. (1980). *Advanced Bacterial Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Ellermeier, C. D., Janakiraman, A. & Slauch, J. M. (2002). Construction of targeted single copy *lac* fusions using lambda Red and FLP-mediated site-specific recombination in bacteria. *Gene* **290**, 153–161.

Franke, S., Grass, G., Rensing, C. & Nies, D. H. (2003). Molecular analysis of the copper-transporting efflux system CusCFBA of *Escherichia coli*. *J Bacteriol* **185**, 3804–3812.

Kershaw, C. J., Brown, N. L., Constantinidou, C., Patel, M. D. & Hobman, J. L. (2005). The expression profile of *Escherichia coli* K-12 in response to minimal, optimal and excess copper concentrations. *Microbiology* **151**, 1187–1198.

Kim, J. S., Kim, M. H., Joe, M. H., Song, S. S., Lee, I. S. & Choi, S. Y. (2002). The *sctR* of *Salmonella enterica* serovar Typhimurium encoding a homologue of MerR protein is involved in the copper-responsive regulation of *cuiD*. *FEMS Microbiol Lett* **210**, 99–103.

Kuhlbrandt, W. (2004). Biology, structure and mechanism of P-type ATPases. *Nat Rev Mol Cell Biol* **5**, 282–295.

Lejona, S., Aguirre, A., Cabeza, M. L., García Véscovi, E. & Soncini, F. C. (2003). Molecular characterization of the Mg^{2+}-responsive PhoP-PhoQ regulon in *Salmonella enterica*. *J Bacteriol* **185**, 6287–6294.

Lim, S. Y., Joe, M. H., Song, S. S., Lee, M. H., Foster, J. W., Park, Y. K., Choi, S. Y. & Lee, I. S. (2002). CuiD is a crucial gene for survival at high copper environment in *Salmonella enterica* serovar Typhimurium. *Mol Cells* **14**, 177–184.

Macomber, L., Rensing, C. & Imlay, J. A. (2007). Intracellular copper does not catalyze the formation of oxidative DNA damage in *Escherichia coli*. *J Bacteriol* **189**, 1616–1626.

Magnani, D. & Solioz, M. (2005). Copper chaperone cycling and degradation in the regulation of the *cop* operon of *Enterococcus hirae*. *Biometals* **18**, 407–412.

Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Moore, C. M. & Helmann, J. D. (2005). Metal ion homeostasis in *Bacillus subtilis*. *Curr Opin Microbiol* **8**, 188–195.

Munson, G. P., Lam, D. L., Outten, F. W. & O'Halloran, T. V. (2000). Identification of a copper-responsive two-component system on the chromosome of *Escherichia coli* K-12. *J Bacteriol* **182**, 5864–5871.

Nies, D. H. (2003). Efflux-mediated heavy metal resistance in prokaryotes. *FEMS Microbiol Rev* **27**, 313–339.

O'Halloran, T. V., Frantz, B., Shin, M. K., Ralston, D. M. & Wright, J. G. (1989). The MerR heavy metal receptor mediates positive activation in a topologically novel transcription complex. *Cell* **56**, 119–129.

Outten, C. E. & O'Halloran, T. V. (2001). Femtomolar sensitivity of metalloregulatory proteins controlling zinc homeostasis. *Science* **292**, 2488–2492.

Outten, C. E., Outten, F. W. & O'Halloran, T. V. (1999). DNA distortion mechanism for transcriptional activation by ZntR, a Zn(II)-responsive MerR homologue in *Escherichia coli*. *J Biol Chem* **274**, 37517–37524.

Outten, F. W., Outten, C. E., Hale, J. & O'Halloran, T. V. (2000). Transcriptional activation of an *Escherichia coli* copper efflux regulon by the chromosomal MerR homologue, CueR. *J Biol Chem* **275**, 31024–31029.

Outten, F. W., Huffman, D. L., Hale, J. A. & O'Halloran, T. V. (2001). The independent *cue* and *cus* systems confer copper tolerance during aerobic and anaerobic growth in *Escherichia coli*. *J Biol Chem* **276**, 30670–30677.

Parkhill, J. & Thomson, N. (2003). Evolutionary strategies of human pathogens. *Cold Spring Harb Symp Quant Biol* **68**, 151–158.

Petersen, C. & Moller, L. B. (2000). Control of copper homeostasis in *Escherichia coli* by a P-type ATPase, CopA, and a MerR-like transcriptional activator, CopR. *Gene* **261**, 289–298.

Rensing, C. & Grass, G. (2003). *Escherichia coli* mechanisms of copper homeostasis in a changing environment. *FEMS Microbiol Rev* **27**, 197–213.

Singh, S. K., Grass, G., Rensing, C. & Montfort, W. R. (2004). Cuprous oxidase activity of CueO from *Escherichia coli*. *J Bacteriol* **186**, 7815–7817.

Stoyanov, J. V., Hobman, J. L. & Brown, N. L. (2001). CueR (YbbI) of *Escherichia coli* is a MerR family regulator controlling expression of the copper exporter CopA. *Mol Microbiol* **39**, 502–511.

Stoyanov, J. V., Magnani, D. & Solioz, M. (2003). Measurement of cytoplasmic copper, silver, and gold with a *lux* biosensor shows copper and silver, but not gold, efflux by the CopA ATPase of *Escherichia coli*. *FEBS Lett* **546**, 391–394.

Tree, J. J., Kidd, S. P., Jennings, M. P. & McEwan, A. G. (2005). Copper sensitivity of *cueO* mutants of *Escherichia coli* K-12 and the biochemical suppression of this phenotype. *Biochem Biophys Res Commun* **328**, 1205–1210.

Wiethaus, J., Wildner, G. F. & Masepohl, B. (2006). The multicopper oxidase CutO confers copper tolerance to *Rhodobacter capsulatus*. *FEMS Microbiol Lett* **256**, 67–74.

Yamamoto, K. & Ishihama, A. (2005). Transcriptional response of *Escherichia coli* to external copper. *Mol Microbiol* **56**, 215–227.

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