Molecular Microbiology (2007) **66**(3), 814–825
doi:10.1111/j.1365-2958.2007.05963.x
First published online 4 October 2007

# GolS controls the response to gold by the hierarchical induction of *Salmonella*-specific genes that include a CBA efflux-coding operon

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## Summary

***Salmonella*** **employs a specific set of proteins that allows it to detect the presence of gold salts in the environment and to mount the appropriate resistance response. This includes a P-type ATPase, GolT, and a small cytoplasmic metal binding protein, GolB. Their expression is controlled by a MerR-like sensor, GolS, which is highly selective for Au ions. Here, we identify a new GolS-controlled operon named** ***gesABC*** **which codes for a CBA efflux system, and establish its role in Au resistance. GesABC can also mediate drug resistance when induced by Au in a GolS-dependent manner, in a strain deleted in the main drug transporter** ***acrAB*****. The GolS-controlled transcription of** ***gesABC*** **differs from the other GolS-regulated loci. It is activated by gold, but not induced by copper, even in a strain deleted of the main Cu transporter gene** ***copA*****, which triggers a substantial GolS-dependent induction of** ***golTS*** **and** ***golB*****. We demonstrate that the Au-dependent induction of** ***gesABC*** **transcription requires higher GolS levels than for the other members of the** ***gol*** **regulon. This correlates with a divergent GolS operator in the** ***gesABC*** **promoter. We propose that the hierarchical induction within the** ***gol*** **regulon allows** ***Salmonella*** **to cope with Au-contaminated environments.**

## Introduction

To avoid toxicity, bacteria have developed systems to control the intracellular concentration of highly reactive transition metal ions (Silver and Phung, 2005; Nies, 2007). Cellular damage can result either from overload of essential or beneficial metal ions, such as Fe, Co, Ni, Cu, Mo, Mn and Zn, or from exposure to transition elements with no known biological role, such as Au, Ag, Al, Bi, Cd, Cr, Hg and Pb. The main mechanism of defence that bacteria employ to overcome overexposure to transition elements relies on their elimination from the cell by active transport, sequestration and/or enzymatic modification to a less toxic form (Nies, 2007; Tottey *et al*., 2007). In most bacteria, metal efflux is mediated by the co-ordinated action of P-type ATPases, CBA efflux systems and cation diffusion facilitators (Silver and Phung, 2005). For example, in *Escherichia coli* the copper excess is removed from the cytoplasm by the P-type ATPase CopA, and from the periplasm by the action of the CBA efflux system CusCFBA and the multicopper oxidase CueO (Outten *et al*., 2001; Finney and O'Halloran, 2003; Franke *et al*., 2003).

CBA efflux systems are tripartite protein complexes that direct efflux of metal ions, xenobiotics or drugs from either the cytoplasm, the inner membrane or the periplasm, across the outer membrane into the extracellular space (McKeegan *et al*., 2003; Nies, 2003; Eswaran *et al*., 2004). The inner-membrane protein of the complex, the resistance–nodulation–cell division (RND) protein, is the central component of the efflux system. It mediates the active transport process and determines substrate specificity (Nies, 2003; Eswaran *et al*., 2004; Hernandez-Mendoza *et al*., 2007). The other two components in the complex are the periplasmic membrane-fusion protein (MFP) and the outer-membrane factor (OMF). During transport, the substrate initially bound to the RND protein is transferred directly to the OMF component for export to the outside (Nies, 2003; Eswaran *et al*., 2004; Lomovskaya *et al*., 2007). The MFP protein serves as a linker to attach the RND protein to the OMF, which is anchored in the outer membrane and projects across the periplasm, forming a 'molecular tube' (Koronakis *et al*., 2000; Murakami and Yamaguchi, 2003; Eswaran *et al*., 2004; Lomovskaya *et al*., 2007).

Gram-negative bacteria typically encode more than one tripartite efflux system with broad and often overlapping substrate specificities, which are required for survival in

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their ecological niches (Nies, 2003; Piddock, 2006a). Recent studies demonstrated a role of some CBA systems from *Salmonella enterica* and other Gram-negative bacteria in pathogenesis (Piddock, 2006b). Usually, the genes coding for the RND and MFP components are transcriptionally coupled, and occasionally associated with a gene coding for the OMF (Grkovic *et al*., 2002; Lomovskaya *et al*., 2007). Most *E. coli* RND exporters cooperate with TolC, the major OMF protein (Nishino *et al*., 2003). The only known exception is the Cu-efflux pump CusCFBA, which codes for its own outer-membrane component that could not be substituted for TolC (Franke *et al*., 2003). By contrast, in the *Pseudomonas aeruginosa* genome, a specific OMF gene is encoded for every RND gene (Poole, 2001).

*Salmonella enterica* serovar Typhimurium harbours five putative CBA efflux systems encoded in its genome (McClelland *et al*., 2001). Four of them, *acrAB*, *acrD*, *acrEF* and *mdtABC*, also present in *E. coli*, affect drug resistance and virulence (Nikaido *et al*., 1998; Nagakubo *et al*., 2002; Eaves *et al*., 2004; Olliver *et al*., 2005). They all require TolC as the outer-membrane channel (Nishino *et al*., 2003). The fifth one is a complete CBA efflux system encoded by a *Salmonella*-specific operon composed by STM0352, STM0351 and STM0350. Deletion of this operon, originally named *mdsABC*, did not have an effect on *Salmonella* susceptibility to toxic compounds. On the other hand, its overexpression from a multicopy plasmid in a strain deleted of the main efflux system, *acrB*, confers some degree of resistance to different xenobiotics (Nishino *et al*., 2006). This *Salmonella*-specific CBA efflux-coding operon is located next, but transcribed in the opposite direction to the *golTS* operon, which encodes for the P-type ATPase GolT and the MerR-like transcriptional regulator GolS (Checa *et al*., 2007). We have recently demonstrated that, in the presence of Au ions, GolS induces the transcription of the *golTS* operon and of *golB*, a small neighbouring gene which codes for a putative metal binding protein (Checa *et al*., 2007). Furthermore, deletion of each individual gene of the *gol* regulon affects *Salmonella* resistance to Au (Checa *et al*., 2007). [Although not entirely clarified, Au toxicity could be due to the intracellular reduction to the thiophilic ion Au(I), which interacts with sulphydryl ligands with high affinity (Hobman *et al*., 2007).] Interestingly, the sole deletion of *golS* renders the most susceptible strain to Au salts, while single or simultaneous deletions of *golT* and *golB* display intermediate phenotypes, suggesting the presence of yet unidentified GolS-regulated loci whose products are involved in Au resistance. Here we demonstrate that transcription of the *Salmonella*-specific CBA efflux-coding operon (renamed as *gesABC* for GolS-induced CBA efflux system-coding operon) is induced by Au ions in a GolS-dependent manner and that it is required for gold resistance. As predicted, we show that transcription of the *gesABC* operon is driven by a typical MerR-like promoter, in which we identify a GolS-recognized operator. Transcription of *gesABC* is delayed compared with the Au-induced expression of *golTS* or *golB*, and requires higher GolS intracellular levels, suggesting a hierarchical activation of the *gol* regulon to cope with contaminated environments. In addition, induction by Au of *gesABC* in a Δ*acrAB* strain increases the resistance to different chemical compounds. To our knowledge, this is the first reported CBA efflux system conferring cross-resistance to metals and other xenobiotics regulated by a metal-responsive MerR-like regulator.

## Results

### *The* Salmonella*-specific CBA efflux system is part of the* gol *regulon*

We have recently demonstrated that the Au sensor GolS induces the expression of GolT and GolB, required for Au resistance in *Salmonella*. However, a strain deleted only in *golS* was more susceptible to gold salts than a Δ*golT* Δ*golB* double mutant strain (Checa *et al*., 2007), suggesting the presence of other unidentified GolS-regulated factors involved in Au resistance. To search for those factors, a consensus motif for the GolS-operator (Fig. 1A) was generated by the MEME tool (Bailey and Gribskov, 1998), using GolS-operator sequences from *golTS* and *golB* and the putative GolS binding sites in the promoter regions of ATPase encoding genes located next to the 10 most similar GolS orthologue genes in the database (see *Experimental procedures*). We then screened the *Salmonella* LT2 genome for matching motifs using the MAST program (Bailey and Gribskov, 1998). From the 684 matches detected by the program, we arbitrary selected those that (i) matched with position *P*-value below 1.0 $e^{-7}$, (ii) were located within an intergenic region, and (iii) were upstream and in the correct orientation of the downstream open reading frames. By using these criteria, only five candidates were selected (Fig. 1B). Among these, we identified the GolS-target operators from the *golTS* operon and the *golB* gene (Checa *et al*., 2007), as well as the CueR-controlled operators from *cueO* and *copA* genes (Kim *et al*., 2002; Espariz *et al*., 2007). The fifth putative operator sequence was located upstream of STM0352, the first gene of an operon encoding for a *Salmonella*-specific CBA efflux system (McClelland *et al*., 2001). Interestingly, this operon is separated 276 bp of *golTS*, forming a divergon (Fig. 2A and B).

Because genes under either GolS or CueR control appeared in the *in silico* analysis, we tested whether STM0352 transcription was controlled by any of these two

A

| Gene | Sequence |
|---|---|
| *golT* | 5'- CTTGACCTTCCCACAATGGCAAGCTTTA-3' |
| *golB* | 5'- CTTGACCTTCCAACACTGGCAAGGTCCA-3' |
| Sh | 5'- CTTGACCTTCCCATCATGGTAAGCCCTA-3' |
| Aci | 5'- CTTTACCTTCCCACGATGGTAAGCCGCA-3' |
| Me | 5'- CTTGACCTTCCCATCGTTGGAAGCCTCA-3' |
| At | 5'- CTTGACCTTGCCACGATGGGAAGCCCTA-3' |
| Bs | 5'- CTTGACCTTCCAACGGTTGGAAGGGCTA-3' |
| Ca | 5'- CTTGACCTTCCCATGATGGGAGGGATCA-3' |
| Rl | 5'- CTTGACCTTCCCATCATAGGAAGGATTA-3' |
| Bpp | 5'- CTTGACCTTCACATCATGGGAAGGTTTA-3' |
| Pss | 5'- CTTGACCTTGCCATCATGACAAGGTTGA-3' |
| Sme | 5'- CTTGACCTTCCCATTATTGGAAGGATTA-3' |

B

| Gene | Sequence | position p-value |
|---|---|---|
| *golT* | 5'- CTTGACCTTCCCACAATGGCAAGCTTTA-3' | 6.3 $e^{-15}$ |
| *golB* | 5'- CTTGACCTTCCAACACTGGCAAGGTCCA-3' | 5.4 $e^{-13}$ |
| *cueO* | 5'- CTTGACCTTCCCGTTAGGGCAGGGTCTA-3' | 8.0 $e^{-10}$ |
| STM0352 | 5'- CTTGACCTTTCCTTCGTTGTAACGCCTA-3' | 1.1 $e^{-08}$ |
| *copA* | 5'- CTTGACCTTAACCTTGCTGGAAGGTTTA-3' | 2.5 $e^{-08}$ |

**Fig. 1.** Discovery of a new GolS-operator sequence in the *Salmonella* genome.
A. A consensus motif for GolS operator was generated using the multiple expectation maximization for motif elicitation, MEME tool (Bailey and Gribskov, 1998) as described in *Experimental procedures*, using the *Salmonella* GolS-operator sequences present in *golTS* and *golB*, and the predicted GolS binding sites identified upstream of genes encoding GolT orthologues in *Shewanella* sp. W3-18–1 (Sh), *Acidovorax* sp. JS42 (Aci), *Mesorhizobium* sp. BNC1 (Me), *Agrobacterium tumefaciens* str. C58 (At), *Brucela suis* 1330 (Bs), *Caulobacter* sp. K31 (Ca), *Rhizobium leguminosarum* bv. viciae 3841 (R1), *Bordetella parapertussis* 12822 (Bpp), *Pseudomonas syringae* pv. phaseolicola 1448 A (Pss), and *Sinorhizobium meliloti* 1021 (Sme). The program *Weblogo* (Crooks *et al*., 2004) was used to generate a logo representing the consensus motif for a GolS operator.
B. List of the putative GolS-operator sequences identified in the *S. enterica* serovar Typhimurium LT2 genome by MAST (Bailey and Gribskov, 1998). The name of the gene, their sequence and the position *P*-value are indicated.

transcriptional regulators. Using a chromosomal *lacZ* transcriptional fusion to the promoter of STM0352, we analysed its expression in Luria–Bertani (LB) without or with the addition of either 10 μM $AuHCl_4$ or 1 mM $CuSO_4$ (Fig. 2C). Only background expression of the STM0352 reporter fusion was observed in the wild-type strain grown in LB, coincident with previous published results (Nishino *et al*., 2006). Addition of Au, but not Cu, provoked an 80-fold induction of STM0352 transcription. This Au-mediated induction was completely dependent on the presence of a functional GolS. On the other hand, deletion of *cueR* had no effect on the transcription of the reporter gene. These results indicate that the operon formed by the STM0350, STM0351 and STM0352 genes (originally named as *mdsABC*), is a new member of the *gol* regulon of *Salmonella*, and we propose to rename it as *gesABC*, for GolS-induced CBA efflux system operon.

### *Analysis of the GolS operator in gesABC*

To examine the interaction of GolS with its operator in the *gesABC* promoter region, we first identified the transcription start site of the operon by primer extension analysis. A major primer extension product was detected, corresponding to an A residue located 55 nucleotides upstream of the *gesA* start codon, with RNA isolated from the wild-type strain grown in the presence of 10 μM $AuHCl_4$ but not with RNA from the Δ*golS* strain (Fig. 3A).

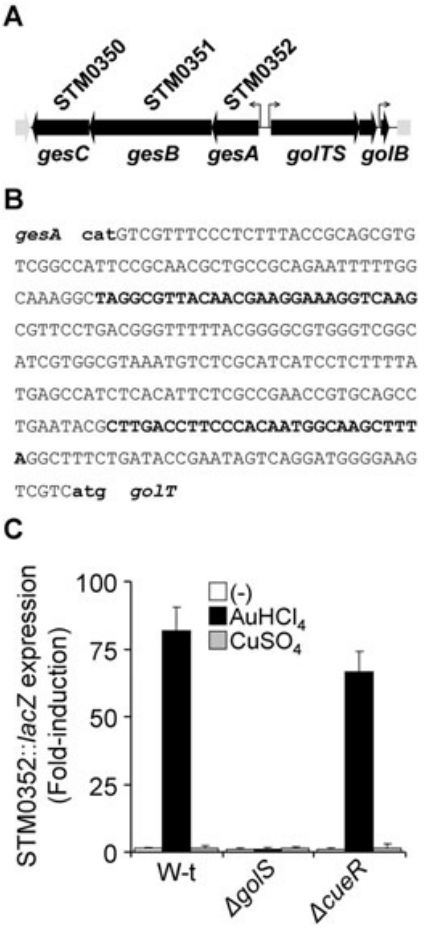


**Fig. 2.** STM0352 transcription is controlled by the Au sensor GolS.
A. Genetic organization of the GolS-controlled genes in the *S. typhimurium* LT2 genome.
B. DNA sequence of the *gesABC-golTS* divergon intergenic region. The consensus GolS-operator sequences for both promoters are shown in boldface.
C. Relative β-galactosidase activity from a STM0352::*lacZ* transcriptional fusion expressed on wild-type (W-t), Δ*golS* and Δ*cueR* cells grown overnight in LB broth with or without the addition of 10 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.

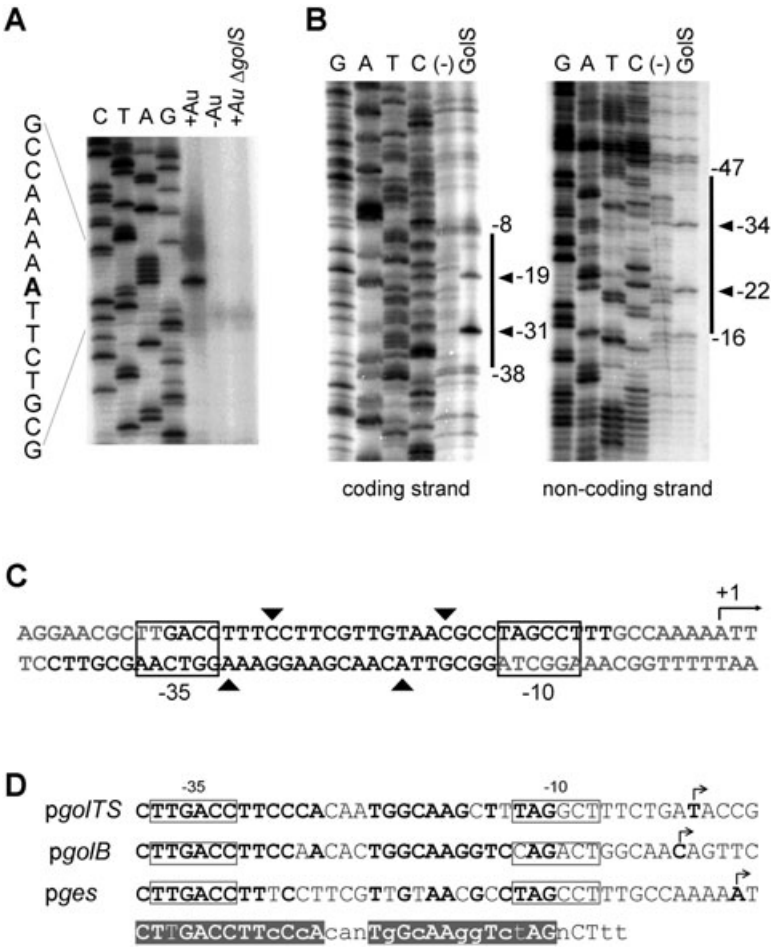


**Fig. 3.** GolS binds to the promoter region of *gesABC*.
A. Primer extension analysis of *gesABC* using RNA isolated from wild-type cells grown after mid-exponential phase in SM9 in the presence (+Au) or absence (–Au) of 10 μM $AuHCl_4$, or RNA isolated from a Δ*golS* strain in the presence of 10 μM $AuHCl_4$ (Δ*golS*). The sequence spanning the transcription start site (bold) is shown on the left.
B. DNA footprinting analysis of the promoter region of *gesABC* performed on both end-labelled coding and non-coding strands. The DNA fragments were incubated with purified GolS at a final concentration of 6 μM. Solid lines and arrows indicate the GolS-protected region and hypersensitive sites respectively.
C. DNA sequence of the *gesABC* promoter region. The GolS-protected region (bold) and the DNase-hypersensitive sites (arrows), as well as the predicted –10 and –35 elements (boxes), are indicated.
D. Alignment of *golTS*, *golB* and *gesABC* promoter regions. The predicted –10 and –35 elements, the transcriptional start sites, and the conserved bases (bold) are indicated. The sequence for a consensus GolS operator (grey boxes) is shown at the bottom.

To experimentally define the DNA sequence that GolS recognizes in the *gesABC* promoter region, DNase I footprinting analysis was performed on both the coding and non-coding strands of the *gesABC* promoter fragment using purified GolS (Fig. 3B and C). The transcriptional regulator protected from nt –8 to nt –38 relative to the transcription start site of the promoter in the coding strand, whereas bases –19 and –31 showed hypersensitivity to cleavage. On the non-coding strand, GolS protected from nt –16 to nt –47, and bases –22 and –34 showed hypersensitivity to DNase I cleavage. Thus, there was an overlap of 23 bp between the two strands which were protected by the regulator protein, which essentially matched with the *in silico* predicted GolS operator (Fig. 1). The size of the footprint, its location within the –35 and –10 spacer, as well as the internal DNase I hypersensitive sites, are hallmarks which distinguish the MerR family of transcriptional regulators (O'Halloran *et al*., 1989; Ansari *et al*., 1995; Outten *et al*., 1999; Espariz *et al*., 2007).

Alignment of *gesABC*, *golTS* and *golB* promoter regions (Fig. 3D) highlights the presence of an inverted repeat sequence that defines the GolS operator as 5′-CTT GACCTTcCcAcanTgGcAAggTctAG-3′, albeit less conserved in the *gesABC* promoter than in the other two loci.

### *The GesABC system is required for Au resistance*

A Δ*gesABC* mutant strain was tested for susceptibility to Au salts. Deletion of the *gesABC* operon rendered a strain similarly susceptible to Au than a Δ*golT* Δ*golB* double mutant, and intermediate between the wild-type strain and the Δ*golS* mutant (Fig. 4A). Wild-type resistance phenotype was restored after complementation of the Δ*gesABC* mutant strain with the expression plasmid pGESAB, which harbours the RND and the MFS coding genes of the *gesABC* operon (Fig. 4A).

To test whether the high susceptibility to Au ions of the Δ*golS* mutant (Fig. 3A and Checa *et al*., 2007) is due to the lack of expression of *gesABC*, besides *golT* and *golB*, we generated an otherwise wild-type strain with non-polar deletions of all GolS-controlled effector genes, i.e. Δ*gesABC* Δ*golT* Δ*golB* (as described in *Experimental procedures*). Au-induced expression of GolS in this strain remains under control of the *golTS* promoter (data not shown). We observed that the Δ*gesABC* Δ*golT* Δ*golB*

 

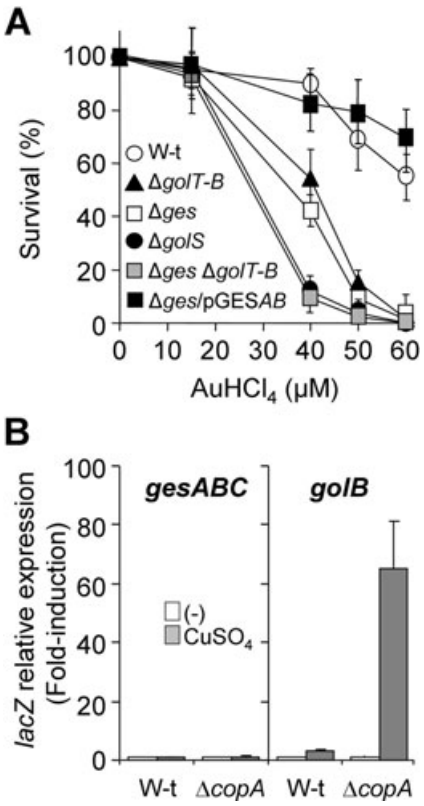


**Fig. 4.** The GesABC efflux system is required for Au resistance.
A. Relative survival of *Salmonella* strains on LB agar plates containing the specified concentrations of $AuHCl_4$. Wild-type strain (W-t) or the mutants in the indicated genes are shown here. Survival of a Δ*gesABC* (Δ*ges*) mutant strain carrying the plasmid pGESAB is also shown. Data correspond to mean values of three independent experiments performed in triplicate. Error bars correspond to standard deviation.
B. The *gesABC* operon is not expressed in the presence of Cu ions. Relative β-galactosidase activity (Miller units) from *gesA* or *golB* transcriptional fusions expressed in the wild-type (W-t) or in a Δ*copA* mutant strain grown overnight in LB broth with or without the addition of 1 mM $CuSO_4$. Data correspond to mean values of three independent experiments performed in triplicate. Error bars correspond to standard deviation.

strain was as susceptible to Au salts as the Δ*golS* strain (Fig. 4A). From these results, we conclude that the residual Au-resistance level displayed by the Δ*golT* Δ*golB* strain is due to the GolS-induced expression of the GesABC efflux system.

### *The* gesABC *operon is neither activated by copper nor required for copper resistance*

We have previously reported that copper has a moderate effect on GolS-mediated *golB* and *golTS* induction in a *Salmonella* wild-type strain, although none of these genes have a primary role on copper tolerance (Checa *et al*., 2007; Espariz *et al*., 2007). On the other hand, in a strain deleted of the main Cu-transporter gene *copA*, an increased Cu-mediated activation of GolS enhances *golB* and *golTS* transcription. In this mutant strain, we also showed that GolT, and to a lesser extent GolB, contribute to eliminate the excess of copper ions (Espariz *et al*., 2007).

We measured the effect of copper on *gesABC* transcription either in the wild-type or in a Δ*copA* strain (Fig. 4B). No increase in *gesABC* expression was detected even in the Δ*copA* strain grown in the presence of 1 mM $CuSO_4$. In the same condition, transcription of *golB* increased almost 65-fold. We also observed that the Δ*gesABC* mutant strain retained wild-type levels of Cu tolerance, even in the presence of single or multiple mutations in *cuiD*, *copA* or *golT*, either in aerobic or anaerobic conditions (Table 1). These results argue against a role of GesABC in Cu resistance. The differential regulation observed among GolS-controlled genes, together with the observation of a less conserved GolS operator in the *gesABC* promoter, suggest that there is a hierarchy in the GolS-controlled transcription of its target genes.

### *Hierarchical induction of the GolS-controlled genes*

To gain insight into the proposed hierarchical induction among GolS-controlled genes, we first compared *in vitro* the interaction of GolS with DNA fragments containing each of its target operators (Fig. 5A). By gel shift analysis, retardation of the fragment containing the *gesABC* operator was detected when incubated with $\geq 0.1$ μM of purified GolS, with an apparent $K_D$ of $1.4 \pm 0.4 \times 10^{-6}$ M. On the other hand, concentrations as low as 0.005 μM of purified GolS were required for band retardation of both *golB* and *golTS* promoter fragments, with apparent $K_D$ of $5.8 \pm 1.2 \times 10^{-8}$ and $9.9 \pm 2.1 \times 10^{-8}$ M respectively.

**Table 1.** Copper tolerance phenotype of the *S*. Typhimurium mutant strains.

| | MIC (mM)[a] | |
|---|---|---|
| Strain | $+O_2$ | $-O_2$ |
| Wild type | 5.50 | 0.70 |
| *gesABC* | 5.50 | 0.70 |
| *cuiD* | 1.25 | 0.50 |
| *cuiD gesABC* | 1.25 | ND |
| *cuiD copA* | 1.00 | ND |
| *cuiD copA gesABC* | 1.00 | ND |
| *cuiD copA golT* | 0.75 | 0.23 |
| *cuiD copA golT gesABC* | 0.75 | 0.23 |
| *copA golT* | 2.75 | ND |
| *copA golT gesABC* | 2.75 | ND |

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

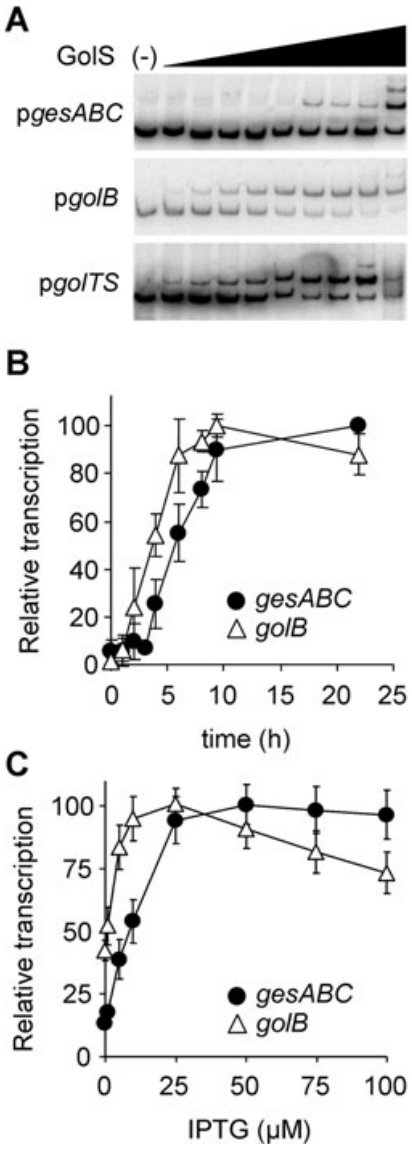


**Fig. 5.** Hierarchical induction of the GolS-controlled genes.
A. Comparative EMSA was performed using 6 fmol of $^{32}$P 3′-end-labelled PCR fragment from *gesABC*, *golB* or *golTS* promoter regions and purified GolS at final concentrations of 0, 0.005, 0.01, 0.02, 0.05, 0.1, 0.2, 0.4, 0.8 and 1.5 μM respectively.
B. Time-course of GolS-controlled induction of *gesABC* and *golB* transcription in the presence of Au. Overnight cells grown in LB were incubated in the same media containing 10 μM $AuHCl_4$. At the times indicated, aliquots were withdrawn from the culture to determine $OD_{630}$ and β-galactosidase activity. The activity was normalized against the maximal response obtained for each transcriptional fusion (278 and 10 090 Miller units were the maximal values obtained for *gesABC*::*lacZ* and *golB*::*lacZ* in these conditions respectively).
C. Relative *gesABC* and *golB* transcription in a Δ*golS* strain harbouring plasmid pGOLS. Cells were grown overnight in LB media containing 10 μM $AuHCl_4$ and the indicated final concentrations of IPTG. β-Galactosidase activity was normalized against the maximal response obtained in each case (363 Miller units for *gesABC*::*lacZ* at 50 μM IPTG, and 7830 Miller units for golB::lacZ at 25 μM IPTG). Data correspond to mean values of at least four independent experiments performed in duplicate. Error bars correspond to standard deviation.

We then compared the kinetics of the Au-induced transcription of both *gesABC* and *golB* in wild-type cells grown in the presence of 10 μM of $AuHCl_4$ (Fig. 5B). To ensure that the response does not depend on the promoter strength, the activities were normalized against the maximal response obtained in each case. We estimated the time required for half-maximal induction of *gesABC* and *golB* reporter fusions as 5.8 and 3.6 h respectively. Because GolS autoinduces its own expression in response to Au, these results strongly suggest that higher intracellular concentrations of the regulator are needed to stimulate *gesABC* transcription than for the induction of *golTS* or *golB*. To confirm this, we determined *gesABC* and *golB* relative transcription in a Δ*golS* strain expressing GolS from pPB1205, a pUHE21-2*lacI*$^q$-based plasmid. Thus, expression of the transcriptional regulator from this plasmid was controlled by the $P_{lacO3/O4}$ promoter and, in consequence, by the concentration of IPTG added to the Au-containing growth medium (Fig. 5C). In these conditions, we estimated that the half-maximal induction of *gesBAC* was reached at 8.7 μM IPTG, while the half-maximal induction of *golB* was attained at 0.8 μM IPTG. These observations not only corroborate the occurrence of a hierarchical induction among GolS-controlled genes, but also suggest that in resting conditions, GolS is not interacting with the *gesABC* promoter region.

### *GesABC can direct the efflux of different compounds after GolS-induced expression*

A recent report indicates that overexpression of GesABC (previously named MdsABC) from a heterologous promoter mediates resistance to different antimicrobial agents and chemical compounds in a Δ*acrB* mutant strain (Nishino *et al*., 2006). Nevertheless, it has been reported that the operon is not expressed under laboratory conditions and, in consequence, its deletion does not affect drug resistance. We evaluated whether this efflux system mediates cross-resistance to toxic compounds when expressed from its indigenous GolS-controlled promoter. Wild-type levels of resistance to both crystal violet (CV) and methylene blue (MB) were observed for the Δ*gesABC* mutant strain, when pre-incubated both in the presence and in the absence of Au salts (Table 2). In contrast, overnight pre-incubation of the Δ*acrAB* mutant strain with Au ions resulted in an increased resistance to both CV and MB, reaching similar levels to those reported by GesABC overexpression (Nishino *et al*., 2006). Moreover, the observed Au-induced resistance phenotype to these compounds was completely lost if either *gesABC* or *golS* was deleted, indicating that it depends on the Au- and GolS-mediated expression of GesABC. As expected, no GesABC-dependent increase of resistance was detected after overnight pre-incubation with either CV, MB or even

 

**Table 2.** Susceptibility to toxic compounds and copper of *S.* Typhimurium mutant strains after grown under GolS-inducing and non-inducing conditions.

| Strain genotype | ON growth conditions[a] | MIC values[b] | |
|---|---|---|---|
| | | CV (μg ml$^{-1}$) | MB (μg ml$^{-1}$) |
| Wild type | – | > 16 | > 256 |
| | Au | > 16 | > 256 |
| *acrAB* | – | 2 | 32 |
| | Au | 4 | 128 |
| | Cu | 2 | 32 |
| | CV | 2 | 64 |
| | MB | 2 | 64 |
| *gesABC* | – | > 16 | > 256 |
| | Au | > 16 | > 256 |
| | Cu | > 16 | > 256 |
| | CV | > 16 | > 256 |
| | MB | > 16 | > 256 |
| *acrAB gesABC* | – | 2 | 32 |
| | Au | 2 | 32 |
| | Cu | 2 | 32 |
| | CV | 2 | 64 |
| | MB | 2 | 64 |
| *acrAB golS* | – | 2 | 32 |
| | Au | 2 | 32 |
| | Cu | 2 | 32 |
| | CV | 2 | 64 |
| | MB | 2 | 64 |

**a.** Bacteria were grown overnight at 37°C in LB supplemented either with 10 μM $AuHCl_4$ (Au), 0.5 μg ml$^{-1}$ CV (CV), 8 μg ml$^{-1}$ MB, or without addition (–), prior to the susceptibility assay.
**b.** MIC values were determined in LB plates containing increasing amounts of CV or MB. The data correspond to mean values of five independent experiments done in triplicate.

Cu ions. (We noticed a *gesABC*- and *acrAB*-independent increase in MB resistance after overnight pre-incubation with either CV or MB.) Finally, and as it was observed for copper, neither CV nor MB could induce the expression of either *gesABC* or *golB* (Table 3).

In sum, our results demonstrate that *gesABC* encodes for a functional efflux system able to mediate resistance to other chemical compounds after its Au-induced and GolS-mediated expression.

## Discussion

Bacteria are able to resist to a wide range of poisonous compounds, including dangerous heavy metals (Nies, 2003). *S. enterica*, as a pathogen, spends a good part of its life within the infected host, but it is also able to survive as stable, dividing populations in the environment. This bacterium harbours several genes which are absent from related enteric species and are not required for virulence. Then, it has been suggested that they may be required for survival in certain niches, outside the host (Winfield and Groisman, 2003). In previous reports (Checa *et al.*, 2007; Espariz *et al.*, 2007), we have characterized a *Salmonella*-specific locus involved in Au resistance, which encodes a MerR-like Au sensor, GolS, and its regulated factors, the P-type ATPase GolT, and metal binding protein GolB. In this work, we identified and characterized a new member of the *gol* regulon, the *Salmonella*-specific *gesABC* operon, coding for a CBA efflux system required for Au resistance. Although MerR-like proteins have been shown to regulate the transcription of genes encoding ATPase and MFS transporters (Grkovic *et al.*, 2002; Brown *et al.*, 2003), to our knowledge, this is the first report of a CBA efflux system shown to be controlled by a MerR regulator.

Some unique features emerged in the characterization of the GolS control of *gesABC* transcription. While it was activated in the presence of micromolar concentrations of $AuHCl_4$ in a similar manner than the other GolS-regulated genes, it was not induced in the presence of copper (Figs 2C and 4B) or silver (our unpublished observation) ions. Unexpectedly, no Cu induction of GesABC was observed even in the Δ*copA* mutant strain, which was shown to trigger a substantial GolS-dependent induction of *golTS* and *golB* (Fig. 4B and Espariz *et al.*, 2007). By *in silico* analysis, we noticed that the GolS operator in the *gesABC* promoter is less conserved than those present in the other two GolS-regulated loci (Fig. 3). The predicted differences in binding affinities between GolS and its target operators were confirmed *in vitro* as well as *in vivo* (Fig. 5). These studies establish that in the presence of Au ions, higher intracellular concentrations of GolS are required to induce a similar level of expression of GesABC than the other two regulated factors, which reflects a hierarchical pattern of induction among

**Table 3.** Au-dependent induction of GolS-controlled genes.

| | β-Galactosidase activity (Miller units)[a] | | | | | |
|---|---|---|---|---|---|---|
| | | | CV (μg ml$^{-1}$) | | MB (μg ml$^{-1}$) | |
| Reporter gene | (–) | Au (10 μM) | 0.5 | 1 | 8 | 16 |
| *ges::lacZ* | 8 ± 2 | 280 ± 33 | 11 ± 4 | 8 ± 2 | 8 ± 3 | 8 ± 3 |
| *golB::lacZ* | 72 ± 5 | 9360 ± 413 | 73 ± 4 | 68 ± 9 | 63 ± 4 | 52 ± 7 |

**a.** β-Galactosidase activity was measured in cells after overnight growth in LB broth without (–) or with the addition of the indicated amounts of $AuHCl_4$ (Au), CV or MB. The data correspond to mean values of four independent experiments performed in duplicate. Standard deviations are indicated in each case.

GolS-regulated loci. Although the *gesABC* operator/promoter region has a predicted suboptimal spacing of 19 bp between the –35 and –10 elements, which is probably responsible of the low-level expression of this operon in the absence of the inducer, our findings strongly suggest that in resting conditions, GolS is not interacting with the *gesABC* promoter.

It has been observed that bacteria usually employ different strategies to reduce the intracellular concentration of poisonous compounds, that generally involve low- and high-affinity transporters (Chamnongpol and Groisman, 2002; Finney and O'Halloran, 2003; Yamamoto and Ishihama, 2005). We demonstrate here that this control can also be exerted at a transcriptional level by the hierarchical induction of expression of different transporters. The above data allow us to propose a model which accounts for a sequential control of transcription of GolS-target genes in Au-contaminated environments. According to our results, in the absence of stimulus, background expression of the sensor protein is ensured by basal transcription of *golTS* (Fig. 6A). In the presence of Au ions (Fig. 6B), Au-GolS actively induces the expression of GolT and GolB, both required for the removal of free Au ions from the cytoplasm, as well as its own expression. In addition, the raise in GolS concentration could also help to lower intracellular free Au levels by direct sequestration. Nevertheless, at this point, the intracellular concentration of the sensor protein has not reached the threshold required for *gesABC* activation. If the exposure to the toxic metal persists, GolS will accumulate, inducing *gesABC* transcription (Fig. 6C). The CBA efflux system can then contribute to eliminate the excess of toxic metal either from the cytoplasm or from the periplasm, alleviating the metal stress (Fig. 6C).

To cope with Cu excess, *Salmonella* employs the products of the ancestral *cue* regulon (Kim *et al*., 2002; Espariz *et al*., 2007). In these conditions, low-level induction of the loci harbouring high-affinity GolS operators in their promoters, i.e. *golTS* and *golB*, is attained by Cu-activated GolS (Fig. 6D). In the absence of a functional CopA, the intracellular Cu concentration would increase. Cu-activated GolS will in turn increase the

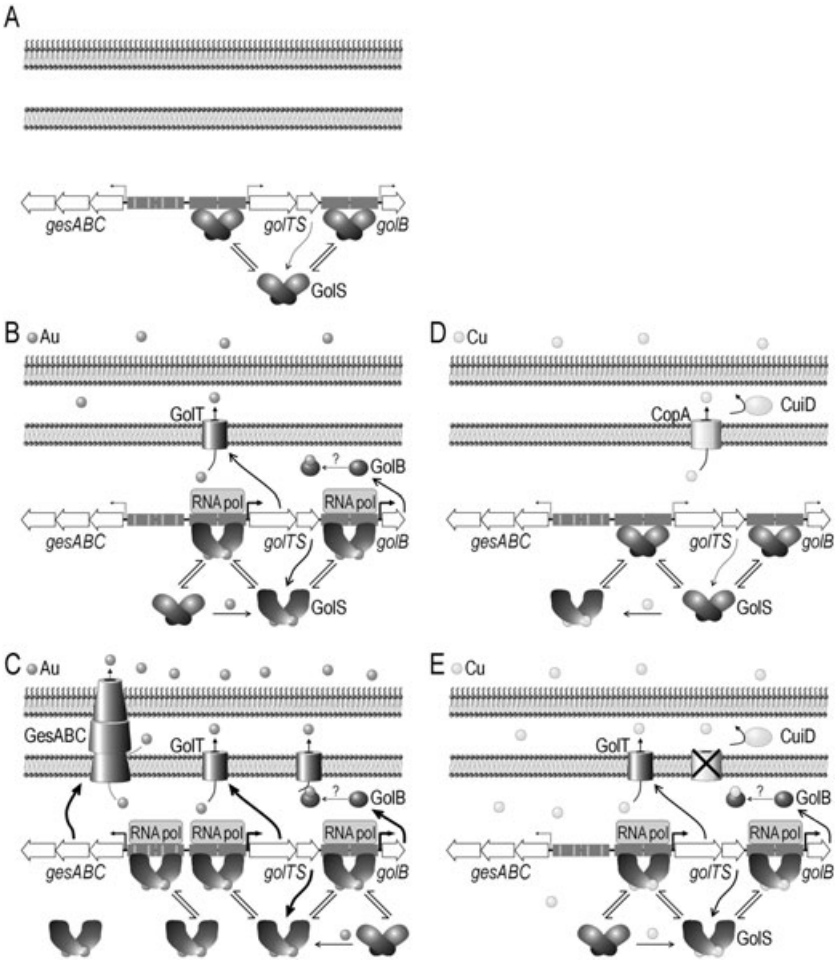


**Fig. 6.** Proposed model for GolS-controlled transcription of the *gol* regulon in the presence of monovalent metal ions.
A. In the absence of stimulus, GolS does not stimulate the transcription of its target genes.
B. In the presence of micromolar concentrations of Au ions, Au-GolS will induce the transcription of *golTS* and *golB* (Checa *et al*., 2007). According to the current model of MerR regulation, the metal-activated regulator allosterically stimulates transcription initiation by remodelling by $\sigma^{70}$ RNA polymerase-dependent promoter structure (Giedroc and Arunkumar, 2007). Metal recognition by the sensor protein does not affect its DNA affinity.
C. At latter times after contact with the toxic metal, or when the exposure to the toxic metal persists, the intracellular accumulation of GolS will reach the threshold required to recognize the *gesABC* operator and induce its transcription. Expression of GolS could also help to lower intracellular free Au levels by direct sequestration.
D. *Salmonella* employs the ancestral *cue* regulon to cope with cytoplasmic Cu excess (Kim *et al*., 2002; Espariz *et al*., 2007). Then, free intracellular Cu will remain negligible. In this condition, the *gol* regulon is not induced.
E. In the absence of a functional CopA, GolS will detect the increase in the intracellular Cu concentration, inducing the expression of *golTS* and *golB* (Espariz *et al*., 2007), which harbour high-affinity GolS operators. Expression of these proteins will compensate the CopA deficiency, directing active copper efflux. Still, in these conditions the intracellular concentration of GolS would be under the threshold necessary for interacting with its operator in *gesABC*. See the text for further details.

 

expression of *golTS* and *golB*, which will compensate for the deficiency, directing active copper efflux. Still, in these conditions the intracellular concentration of GolS attained would be insufficient for interacting with its operator in *gesABC* to promote transcription of the efflux transporter operon. Therefore, even in the absence of a functional *cue* regulon the efflux pump GesABC does not contribute to eliminate Cu excess. It is worth mentioning that Au-induced GesABC expression does not confer copper resistance to a wild-type strain, to Δ*golT* Δ*copA* or to Δ*golT* Δ*copA* Δ*cuiD* strains (our unpublished observation), establishing that this system is not functional for mediating Cu efflux.

The demonstrated role of GesABC in Au resistance (Fig. 4A) strongly suggests a direct role of the CBA system in Au efflux. GesB, the RND component of the tripartite efflux pump, displays a substrate motif which differs from the consensus sequence $AVGX_3DAAX_3IEN$ present in monovalent heavy metal ion transporters such as the Cu transporter CusA and the silver transporter SilA (Gupta *et al*., 1999; Franke *et al*., 2003), and shows higher similarities to RND proteins which export organic substances, such as AcrB and MexF (Nies, 2003; Hernandez-Mendoza *et al*., 2007). In this sense, we and others have shown that GesABC can also detoxify different antimicrobial agents and chemical compounds either in Δ*acrAB* or in Δ*acrB* mutant strains (Table 2 and Nishino *et al*., 2006). However, neither of these compounds could induce the expression of the efflux system (Table 3), and the resistance phenotype could only be detected after Au- and GolS-mediated induction (Table 2) or by ectopic *gesABC* overexpression (Nishino *et al*., 2006).

Metal contamination represents an ancient, widespread and persistent selection pressure for the onset, maintenance and spread of antibiotic resistance with both environmental and clinical importance (Baker-Austin *et al*., 2006, and references therein). It has been shown that several efflux systems can confer cross-resistance to metals and chemical compounds such as antibiotics. i.e. *P. aeruginosa* MexGHI-OpmD confers resistance to vanadium, antibiotics and it is able to transport a quinolone derivative involved in cell-to-cell communication (Aendekerk *et al*., 2002; Aendekerk *et al*., 2005). The *Listeria monocytogenes* multidrug efflux transporter MdrL is required for resistance to ethidium bromide, macrolides, cefotaxime and heavy metals (Mata *et al*., 2000). Microarray analysis of chemostat-cultured *E. coli* cells demonstrated that the RpoS-controlled *mdtABC* operon is upregulated in response to zinc excess (Lee *et al*., 2005).

In view of the Au and chemical compounds' cross-resistance displayed by GesABC (Table 3), it is also possible that this efflux system would function to eliminate cellular metabolites damaged by or forming complexes with Au, rather than the direct transport of the toxic metal ion. The elucidation of the substrate selectivity of this CBA efflux will be fundamental to reveal its unique role in *Salmonella*.

## Experimental procedures

### *Bacterial strains and growth conditions*

Bacterial strains and plasmids used in this study are listed in Table 4. Bacterial strains were grown overnight at 37°C in LB broth or LB agar plates. Ampicillin was used at 100 μg $ml^{-1}$, kanamycin at 25 μg $ml^{-1}$, and chloramphenicol at 10 μg $ml^{-1}$. IPTG was added, when necessary, to induced GolS expression. All reagents, chemicals and oligonucleotides were from Sigma.

### *Bacterial genetics and molecular biology techniques*

Strains carrying gene disruptions or *lacZ* reporter fusion to promoters on the chromosome were constructed using Lambda Red-mediated recombination (Datsenko and Wanner, 2000; Ellermeier *et al*., 2002) and moved into the wild-type 14028s background by P22 transduction as previously described (Checa *et al*., 2007). The sequences of the primers employed are available upon request. When necessary, the antibiotic resistance cassette inserted at the deletion point was removed using the temperature-sensitive plasmid pCP20 carrying the FLP recombinase (Cherepanov and Wackernagel, 1995).

To construct plasmid pPB1211 (Table 4), a region of 4409 bp containing the gesA and gesB genes was amplified from *Salmonella* chromosome by polymerase chain reaction (PCR), using primers *gesAB*-F (5′-CGCGGATCCATGCGTA GAACATTC-3′) and *gesAB*-R (5′-CCCAAGCTTATGCTTGC TGATCATG-3′). The fragment obtained was cloned into the BamHI–HindIII-digested pUH21-2$lacI^q$ vector to generate the *gesAB* expression plasmid. Plasmid DNA was introduced into bacterial strains by electroporation (Checa *et al*., 2007).

### *Metal induction and inhibition assays*

β-Galactosidase assays and minimum concentration inhibitory (MIC) determination were carried out basically as described previously (Checa *et al*., 2007). In total, 30 μl of aliquots from a $5 \times 10^{-7}$ dilution in PBS of each strain was applied to LB plates containing varying concentrations of $AuHCl_4$, $CuSO_4$, MB or CV. After 24–48 h of incubation at 37°C, the MIC values were determined as the minimal concentration of compound in which no growth was observed. When necessary, colony-forming units per ml were calculated and the percentage of survival estimated, based on the count of the corresponding strain grown in the absence of added compound. Protein concentration was determined by the Bradford assay, using bovine serum albumin as standard.

### *Primer extension*

Total RNA was extracted from wild-type or Δ*golS* strains grown in SM9 medium without or with the addition of

 

**Table 4.** Bacterial strains and plasmids.

| | Relevant properties | Reference or source |
|---|---|---|
| Strain | | |
| 14028s | Wild type | ATCC |
| PB3140 | *golB*::*lacZY*$^+$ | Checa *et al.* (2007) |
| PB3167 | *cuiD*::Mud*J* | Checa *et al.* (2007) |
| PB4026 | Δ*gesABC* | This study |
| PB4110 | Δ*golT* golB::*Cm* | Checa *et al.* (2007) |
| PB4861 | *cuiD*::Mud*J* Δ*gesABC* | This study |
| PB4865 | *cuiD*::MudJ Δ*copA* Δ*gesABC* | This study |
| PB5143 | Δ*copA golB*::*lacZY*$^+$ | Espariz *et al.* (2007) |
| PB5257 | Δ*golS* | Checa *et al.* (2007) |
| PB5259 | Δ*golS golB*::*lacZY*$^+$ | This study |
| PB5449 | Δ*cueR* | Espariz *et al.* (2007) |
| PB5557 | Δ*copA* Δ*golT* | Espariz *et al.* (2007) |
| PB5662 | *gesA*::*lacZY*$^+$ | This study |
| PB5810 | Δ*copA* Δ*golT* Δ*gesABC* | This study |
| PB5826 | *cuiD*::MudJ Δ*golT* Δ*copA* | Espariz et al. (2007) |
| PB5828 | *cuiD*::MudJ Δ*copA* Δ*golT* Δ*gesABC* | This study |
| PB5962 | Δ*copA gesA*::*lacZY*$^+$ | This study |
| PB6149 | *cuiD*::Mud*J* Δ*copA* | Espariz *et al.* (2007) |
| PB6344 | Δ*acrAB*::*Cm* | This study |
| PB6601 | Δ*golB* Δ*golT* Δ*gesABC* | This study |
| PB6620 | Δ*acrAB*::*Cm* Δ*gesABC* | This study |
| PB6621 | Δ*acrAB*::*Cm* Δ*golS* | This study |
| Plasmid | | |
| pUH21-2*lacI*$^q$ | rep$_p$MB1 Ap$^r$ *lacI*$^q$ | Soncini *et al.* (1995) |
| pPB1205 (pGOLS) | rep$_p$MB1 Ap$^r$ *golS*$^+$ | Checa *et al.* (2007) |
| pPB1211 (pGesAB) | rep$_p$MB1 Ap$^r$ *gesAB*$^+$ | This study |

10 μM $AuHCl_4$ as previously described (Checa *et al.*, 2007). A total of 2 pmol $^{32}$P-end-labelled primer PROM-*ges*-R (5′-CAAGCGTATTCAGGCTGCACGGTTC-3′), 50 μg of total RNA and 1 U of SuperScript II RNase H2 reverse transcriptase (Invitrogen) were used for cDNA synthesis. The extension product was analysed by electrophoresis on a 6% polyacrylamide-8 M urea gel and compared with sequence ladders initiated with the same $^{32}$P-labelled primer which was used for primer extension.

### *Protein–DNA interaction analysis*

Electrophoretic gel mobility shift assays (EMSA) was performed essentially as previously described (Lejona *et al.*, 2003; Checa *et al.*, 2007). The DNA fragments corresponding to the *gesABC*, *golTS* and *golB* promoter regions were amplified by PCR using the primers PROM-*ges*-F (5′-CTCCCGGGAATTTTGAATGTTCTAC-3′) and PROM-*ges*-R (see above); PROM-*golT/S*-F (5′-GGCGTGGGTCGGCAT CGTGGC-3′) and PROM-*golT/S*-R (5′-TCCCCCGGGAGCT TATCGTGTCGTG-3′); and PROM-*golB*-F (5′-AGGAATTC ACGTATCCAGAACATGC-3′) and PROM-*golB*-R (5′-TCC CCCGGGCAGCCGCCGCAGGTC-3′) respectively. Approximately 6 fmol of each labelled DNA fragment was incubated with purified GolS (in the amounts indicated in the legend to Fig. 5A) at room temperature for 20 min. Samples were run on an 8% non-denaturing Tris-glycine polyacrylamide gel at room temperature. After electrophoresis, the gel was dried and autoradiographed. Three individual experiments were densitometrically scanned to determine the apparent $K_D$, which is the concentration of GolS that shifts 50% of the DNA.

DNase I footprinting assays were performed for both DNA strands essentially as described (Espariz *et al.*, 2007), using 120 pmol of purified GolS, 6 fmol of labelled DNA corresponding to the *ges* promoter region (obtained as described for EMSA) and 0.05 U of DNase I (Promega). In total, 5 μl of samples was analysed by denaturing 6% polyacrylamide gel electrophoresis by comparison with a DNA sequence ladder generated with either PROM-*ges*-F or PROM-*ges*-R primers. GolS was purified from *E. coli* XL1-Blue strain carrying plasmid pGOLS as essentially described (Checa *et al.*, 2007).

### *Bioinformatics*

A consensus motif of GolS-operator sequences was generated using the multiple expectation maximization for motif elicitation tool, MEME (Bailey and Gribskov, 1998). To train the program, we first selected the 10 most homologous proteins to the first 60 amino acid residues of the N-terminal sequence of GolS (DNA binding domain) by BLASTP analysis. This included GolS-like proteins from *Shewanella* sp. W3-18–1 (ZP_00905420), *Acidovorax* sp. JS42 (ZP_01384733), *Mesorhizobium* sp. BNC1 (YP_676424), *Agrobacterium tumefaciens* str. C58 (NP_354214), *Brucela suis* 1330 (NP_697255), *Caulobacter* sp. K31 (ZP_01419023), *Rhizobium leguminosarum* bv. viciae 3841 (YP_771363), *Bordetella parapertussis* 12822 (NP_885229), *Pseudomonas syringae* pv. phaseolicola 1448 A (YP_276749), and *Sinorhizobium meliloti* 1021 (NP_437559). The genes encoding these GolS orthologues are contiguous to, and forming predicted operons with, a P-type ATPase coding genes. We identified a GolS-like operator sequence upstream each of these predicted transcriptional units that also show the conserved 5′-TTGACC-3′ –35 element (Fig. 1A). A 28 nt sequence harbouring the –35 element and the predicted

GolS operator of each of the above operons, and the corresponding sequence from *golTS* and *golB* promoters, were used as the training set for the MEME program, to identify one motif per sequence. The position-specific scoring matrix obtained was then used to search for matching motifs by MAST in the *S. enterica* serovar Typhimurium LT2 genome sequence as the database (Bailey and Gribskov, 1998). Matches with *P*-values below 1.0 $e^{-7}$, in intergenic regions, and in the correct orientation (taking into account the presence of the −35 element) to the downstream genes were considered for further analysis.

## Acknowledgements

We thank Néstor Carrillo and Eleonora García Véscovi for critical reading and comments on the manuscript, and Esteban Serra for technical assistance in the bioinformatic analysis. This work was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica and from the National Research Council CONICET to F.C.S. and S.K.C., who are career investigators of the CONICET. M.E. and M.E. P.A. are fellows of the same institution. F.C.S. is also a career investigator of the Rosario National University Research Council (CIUNR).

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