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Protein signatures that promote operator selectivity among paralog MerR monovalent metal-ion regulators

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*Running title: *Protein signatures for DNA-selectivity among MerR-regulators*

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Background: Two nucleotide bases distinguish promoters controlled by paralog MerR monovalent metalloregulators avoiding cross-activation.

Results: Specific residues within the DNA-binding region of the regulators were identified as responsible for the selectivity in the operator recognition.

Conclusion: Co-evolution of both the regulator and its target operator sequences prevents cross-activation of paralog regulatory circuits.

Significance: The basis for regulator/operator specificity among MerR monovalent metalloregulators is described.

SUMMARY

Two paralog transcriptional regulators of the MerR family, CueR and GolS, are responsible for monovalent metal ion sensing and resistance in *Salmonella enterica*. Although similar in sequence and also in their target binding sites these proteins differ in signal detection, and in the set of target genes they control. Recently, we demonstrated that selective promoter recognition depends on the presence of specific bases located at positions 3' and 3 within the operators they interact with. Here, we identify the amino acid residues within the N-terminal DNA-binding domain of these sensor proteins that are directly involved in operator discrimination. We demonstrate that a methionine residue at position 16 of GolS, absolutely conserved among GolS-like proteins, but absent in all CueR-like xenologs, is key to selectively recognize operators that harbor the distinctive GolS-operator signature, while residue at position 19 finely tunes the regulator/operator interaction. Furthermore, swapping these residues switches the set of genes recognized by these transcription factors. These results indicate that co-evolution of a regulator and its cognate operators within the bacterial cell provides the conditions to avoid cross-recognition and guarantees the proper response to metal injury.

Transcriptional regulators of the MerR family modulate transcription in response to different environmental signals including heavy metal ions, organic compounds or oxidative stress (1). These proteins have a structurally-conserved N-terminal DNA-binding domain with two helix-turn-helix (HTH) motifs separated by a two-stranded antiparallel β sheet, in a α1-α2-β1-β2-α3-α4 topology. This region is connected to a variable C-terminal effector-binding domain by an extended α-helix forming a coiled-coil dimerization region.

Usually these regulators recognize pseudopalindromic operator sequences in σ^{70}-targeted promoters with longer (19- or 20- bp) spacers between the -35 and -10 elements that prevent open complex formation by RNAP without an activator (2-4). According to the current model on MerR-mediated induction, after the signal is detected by the regulator, the information is transduced to the DNA-binding domain triggering localized base-pair breaking and base sliding in the operator. This results in a realignment of the promoter elements that now allows proper RNAP-DNA interaction and transcription initiation. The solved structure of three MerR homologues bound to their target operators, the drug-binding BmrR and Mta proteins and the oxidative stress sensor SoxR, envisages a conserved mechanism for DNA recognition (3-5). Protein-DNA interactions involve hydrogen bonds and van der Waals contacts between amino acid residues mostly belonging to the α2-helix and the β2 strand of the N-terminal DNA-binding domain and the DNA backbone of the target operator sequence (3-5). It has been proposed that these contacts serve both to stabilize the distorted DNA conformation as well as to provide regulator/operator selectivity.

In *Salmonella enterica* serovar Typhimurium, two metal ion sensors of the MerR family, CueR and GolS, control the transcription of genes coding for factors responsible for monovalent metal ion resistance (6-8). These paralog transcription factors probably evolved from a common ancestor by gene duplication followed by divergence that rewired both signal recognition and the set of controlled genes (9,10). The Cu sensor CueR, present in most Gram negative bacteria induces the expression of its target genes in response to either Cu(I), Ag(I) or Au(I) (11,12), while the horizontally acquired GolS regulator evolved to preferentially sense Au(I) ions (6,7). As we mentioned before, the set of genes controlled by each transcription factor also differs. CueR activates the expression of the P-type ATPase CopA, the multicopper oxidase CueO/CuiD and the periplasmic Cu-binding protein CueP (7,8,13), while GolS induces transcription of the *Salmonella* specific genes encoding for another P-type ATPase, named GolT, the metal binding protein GolB and the CBA-type GesABC efflux system (6,14). We recently demonstrated that although these regulators recognize similar operator sequences at their target promoters, particularly at the -35 promoter region, the presence of only two distinctive nucleotide bases at the 3' and 3 positions relative to the centre of the operator determines regulator/operator selective recognition (15).

GolS-controlled promoters have an A at the 3' position and a T at the 3 position relative to the centre of the operator, while operators recognized by CueR have either a C or a G at these positions (Fig. 1A). Switching these nucleotide bases in *gol*-like or *cue*-like operators is sufficient to swap the regulator dependency (15). In other words, the mutant $golB_{CC}$ promoter which harbours a C at the operator positions 3' and 3 as P*copA* decreases the affinity for its native regulator GolS, but has an increased binding to CueR, compared to the wild-type *golB* promoter. A similar switch in regulator dependency is observed when the mutant $copA_{AT}$ and the wild-type *copA* promoters were compared. In fact, the A and T signature nucleotide bases at 3' and 3 positions from the centre of the dyad operator sequence are conserved in promoters that are proposed to be transcriptionally controlled by GolS-like regulators while operators predicted to be controlled by the CueR group harbor C or G at these positions (15).

The presence of selective nucleotide bases at the operators must correlate with specific amino acid residues within the DNA-binding domain of the transcription factors that direct selective recognition. To identify the amino acid residues involved in operator discrimination, here we constructed a series of GolS and CueR hybrid proteins replacing different portions of the DNA binding domain of each regulator by the same region of the paralogous protein and tested their ability to directed expression from the *golB* or *copA* promoter. Together with site-directed mutagenesis and *in silico* modeling these studies demonstrate that the residue at position 16 from the α2-helix of both CueR and GolS is essential for selective recognition. Our results provide additional evidence on the co-evolution of both the MerR regulators and the regulated genes to avoid cross-recognition and guarantee a proper response to metal injury.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions-Bacterial strains (all derivatives of *E. coli* W3110 strain or *Salmonella enterica* serovar Typhimurium 14028s, except when indicated) and plasmids used in this study are listed in Table 1. Oligonucleotides are listed in Table 2. Cells were grown at 37°C in Luria broth (LB) or on LB agar plates. Ampicillin, tetracycline, kanamycin and chloramphenicol were used when necessary at 100, 15, 50 and 20 μg ml^{-1}, respectively. Reagents, chemicals and oligonucleotides were from Sigma, except the Luria–Bertani culture media that was from Difco.

Bacterial genetic and molecular biology techniques-*E. coli* strains carrying deletions on the *lacZ*, *cueR* or *copA* genes were generated in the W3110 strain by Lambda Red-mediated recombination (16) using the appropriate primer pairs listed in Table 2. The deletions were individually transferred to different W3110 derivatives by P1-mediated transduction (17) to generate PB10305 strain. When necessary, the antibiotic-resistance cassette inserted at the deletion point was removed using the temperature sensitive plasmid pCP20 carrying the FLP recombinase (18).

Construction of hybrid *cueR-N_S*, *cueR-$HTH1_S$*, *cueR-$(HTH1+L)_S$* and *golS-N_R* alleles was carried out by using mutagenesis by PCR overlap extension or SOE-PCR (19) (for the chimeric proteins nomenclature used, see Fig. 1B and 2A). Briefly, we performed two independent PCR reactions using the complementary primers carrying the desired hybrid junction and oligonucleotides homologous to 5' and 3' wild-type gene of interest harbouring *Bam*HI or *Hind*III restriction sites (Table 2). Then the products of both PCRs were purified and combined in a third PCR with the appropriated forward or reverse primers to generate the final product. To construct the *cueR-$(L+HTH2)_S$ and cueR-$HTH2_S$ alleles*, we amplified a first PCR product using GolS-Loop-Fw/GolS-HTH2-Rv or GolS-HTH2-Fw/GolS-HTH2-Rv primer pairs (Table 2). Each product was employed as primer in two independent PCR reactions along with CueR-ORF-Fw or CueR-ORF-Rv to generate the overlapping fragments used in the final PCR reaction as described above. The *cueR-$\alpha2_S$*, *golS-$\alpha2_R$* or the alleles carrying point mutations were amplified in a single PCR using the forward oligonucleotides harbouring the modification and the *Bam*HI restriction site and the appropriated reverse primer. The final PCR fragments were purified and individually cloned into *Bam*HI/*Hind*III digested pUH21–2*laqI*^{q} or the pSU36 vector to generate the expression plasmids listed in Table 1.

The plasmids and the linear DNA fragments were introduced to *E. coli* strains by electroporation using a Bio-Rad device following the manufacturer`s recommendations. All constructs were verified by DNA sequencing.

Metal induction assays-The levels of expression of the *lacZ* reporter gene under the control of the native (P*copA* or P*golB*) or mutated (P*copA*$_{AT}$ or P*golB*$_{CC}$) promoters in the presence of 10 μM $AuHCl_3$, 100 μM $CuSO_4$ or without metal added were done essentially as previously described (15) using overnight cultures on LB. β-galactosidase assays were carried out essentially as described (17).

Protein purification-GolS, CueR or the hybrid proteins GolS-$\alpha2_R$ and CueR-$\alpha2_S$ were over-expressed and purified from *E. coli* XL1-Blue strain essentially as previously described (14). 0.1 mM or 0.5 mM IPTG (for CueR or GolS variants, respectively) was added to the cultures at an OD_{600}=0.6 to promote protein expression. All procedures were carried out at 4°C. The protein profile of purified samples was determined by SDS-PAGE and the concentration was calculated recording absorbance at 280 nm with an epsilon of 4320 $M^{-1}cm^{-1}$ (CueR), 6585 M^{-1} cm^{-1} (CueR-$\alpha2_S$), 11835 M^{-1} cm^{-1} (GolS) or 10345 M^{-1} cm^{-1} (GolS-$\alpha2_R$); or by Bradford assay, using bovine serum albumin as standard.

Protein–DNA interaction analysis-Electrophoretic gel mobility shift assays were performed using purified wild type or mutant regulators as previously described (15). The primers used to amplify the P*copA* or P*golB* promoter region are listed in Table 2.

Fluorescence anisotropy assays were carried out essentially as described (20,21). Fluorescein-labeled double-strand DNA fragments harboring the *copA* or *golB* operator sequences were generated by incubating pairs of single strand oligonucleotides (Table 2) at 85°C for 10 min and then allowing the mix to cool at room temperature.

The binding of native and mutant transcriptional regulators to P*copA* and P*golB* promoters were measured using a Varian Cary Eclipse fluorescence spectrophotometer (Agilent Technologies). All fluorescence anisotropy titrations were done in 10 mM Tris buffer with 10 mM NaCl, 2 mM $MgCl_2$ and 5mM DTT, pH 7.3. The fluorescence was excited at 495 nm. Anisotropy (r) was calculated as $r = (I_{\parallel}-G*I_{\perp})/(I_{\parallel}+2*G*I_{\perp})$, where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensity parallel and perpendicular to the excitation polarization, respectively, and G is the correction factor for the instrument's different responses to light of parallel and vertical polarizations. Binding isotherms were fitted to Eq. $r = r_f+(r_b-r_f)*(k_A*x/(1+k_A*x))$ [where r_f and r_b are the anisotropy values for free and protein-bound DNA, respectively, x is the total protein concentration and k_A is the association constant of the protein-DNA complex], by nonlinear regression using the Sigma Plot software. Dissociation constants (k_D) were estimated from Eq. $r = r_f + a*x/(b+x)$ [were $a = (r_f-r)*(k_A*x)/(1+k_A*x)$ and $b = k_D$].

In silico modelling-A structural model for CueR, CueR-$\alpha2_S$ and GolS were generated by homology modelling using Rosetta 3.1. The structure of BmrR transcription factor bound to its promoter (PDB 3Q5R) was used as a template. 100 initial structures were generated using a cyclic coordinate descent algorithm combined with fragment assembly for modelling the CueR sequence with gaps. The 10 lowest energy structures were subsequently refined and the lowest score structure was used as a model. The model structures were then superimposed on the structure of the complex used as a template in order to locate the position of the residues of interest (Ala-16 and Phe-19) with respect to the promoter DNA and compare their relative conformation in the different proteins.

RESULTS

Selective regulator/operator recognition is directed by amino acids within the α2-helix of the sensor protein-Salmonella CueR and GolS can distinguish their target operators from those of the paralog regulator by selectively recognizing two nucleotide bases located at the 3' and 3 positions from the centre of the operator [Fig. 1A and (15)]. To identify the amino acid residues within the DNA-binding domain of the transcription factor that direct operator discrimination, we designed chimeric proteins, in which the N-terminal DNA-binding domain of CueR (from Met-1 to Asn-68) was replaced by the equivalent region of GolS and vice versa (Fig. 1B). The encoding mutant alleles were cloned in plasmids and introduced either into a *S.* Typhimurium Δ*gol* Δ*cueP* Δ*cueR-copA* or into an *E. coli* W3110 Δ*cueR-copA* Δ*lacZ* strain harbouring *lacZ* reporter fusions to *copA* or *golB* promoters. (The use of strains deleted in *copA* helped to minimize differences in intracellular Cu levels derived from partial activation of the Cu transporter by the regulator variants.) The cells were grown in Luria–Bertani medium (LB) in the absence or presence of either 10 μM $AuHCl_4$ or 100 μM $CuSO_4$, concentrations required to attain the maximal induction of the reporter genes (7,14). The chimeric CueR-N_S and GolS-N_R regulators were functional to activate transcription of the reporter genes in response to the metals but their induction pattern resembled that attained by the paralog regulator (Fig. 1C and data not shown). That is, CueR-N_S more efficiently activated the expression of the reporter gene under the P*golB* promoter than under the native P*copA* promoter, resembling wild-type GolS, and GolS-N_R switched its operators recognition preference acting as a better inducer of CueR-regulated promoters. Because, essentially identical results were obtained using either *Salmonella* or *E. coli*, we choose to continue the analysis in the latter species.

To delimit the region of CueR responsible for operators discrimination, we replaced different portions from the N-terminal DNA-binding domain of CueR by the equivalent regions of GolS and analysed the ability of each chimeric regulator to activate the expression of the reporter genes under either a GolS or a CueR-controlled promoter (Fig. 2). Like CueR-N_S, CueR-$HTH1_S$ and CueR-$\alpha2_S$, carrying the first HTH motif (from Met-1 to Lys-23) or the α2-helix (from Thr-13 to Lys-23) of GolS, respectively, drove a reporter gene expression like GolS (Fig. 2B). These hybrid proteins showed an improved activation of P*golB*-driven expression, and a decreased induction of transcription from the native P*copA* promoter compared with wild type CueR, suggesting that

the operator selectivity resides within the α2-region. Furthermore, the equivalent α2-helix replacement in GolS resulted in a transcriptional regulator that exhibited a CueR-like expression pattern with an increased metal-activated expression of the reporter gene from P*copA*, and almost no induction of transcription from the P*golB* promoter (Fig. 2B). The other CueR chimeric constructions with the replacement of either the β1-β2 loop or of the HTH2 domain from GolS, i.e., CueR-HTH2$_S$, CueR-(HTH1+L)$_S$, or CueR-(L+HTH2)$_S$, resulted in less active or inactive regulators that could not induce the expression from either native P*copA* or P*golB* (Fig. 2B).

To verify the role of the α2-helix in the selectivity of operator recognition, we performed electrophoresis mobility shift assays (EMSA) using amplified fragments derived from *copA* or *golB* promoter regions and the purified wild-type regulators or chimeric CueR-α2$_S$ or GolS-α2$_R$ proteins (Fig. 3A). A 7-fold increase in CueR-α2$_S$ amounts were required to similarly affect the mobility of the P*copA* promoter compared to CueR. On the other hand, CueR-α2$_S$ substantially gained an apparent affinity for the P*golB* promoter. GolS-α2$_R$ shows much lower affinity for the P*golB* promoter compared to the parental GolS regulator, while it gained affinity for the P*copA* region, resembling wild type CueR (Fig. 3A).

The binding affinity for each regulator/operator pair were estimated by fluorescence anisotropy titration. In these assays we used fluorescein-labeled 41-base pair double-strand DNA containing the operator sequences from *copA* or *golB*. Each labelled ds-DNA probe was titrated with increasing amounts of CueR, GolS, or the mutant variants CueR-α2$_S$ or GolS-α2$_R$. Representative titration curves for each pair are shown in Fig. 3B. The curves for GolS/PgolB and GolS-α2$_R$/PcopA did not reach saturation because these proteins aggregate at high concentrations. This behavior is evidenced by a sharp increase in the anisotropy values induced by the contribution of light scattering from higher molecular weight particles to the measured anisotropy at protein concentrations higher than those reported. The saturation levels of anisotropy observed in both CueR variants (that reached saturation), are 0.102 and 0.133. The values obtained from fitting the available data on the complexes with GolS variants are 0.133 and 0.147, which are close to the values obtained for CueR. Assuming that similar kind of complexes are formed by both proteins, we can conclude that the extrapolated saturation values are in good agreement with the physical system. With all this information, the dissociation equilibrium constants (k_D) were calculated for the interaction of the CueR/P*copA* and GolS-α2$_R$/P*copA* as 141 ± 15 nM and 198 ± 49 nM, respectively. Similarly, the estimated k_D values for GolS/P*golB* and CueR-α2$_S$/P*golB* interactions were 12 ± 3 nM and 52 ± 12 nM, respectively.. The equilibrium constants for the GolS/P*copA*, CueR-α2$_S$/P*copA*, CueR/P*golB* and GolS-α2$_R$/P*golB* pairs could not be estimated because of the low affinity of these interactions, precluding the acquisition of binding data to saturating concentrations of the protein and thereby hindering fitting of the experimental points (Fig. 3B). Nevertheless, previously estimated binding affinities for the GolS/P*copA* and CueR/P*golB* heterologous interactions (by resonant mirror biosensor technology) were at least 1 and 2 orders of magnitude lower than those of the native CueR/P*copA* and GolS/P*golB* interactions, respectively (15). These data indicate that, compared to the parental proteins, both the CueR-α2$_S$ and the GolS-α2$_R$ mutants exhibit stronger binding affinities for the otherwise heterologous promoters in detriment of the native target sequences, resembling the paralog regulators. Overall, these results indicate that the regulator/operator specificity resides in the α2-helix of these transcription factors.

The amino acid residues at positions 16 and 19 of the regulator determine target recognition-We analysed the sequence differences within CueR or GolS α2-helix in order to identify the amino acid residues that direct operator recognition specificity (Fig. 4A). Considering that different members of the MerR family employ a similar DNA-distortion mechanism for transcriptional activation [reviewed by (1,22-24)], we also included in the analysis the sequence for the predicted α2-region from MtaN, BmrR and SoxR, non-metal binding MerR proteins for which detailed structural information of the regulator bound to their target DNA sequences is available (3-5), as well as the predicted region from the metal-responsive

regulators MerR and ZntR. We focused on those residues that, according to the crystallographic studies performed on MtaN, BmrR and SoxR, could establish hydrogen bonds or van der Waals contacts with DNA [residues at position 15, 16, 18, 19 and 20 relative to CueR (Fig. 4A)]. We observed that the Tyr-20 of CueR is conserved in these proteins, the Ile-17 and Arg-18 residues are present in all metal-binding sensors (and not in the homologs responding to other signals) while the Lys residue at position 15 of both CueR and GolS is not conserved in other metal sensors. The residues at position 16 and 19 differ between GolS and CueR. Thus, we carefully examined the identity of these residues in the different CueR-like and GolS-like homologs that we previously characterized for their operators selectivity (15). As shown in Fig. 5, all GolS-like proteins that were shown or proposed to recognize *gol*-like operators' sequences harbour a conserved Met residue at position 16, while Ala, Ser or Thr, but not Met, is present in all CueR-like proteins. The identity of the residue at position 19 was less conserved, but interestingly, the more close CueR homologs harbour a Phe at this position, while close GolS xenologs have a Tyr.

In view of these observations, we constructed single and double mutant versions of CueR and GolS at these positions, replacing GolS residues at positions 16 and/or 19 by those present in CueR and vice versa, and assayed their ability to activate transcription from P*copA* and P*golB* in the presence of Cu. Although residues at positions 14 and 22 are not predicted to interact with the DNA we also analyzed the role of these residues in the selective operator recognition as they differ between CueR and GolS (Fig. 4A).

The activation profile driven by either $CueR_{A16M-F19Y}$ or $GolS_{M16A-Y19F}$ mutants resembled the α2-variants of CueR and GolS, respectively. These mutants have an improved induction of transcription from the heterologous promoter and a diminished induction from their innate target promoter compared to the wild-type regulators (Fig. 4B). Furthermore, $CueR_{A16M}$ and $GolS_{M16A}$ displayed patterns of transcriptional induction similar to the double mutant proteins. Replacement of the residue at position 19 ($CueR_{F19Y}$ and $GolS_{Y19F}$) had only minor effects on their operator´s recognition pattern (Fig. 4B). These results clearly indicate that the identity of the residue at position 16 is a main determinant of regulator/operator´s selectivity among *gol* and *cue* regulons. Moreover, our observations also pointed out that the residue at position 19 finely tunes the selectivity. As expected, mutant regulators with replacements at positions 14 and 22 displayed wild-type patterns of transcriptional induction (Fig. 4B) indicating that these residues are not involved in operator discrimination.

The residues at position 16 and 19 of the regulator recognize the selective operator bases 3' and 3-There is currently no structural information about the target operator recognition by MerR-metalloregulators. Thus, assuming that all MerR homologs interact with DNA in a similar manner, we used the available crystallographic structure of the drug-binding homolog BmrR bound to its target promoter (3) to simulate the interaction of CueR or GolS with DNA. We selected BmrR because, like CueR and GolS, it recognizes target promoters with a 19-bp spacer between the -35 and -10 elements and interacts with dyad-symmetric sequences separated by 1-bp (4,15). In the models, we replaced the amino acid residues present in the N-terminal region of BmrR (from residue 1 to 88) by those present either in CueR, CueR-$\alpha 2_S$ or in GolS. As shown in Fig. 4C, the side chain of the residue at position 16 in both CueR and GolS approached the DNA backbone toward the nucleotide bases located at position 3 from the centre of the operator (15). Substitution of the small methyl side-chain of the alanine by a bulkier methionine will likely shift the position of the HTH motif with respect to the operator DNA. The side chain of the residue at position 19 was also oriented toward the interface between nucleotide bases at position 3 and 4, supporting the experimental data about the role of the residue at position 16 in directing selectivity in the operator recognition.

To verify the role of amino acid residues at position 16 and 19 in the distinction of the operator nucleotide bases at positions 3' and 3, we compared the promoter preferences of the mutant regulators $CueR_{A16M-F19Y}$ and $GolS_{M16A-Y19F}$ with the wild-type regulators, using innate P*copA* and P*golB* promoters or the mutated versions, P$copA_{AT}$ and P$golB_{CC}$, in which the operator nucleotide bases at positions 3' and 3 were switched by those

present in the heterologous operators [(15); see also Fig. 6A]. Here again, we observed that the induction of expression from the wild-type and mutated *gol* and *cue* promoters by $CueR_{A16M-F19Y}$ is similar to that obtained using GolS, while the induction of the analyzed promoter by $GolS_{M16A-Y19F}$ mimicked that of wild-type CueR (Fig. 6B). In other words, $CueR_{A16M-F19Y}$ was more efficient in activating expression from P*copA*$_{AT}$ than from wild-type P*copA*. Conversely, it better recognized wild type P*golB*, that harbours A and T at position 3' and 3, respectively, than P*golB*$_{CC}$ that harbours C at these two positions, exhibiting a pattern of induction similar to GolS. Similarly, $GolS_{M16A-Y19F}$ better recognized promoters with Cs at position 3' and 3, such as the native P*copA* or the mutant P*golB*$_{CC}$ promoters, than promoters with A and T at these positions, i.e., P*golB* and P*copA*$_{AT}$. Overall, these results established the importance of the amino acid residue at position 16, and to a lesser extent at position 19, in the selection of the *Salmonella cue* and *gol* regulons target operators.

DISCUSSION

Transcription factors must be capable of locating their specific target sequences along the chromosome, avoiding their unproductive or even harmful interaction at ectopic places. This appears particularly relevant when similar regulatory proteins with almost identical target recognition operators coexist in a single cell. *Salmonella* has two structurally related but functional distinctive metalloregulators of the MerR family that orchestrate the cellular response to the presence of toxic amounts of monovalent metal ions in the environment (25). By rewiring both the input-signal detection and the recognized operator sequences, the horizontally acquired Au-sensor GolS is able to induce the expression of its target genes without interfering with the function of the Cu-homeostasis *cue* regulon, controlled by the enterobacterial-ancestral regulator CueR. In a previous report we demonstrated that selectivity in the recognition of GolS- or CueR-target operators is achieved by subtle modifications of the operator sequences [regulon-signature nucleotide bases are displayed at the 3 and 3' operator positions, (15)] which is accompanied, as it is shown in this work, by subtle modifications at the DNA-binding motif of these transcriptional regulators.

We constructed a set of hybrid proteins between GolS and CueR to identify the region that directs operator recognition (Fig. 1 and 2). These studies allowed us to focus on the α2-helix (from residue 14 to 22), which can be defined as the minimal region necessary for operator discrimination among the *gol* and *cue* regulons. *In vitro* experiments confirmed these observations showing that the solely replacement of this motif in each regulator lowered its affinity for its innate promoters and increased its affinity for the paralog operators (Fig. 3).

There is not structural information of any metal-sensing MerR regulator bound to its target operator, and based on the available biochemical and genetic data, it was postulated that the DNA distortion mechanism for transcriptional activation is conserved among all family members [reviewed by (1,22-24)]. Therefore, we assumed that CueR and GolS interact with their target sequences in a similar manner than the non-metal binding MerR homologs BmrR and MtaN, from which the crystallographic structure of protein-DNA complexes is available (3,5). That is, the axis of symmetry of the CueR/GolS dimer is facing the minor groove at the center of the palindrome (position 1), while the α2-helix approaches the DNA at the second minor groove near the adjacent major grooves, where the signature 3 and 3' bases are located. An *in silico* modeling performed for both CueR and GolS pointed out residues 16 and 19 as candidates for directing selectivity towards target operators (Fig. 4C). Amino acid swapping demonstrated that the residue 16 is key in directing the selective recognition of the signature operator base 3 (Fig. 4). In the presence of Cu ions the mutant $CueR_{A16M}$ induced the expression of P*golB*::*lacZ* and P*copA*::*lacZ* like GolS (Fig. 4B). A similar trend of operator switch was detected with $GolS_{M16A}$. P*copA*::*lacZ* achieved higher levels of induction with $GolS_{M16A}$ than with GolS, while there was a lower metal-dependent induction of P*golB*::*lacZ* with $GolS_{M16A}$ than with the native gold sensor. On the other hand, the contribution of the residue at position 19 was less notorious *per se*. $CueR_{F19Y}$ and $GolS_{Y19F}$ regulators exhibited patterns of metal-induction intermediate between the parental regulator and the mutant that harbors the α2-region replaced. Nevertheless, their contribution to fine-tuning the regulator/operator

interaction is corroborated by the observation that double mutant regulators $CueR_{A16M-F19Y}$ and $GolS_{M16A-Y19F}$ were as effective as the hybrid CueR-$\alpha2_S$ and GolS-$\alpha2_R$, respectively, to activate the promoters controlled by the paralog regulator (Fig. 4B). In addition, $CueR_{A16M-F19Y}$ and $GolS_{M16A-Y19F}$ activated transcription of the reporter gene from the promoter having the 3' and 3 bases replaced more efficiently than from the wild-type promoters (Fig. 6). We hypothesize that the failure of GolS to recognized operators bearing a C/G substitutions at positions 3 and 3' could be explained by the formation of a third hydrogen bond between the pairing C-G bases which renders a base pair less deformable than the A-T base pair. This would result in either steric or electrostatic interferes with interaction of GolS which have the more voluminous, yet hydrophobic, methionine residue at position 16.

The presence of the distinctive Met residue at the α2 helix of the DNA binding domain of GolS is extended to all GolS-like proteins having the 3'-3 A-T signature in the operators of genes predicted to be controlled by them [Fig. 5; see also (15)]. By contrast, CueR homologs that recognize promoters with the signature C/G-C/G have Ala, Ser or Thr at position 16 and indeed, replacement of the Ala_{16} residue of CueR by Thr did not affect its operator selectivity (Fig. 4B). As expected, the residue at position 19 is not conserved, but interestingly closely GolS homologs harbor a Tyr at this position, while all CueR xenologs have a Phe. Indeed, the recently characterized *Cupriavidus metallidurans* CH34 gold sensor CupR, which controls genes with the characteristic 3'-3 A-T operators (26), harbors the GolS-like Met-Phe signature at the N-terminal DNA-binding domain. Notoriously, several MerR homologs that recognized one central-base pair separation between the pseudopalindromic sequences and have the 3-3' signature C/G-C/G like CueR (e.g. the non-metal sensors BmrR, Mta and TipA, and the metal sensors CadR and PbrR), have Ala or Thr at position 16, but not Met, while either and Phe or a Tyr at position 19 (4). Therefore, it is evident that specific operator recognition in CueR-like and GolS-like regulators relies exclusively on the interaction of the amino acid residue at position 16 and the signature nucleotide base at position 3 of the operator.

Overall, these studies suggest that, along evolution, rewiring of both the transcriptional regulators GolS and CueR, and the regulatory elements in their target genes, confer novel abilities to detect distinct environmental cues, avoiding at the same time cross-regulation that would jeopardize the adequate response to a specific stress.

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FOOTNOTES

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FIGURE LEGENDS

FIGURE 1. The N-terminal DNA-binding domain is responsible for selective sensor/operator recognition. A. Sequences of the *Salmonella* GolS- and CueR-controlled operators. The predicted -10 and -35 elements are boxed and the nucleotide bases at 3´and 3 position are highlighted. B. Schematic representation of GolS, CueR, and the hybrid proteins CueR-N_S and GolS-N_R. The region corresponding to CueR or GolS are colored in black or grey, respectively. C. β-galactosidase activities (Miller units) from pMC1871 derived plasmids carrying *lacZ* fusions to CueR- or GolS-controlled promoters P*golB* or P*copA*, respectively. The cells (all derivatives of W3110 Δ*lacZ* Δ*copA cueR*::*cat*) expressing the indicated CueR or GolS variant from a pSU36 derivative plasmid were grown overnight in LB broth without (-) or with the addition of 10 μM $AuHCl_4$ (Au) or 100 μM $CuSO_4$ (Cu). The data correspond to mean values of four independent experiments performed in duplicate. Error bars correspond to standard deviations.

FIGURE 2. The α2-helix determines operator specificity. **A.** Schematic representation of the generated hybrid proteins. The different motifs or regions from the N-terminal DNA binding region of CueR and GolS are indicated. The motifs swapped in each cased are shown by either black (CueR) or grey (GolS). Chimeric proteins were constructed as indicated. B. β-galactosidase activity (Miller units) from the P*copA* or P*golB* reporter fusions as in Fig. 1 expressed on the W3110 Δ*lacZ* Δ*copA cueR*::*cat* cells carrying the expression plasmids for the indicated CueR or GolS hybrid proteins were grown overnight in LB broth without (-) or with the addition of 100 μM $CuSO_4$ (Cu). The data correspond to mean values of at least six independent experiments done in duplicate. Error bars correspond to the standard deviations.

FIGURE 3. The CueR-$\alpha2_S$ and GolS-$\alpha2_R$ proteins change operator preference. A. Electrophoretic gel mobility shift assay (EMSA) using 6 fmol of ^{32}P 3′-end-labelled PCR fragment from the *golB* or *copA* promoter regions and purified CueR, CueR-$\alpha2_S$, GolS or GolS-$\alpha2_R$, as indicated. Wild-type and mutant regulators were used at 0, 0.025, 0.050, 0.075, 0.100, 0.125, 0.250, 0.500, 0.750 and 1.000 μM final concentrations with P*copA*, and at 0, 0.002, 0.004, 0.012, 0.025, 0.050, 0.100, 0.250, 0.500 and 1.000 μM final concentrations with P*golB*. The DNA-protein complex is indicated in each case. B. Fluorescence anisotropy titration curves of the fluoresceine-labeled P*copA* (black circles) or P*golB* (white circles) promoter with increasing concentrations of native or mutant transcriptional regulators, as indicated. Binding isotherms were fitted to Eq. $r = r_f+(r_b-r_f)*(k_A*x/(1+k_A*x))$ by nonlinear regression. Association equilibrium constants (k_A) for each sensor/operator duplex are indicated. The binding isotherm curves are shown at the bottom. Dissociation equilibrium constants (k_D) were determined expressing normalized anisotropy values $[(k_A*x)/(1+k_A*x)]$ against protein concentration at logarithmic scale.

FIGURE 4. Amino acid residues at position 16 and 19 of the α2-helix are essential for selective operator recognition. A. Consensus motif for the α2-helix region of different metal-binding and non-metal binding MerR proteins. The residues at position 16 and 19 are shaded in black and highlighted (▼) while those residues conserved in the majority of the sequences are indicated in bold case. DNA-contacting residues identified in the crystal structures of MtaN-DNA, BmrR-DNA and SoxR-DNA complexes are highlighted by an asterisk (*****). B. β-galactosidase activities (Miller units) from the P*copA* or P*golB* reporter fusions as in Fig. 1 expressed on the W3110 Δ*lacZ* Δ*copA cueR*::*cat* cells carrying the expression plasmids for the indicated CueR or GolS hybrid or mutant proteins. Bacteria were grown overnight in LB broth (-) or in LB broth supplemented with 100 μM $CuSO_4$ (Cu). The data correspond to mean values of at least three independent experiments done in duplicate. Error bars correspond to the standard deviations. C. Structural model for CueR/, CueR-$\alpha2_S$/ or GolS/DNA complex. The amino acid residues at positions 16 and 19 approached the signature nucleotide base at the operator sequences. The side chain for the residues at position 16 and 19 in each protein is shown.

FIGURE 5. The identity of the residues at position 16 and 19 is conserved among CueR-like and GolS-like proteins. Phylogenetic tree obtained by comparison of the full length CueR-like and GolS-like regulators. The tree was constructed by Bayesian inference as previously described in (15). The α2-helix sequence for each CueR or GolS homologues was extracted and listed on the right. The residues 16 and 19 are highlighted.

FIGURE 6. Operator discrimination among CueR- and GolS-controlled promoters depends on key α2-residues and signature nucleotide bases. A. The sequences of the native and mutant *golB* and *copA* promoters, as well as the nucleotide bases that have been modified in each case are indicated. B. β-galactosidase activities (Miller units) were determined on Δ*cueR* Δ*copA* Δ*lacZ* strains carrying each of the reporter plasmids harboring the native (pP*golB* or pP*copA*) or mutant (pP$golB_{CC}$ or pP$copA_{AT}$) versions of the promoters and expression plasmids for the indicated regulator protein. Bacteria were grown overnight in LB (-) or in LB plus 100 μM $CuSO_4$ (Cu). The data correspond to mean values of at least three independent experiments done in duplicate. Error bars correspond to the standard deviations.

Table 1. Bacterial strains and plasmids used in this study.

Strain	Relevant genotype	Reference or source
XL1-Blue	*recA1 endA1 gyrA96 thi*-1 *hsdR17 supE44 relA1 lac* [F´ *proAB lacI*^{q} *ZΔM15 Tn10* (Tet^{R})]	Stratagene, CA, USA.
W3110	F^{-} λ^{-} IN(*rrnD-rrnE*)1 *rph*-1	(27)
PB8885	W3110 *lacZ*::*cat*	This study
PB6731	W3110 *cueR*::*cat*	(13)
PB10285	W3110 Δ*lacZ copA*::*kan*	This study
PB10295	W3110 Δ*lacZ* Δ*copA*	This study
PB10305	W3110 Δ*lacZ* Δ*copA cueR*::*cat*	This study
PB10683	14028 Δ*gol* Δ*gesABC* Δ*cueP* Δ*copA cueR*::*cat*	Laboratory stock

Plasmid	Relevant genotype	Reference or source
pKD46	oriR_{pSC101}ts P_{araB}*exo-bet-gam* Amp^{R}	(16)
pKD3	ori$_{\gamma}$ Amp^{R} FRT Cm^{R} FRT	(16)
pKD4	ori$_{\gamma}$ Amp^{R} FRT Km^{R} FRT	(16)
pCP20	λ-cI857-ts Pr-*flp* Amp^{R} Cm^{R}	(16)
pUH21-2*lacI*^{q}	ori$_{pMB1}$ Amp^{R} *lacI*^{q}	(28)
pPB1205	pUH::*golS*	(6)
pPB1304	pUH::*cueR-α2*$_{S}$	This study
pPB1209	pUH::*cueR*	(6)
pPB1291	pUH::*golS-α2*$_{R}$	This study
pSU36	Ori$_{p15A}$, Km^{R}	Addgene
pPB1389	pSU36::*cueR*	This study
pPB1395	pSU36::*cueR-N*$_{S}$	This study
pPB1391	pSU36::*cueR-(HTH1+L)*$_{S}$	This study
pPB1392	pSU36::*cueR-HTH1*$_{S}$	This study
pPB1393	pSU36::*cueR-(L+HTH2)*$_{S}$	This study
pPB1394	pSU36::*cueR-HTH2*$_{S}$	This study
pPB1396	pSU36::*cueR-α2*$_{S}$	This study
pPB1423	pSU36::*cueR*$_{S14A}$	This study
pPB1399	pSU36::*cueR*$_{A16M}$	This study
pPB1424	pSU36::*cueR*$_{A16T}$	This study
pPB1400	pSU36::*cueR*$_{F19Y}$	This study
pPB1425	pSU36::*cueR*$_{E22Q}$	This study
pPB1401	pSU36::*cueR*$_{A16M-F19Y}$	This study
pPB1390	pSU36::*golS*	(6)
pPB1397	pSU36::*golS-N*$_{R}$	This study
pPB1398	pSU36::*golS-α2*$_{R}$	This study
pPB1414	pSU36::*golS*$_{A14S}$	This study
pPB1402	pSU36::*golS*$_{M16A}$	This study

pPB1403	pSU36::*golS*$_{Y19F}$	This study
pPB1416	pSU36::*golS*$_{Q22E}$	This study
pPB1404	pSU36::*golS*$_{M16A\text{-}Y19F}$	This study
pMC1871	pBR322, Tet^{R}, *lacI*^{q}	Amersham Pharmacia
pPB1225 (p*copA*)	pMC1871::P*copA*	(15)
pPB1222 (p*golB*)	pMC1871::P*golB*	(15)
pPB1233 (p*copA*$_{AT}$)	pMC1871::P*copA*$_{AT}$	(15)
pPB1230 (p*golB*$_{CC}$)	pMC1871::P*golB*$_{CC}$	(15)

Table 2. Oligonucleotides used in this study.

Primer name	Sequence (5'-3')	5'-restriction site	Purpose
lacZ-P1	TTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGATGTGTAGGCTGGAGCTGCTTC		Deletion of the *lacZ* gene
lacZ-P2	GCGAAATACGGGCAGACATGGCCTGCCCGGTTATTATTATCATATGAATATCCTCCTTA		Deletion of the *lacZ* gene
copA-P1	CACAGCCAGTCAAAACTGTCTTAAAGGAGTGTTTTATGGTGTAGGCTGGAGCTGCTTCG		Deletion of the *copA* gene
copA-P2	CTAAAGCAGCGCATCCGCAATGATGTACTTACATATGAATATCCTCCTTA		Deletion of the *copA* gene
cueR-P1	CCCTTTAACAAAGCACAGGAGGCGTTGCGCGAACGATGGTGTAGGCTGGAGCTGCTTCG		Deletion of the *cueR* gene
cueR-P2	GGGATAACCCTACATATCCGAGCCGTCTCGTCTTAATCACATATGAATATCCTCCTTA		Deletion of the *cueR* gene
CueR-ORF-Fw	GAGGATCCATATGAATATTAGCG	*Bam*HI	Amplification of wild-type or hybrid *cueR*
CueR-ORF-Rv	CGCAAGCTTGATCAACGTGGCTTTTGCGCC	*Hind*III	Amplification of wild-type or hybrid *cueR*
GolS-ORF-Fw	GAGGATCCATATGAACATCGGTAAAGCAGC	*Bam*HI	Amplification of wild-type or hybrid *golS*
GolS-ORF-Rv	ACCCAAGCTTACAGACGCTTTGCCAG	*Hind*III	Amplification of wild-type or hybrid *golS*
CueR-N-Fw	GTTGCTGAAATCAGCGACTTACTGAATCTGTTTAACGATCCGCG		Generation of *cueR-*N_S
GolS-N-Rv	CGCGGATCGTTAAACAGATTCAGTAAGTCGCTGATTTCAGCAAC		Generation of *cueR-*N_S
CueR-HTH1+L-Fw	GGCAAGTCGGACGGATTCCGGCTATCGCACCTACACGCAGAAGC		Generation of *cueR-(HTH1+L)*$_S$
GolS-HTH1+L-Rv	GCTTCTGCGTGTAGGTGCGATAGCCGGAATCCGTCCGACTTGCC		Generation of *cueR-(HTH1+L)*$_S$
CueR-HTH1-Fw	CGCTACTATGAACAGATTGGGCTGGTGACGCCGCCATTACG		Generation of *cueR-HTH1*$_S$
GolS-HTH1-Rv	CGTAATGGCGGCGTCACCAGCCCAATCTGTTCATAGTAGCG		Generation of *cueR*-HTH1
GolS-Loop-Fw	AAGAGAAAGGGCTGGTGACGATTGGTCTGA		Generation of *cueR-(L+HTH2)*$_S$

GolS-HTH2-Fw	ACACGCAGAAGCATTTAAACCAGGCTGATG		Generation of *cueR-HTH2S*
GolS-HTH2-Rv	ACATCTGCGCTATGGCGTCGATTCAGTAAG		Generation of *cueR-(L+HTH2)$_S$* and cueR-*HTH2$_S$*
CueR-α2-Fw	GAGGATCCATATGAATATTAGCGATGTGGCGAAAAAA ACCGGTTTATCGGCCAAAATGATTCGCTACTATGAACA GATTGGGCTGGTGACGCCGCCATTACG	*Bam*HI	Amplification of *cueR-α2$_S$*
GolS-N-Fw	GGGGTTTAATCTGGAAGAGTGTGGCGAACTGGTCAAT CTTTGGAATAACCAGTCGCGGC		Generation of *golS-N$_R$*
CueR-N-Rv	GCCGCGACTGGTTATTCCAAAGATTGACCAGTTCGCCA CACTCTTCCAGATTAAACCCC		Generation of *golS-N$_R$*
GolS-α2-Fw	GAGGATCCATATGAACATCGGTAAAGCAGCTAAAGCA TCGAAAGTCTCGTTTTATGAAGAGAAAGGGCTGGTGA CGATTGGTCTGATTCCCGCGGC	*Bam*HI	Amplification of *golS-α2$_R$*
CueR(A14)-Fw	GAGGATCCATATGAATATTAGCGATGTGGCGAAAAAA ACCGGTTTAACCGCCAAAGCCATTCGGTTTTATGAAGA GAAAGGGCTGGTGACGC	*Bam*HI	Amplification of *cueR$_{S14A}$*
CueR(M16)-Fw	GAGGATCCATATGAATATTAGCGATGTGGCGAAAAAA ACCGGTTTAACCAGCAAAATGATTCGGTTTTATGAAGA GAAAGGGCTGGTGACGC	*Bam*HI	Amplification of *cueR$_{A16M}$*
CueR(T16)-Fw	GAGGATCCATATGAATATTAGCGATGTGGCGAAAAAA ACCGGTTTAACCAGCAAAACGATTCGGTTTTATGAAG AGAAAGGGCTGGTGACGC	*Bam*HI	Amplification of *cueR$_{A16T}$*
CueR(Y19)-Fw	GAGGATCCATATGAATATTAGCGATGTGGCGAAAAAA ACCGGTTTAACCAGCAAAGCCATTCGGTACTATGAAG AGAAAGGGCTGGTGACGC	*Bam*HI	Amplification of *cueR$_{F19Y}$*
CueR(Q22)-Fw	GAGGATCCATATGAATATTAGCGATGTGGCGAAAAAA ACCGGTTTAACCAGCAAAGCCATTCGGTTTTATGAACA GAAAGGGCTGGTGACGC	*Bam*HI	Amplification of *cueR$_{E22Q}$*
CueR(M16-Y19)-Fw	GAGGATCCATATGAATATTAGCGATGTGGCGAAAAAA ACCGGTTTAACCAGCAAAATGATTCGGTACTATGAAG AGAAAGGGCTGGTGACGC	*Bam*HI	Amplification of *cueR$_{A16M\text{-}F19Y}$*
GolS(S14)-Fw	GAGGATCCATATGAACATCGGTAAAGCAGCTAAAGCA TCGAAAGTCTCGAGCAAAATGATTCGCTACTATGAAC AGATTGGTCTGATTCCC	*Bam*HI	Amplification of *golS$_{A14S}$*
GolS(A16)-Fw	GAGGATCCATATGAACATCGGTAAAGCAGCTAAAGCA TCGAAAGTCTCGGCCAAAGCCATTCGCTACTATGAAC	*Bam*HI	Amplification of *golS$_{M16A}$*

	AGATTGGTCTGATTCCC		
GolS(F19)-Fw	GAGGATCCATATGAACATCGGTAAAGCAGCTAAAGCA TCGAAAGTCTCGGCCAAAATGATTCGCTTTTATGAACA GATTGGTCTGATTCCC	*Bam*HI	Amplification of $golS_{Y19F}$
GolS(E22)-Fw	GAGGATCCATATGAACATCGGTAAAGCAGCTAAAGCA TCGAAAGTCTCGGCCAAAATGATTCGCTACTATGAAG AGATTGGTCTGATTCCC	*Bam*HI	Amplification of $golS_{Q22E}$
GolS(A16-F19)-Fw	GAGGATCCATATGAACATCGGTAAAGCAGCTAAAGCA TCGAAAGTCTCGGCCAAAGCCATTCGCTTTTATGAACA GATTGGTCTGATTCCC	*Bam*HI	Amplification of $golS_{M16AY19F}$
P*copA*-Fw	TTTCCCCCTTGACCTTAACCTTGCTGGAAGGTTTAACC TTT		Fluorescence anisotropy
P*copA*-Rv-F	AAAGGTTAAACCTTCCAGCAAGGTTAAGGTCAAGGGG GAAA		Fluorescence anisotropy
P*golB*-Fw	AAAGGTTAAACCTTCCAGCAAGGTTAAGGTCAAGGGG GAAA		Fluorescence anisotropy
P*golB*-Rv-F	GCCAGTCTGGACCTTGCCAGTGTTGGAAGGTCAAGCG TAAT		Fluorescence anisotropy
P*copA*/*cueR*-Fw	GACCCGGGCAAACCGTCCAGGGTCAGG	*Xma*I	EMSA
P*copA*/*cueR*-Rv	CTCCCGGGTAAACCGGTTTTTTTCGC	*Xma*I	EMSA
P*golB*-Fw	GACCCGGGACGTATCCAGAACATGC	*Xma*I	EMSA
P*golB*-Rv	TCCCCCGGGGCAGCCGCCGCAGGTC	*Xma*I	EMSA

Figure 1

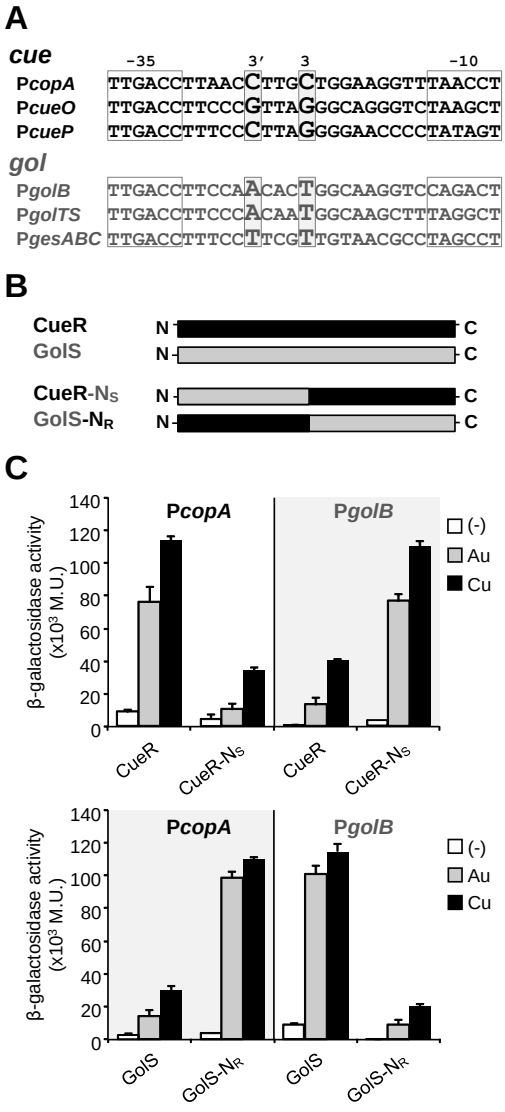


Figure 2

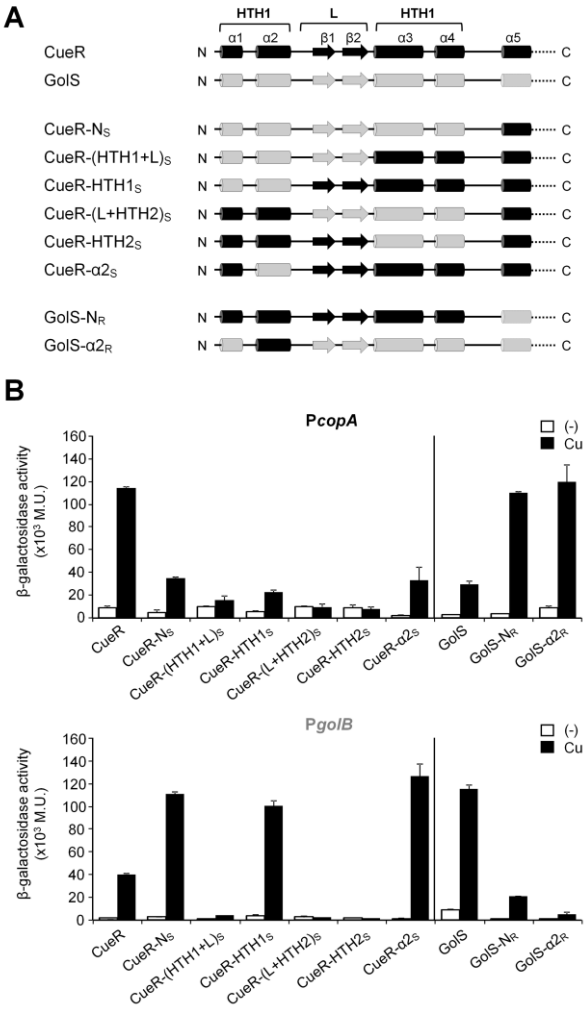


Figure 3

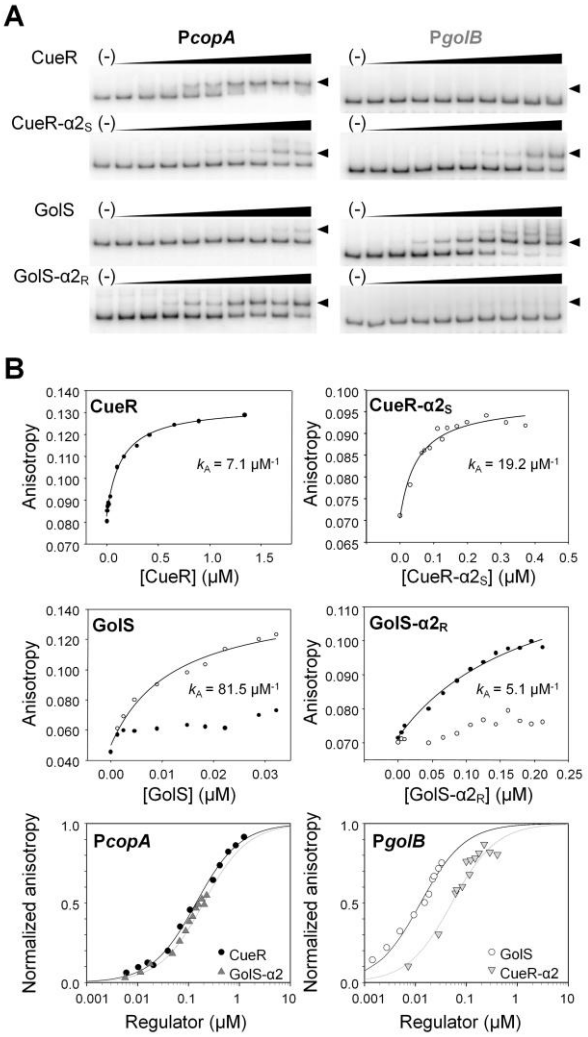


Figure 4

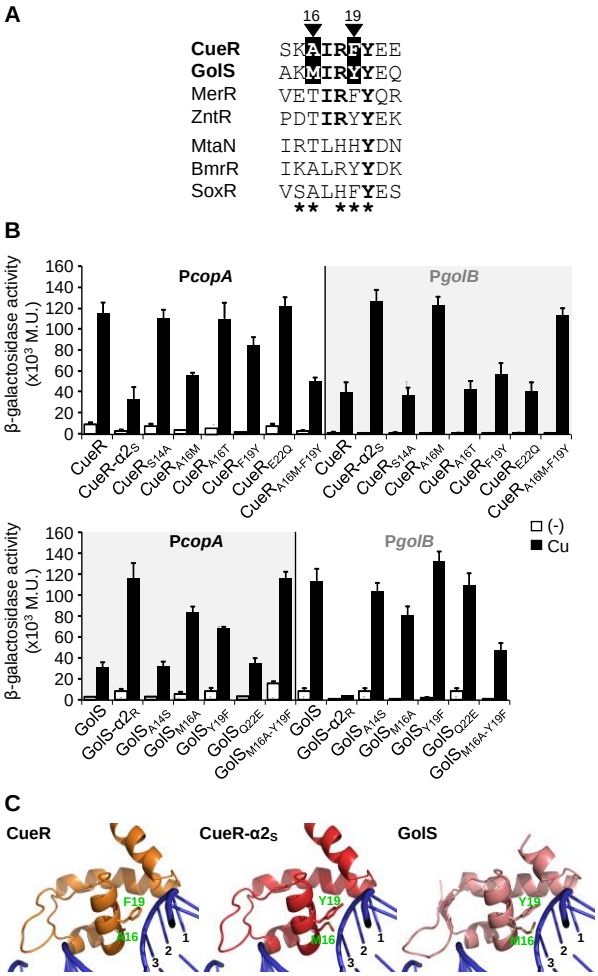


Figure 5

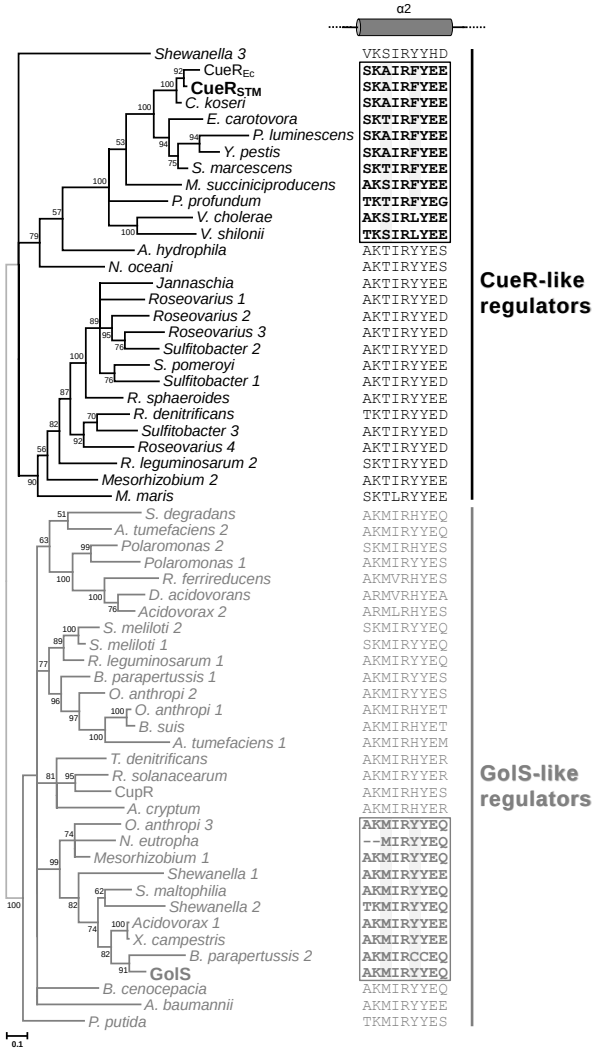
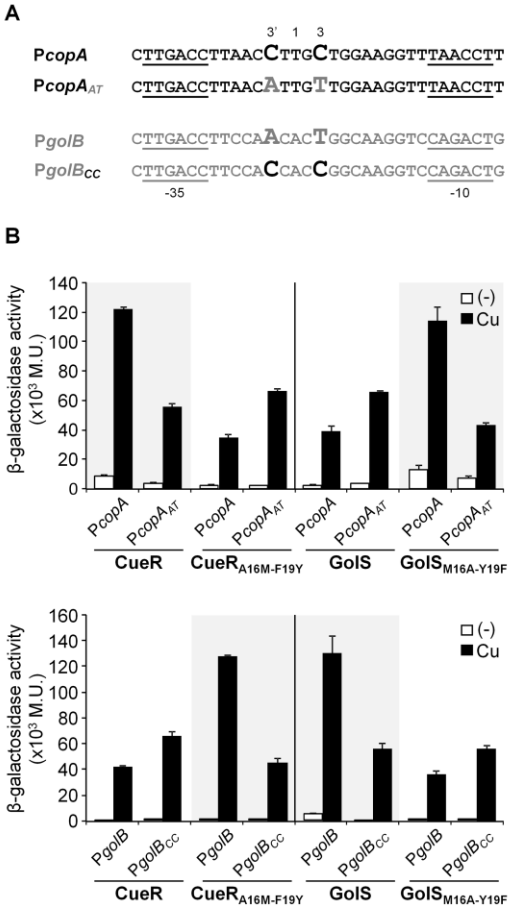


Figure 6



Protein signatures that promote operator selectivity among paralog MerR monovalent metal-ion regulators

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