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Dissecting the Metal Selectivity of MerR Monovalent Metal Ion Sensors in *Salmonella*

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Two homologous transcription factors, CueR and GolS, that belong to the MerR metalloregulatory family are responsible for *Salmonella* Cu and Au sensing and resistance, respectively. They share similarities not only in their sequences, but also in their target transcription binding sites. While CueR responds similarly to Au, Ag, or Cu to induce the expression of its target genes, GolS shows higher activation by Au than by Ag or Cu. We showed that the ability of GolS to distinguish Au from Cu resides in the metal-binding loop motif. Here, we identify the amino acids within the motif that determine *in vivo* metal selectivity. We show that residues at positions 113 and 118 within the metal-binding loop are the main contributors to metal selectivity. The presence of a Pro residue at position 113 favors the detection of Cu, while the presence of Pro at position 118 disfavors it. Our results highlight the molecular bases that allow these regulators to coordinate the correct metal ion directing the response to a particular metal injury.

Transition metal homeostasis influences many fundamental aspects of bacterial cell physiology and pathogenesis (1–3). The intracellular concentration of essential metals or the presence of harmful elements is monitored by a set of transcriptional regulators that modulate the expression of factors that rapidly restore metal homeostasis (4, 5). A large class of these metalloregulators belongs to the MerR family, a group of proteins that share similarity at the N-terminal DNA-binding domain (6–9). According to the current model, dimeric metal-sensing MerR regulators control gene transcription via a DNA distortion mechanism. Both the apo- and the metal-bound regulator recognize and interact with their target operators (a dyad-symmetric DNA sequence at the promoter region of their target genes). Binding of the metal ion at the C-terminal inductor-binding site would provoke an allosteric change at the N-terminal DNA binding region of the protein, which in turn transduces changes in the promoter structure resulting in transcription activation of the expression of genes coding mostly for efflux or detoxification systems (10–12).

Most of the metal ion sensors of the MerR family are poorly selective because they cannot distinguish between cognate metals with similar physicochemical properties, including charge and coordination chemistry. For example, the Cu sensor CueR can discriminate between metal ions with +1 and +2 charges, but it cannot distinguish between monovalent metal ions of group 1B—i.e., Cu(I), Ag(I), and Au(I) (13). Structural studies indicate that CueR has only two coordinating ligands, the S-atoms of the conserved C112 and C120 residues which are appropriate for the interaction with the +1 metal ion in a linear array but not to coordinate metal ions with a +2 charge, which requires higher number of ligands (14). The recent identification of two Au-selective MerR sensors, first in the bacterial pathogen *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) and then in *Cupriavidus metallidurans*, a betaproteobacterium associated with Au grains (15–17), is useful to understand the molecular bases that allow metalloregulators of the MerR family to distinguish between cognate metal ions. Although there is no structural information available for the Au sensors, a number of studies suggest that these sensors coordinate the metal by using the conserved C112 and C120 residues of the metal-binding loop (MBL) apparently in a bis-thiolate geometry, similar to CueR, but unlike the Cu sensor, they can distinguish Au(I) from Cu(I) or Ag(I) (15, 16). Both *Salmonella* GolS and *Cupriavidus* CupR activate the expression of their target genes mainly in the presence of Au(I) ions. Recently reported *in vitro* experiments show that GolS and CueR have similar affinities for Cu(I) (18); nevertheless, *in vivo* evidence shows that GolS distinguishes Au(I) from Cu(I) or Ag(I) in the induction of its target genes (15, 19–21). Mutant strains with deletions in genes controlled by GolS, including the transcriptional regulator itself, which is autoregulated in *Salmonella*, have increased susceptibility to gold ions while retaining wild-type copper resistance in the presence of the intact copper resistance *cue* regulon (15, 21, 22). In this case, it became clear that metal selectivity of GolS is achieved by the combination of subtle modifications in the sensing domain of the gold sensor and the presence of an efficient copper resistance system operating in the cell. Indeed, GolS is activated by copper in a mutant strain deleted in both the copper sensor CueR and the copper transporter CopA, inducing the expression of part of its regulon (20–22). Expression of the P-type ATPase GolT and probably the metal-binding protein GolB serves to alleviate the toxic effect of Cu excess in the absence of CopA and/or CueR (18, 20, 22, 23). Interestingly, one of the components of the *gol* regulon, the *gesABC* operon, is solely induced by gold and not by copper, even in a strain deleted of the entire copper resistance *cue* regulon (21; our unpublished results). These observations strengthened the physiological role of the *gol* regulon in gold sens-

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ing and resistance and prompted us to investigate the determinants of metal selectivity in GolS.

Previously, we showed that the expression of the GolS-regulated *golB* gene is similarly induced by Au, Cu, or Ag in a mutant that codes for a GolS chimeric protein with the metal-binding loop of CueR (from I109 to C120 and encompassing the two conserved cysteine residues directly involved in metal coordination), resembling the metal response of the wild-type Cu sensor (15). In this study, we dissect the metal-binding loop of GolS and CueR to identify the amino acid residues that direct metal discrimination. We show that residues at positions 113 and 118 within the metal-binding loop are the main contributors to metal selectivity, while other nonconserved amino acids within the loop also cooperate to fine-tune metal selectivity.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains (all derivatives of *Salmonella enterica* serovar Typhimurium 14028s, except when indicated), plasmids, and oligonucleotides used in this study are listed in Tables S1 and S2 in the supplemental material. Bacterial strains were grown at 37°C in Luria-Bertani (LB) broth, except when indicated. Ampicillin, kanamycin, chloramphenicol, and tetracycline were used at 100, 25, 10, and 15 μg ml^{-1}, respectively. The cell culture medium reagents, chemicals, and oligonucleotides were from Sigma, except for the LB culture medium, which was from Difco.

Genetic and molecular biology techniques. Gene disruptions, point mutations, or *lacZ* reporter fusions to promoters were carried out using previously described protocols (24, 25). The constructions were initially done on *Salmonella* strain LB5010 (26) or the indicated mutant derivative of this strain and transferred to the wild-type ATCC 14028s strain by P22 transduction (27). When necessary, the antibiotic resistance cassette was removed using the temperature-sensitive plasmid pCP20 carrying the FLP recombinase (24).

The desired mutations were introduced on *golS* or *cueR* by PCR overlap extension using appropriate primers containing either mismatched bases or regions. To construct *golS* mutant alleles, a megaprimer was generated using primer *golS*-F and the corresponding reverse primer for each mutant version. The megaprimer was extended using the oligonucleotide RvP1-*golB*-R, and the final product of 556 bp was fused to a chloramphenicol resistance cassette (amplified from the plasmid pKD3 using *golB*-P1-F and *golB*-P2-R oligonucleotides) by splicing by overlap extension (SOE)-PCR (28). The final product of the SOE-PCR was introduced by linear transformation in the chromosome of *Salmonella* LB5010 Δ(*golS-golB*)::*kan* by the one-step method described by Datsenko et al. (24). To generate the *golB*::*lacZ* transcriptional fusion, the chloramphenicol resistance cassette was removed using FLP-mediated recombination followed by site-specific integration of a *lac* fusion plasmid, pKG136 (see Table S1 in the supplemental material) into the remaining FLP recombination target (FRT) site.

In order to construct *cueR* mutant alleles, a megaprimer was amplified using primer *cueR*-F and the corresponding reverse primer for each mutant version. The extension of the megaprimer was done using the oligonucleotide RvP1-*cueR*-R, and the final product of 577 bp was fused to a chloramphenicol resistance cassette (amplified from plasmid pKD3 using cueR-P1-F and *cueR*-P2-R). The *cueR* mutant fused to the antibiotic resistance gene was introduced in the chromosome of *Salmonella* LB5010 Δ*cueR*::*kan* using the one-step method.

The mutant $golS_{P118A}$ allele (*golS* with a P-to-A change at position 118) was PCR amplified from the *Salmonella* chromosome using the *golS*-ORF-F and *golS*-ORF-R primers and cloned into pUH21-2$laqI^q$ to generate the pPB1353 expression plasmid. The plasmids and the linear constructions were introduced into *Salmonella* strains by electroporation using a Bio-Rad device following the manufacturer's recommendations. All constructs were verified by DNA sequencing.

Au and Cu induction assays. β-Galactosidase assays were carried out essentially as described previously (29). Metal induction assays of GolS- and CueR-controlled genes were done on mid-log-phase (not shown) or overnight cell cultures in LB medium with 40 μM $AuHCl_4$ or 1 mM $CuSO_4$ or without metal (15). When indicated, induction assays were done in SM9 medium or M9 minimal medium without NaCl (15) or with increasing amounts of $AuHCl_4$ or $CuSO_4$. Statistical analysis was performed by one-way analysis of variance and the Holm-Sidak test.

Protein stability assays. *S.* Typhimurium 14028s strains PB7116 and PB7171, which carried either the wild-type *golS* or the $golS_{P118A}$ allele, respectively, were grown at 37°C with shaking in LB medium with 40 μM $AuHCl_4$ overnight. Cells were washed twice with LB medium, resuspended in fresh LB medium, and incubated at 37°C with shaking. Samples were collected after 0, 4, and 12 h, resuspended in a mixture of 50 mM Tris-HCl (pH 7), 2 mM EDTA, and 5 mM dithiothreitol (DTT), and disrupted by sonication. In each case, 20 μg of total soluble protein was subjected to SDS-PAGE. Detection of GolS or $GolS_{P118A}$ in whole-cell extracts was performed by Western blotting using rabbit polyclonal anti-GolS antibodies as described previously (15). The protein concentration was determined by Bradford assay, using bovine serum albumin as the standard.

Protein-DNA interaction analysis. Electrophoretic gel mobility shift assays were performed essentially as previously described (15). Approximately 6 fmol of labeled DNA fragment containing the *golB* promoter region was incubated at room temperature for 20 min with purified GolS or $GolS_{P118A}$. Both proteins were overexpressed and purified from the *Salmonella* PB5257 strain carrying the corresponding expression plasmids as described previously (15).

Bioinformatics. The GolS or CueR homologs shown in Table 1 were extracted from the phylogenetic analysis previously performed (20). Complete protein sequences of the GolS or CueR homologues, as well as accession numbers and species names, are shown in a previous publication (20). The alignment of the C-terminal inducer-binding domains was performed using MEGA version 3.0 software (30).

RESULTS

The GolS metal-binding loop is essential for Au selectivity. We have previously demonstrated that the Au sensor GolS shows higher activation by Au than by Ag or Cu and that a chimeric protein with a replacement of the region between I109 and C120 of GolS for the same region of CueR ($GolS_L$) induces the transcription of the GolS-controlled *golB* gene in the presence of either Au or Cu ions, achieving similar transcription levels with both metal ions, resembling the activation pattern of CueR-dependent genes (15) (Fig. 1). To further examine the role of this region in metal selectivity, we modified *cueR* to code for a chimeric sensor, $CueR_L$, in which the region between A109 and C120 was replaced by the equivalent region from GolS (Fig. 1A). $cueR_L$ was introduced into the chromosome of the *S.* Typhimurium 14028 strain replacing wild-type *cueR*, and the expression of the CueR-dependent *copA*::*lacZ* reporter in cells grown in Luria-Bertani (LB) medium was determined in the presence or absence of either 40 μM $AuHCl_4$ or 1 mM $CuSO_4$ (Fig. 1B). We noticed that the response to Cu decreased in cells with the $cueR_L$ allele, in comparison with the induction observed in the wild-type strain, while conserving similar levels of activation by Au ions. These results confirmed that specific amino acid residues within the region from positions 109 to 120 of CueR and GolS determine the ability of these sensor proteins to respond to Cu and in consequence to discern between Au and Cu.

GolS and CueR metal-binding loops differ in four out of the seven residues between C112 and C120, the two cysteine residues responsible for the metal binding. Besides, GolS has an additional

TABLE 1 The amino acid residues at positions 113 and 118 are highly conserved within the GolS- and CueR-like groups

Locus tag or product	Bacterial species	Metal-binding loop sequence[a]
GolS-like		
GolS (STM0354)	*Salmonella enterica* serovar Typhimurium LT2	CAGDALPDC
XCV2319	*Xanthomonas campestris* pv. vesicatoria strain 85-10	CAGDDRPDC
Ajs_1373	*Acidovorax* sp. strain JS42	CAGDDRPDC
Sputw3181_1111	*Shewanella* sp. strain W3-18-1	CAGDERSEC
Smal_1771	*Stenotrophomonas maltophilia* R551-3	CAGDERPEC
Sputw3181_1120	*Shewanella* sp. strain W3-18-1	CAGNEKPDC
Meso_3892	*Mesorhizobium* sp. strain BNC1	CSGDNRPDC
Neut_0068	*Nitrosomonas eutropha* C91	CSGDDRPDC
Oant_4049	*Ochrobactrum anthropi* ATCC 49188	CHGDHRPHC
CupR (Rmet_3523)	*Cupriavidus metallidurans* CH34	CTGDDRPDC
CueR-like		
CueR (b0487)	*Escherichia coli* K-12 MG1655	CPGDDSADC
CueR (STM0499)	*Salmonella enterica* serovar Typhimurium LT2	CPGDDSADC
CKO_02653	*Citrobacter koseri* ATCC BAA-895	CPGDDSADC
ECA1194	*Erwinia carotovora* subsp. *atroseptica* SCRI1043	CPGDGGSEC
plu3823	*Photorhabdus luminescens* subsp. *laumondii* TTO1	CPGDDGAAC
CueR (y1094)	*Yersinia pestis* KIM	CPGDEGADC
CueR (peg.5396)	*Serratia marcescens* subsp. *marcescens* Db11	CPGDEGAEC
MS0886	*Mannheimia succiniciproducens* MBEL55E	CPGDGSEHC
PBPRA2823	*Photobacterium profundum* SS9	CPGNEGAAC
CueR (VC0974)	*Vibrio cholerae* O1 biovar El Tor strain N16961	CPGDQGSDC
VSAK1_08833	*Vibrio shilonii* AK1	CPGDTNSAC

[a] The amino acid residues at positions 113 and 118 are underlined.

cysteine residue at position 111, while *S.* Typhimurium CueR has a Ser and *Escherichia coli* CueR has an Ala at this position. To investigate the role in the metal response of the residue at position 111, two additional mutant versions of GolS were constructed and the genes coding for them introduced into the *S.* Typhimurium chromosome to replace the wild-type gene. These are $GolS_{C111S}$, which only differs from GolS in the residue at position 111, and $GolS_{sL}$, which keeps the C111, but has the 7 residues between C112 and C120 present in CueR (Fig. 1A). Like $GolS_L$, $GolS_{sL}$ induced transcription of *golB*::*lacZ* in the presence of either Au or Cu ions (Fig. 1B). In contrast, $GolS_{C111S}$ exhibited the wild-type activation pattern, confirming that residue 111 is involved in neither metal recognition nor metal selectivity.

These results allowed us to focus on the residues within the metal-binding loop that are not conserved between GolS and CueR (i.e., the residues at positions 113, 116, 117, and 118) to search for determinants of metal selectivity.

The amino acid residue at position 113 plays a key role in copper sensitivity. To analyze the molecular bases that favor the activation of GolS by Au over Cu, the amino acid residues at position 113, 116, 117, or 118 in the Au sensor were individually replaced for the corresponding residues present in CueR (Fig. 2A). The mutant alleles were separately introduced into the *S.* Typhimurium chromosome, replacing the wild-type *golS*, and the response of each mutant sensor to Au or Cu ions was evaluated, measuring the metal-dependent expression of *golB*::*lacZ*. Almost all mutant sensors have wild-type response to Au, both in rich medium (Fig. 2B) and in minimal medium (Fig. 3A), except for $GolS_{P118A}$, which had a slightly reduced response to the metal ion (Fig. 3A).

Conversely, $GolS_{A113P}$ showed an increased response to Cu ions compared to wild-type GolS, resembling the response attained by $GolS_L$, although the level of expression of *golB*::*lacZ* was always lower in the $golS_{A113P}$ strain than in the $golS_L$ strain (Fig. 2B and 3C). The $GolS_{L117S}$ sensor also showed an increased response to Cu compared to the wild-type GolS, but it was only appreciable when concentrations higher than 9 μM $CuSO_4$ were used in the culture medium (Fig. 3C), suggesting that this substitution also favors the detection of the metal ion, although to a lesser extent than the A113P substitution. Under these conditions, neither $GolS_{A116D}$ nor $GolS_{P118A}$ was able to induce the expression of *golB*::*lacZ* even in high Cu concentrations, similar to wild-type GolS (Fig. 3C).

We have previously shown that in a strain deleted of both *cueR* and *copA*, GolS induces the expression of part of the *gol* regulon in the presence of Cu, reaching expression levels comparable to those attained by Au (21, 22). Although not determined, this suggests that in the absence of CueR and CopA the intracellular level of Cu increases, reaching the detection threshold of GolS for this metal ion. Therefore, this strain can be used to expand the sensitivity of the mutant sensors to Cu. It is also worth noting here that deletion of *cueR* and *copA* only slightly affected the response of all the mutant sensors to Au ions (Fig. 2C and B). This background allowed us to observe that $GolS_{A116D}$ has a wild-type response to Cu (Fig. 2C and 3D). Even under this condition, $GolS_{P118A}$ was unable to induce *golB*::*lacZ* expression in the presence of Cu (Fig. 2C and D). This observation and the reduced response to Au ions observed for $GolS_{P118A}$ both in wild-type *cue* and in the Δ(*cueR-copA*) backgrounds (Fig. 2 and 3) suggest that the presence of an Ala at position 118 in the metal-binding loop is detrimental either for metal binding or for the intramolecular transduction of the activation state from the metal-binding site to the DNA-binding domain. The possibility that this substitution could affect the stability of the sensor was discarded by comparing the kinetics of

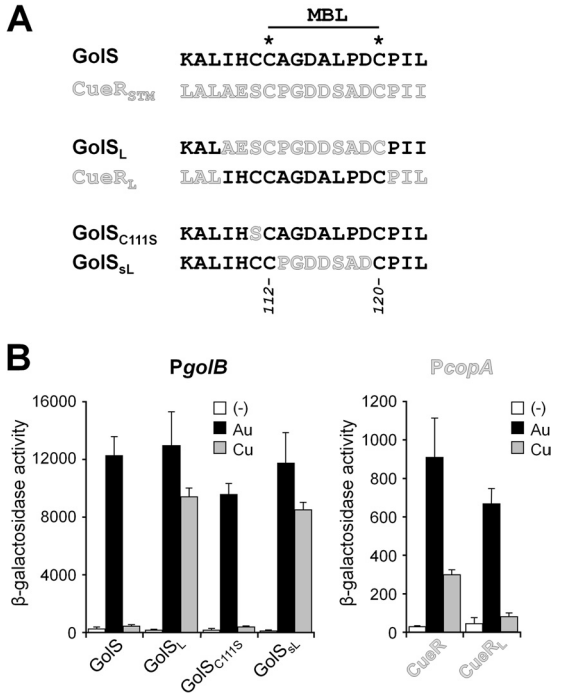

FIG 1 The metal-binding loop is responsible for metal selectivity in *Salmonella* GolS and CueR. (A) Schematic representation of *Salmonella* Typhimurium GolS, CueR (CueR$_{STM}$), and the mutant proteins GolS$_L$, CueR$_L$, GolS$_{C111S}$, and GolS$_{sL}$. The MBL sequence is shown in each case, and the conserved cysteine residues are depicted. (B) β-Galactosidase activity from GolS- or CueR-regulated transcriptional fusions *golB*::*lacZ* (P*golB*) or *copA*-*lacZ* (P*copA*), respectively, on cells carrying either wild-type *golS* and *cueR*, or *golS$_L$*, *golS$_{sL}$*, or *golS$_{C111S}$*, replacing the wild-type copy of *golS*, or *cueR$_L$*, replacing *cueR*. The *lacZ* reporter gene was inserted as an operon fusion 3′ of *copA*, so the expression of the CopA transporter was not affected (22). Cells were grown overnight in LB broth without (−) or with the addition of 40 μM $AuHCl_4$ (Au) or 1 mM $CuSO_4$ (Cu). The data correspond to mean values of four independent experiments performed in duplicate. Error bars correspond to the standard deviations. The β-galactosidase activity values of the GolS$_L$, GolS$_{sL}$, or CueR$_L$ strains grown in the presence of Cu differ significantly ($P < 0.001$) from those carrying the wild-type alleles. No significant differences were observed in the presence of Au.

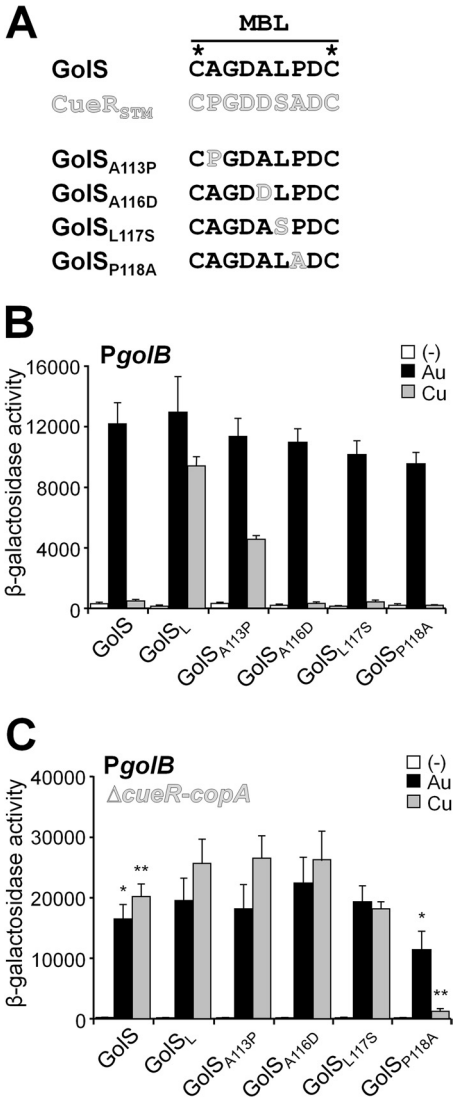

FIG 2 Contribution to metal selectivity of different residues within the GolS metal binding loop. (A) Sequence of the MBL of GolS, CueR, and the mutants GolS$_{A113P}$, GolS$_{A116D}$, GolS$_{L117S}$, and GolS$_{P118A}$. (B and C) β-Galactosidase activity from a *golB*::*lacZ* transcriptional fusion expressed by cells carrying either *golS* (GolS) or the mutant alleles coding for GolS$_L$, GolS$_{A113P}$, GolS$_{A116D}$, GolS$_{L117S}$, and GolS$_{P118A}$ in otherwise wild-type (B) or Δ(*cueR-copA*) (C) genetic backgrounds. The cells were grown overnight in LB broth without (−) or with the addition of 40 μM $AuHCl_4$ (Au) or 1 mM $CuSO_4$ (Cu). The data correspond to mean values of four independent experiments performed in duplicate. Error bars correspond to standard deviations. β-Galactosidase activity values of the GolS$_{P118A}$ strain grown in the presence of Au or Cu differ significantly (*, $P = 0.02$; **, $P < 0.001$) from the wild-type strain.

decay of the wild-type GolS and GolS$_{P118A}$ expressed from the P*golTS* chromosomal promoter after removal of the inducer, Au, from the medium (Fig. 4A). We also compared the abilities of GolS and GolS$_{P118A}$ to interact with the P*golB* promoter (Fig. 4B). Neither differences in relative protein stability nor differences in DNA affinity were observed.

To verify the relevance of residue 113 in directing signal discrimination, the *S.* Typhimurium CueR$_{P113A}$ and CueR$_{A118P}$ mutants were constructed and tested (Fig. 5). The CueR$_{P113A}$ mutant sensor is expected to be less responsive to Cu than the native CueR sensor, while CueR$_{A118P}$ would have little or no effect on the response of the sensor protein to the metals. The mutant genes were inserted into the chromosome of the *S.* Typhimurium 14028 strain, replacing the wild-type *cueR*. The sensor proteins' response was followed by measuring the expression of the CueR-dependent *copA*::*lacZ* reporter in LB medium in the absence or presence of either Au or Cu. As expected, the CueR$_{P113A}$ sensor exhibited a metal activation pattern similar to the chimeric CueR$_L$ sensor; that is, there was less response to Cu than that observed with wild-type CueR, but there were similar levels of Au-mediated induction (Fig. 5B). Introduction of the A118P mutation in CueR showed a wild-type response to the metal ions. Similar results were obtained in a strain deleted in the Au sensor GolS (Fig. 5C).

Most sensors that are phylogenetically linked to GolS (15, 20) have Ala, Ser, or His at position 113, and the recently characterized gold sensor CupR from *C. metallidurans* has a Thr at this position (Table 1). We constructed GolS variants with Ser, His, or Thr at this position and determined their ability to induce the expression of *golB*::*lacZ* in the presence of Au or Cu, either in the wild-type *cue* background or in the Δ(*cueR-copA*) mutant strain (Fig. 6). The

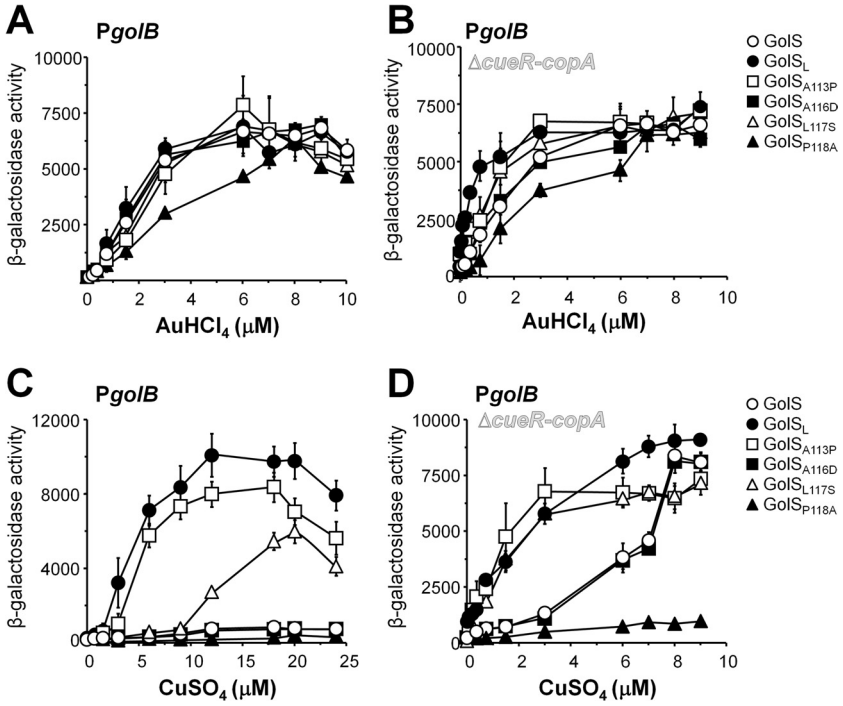

FIG 3 The residue at position 113 affects the copper response in GolS. β-Galactosidase activity from a *golB*::*lacZ* transcriptional fusion expressed by cells carrying either *golS* (GolS) or the mutant alleles coding for $GolS_L$, $GolS_{A113P}$, $GolS_{A116D}$, $GolS_{L117S}$, or $GolS_{P118A}$, in an otherwise wild-type genetic background (A and C) or in a Δ(*cueR-copA*) genetic background (B and D). Cell cultures were grown overnight in SM9 containing the indicated concentrations of $AuHCl_4$ (A and B) or $CuSO_4$ (C and D). The data correspond to mean values of four independent experiments performed in duplicate. Error bars correspond to the standard deviations.

metal-dependent *golB* induction pattern attained with $GolS_{A113S}$ and $GolS_{A113H}$ variants was similar to that of the wild-type GolS in both genetic backgrounds (Fig. 6A and B). Interestingly, $GolS_{A113T}$, the *C. metallidurans* CupR-like variant, responded to Au like the wild-type GolS but exhibited even a lower response to Cu in a Δ(*cueR-copA*) strain (Fig. 6B).

Residues at positions 116, 117, and 118 contribute to fine-tuning the metal response. The above observations suggest that the GolS residue 113 plays a key role in the response to copper, and residues at positions 117 and 118 may also modulate the metal selectivity. We combined the above mutations to generate double and triple mutant forms of GolS and tested their response to $CuSO_4$ (Fig. 7). As predicted by the single mutations, the simultaneous modification of the residues at positions 113 and 117 increased the response to copper of $GolS_{A113P\ L117S}$ (Fig. 7B). Interestingly, the introduction of the apparently neutral replacement at position 116 (A116D) in $GolS_{A113P}$ also enhanced the response to Cu to levels similar to those attained by $GolS_{A113P\ L117S}$ (Fig. 7B). Both the $GolS_{A113P\ L117S}$ and $GolS_{A113P\ A116D}$ reached the maximal *golB*::*lacZ* expression at lower Cu concentrations compared to $GolS_L$, suggesting that other amino acids in the $GolS_L$ loop negatively affect the Cu response. Indeed, introduction of Ala at position 118 in $GolS_{A113P\ L117S}$ or in $GolS_{A113P\ A116D}$ sensors lowered their response to copper to the level observed with $GolS_L$ (compare in Fig. 7B and C, $GolS_{A113P\ L117S}$ with $GolS_{A113P\ L117S\ P118A}$ and $GolS_{A113P\ A116D}$ with $GolS_{A113P\ A116D\ P118A}$) and in all of the loop mutant sensors tested (compare also $GolS_{A113P}$ with $GolS_{A113P\ P118A}$ and $GolS_{A116D\ L117S}$ with $GolS_{A116D\ L117S\ P118A}$ in Fig. 7B and C). In contrast, the introduction of the C111S mutation in GolS with the simultaneous modification of the residues at position 113, 118, or both did not affect the metal response of the mutant sensors (Fig. 8), confirming that the C111 of GolS is dispensable for both its activity and for metal recognition.

Similar results were observed for CueR. The A118P replacement in the $CueR_{P113A}$ mutant partially counteracted the effect of the P113A mutation (Fig. 5B), resulting in a sensor protein with a pattern of induction in the presence of Cu intermediate between wild-type CueR or $CueR_{A118P}$ and $CueR_L$ or $CueR_{P113A}$.

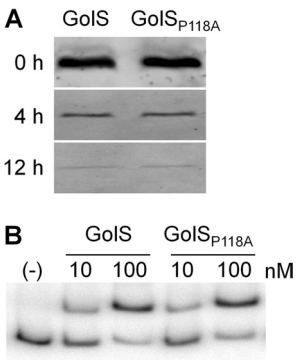

FIG 4 The P118A replacement in GolS does not affect its stability or its DNA affinity. (A) GolS or $GolS_{P118A}$ was expressed from the GolS-dependent P*golTS* promoter in the chromosome by the addition of 40 μM $AuHCl_4$ to the growth medium (LB). Overnight cultures were washed and suspended in with fresh LB without $AuHCl_4$ and incubated for 0, 4, or 12 h. Detection of intracellular GolS or $GolS_{P118A}$ was carried out by Western blotting in whole-cell extracts. (B) Electrophoretic gel mobility shift assay analysis using 6 fmol of ^{32}P 3′-end-labeled PCR fragment from the *golB* promoter region and the purified GolS variant at the indicated concentrations. (−), no protein addition.

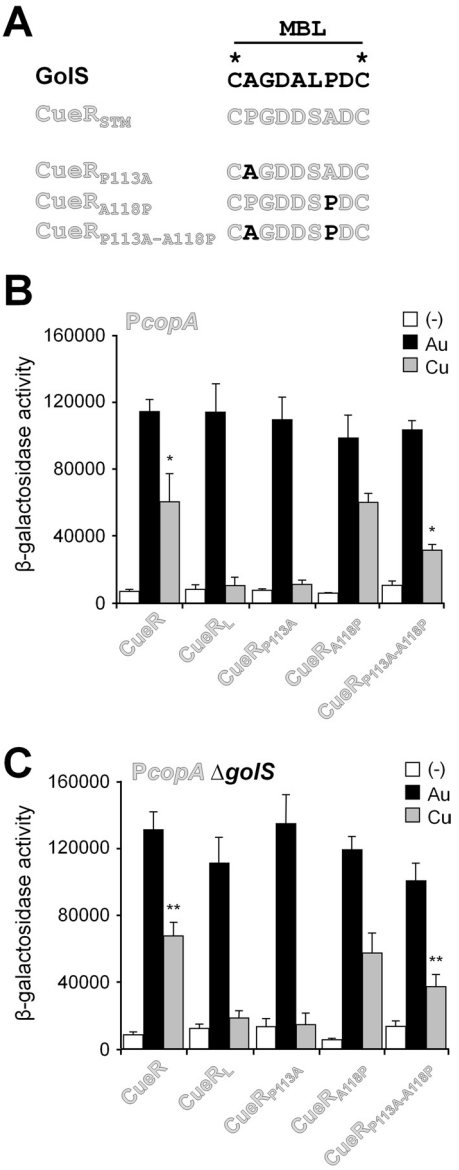

FIG 5 Analysis of the roles of residues at positions 113 and 118 in *Salmonella* CueR. (A) Sequence of the MBL of GolS, CueR, and the mutant alleles $CueR_{P113A}$, $CueR_{A118P}$, and $CueR_{P113A\ A118P}$. (B and C) β-Galactosidase activity from a *copA*::*lacZ* transcriptional fusion expressed from a reported plasmid by cells carrying *cueR* (CueR) or the mutant alleles coding for $CueR_L$, $CueR_{P113A}$, $CueR_{A118P}$, or $CueR_{P113A\ A118P}$ in an otherwise wild-type (B) or Δ*golS* (C) genetic background. Cells were grown overnight in LB broth without (−) or with the addition of 40 μM $AuHCl_4$ (Au) or 1 mM $CuSO_4$ (Cu). The data correspond to mean values of four independent experiments performed in duplicate. Error bars correspond to the standard deviations. Differences in β-galactosidase activity values from the wild-type CueR and $CueR_{P113A\ A118P}$ in the presence of Cu were statistically significant (*, $P = 0.029$; **, $P = 0.001$). No significant differences were observed from wild-type CueR and $CueR_{A118P}$ in the presence of Cu.

Overall, these results indicate that the Pro residues present at position 113 or 118 are the main contributors of the copper detection ability of the *Salmonella* MerR monovalent metal ion regulators, while residues at position 117 and, to a lesser extent, at position 116 are required to fine tune the metal-induced activation of these sensors.

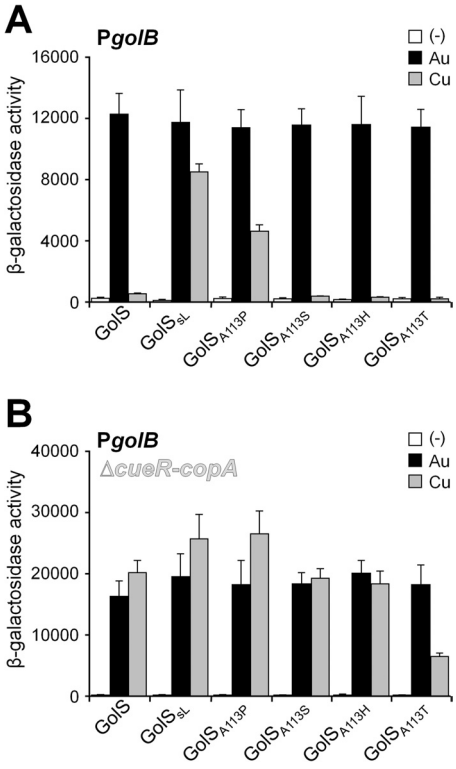

FIG 6 Proline at position 113 in GolS enhances the response to copper. β-Galactosidase activity from a *golB*::*lacZ* transcriptional fusion expressed by cells carrying the gene coding either for GolS, $GolS_{sL}$, $GolS_{A113P}$, $GolS_{A113S}$, $GolS_{A113H}$, or $GolS_{A113T}$ in a wild-type (A) or Δ(*cueR-copA*) (B) background. Cells were grown overnight in LB broth without (−) or with the addition of 40 μM $AuHCl_4$ (Au) or 1 mM $CuSO_4$ (Cu). The data correspond to mean values of four independent experiments performed in duplicate. Error bars correspond to the standard deviations. β-Galactosidase activity values from $golS_{A113T}$ in a Δ(*cueR-copA*) strain grown in the presence of Cu differ significantly ($P < 0.001$) from that of the wild-type *golS* in the same genetic background.

DISCUSSION

Metal-binding specificity in MerR sensors is mainly influenced by the intrinsic properties of a metal ion—i.e., charge, size, and coordination chemistry, its intracellular availability, as well as by the protein environment (that is, the array of ligands forming the first and second coordination spheres within the folded protein). MerR-like metal sensors, like CueR, cannot differentiate between metal ions with similar charges and coordination chemistries, like Cu(I), Ag(I), or Au(I) ions (14). In contrast, its homologs, GolS from *Salmonella* Typhimurium and CupR from *Cupriavidus metallidurans*, were shown to be distinctively activated by Au(I) (15, 16). Here, we demonstrated the role of individual residues within the C112-to-C120 metal-binding loop in the metal-induced activation of GolS and CueR, the two MerR homologs that detect monovalent metal ions in *Salmonella*.

As modules of evolutionary exchange, loops play important roles in molecular function and biological recognition. It has been postulated that the primary sequence of a loop affects not only the dynamics of folding of the nascent polypeptide chain but also the mobility and flexibility of the region in the folded protein. In fact, the modification of the length or sequence of a protein loop in immunoglobulins and enzymes has been shown to alter their sub-

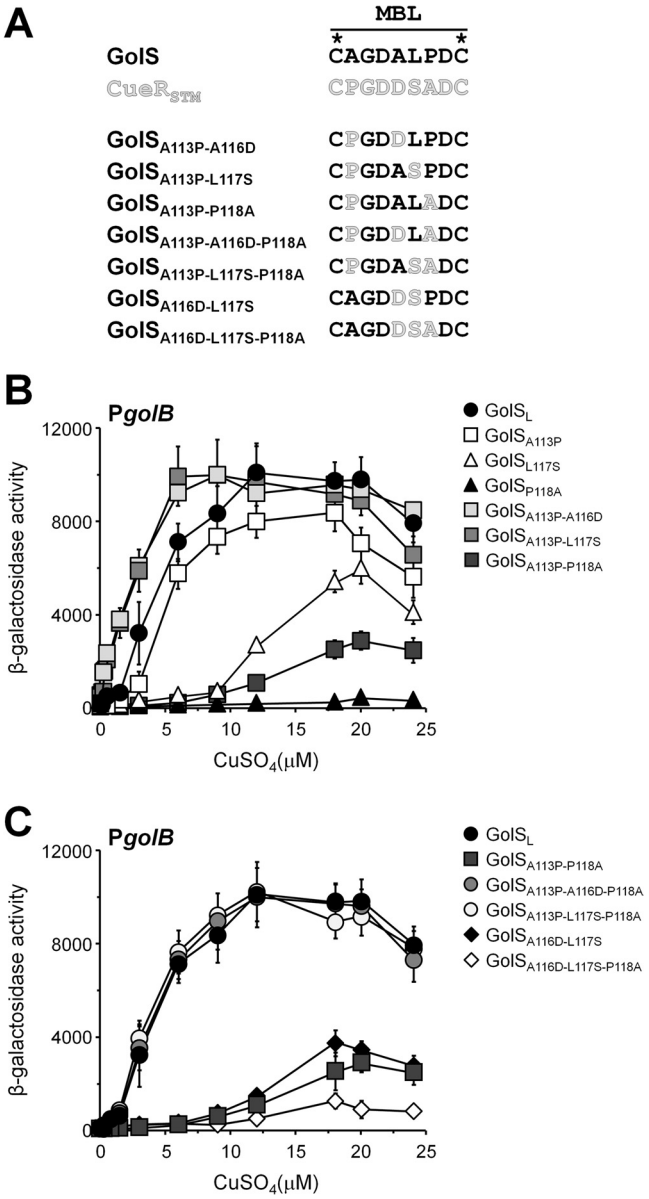

FIG 7 Role of residues at positions 116, 117, and 118 in tuning metal-induced activation of the *gol* regulon. (A) Sequence of the MBL of the different GolS alleles analyzed in this figure. (B and C) β-Galactosidase activity from a *golB*::*lacZ* transcriptional fusion expressed by cells carrying the different GolS alleles depicted in panel A. Cell cultures were grown overnight in SM9 containing the indicated concentrations of $CuSO_4$. The data correspond to mean values of four independent experiments performed in duplicate. Error bars correspond to the standard deviations.

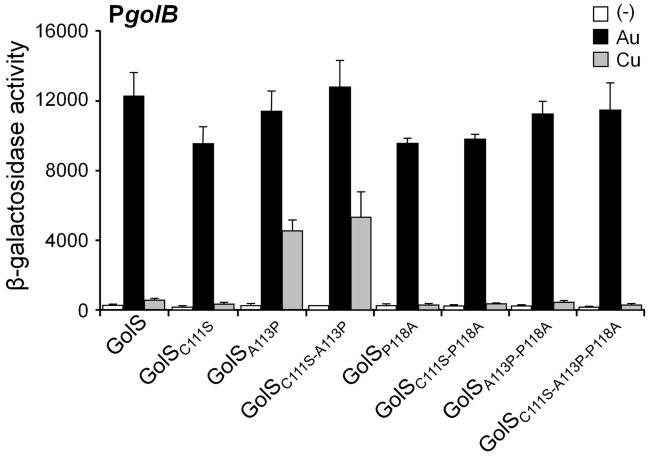

FIG 8 The residue Cys111 of GolS is dispensable for metal recognition. β-Galactosidase activity from a *golB*::*lacZ* transcriptional fusion expressed by cells carrying the wild-type copy of *golS* (GolS) or the allele coding for the indicated variant of GolS. Cells were grown overnight in LB broth without (−) or with the addition of 40 μM $AuHCl_4$ (Au) or 1 mM $CuSO_4$ (Cu). The data correspond to mean values of four independent experiments performed in duplicate. Error bars correspond to standard deviations.

strates' specificity (31–34). In all monovalent metal ion sensors of the MerR family, the two conserved cysteine residues that coordinate the metal ion are separated by a 7-residue-long loop (15) (Table 1). Our results allow us to postulate that the loop in CueR must be sufficiently flexible to accommodate cognate monovalent metal ions that greatly differ in ionic ratio (from the smallest Cu to the larger Au). In contrast, in GolS, and probably in CupR, the loop might not have the same plasticity, and in this way it interacts better with Au(I), which exhibits a softer nature and a larger ionic size than Cu(I) (35). In this context, we observed a change in metal preference of the *Salmonella* MerR monovalent metal ion sensors just by swapping their metal-binding loop (between C112 and C120); i.e., GolS$_{sL}$, which harbors the CueR loop region, responds to either Au(I) or Cu(I), while CueR$_L$, the CueR sensor with the metal-binding loop of GolS, is less responsive to Cu ions but conserves its Au induction capability (Fig. 1).

A recent report on the redox-sensing members of the MerR family (36) supports our observation about the role of the loop in signal selectivity. It is well known that the SoxR sensors from enteric bacteria are able to trigger a global stress response by sensing a broad spectrum of redox-cycling compounds. In contrast, orthologues from soil-dwelling organisms, such as *Pseudomonas aeruginosa* and *Streptomyces coelicolor*, only regulate the expression of a small set of genes in response to a reduced number of redox-active small molecules. In order to understand the molecular bases of these differences, the latter sensors increased their sensitivity to other redox compounds by replacing three amino acid residues present within the 2Fe-2S cluster binding region (36), which is equivalent to the metal-binding loop of the monovalent metal ion sensors.

Using a competitive Cu(I) binding approach, Osman and colleagues recently reported that GolS and CueR share similar *in vitro* affinities for Cu(I) (18). In view of our results, we proposed that the differences in Cu sensitivity between these sensors observed in living cells would not rely in the metal affinity but rather in the ability of the metal ion to properly activate the sensor protein (4, 37, 38), differences subtle enough that can only be perceived *in vivo*. Our analysis of the contribution to Cu sensitivity of each of the four nonconserved amino acids within the metal-binding loop between GolS and CueR clearly revealed the importance of the residue at position 113. Replacement of the Ala113 residue by Pro, present in CueR as well as in its xenologues (Table 1), rendered a GolS mutant sensor with an increased sensitivity to Cu, without affecting the response to Au ions (Fig. 2 and 3). Conversely, replacement of Pro113 by Ala in CueR lowered its response to Cu without affecting its activation by Au (Fig. 5). Replacement of the

GolS Ala113 residue by Ser, His, or Thr, the residues present in other GolS xenologues (20) (Table 1) had either no effect on metal response compared with wild-type GolS (Fig. 6) or, as observed for $GolS_{A113T}$, with the Thr residue present in *C. metallidurans* CupR, becomes less sensitive to Cu ions (Fig. 6B). The latter observation could be interpreted as an adaptation in the case of CupR to better discriminate between monovalent metal ions, although as we have demonstrated here, there are other residues in the loop (residues 116 and 117, for example) not conserved between GolS and CupR (Table 1) that can also influence the metal selectivity.

The presence of an Ala at position 118 in GolS is of note because of its negative effect on the response to Cu. The $GolS_{P118A}$ sensor showed a slight decrease in its response to Au, but it became almost insensitive to Cu, even in the Δ(*cueR-copA*) strain (Fig. 2 and 3). Although we showed that the P118A substitution in GolS does not have any detectable effect on the protein stability or its ability to bind a GolS operator (Fig. 4), this replacement was always detrimental for Cu sensing in the different GolS variants tested (Fig. 7). Furthermore, the A118P substitution increased the sensitivity of $CueR_{P113A}$ to Cu (Fig. 5B and C), strengthening the role of position 118 residue in metal selectivity. Remarkably, almost all of the putative monovalent metal ion sensors detected in bacterial genomes sequenced to date harbor a Pro residue at either position 113 or 118, but none harbors both simultaneously (20) (Table 1). CueR-like proteins have Pro at position 113, while GolS xenologues harbor a conserved P118 (Table 1). Proline is frequently found in turns and loop structures of proteins and, together with glycine residues, is predicted to affect chain compaction early in folding (39). Also, the side chain of proline can wrap around to form a covalent interaction with the backbone, restricting its flexibility and limiting the conformation of neighboring residues (40). Therefore, proline has a unique role in determining local conformation and movement freedom in the folded protein, functioning as a molecular switch in the regulation of a number of cellular processes (39, 41, 42). In the metal sensors, the restricted set of conformations adopted by Pro at position 113 or 118 may differentially affect chain dynamics and the range of conformations the metal-binding loop can assume. This would alter the flexibility of the region, and in consequence, the protein environment around the metal ion that determines metal selectivity. Nevertheless, structural studies are needed to confirm this hypothesis.

Concerning the other amino acids of the metal-binding region that could modulate the inducer's selectivity or accessibility, we show that individual replacements of the amino acids at position 116 or 117 had minor effects on the metal-induced expression of the reporter genes (Fig. 2 and 3). Only the replacement of Leu_{117} for Ser increased the response to Cu compared with wild-type GolS, and this was only detectable at high Cu concentrations. Interestingly, most GolS-like sensors have Arg at this position, while all CueR-like sensors have either Ser or Gly, but not Arg at this position (Table 1). High variability is also extensive to the amino acid residues present at position 116. Most sensors have Asp at this position, including homologues to both GolS and CueR (Table 1). Nevertheless, the replacement of the Ala for Asp at this position in GolS ($GolS_{A116D}$) did not affect its response to either Cu or Au (Fig. 2 and 3), although it increased the response to Cu when combined with the A113P replacement (Fig. 7B). In contrast, mutations at residue 111 of GolS did not alter metal selectivity either alone or in combination with other mutations within the loop (Fig. 1 and 8).

In sum, our results demonstrate that the metal ion response in monovalent metal sensors of the MerR family is affected by the combination and arrangement of the different residues that constitute the metal-binding loop. Furthermore, these studies will also provide information to help understand how an existing allosteric metal-binding site can be reengineered to allow the design of customized metal sensors with different ranges of selectivity and sensitivity against cognate metal ions.

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