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The CpxR/CpxA system contributes to *Salmonella* gold-resistance by controlling the GolS-dependent *gesABC* transcription

Sebastián Cerminati[1], Germán F. Giri, Julián I. Mendoza, Fernando C. Soncini and Susana K. Checa

Instituto de Biología Molecular y Celular de Rosario (IBR), Universidad Nacional de Rosario (UNR), CONICET y Departamento de Microbiología, Facultad de Ciencias Bioquímicas y Farmacéuticas, UNR, Ocampo y Esmeralda, Rosario, Argentina

Corresponding author: Susana K. Checa; Instituto de Biología Molecular y Celular de Rosario (IBR-UNR/CONICET), Ocampo y Esmeralda, 2000-Rosario, Argentina; Phone: 54-341-4237070 ext 637; Fax: 54-341-4237070 ext 607; e-mail: checa@ibr-conicet.gov.ar

[1]Present address: Instituto de Procesos Biotecnológicos y Químicos (IPROBYQ- UNR/CONICET), Rosario, Argentina.

Running Title: Gold triggers CpxRA activation

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1111/1462-2920.13837

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Summary

Several regulatory systems contribute to bacterial resistance to heavy metals controlling the expression of factors required to eliminate the intoxicant and/or to repair the damage caused by it. In *Salmonella*, the response to Au ions is mediated by the specific metalloregulator GolS that, among other genes, controls the expression of the RND-efflux pump GesABC. In this work, we demonstrate that CpxR/CpxA, a main cell-envelope stress-responding system, promotes *gesABC* transcription in the presence of Au ions at neutral pH. Deletion of either *cpxA* or *cpxR*, or mutation of the CpxR-binding site identified upstream of the GolS-operator in the *gesABC* promoter region reduces but does not abrogate the GolS- and Au-dependent activation of *gesABC*. Au also triggers the activation of the CpxR/CpxA system and deletion of the *cpxRA* operon severely reduces survival in the presence of the toxic metal. Our results indicate that the coordinated action of GolS and CpxR/CpxA contribute to protecting the cell from severe Au damage.

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Introduction

Bacteria usually trigger the expression of specific factors to counteract the presence of pollutants or xenobiotics. Among them, envelope-located tripartite RND-efflux complexes are required for the elimination of dyes, antibiotics, metal ions, and other toxic compounds (Blair et al., 2014; Yamaguchi et al., 2015). *Salmonella enterica* encodes five RND-efflux complexes with broad and often overlapping substrates specificities. Four of them -AcrAB, AcrAD, AcrEF and MdtAB/C- have orthologues in *Escherichia coli* while GesABC -also known as MdsABC - is *Salmonella*-specific (Nishino et al., 2006; Pontel et al., 2007). Expression of these efflux pumps is usually controlled at the level of transcription and frequently involves more than one transcription factor. For example, *acrAB* transcription in *Salmonella* is controlled by AcrR, a TetR-like repressor encoded nearby, and by the AraC/XylS-like regulators RamA, MarA, SoxS and Rob, involved in stress- and virulence-response (Bailey et al., 2010; Nikaido et al., 2011). Similarly, expression of both MdtAB/C and the RND protein ArcD is controlled by the BaeR/BaeS two-component system, encoded within the *mdtABCD* genetic locus, and, at least in *E. coli*, by CpxR/CpxA, a master regulatory system involved in preserving the integrity of the cell envelope (Nishino et al., 2010; Leblanc et al., 2011).

The GesABC-encoding operon, *gesABC*, was characterized as part of a horizontally acquired locus that directs gold (Au) resistance in *Salmonella* (Pontel et al., 2007; Checa and Soncini, 2011). It is barely expressed under all conditions tested (Nishino et al., 2006; Kröger et al., 2013; Srikumar et al., 2015), except when grown in the presence of $AuHCl_4$, AuKCN or auranofin –an organic Au(I) compound that have been widely used in medicine- (Pontel et al.,

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2007; Conroy et al., 2010). Besides increasing the sensitivity to toxic Au ions, deletion of *gesABC* decreases biofilm formation and attenuates virulence in mice (Nishino et al., 2006; Baugh et al., 2012). However, the mechanisms that link GesABC with these phenotypes are still not understood. As other *Salmonella* RND-based complexes, overexpression of GesABC compensates the lack of the major xenobiotic efflux pump AcrAB for antibiotics resistance (Nishino et al., 2006; Pontel et al., 2007; Conroy et al., 2010), and it was recently reported that GesABC overexpression increases the secretion of the bacterial generated phosphatidylserine, a molecule proposed to be important to mitigate oxidative stress during pathogenesis (Song et al., 2015).

We have previously shown that *gesABC* transcription depends on the divergently encoded cytoplasmic Au sensor GolS, a member of the MerR family of transcriptional regulators (Pontel et al., 2007). This metalloregulator interacts with operators located at atypical σ^{70}-dependent promoters with a 19 bp-long spacer between the -35 and -10 elements instead of the optimal 17 ± 1 bp. Binding of Au(I) to GolS is proposed to be essential to remodel the promoter structure aligning the -35 and -10 elements for their simultaneous recognition by the RNA polymerase (Perez Audero et al., 2010; Humbert et al., 2013; Martell et al., 2015; Philips et al., 2015). The sequence of the GolS-operator at the *gesABC* promoter differs from those at *golTS* or *golB*, the other two transcriptional units of the Gol regulon (Checa et al., 2007; Perez Audero et al., 2010). This difference implies that it requires higher GolS concentration than the other genes for its Au-induction (Pontel et al., 2007), which is achieved at later times after Au-addition or at higher Au concentrations because GolS is autoinduced (Checa et al., 2007).

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The CpxR/CpxA two-component system modulates the transcription of more than fifty genes coding for chaperones, proteases and envelope-associated complexes including several RND efflux pumps, many of them linked to metal homeostasis (Raivio, 2014; Grabowicz and Silhavy, 2016). In this work, we report that the Cpx system enhances the GolS-dependent transcription of *gesABC* in response to Au ions, by binding to sequences located upstream from the GolS operator. Besides *gesABC*, other Cpx-controlled genes are required for counteracting the toxic effects of Au at the cell envelope. As we demonstrated here, gold triggers the expression of *cpxP* in a CpxRA-dependent manner at neutral pH. Our results indicate that the stress caused by the presence of the toxic ion activates the CpxR/CpxA system to preserve cell envelope integrity from Au damage.

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Results and discussion

Identification of a putative CpxR-binding site at the *gesABC* promoter

The delayed Au-dependent activation of *gesABC* compared with the rest of the GolS regulon (Pontel et al., 2007; Perez Audero et al., 2010) led us to search for additional regulatory systems controlling *gesABC* transcription. As mentioned in the introduction, the CpxR/CpxA two component system controls the expression of a number of RND efflux complexes, including genes which expression depends mainly on a dominant local regulator, like *gesABC* (Nishino et al., 2010; Srinivasan et al., 2012; Taylor et al., 2014; Acosta et al., 2015; Pletzer et al., 2015). Thus, as a first approach we run a virtual footprinting, http://www.prodoric.de/vfp/ (Münch et al., 2005), at the *gesABC* (P*ges*) promoter, using a 900 bp-long sequence that encompasses the *gesABC-golTS* intergenic region, position 398574-399474 of the *S.* Typhimurium 14028s genome (Jarvik et al., 2010). This analysis revealed the presence of a putative CpxR-binding site at position -47 to -62 relative to the predicted transcription start site of *gesABC* and in the same orientation (Fig. 1A and Fig. S1A). Interestingly, the putative CpxR binding-site at P*ges* has one of the highest position weight matrix (PWM) score, position 16 among the 80 identified sequences located at intergenic regions, when performing a CpxR regulon prediction analysis using the same tool on the whole *S.* Typhimurium LT2 genome (Table S1). This site differs only in two nucleotides to the consensus CpxR-binding sequence 5'-GTAAAN$_5$GTAAA-3' (De Wulf et al., 2002). The presence of the putative CpxR-binding site was also detected by the multiple expectation maximization for motif elicitation and matching, MEME/MAST (Bailey et al., 2009), using as input nine *bona-fide* CpxR-binding sites of *E. coli*

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CpxR-dependent genes (Fig. S1B). No CpxR-binding site was detected at the *golB* promoter, the other transcriptional unit controlled by GolS (Checa et al., 2007; Perez Audero et al., 2010).

(Figure 1, here)

Electrophoretic gel mobility shift assays (EMSA) and DNase I footprinting were performed using a 249 bp-long PCR fragment containing most of the *gesABC-golTS* intergenic region, but excluding the *golTS* promoter. A complete and specific shift in the DNA probe mobility was observed after the addition of 1 μM of phosphorylated-CpxR (CpxR~P) (Fig. 1B and C, see also Fig. S2A). CpxR~P protected a region extending from nt -40 to -66 relative to *gesABC* transcription start site from DNaseI cleavage (Fig. 1D), encompassing the predicted CpxR-binding site, 5'-GTAAAAACCCGTCAG-3' (Pontel et al., 2007) (Fig. 1A). The simultaneous incubation of the DNA fragment with GolS and CpxR extended the protected region from nt -66 to -16, to include the CpxR- and GolS-binding sites (Fig. 1D). No differences were noted if the order of addition of the regulators was inverted (data not shown). These results indicate that CpxR and GolS bind to DNA either in the absence or the presence of the other protein.

CpxR/CpxA increases *gesABC* transcription in the presence of toxic Au

CpxR is activated by conditions that disturb the cell envelope homeostasis and particularly the inner membrane, including alkaline pH, ethanol or metals such as Cu or Zn (Raivio, 2014; Delhaye et al., 2016; Grabowicz and Silhavy, 2016; Pezza et al., 2016; Surmann et al., 2016). To verify the effective CpxR-regulation of *gesABC* transcription, expression from a chromosomally encoded

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P*ges-lacZ* reporter (Pontel et al., 2007) was analyzed in the wild-type strain as well as in mutants deleted in either *cpxR* or *cpxA,* or in both genes (Fig. 2).

(Figure 2, here)

Only basal expression from the *gesABC* promoter was observed either in the absence of Au ions or in a Δ*golS* strain (Fig. 2). This was expected for a gene controlled by GolS (Checa et al., 2007; Pontel et al., 2007) because it requires the metal-activated regulator to initiate transcription (Pezza et al., 2016).
At pH 7.0, a CpxR/CpxA-activating condition, addition of Au induced the expression of the reporter gene either in the wild-type or in the Δ*cpxR*, Δ*cpxA* or Δ*cpxRA* mutants (Fig. 2A). However, the induction attained in the *cpx* mutants was less than a half of that attained the wild-type strain and similar to the induction observed either in the wild-type or in the *cpx* mutant strains at pH 5.5 (Fig. 2B), a condition at which the histidine kinase CpxA and, in consequence, its cognate response regulator are not active (Danese and Silhavy, 1998; Raivio, 2014; Grabowicz and Silhavy, 2016; Pezza et al., 2016). Au-induction was restored by complementing the Δ*cpxR* mutant with the CpxR-expressing multicopy plasmid at pH 7.0 (Fig. 2A).
Overall, these results indicate that besides GolS, the CpxR/CpxA signal transduction system is required to attain maximal expression of *gesABC* operon in the presence of Au ions.

Direct control of *gesABC* transcription by CpxR/CpxA

The *gesABC* and *golTS* operons form a divergon, with their transcriptional start sites separated by less than 300 bp (Fig. 1 and Fig. S1A). The predicted CpxR binding-site is located at 47 bp from the transcription start site of *gesABC*, and

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at 131 bp from the +1 of the divergent *golTS* operon, encoding the P_{1B}-type ATPase transporter GolT and the Au-sensor/regulator GolS. *E. coli* CpxR interacts with either one or multiple sites located usually, but not always, within 100 bp upstream from the transcriptional start site, and in the same or the opposite orientation regarding the regulated gene (De Wulf et al., 2002; Price and Raivio, 2009). Thus, the increased expression of P*ges-lacZ* at pH 7.0 in the presence of Au (see Fig. 2A) could be indirect, due to CpxR-dependent induction of *golS* transcription from the *golTS* promoter (Checa et al., 2007). However, no differences in the Au-induced expression of the P*golTS-lacZ* reporter was observed between the wild-type and the Δ*cpxR* strain in all conditions tested (Fig. 3A), indicating that CpxR-mediated control of the *gesABC* transcription does not depends on *golTS* induction.

(Figure 3, here)

To corroborate the functional interaction of CpxR with the *gesABC* promoter, we constructed reporter *lacZ* plasmids carrying either the native P*ges* promoter or its mutant version, P*ges**, in which the CpxR-binding site (5'-GTAAAAACCCGTCAG-3') was replaced by 5'-**cact**AAACCC**ca**C**t**G-3'. As shown in Fig. 3B, the reporter plasmid carrying the *Pges** promoter was induced by Au ions similarly at pH 7.0 and 5.5, either in the wild-type strain or the Δ*cpxR* mutant, and it exhibited essentially the same levels of β-galactosidase activity than a Δ*cpxR* strain expressing the native P*ges-lacZ* reporter (Fig. 3C). Deletion of *golS* abrogated both P*ges**- and P*ges*-directed expression in response to Au, confirming that disruption of the CpxR-binding site did not affect GolS recognition (Fig. 3B).

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The Cpx regulon contributes to Au resistance

To evaluate whether the CpxR/CpxA-enhanced expression of GesABC contributes to alleviate stress caused by Au ions, the native *gesABC* promoter was replaced by the CpxR-independent *Pges** version in the chromosome of *S.* Typhimurium 14028s. This modification did not affect the expression of GolS from the divergent *golTS* promoter (Fig. S3). Then, the susceptibility to Au salts was compared between the wild-type, the strain deleted in *gesABC* (Δ*ges*) and the strain in which GesABC is expressed from the modified *gesABC* promoter (P*ges**). As shown in Fig. 4, the strain carrying the CpxR-independent promoter was slightly more sensitive to the metal ion than the wild-type strain, confirming that CpxR-mediated activation is required for the maximal effectiveness of GesABC in Au-resistance.

(Figure 4, here)

Interestingly, the Δ*cpxRA* strain was highly susceptible to Au ions, even more than the Δ*ges* mutant (Fig. 4), suggesting that, besides GesABC pump, other factors under CpxR/CpxA-control are involved in handling the stress caused by the toxic metal at the cell envelope. A similar effect has been previously reported when testing the sensitivity to copper, another group IB metal from the periodic table with similar physicochemical properties than Au, that severely impacts the cell envelope (Yamamoto and Ishihama, 2006; Pezza et al., 2016).

Au activates the expression of *cpxP*, the archetypical CpxR/CpxA-regulated gene

To test if in the presence of Au, other members of the Cpx regulon are induced, we measured the expression of *cpxP*, coding for a key component of the

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CpxR/CpxA signal transduction pathway known to acts as a CpxA modulator (Hunke et al., 2012; Raivio, 2014; Grabowicz and Silhavy, 2016)). To avoid disturbance of the Cpx-signaling we used a chromosomal P*cpxP*-*lacZ* transcriptional fusion that conserved the intact *cpxP* gene. As previously reported (DiGiuseppe and Silhavy, 2003), the *cpxP* promoter was induced at neutral pH in the wild-type strain, but not in the Δ*cpxRA* strain (Fig. 5), contrasting with the GolS-dependent *gesABC* promoter that exhibited basal levels of activation in similar conditions (see Fig. 2 and 3). Addition of $AuHCl_4$ stimulates the expression of the reporter gene at pH 7.0, but not at pH 5.5 or in cells lacking a functional Cpx system (Fig. 5).

(Figure 5, here)

Overall, our results established that, as strong envelope stressor, Au provokes the activation of the CpxR/CpxA system. As with other metal ions such as Cu or Zn, the mechanism involved in the activation of CpxA in the presence of Au is still not known. We predict that, as reported for the other metal ions (Lee et al., 2005; Yamamoto and Ishihama, 2006), the sensor kinase does not directly perceive Au, but the damage caused by its presence. Reactive Au(III) and/or Au(I) ions are probably acting on exposed thiol groups of envelope-associated cellular components. In this sense, a recent report showed that Au(III) activates the expression of proteins involved in cell membrane signaling and oxidative stress in *Cupriavidus metallidurans*, a β- proteobacterium adapted to live at biofilm communities on the surface of Au grains (Zammit et al., 2016). Moreover, the formation of metastable Au(I)-S intermediates in the cell wall or the periplasm were observed in this bacterium, as well as in other bacteria with the ability to metabolically reduce and accumulate Au nanoparticles, such as

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Pseudomonas and *Geobacter* spp. (Kashefi et al., 2001; Karthikeyan and Beveridge, 2002; Reith et al., 2007; Reith et al., 2009; Kenney et al., 2012). These observations support our hypothesis that Au disturbs envelope homeostasis in *Salmonella*, leading to the activation of the CpxR/CpxA signal transduction system, which in turn will contribute to Au resistance by enhancing the expression of GesABC as well as other members of the Cpx regulon.

In summary, this work provides clues about the contribution of both a signal-specific (GolS) and a protein quality control regulatory system (CpxR/CpxA) to preserve the integrity of the cell envelope under severe Au stress.

Experimental Procedures

Bacterial strains and growth conditions. *S. enterica* serovar Typhimurium strains and plasmids used in this study are listed in Table 1. Cells were routinely grown at 37 ºC in Luria Broth (LB) or on LB-agar plates, except for metal-induction and inhibition assays (see below). Ampicillin, chloramphenicol and tetracycline were used at 100, 20 and 12.5 μg ml^{-1}, respectively. 100 μM isopropyl-β-thiogalactopyranoside (IPTG) was added to induce the expression of *cpxR* from the plasmid. All reagents and chemicals were from Sigma, except the Luria-Bertani culture media that were from Difco. Oligonucleotides and enzymes were from Life Technologies.

Genetic and molecular biology techniques. The Δ*cpxR* strain (PB10122, Table 1) was constructed by inserting a ~1100 bp-long PCR fragment containing the Cm^R cassette amplified from pKD3 plasmid using cpxR-P1-Fwd and cpxR-int-P2-Rv primers (Table S2) by Lambda Red-mediated

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recombination (Datsenko and Wanner, 2000), essentially as described previously (Checa et al., 2007). This as well as other mutations were transferred to the wild-type ATCC14028s strain or between strains by using P22 transduction. Briefly, a P22 lysate grown in the donor strain was incubated with an overnight culture of the recipient strain. Recombinant Cm resistance bacteria were selected on LBA plates supplemented with 20 μg ml^{-1} chloramphenicol and 25 mM Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA). To avoid polar effects on *cpxA* expression the cassette disrupting the *cpxR* gene was removed by FLP-mediated recombination as described (Datsenko and Wanner, 2000).

Plasmid pPB1433 carrying the CpxR-independent *gesABC* promoter, P*ges** (Table 1), was constructed by PCR-mediated site-directed mutagenesis using the protocol described in Perez Audero et al, 2010 (Perez Audero et al., 2010). Briefly, a 151 bp fragment was amplified using pPB1224 (Table 1) as template and Pges-Fw and Pges*-Rv (Table S2). After purification, this fragment was used as primer along with Pges-Rv-XmaI to amplify a 341 bp product from the same DNA template. The final product was digested with *Xma*I and cloned into plasmid pMC1871 (Amersham) digested with the same restriction enzyme.

For integration of P*ges** into the chromosome of *S.* Typhimurium 14028s (strain PB12795, Table 1), the modified promoter was amplified from the pPB1433 using Pges-Fw and Pges-Rv (Table S2). This PCR product and the chloramphenicol resistance cassette [amplified from plasmid pKD3 (Datsenko and Wanner, 2000) using P1-Pges-F and P2-Pges-R, see Table S2] were fused by PCR-mediated overlap extension as previously described (Ibanez et al., 2013). The final product was introduced into the chromosome of *Salmonella*

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LB5010 strain carrying the pKD46 plasmid, following the protocol described by Datsenko et al. (Datsenko and Wanner, 2000). The control strain (PB12794, Table 1) carrying the chloramphenicol resistance cassette inserted at the same position but conserving the wild-type *gesABC* promoter was constructed in parallel. The resistance cassette was removed from both strains by FLP-mediated recombination (Datsenko and Wanner, 2000).

DNA fragments as well as plasmids were introduced into bacterial cells by electroporation using a Bio-Rad device following the manufacturer`s recommendations. All constructs were verified by DNA sequencing. Western blot analysis of GolS was performed essentially as described (Checa et al., 2007), using rabbit polyclonal antibodies directed against the transcriptional regulator and Alkaline phosphatase-conjugated protein A.

Induction and Inhibition assays. β-galactosidase activity was measured on total cell extracts from cells carrying the indicated *lacZ* reporter gene fusion essentially as previously described (Perez Audero et al., 2010). Cells were cultured in the presence of 10 μM $AuHCl_4$ or in the absence of metal for 18 h at 37°C under vigorous shaking in LB medium adjusted at either pH 7.0 or pH 5.5 by the addition of 100 mM MES and 50 mM MOPS (LB-MOPS/MES), as indicated in each figure.

For metal-sensitivity assays an over-night culture of the indicated strain was diluted in PBS until 10^{-7}. Then, a 10 μl aliquot of the indicated dilution was applied on the top of LB-MOPS/MES agar plates adjusted to pH 7.0 and containing increasing concentrations of $AuHCl_4$. After incubation at 37°C for 24 h, developing colonies were photographically recorded.

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Protein–DNA interaction assays. His-tagged CpxR was purified by Ni^{2+}-NTA-agarose affinity (Pezza et al., 2016). GolS was purified as described (Checa et al., 2007). Protein concentration was routinely determined by Bradford assay, using bovine serum albumin as standard.

EMSA assays and DNase I footprinting assays were done using 6 fmol of a ^{32}P-labeled DNA fragment containing to the *gesABC* promoter and purified CpxR and/or GolS following basically previously described protocols (Pontel et al., 2007; Pezza et al., 2016). Except when indicated, purified CpxR was incubated with 25 mM of acetyl phosphate (AcP) for 1 h at 30 °C prior to adding to the sample to phosphorylate the transcription factor. The specificity of CpxR~P/P*ges* interaction was analyzed by adding the unlabeled P*ges* probe or a ~400 bp PCR fragment corresponding to the *nucA* gene from *Serratia marcescens* (see Table S2) as nonspecific competitor. The DNA sequence ladder was generated in parallel using the reverse primer and the Sequenase DNA Sequencing kit (Affimetrix). After electrophoresis, the gels were dried and exposed to autoradiography.

CpxR-binding site prediction. The free Virtual Footprinting tool (Version 3.0) from PRODORIC (Münch et al., 2005) available at http://www.prodoric.de/vfp/ was used to predict the CpxR-binding sites at the *gesABC-golTS* intergenic region from the *S.* Typhimurium 14028s and to perform a genome wide analysis of CpxR-binding sites of the *S.* Typhimurium LT2 strain chromosome. For all

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predictions, the Position Weight Matrix for the *E. coli* CpxR homolog was used along with default sensitivity parameters. The maximum distance to the downstream gene was set to 500 pb. The search was restricted to intergenic regions with the options for ignoring strand orientation and removing redundant palindromic matches activated. In parallel, we used the multiple expectation maximization for motif elicitation and matching (MEME/MAST) tools (Bailey et al., 2009) to generate a position-specific scoring matrix from genes previously characterized as members of the CpxR regulon in *E. coli* and to match the *S.* Typhimurium LT2 genome, basically as previously described (Pontel et al., 2007).

ACKNOWLEDGMENTS

We thank Julián Lescano for technical assistance. This work was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (PICT-2013-1513) and from the National Scientific and Technical Research Council, CONICET (PIP11220110100329). S.C., G.F.G and J.I.M. are fellows of CONICET. F.C.S. and S.K.C. are career investigators of CONICET. F.C.S. is also a career investigator of the Rosario National University Research Council (CIUNR).

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Table 1. *S. enterica* serovar Typhimurium strains and plasmids used in this study

Strain	Relevant features[a]	References
14028s	Wild type	ATCC®-14028™
PB5257	Δ*golS*	(Checa et al., 2007)
PB4026	Δ*gesABC*	(Pontel et al., 2007)
PB9662	Δ*cpxRA::Cm^R*	(Pezza et al., 2016)
PB10106	Δ*golS* Δ*cpxR*	This work
PB10122	Δ*cpxR*	This work
PB12526	Δ*cpxA::Cm^R*	(Pezza et al., 2016)
PB5662	*gesA::lacZ*	(Pontel et al., 2007)
PB10211	Δ*cpxR gesA::lacZ*	This work
PB12789	Δ*cpxA::Cm^R gesA::lacZ*	This work
PB10008	Δ*cpxRA::Cm^R gesA::lacZ*	This work
PB5962	Δ*golS gesA*::*lacZ*	(Pontel et al., 2007)
PB10384	Δ*cpxR* Δ*golS gesA*::*lacZ*	This work
PB10864	*cpxP-lacZ*	(Pezza et al., 2016)
PB11450	Δ*cpxRA::Cm^R cpxP-lacZ*	This work
PB12794	P*ges*^{WT} (control strain)	This work
PB12795	P*ges** (CpxR-independent *gesABC* promoter)	This work

Plasmids	Relevant features[a]	References
pPB1467	pQE-32::6x-His-*cpxR*	(Pezza et al., 2016)
pUH21-2*lacI^q*	reppMB1 Ap^R *lacI^q*	Laboratory stock
pPB1466	pUH::*cpxR* (pCPXR)	(Pezza et al., 2016)
pPB1223	pMC1871-derived plasmid containing the P*golTS*::*lacZ* reporter fusion (pP*golTS*) Tc^R	(Perez Audero et al., 2010)
pPB1224	pMC1871-derived plasmid containing the P*gesABC*::*lacZ* reporter fusion(pP*ges*) Tc^R	(Perez Audero et al., 2010)
pPB1433	pMC1871-derived plasmid containing the CpxR-independent P*gesABC*::*lacZ* reporter fusion (pP*ges**) Tc^R	This work

[a] Cm^R Ap^R and Tc^R indicate resistance to chloramphenicol, ampicillin and tetracycline, respectively.

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FIGURE LEGENDS

Figure 1. CpxR interacts with the *gesABC* promoter region. (A) Schematic representation of the *gesABC-golTS* intergenic region in *S.* Typhimurium and DNA sequence of the *gesABC* promoter region. The position of each transcription start site (+1) is indicated by an arrow. The -10 and -35 elements at P*ges* are underlined. The GolS-operator (black oval in the scheme) is depicted in bold in the sequence. The predicted CpxR-binding site is indicated as a grey box in both the scheme and the sequence. (B) Electrophoretic gel mobility shift assays (EMSA) using a ^{32}P-labeled DNA fragment containing P*ges* and purified CpxR (at final concentrations of 0.1, 0.5, 1, or 2 μM, as indicated) pre-incubated (+) or not (-) with 25 mM acetyl phosphate (AcP) [(-) also stands for no protein addition]. The free DNA or the protein-DNA complex are indicated by arrows on the left. (C) EMSA assays were done as in B using 1 μM of CpxR~P and the indicated amount (in X-fold excess) of the non-labeled P*ges* (c-P*ges*) or P*nunA* (c-P*nucA*) DNA fragments used as specific or nonspecific competitive unlabeled DNA. (D) DNase I footprinting assays was performed on the end-labelled non-coding strand of the *gesABC* promoter, using 2 μM CpxR~P and/or 6 μM GolS, concentrations required to obtained full retardation of the P*ges* probe, see part B and (Pontel et al., 2007). The grey and black lines indicate the CpxR and GolS protected regions, respectively, and the arrows mark hypersensitive sites.

Figure 2. CpxR/CpxA activates *gesABC* transcription only in the presence of Au ions. β-galactosidase activity (in Miller units, MU) from a P*ges-lacZ* chromosomal transcriptional fusion expressed in the wild-type (W-t), Δ*cpxR,*

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Δ*cpxA*, Δ*cpxRA*, Δ*golS,* or Δ*cpxR* Δ*golS* cells, or in strains carrying pUH21-2*lacI*^{q} (vector) or pPB1466 (pCPXR), as indicated. Bacteria were grown overnight at 37°C under vigorous shaking in LB-MOPS/MES broth at pH 7.0 or pH 5.5, without (-) or with the addition of 10 μM $AuHCl_4$ (Au). When necessary, 100 μM IPTG was added to induce the expression of *cpxR* from the plasmid. The data correspond to mean values of four independent experiments performed in duplicate. Error bars correspond to the standard deviations.

Figure 3. Recruitment of CpxR to its binding site in the promoter of *gesABC* maximizes transcription in the presence of Au ions. β-galactosidase activity (in Miller units, MU) was measured on total cell extracts from wild-type (W-t), Δ*cpxR*, Δ*golS, or* Δ*cpxR* Δ*golS* cells carrying P*golTS-lacZ* (A), P*ges*-lacZ* (B), or P*ges-lacZ* (C) reporter plasmids (see Table 1 and the text for details) as indicated. Bacteria were grown as indicated in Fig. 2. [Residual Au-dependent activation observed in Δ*golS or* Δ*golS* Δ*cpxR* mutants is the result of the cross-activation of the plasmid-encoded *gesABC* promoter by the homolog Cu/Ag/Au sensor CueR as we previously reported (Checa et al., 2007; Perez Audero et al., 2010).]. The data correspond to mean values of four independent experiments performed in duplicate. Error bars correspond to the standard deviations.

Figure 4. Contribution of CpxR/CpxA to Au resistance. Comparative Au-sensitivity assay between the wild-type (W-t), the strain carrying the CpxR-independent *gesABC* promoter (P*ges**) in the chromosome, or mutants deleted in *gesABC* (Δ*ges*) or in *cpxRA* (Δ*cpxRA*). A 10 μl aliquot of the indicated serial

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dilution of each strain were applied on top of LB-MOPS/MES agar plates adjusted to pH 7.0 and containing the indicated $AuHCl_4$ concentrations. The plates were incubated at 37°C for 24 h before photographical recording.

Figure 5. CpxP is activated by Au in a CpxR/CpxA-dependent manner. β-galactosidase activity (in Miller units, MU) from a P*cpxP-lacZ* transcriptional fusion expressed in the wild-type (W-t) or the Δ*cpxRA* strain, as indicated. Bacteria were grown under the conditions described in Fig. 2. The data correspond to mean values of four independent experiments performed in duplicate. Error bars correspond to the standard deviations.

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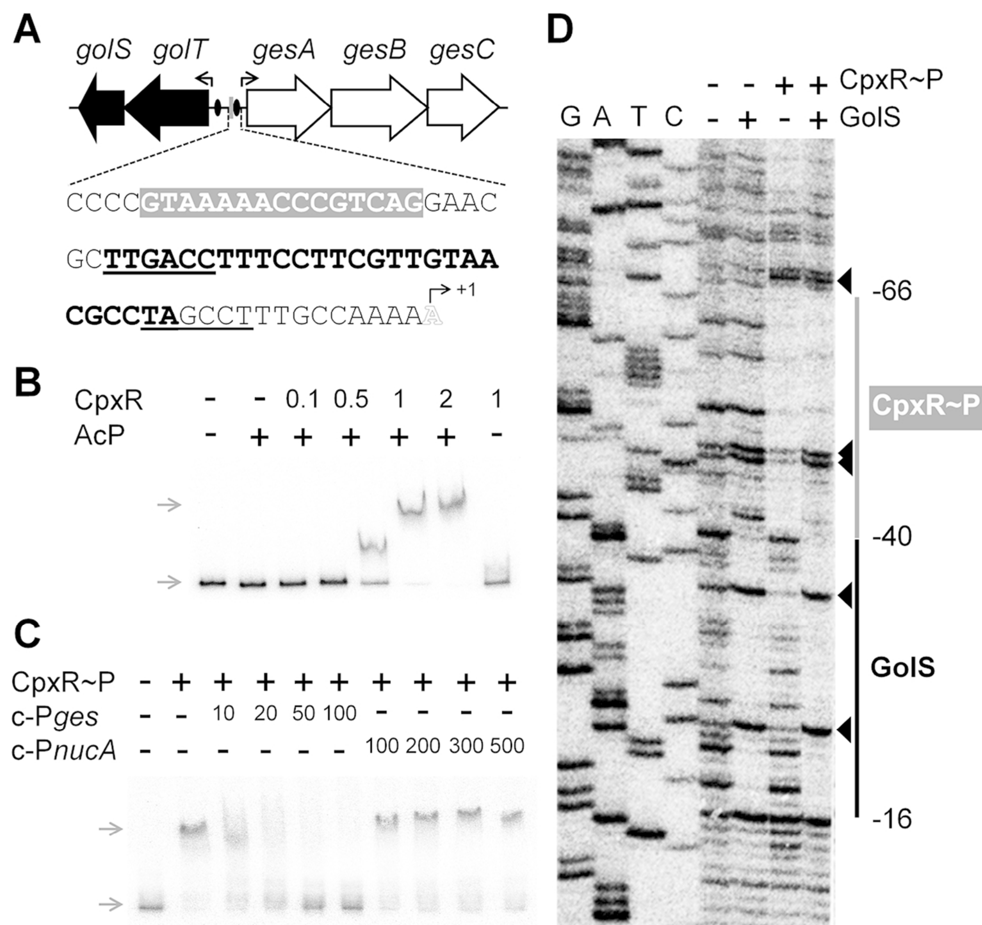

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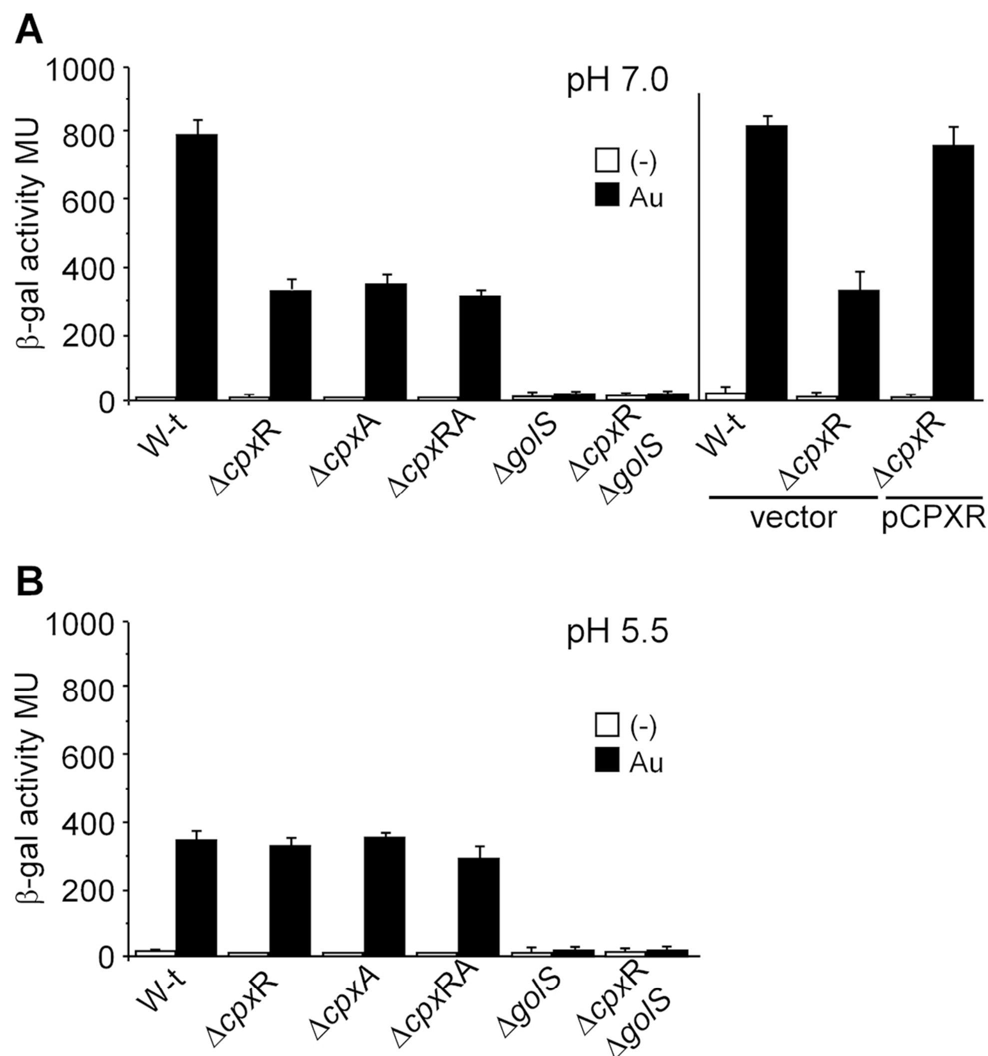

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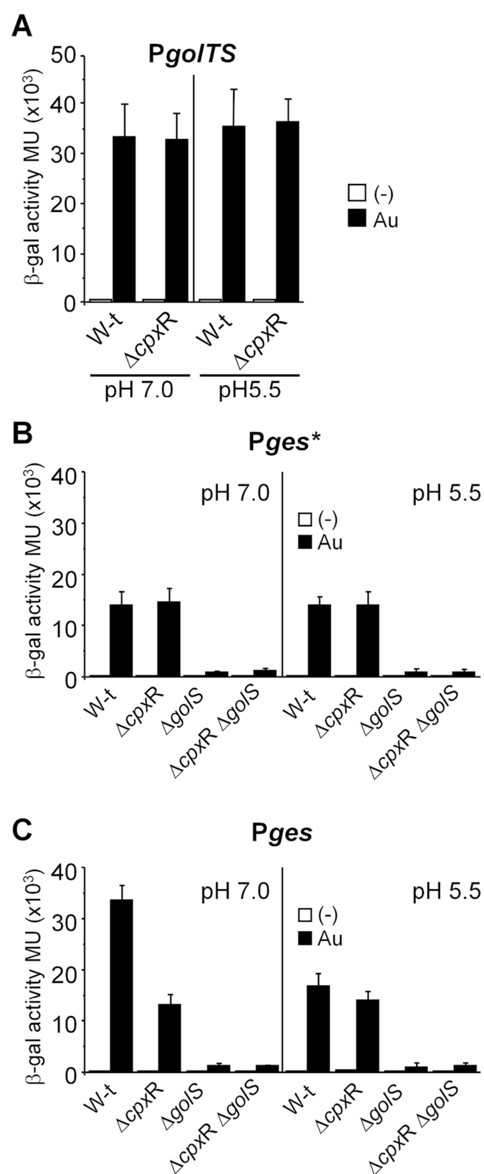

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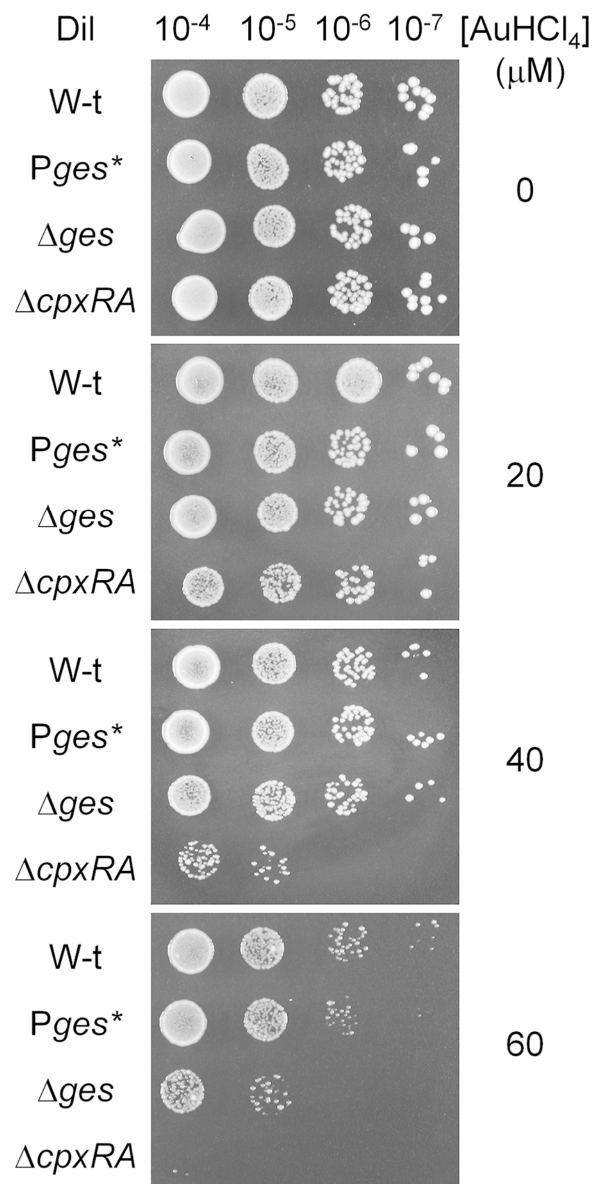

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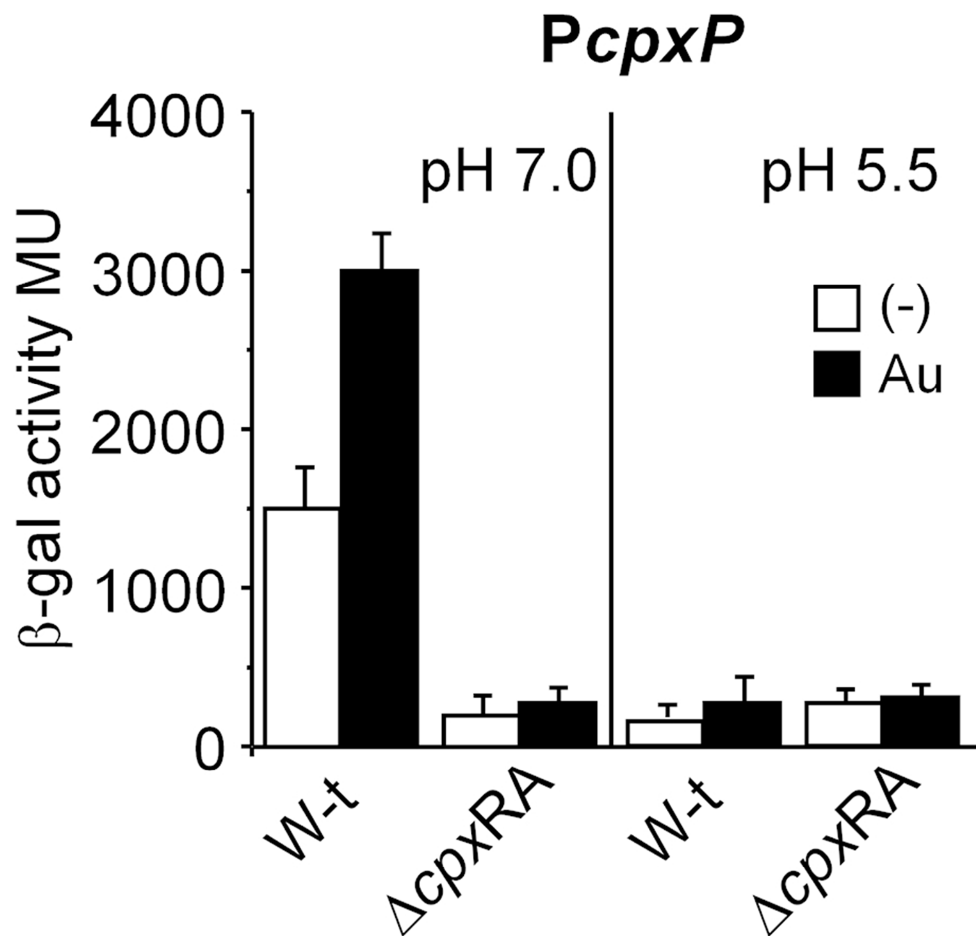

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