Exploring the Role of GolT and GolB Proteins in Copper Homeostasis in *Salmonella*: Generation of *E. coli* Clones for Protein Expression

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Abstract

Salmonella is a bacterial infection that results in an estimated 93.8 million cases and 155,000 deaths annually. Copper is an essential micronutrient that is toxic at high concentrations, and the ability of *Salmonella* to maintain copper homeostasis may be of importance to studying *Salmonella*'s pathogenicity. The GolS system plays a role in copper homeostasis in *Salmonella*, with its components, GolT and GolB, functioning as an inner membrane transporter and a cytoplasmic chaperone, respectively. In this study, we successfully generated *golT* and *golB E. coli* clones and studied the clone's expression of the GolT and GolB proteins using arabinose to induce the pBAD vector. The clones were confirmed to be mutation-free through sequencing analysis. Our study also determined the optimal concentrations of arabinose for inducing protein expression using dot blot analysis. These findings provide a foundation for further investigations into the GolS system and its role in copper homeostasis in *Salmonella*.

Acknowledgments

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Introduction

Salmonella is a rod-shaped, gram-negative bacterium that is highly contagious and causes salmonellosis, an infectious disease that results in an estimated 93.8 million cases worldwide with 155,000 deaths annually (Majowicz et al., 2010). The bacterium is commonly transmitted through contaminated food products such as poultry, meat, and eggs, and can cause an outbreak of foodborne illness (Baron, 1996). There are over 2,500 serovars of *Salmonella enterica*, such as Typhi and Paratyphi, responsible for typhoid and paratyphoid fevers (Kurtz et al., 2017). These fevers can be life-threatening in regions with limited access to appropriate medical care, which can increase the fatality rate of typhoid fever from 1-4% up to 10-30% (Buckle et al., 2012). To be able to infect the host, *Salmonella* must adapt to the host environment and acquire essential nutrients, such as copper. Understanding how *Salmonella* is able to regulate copper homeostasis could lead to the development of novel treatments for salmonellosis and other related diseases.

Copper is required for a wide variety of cellular functions in the form of cofactors for redox proteins, which is important for *Salmonella's* survival. Some examples include copper/zinc-containing superoxide dismutases, or SodC proteins, which can use copper as a cofactor to defend against phagocytic cell superoxide produced by respiratory bursts (De Groote et al., 1997). Another prominent cuproprotein is the Cytochrome c oxidase, which is a membrane protein responsible for accepting and transferring electrons for cellular respiration. In a genomic analysis of 450 bacterial genomes, 72% of genomes contained one or more cuproproteins. Of that 72% containing cuproproteins, the most common cuproprotein was cytochrome c oxidase where 91% utilized the protein. Other common cuproproteins included NADH dehydrogenase-2 and superoxide dismutase 1 (SOD1) at 34% and 21% respectively (Ridge et al., 2008).

While copper is essential to the survival of *Salmonella*, it is also toxic in high concentrations. Copper alloy has been registered as an antimicrobial according to the Environmental Protection Agency and copper sulfate is used in an antifungal pesticide (Samanovic et al., 2012). Copper exists in two forms, Cu^{+} which is highly toxic and reactive due to its ability to produce hydroxyl radicals, and Cu^{2+} which is less toxic and more stable (Rensing & Grass, 2003; Hodgkinson & Petris, 2012). Cu+ is capable of producing reactive oxygen species (ROS), where hydroxyl radicals can damage cellular components (Paiva & Bozza, 2014). Since most cuproproteins are typically located in the membrane or periplasm, there is less demand for copper in the cytoplasm. Thus, the bacteria need to maintain low levels of copper ions in the cytoplasm to prevent cellular damage. (Hodgkinson & Petris, 2012; Changela et al., 2003). To maintain control over copper ion levels within the cell, bacteria have developed various systems, including the Cus, Pco, and Cue systems (Argüello et al., 2013). To prevent cellular damage from fluctuations in copper concentrations, these copper homeostasis systems employ chaperones, transmembrane, and redox proteins to detect and respond to changes in copper levels.

A gold-responsive protein system, the GolS system has been shown to have homology with the Cue system. GolS, a cytoplasmic gold sensor, and GolT, an inner membrane gold transporter, have been shown to have a 42% identity at the protein level with the CueR sensor and the CopA transporter respectively (Checa et al., 2007). In the GolS system, there also exists a cytoplasmic gold-binding chaperone protein (GolB), as seen in Figure 1 (Espariz et al., 2007).

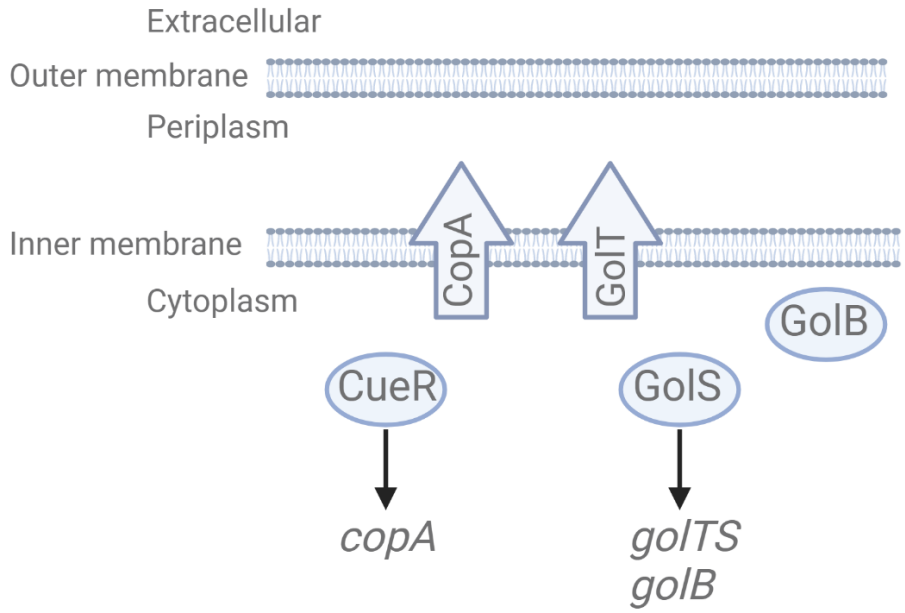

Figure 1. GolS and Cue System. The diagram displays the positions of the proteins involved in the GolS system, as well as two proteins from the Cue system, CopA and CueR.

Research has shown that the GolS system also contributes to copper tolerance in *Salmonella* (Espariz et al., 2007). GolS, a MerR-type sensor, regulates the expression of the proteins GolB and GolT in response to gold ions, as demonstrated by a β-galactosidase assay using a lacZ reporter fusion to the golB promoter (Checa et al., 2007). While gold induces the highest β-gal activity, copper and silver also increase β-gal activity.

Studies have also shown GolT's ability to induce copper-resistant properties where in a *ΔcopA* strain, a *ΔgolT* mutant strain resulted in less copper resistance than a strain with the *golT gene,* suggesting its contribution towards copper resistance (Espariz et al., 2007).

GolB is a small 64 amino acid metal-binding chaperone protein shown in Figure 2. In a *ΔcopA* strain, the deletion of *golB* does not change copper tolerance; however, in a *ΔcopA ΔgolS* mutant, the deletion of *golB* did result in a slight reduction of survivability (Espariz et al., 2007).

To efficiently study the GolB and GolT proteins, the pBAD TOPO TA cloning vector (Invitrogen, Carlsbad, CA) was used, as shown in Figure 3. By inserting the gene of interest into the pBAD expression system, the protein can be produced and purified. This is because the pBAD system allows regulation of the expression of protein through the arabinose inducible promoter, enabling control over the amount of protein produced by changing the arabinose concentration in the growth medium (Guzman et al., 1995). The pBAD expression vector also tags the synthesized protein with a polyhistidine tag, allowing for easy purification of the target protein through a nickel resin using affinity chromatography, and the detection of protein using anti-His antibodies (Hochuli et al., 1988).

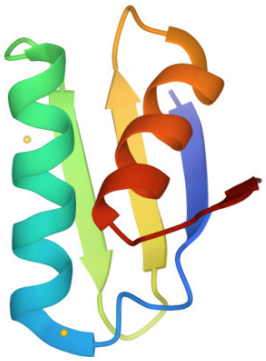

Figure 2. GolB structure using X-ray crystallography (Wei et al., 2015)

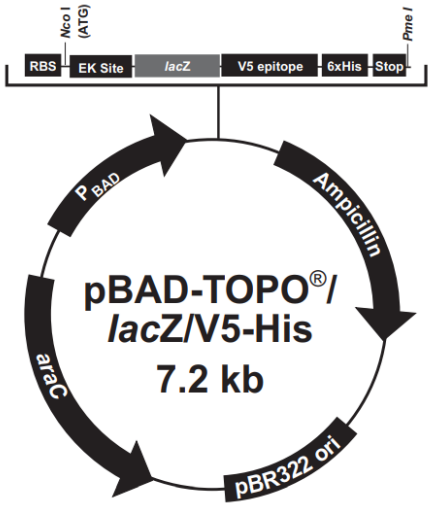

Figure 3. pBAD TOPO Vector Features (Invitrogen, Carlsbad, CA)

Materials and methods

Gene Cloning of GolB and GolT

Genomic DNA extraction was performed on an overnight culture of *Salmonella typhimurium* SL1344 grown in LB broth. The culture was collected by centrifugation in preparation for genomic extraction. Cells were washed twice with TES solution (10 mM Tris, 25 mM EDTA, 150 mM NaCl, pH8). The pellet was resuspended with 275 µl 2 mg/ml Lysozyme solution and incubated for 15 min at 37 ℃. 300 µl of 10% sarkosyl solution (N-Lauroylsarcosine) and 30 µl proteinase K were added and then incubated for 1 hr at 55 ℃. 600 µl of isopropanol and 60 µl NH4-Acetate was added to the solution. The tube was then inverted until DNA precipitate was observed, and then centrifuged at 16,000 x g. The supernatant was then removed and the DNA pellet was washed with 80% ethanol and collected through centrifugation. The ethanol is aspirated and the pellet is dried by evaporation. The pellet is dissolved in 200 µl sterile millipore water.

The golB and golT sequences were amplified using the extracted genomic DNA and the designed forward and reverse primers listed in Table 1 via PCR.

Gene	Direction	Sequence
golT	Forward	5’ - ATGAGTCAGTCAGAAAATCGTCAC -3’
	Reverse	5’ - TGTCGTGGATGTGTCGG - 3’
golB	Forward	5’ - ATGCAGTTCCATATTGATGACATGACCT - 3’
	Reverse	5’ - CCTCTCGCGAGGAGGGAAAC - 3’

Table 1. Primers Used for PCR Amplification.

PCR was conducted using 0.5 µl volumes of each primer, 0.25 µl Q5 High-Fidelity DNA polymerase (New England Biolabs (NEB)), 5 µl Q5 reaction buffer (NEB), 0.5 µl 10 mM dNTP,

and 100 ng genomic DNA from the previous step. The PCR samples were run at different settings based on the unique properties of the primers and gene lengths. For the *golT* clone, the DNA was initially denatured for 30 seconds at 98 ℃, followed by 30 cycles of 10 s denaturation at 98 ℃, 30 seconds of annealing at 65 ℃, and 120 seconds of elongation at 72 ℃. The cycle was ended with a final 2 min extension at 72 ℃. The *golB* clone was initially denatured for 2 min at 98 ℃, followed by 30 cycles of 10 s denaturation at 98 ℃, 30 seconds of annealing at 69 ℃, and 20 seconds of elongation at 72 ℃. The cycle was ended with a final 2 min extension at 72 ℃. The annealing temperatures were determined by utilizing the NEB Tm calculator, while the elongation temperature was set at 30 seconds per kb based on the clone's length. The amplified *golT* and *golB* clones were then verified with gel electrophoresis on a 1% agarose gel with 1 kb and 100 bp ladders (NEB).

Transfection Into Chemically Competent *E. coli*

After PCR amplification, the product was purified using the NucleoSpin Gel and PCR Cleanup Kit (Macherey-Nagel) according to the manufacturer's instructions. The DNA concentration of the purified product was then measured using a spectrophotometer. Taq polymerase was then used to add 3’ Adenosine sticky ends with 0.05 µl 100mm dATP, 0.05 µl Taq polymerase (NEB), 2 µl Taq buffer (NEB), 10 µl purified PCR product, and 4 µl nuclease-free water to fill to 16 µl. The mix was incubated for 20 min at 72 ℃. The Adenosine extended PCR product is ligated into the pBAD TOPO-TA expression vector (Invitrogen) with a 10:1 molar ratio of PCR product to vector. The ligation reaction used 1 µl salt solution (Invitrogen), 1 µl TOPO vector (Invitrogen), 0.5 to 4 µl PCR product to achieve a 10:1 molar ratio of PCR product to vector, and then sterile water to fill up to a final volume of 6 µl. The mix was then incubated for 20 min at room

temperature. 2 µl of the ligated vector was added to a vial of One Shot TOP10 chemically competent *E. coli* (Invitrogen) and inverted to mix. The vials were incubated for 30 min and then heat-shocked for 30 seconds at 42 ℃. Vials were then transferred to ice and 250 µl super optimal broth with catabolite repression medium (S.O.C) (Invitrogen) was added. The vial was then incubated in a shaker at 37 ℃ for 1 hr, and then 50 µl was plated on an LB-amp plate.

Analysis of Insert Orientation

To ensure the pBAD vector will express protein, the orientations of the *golB* and *golT* inserts within the vectors were verified. The orientation of the *golB* and *golT* inserts in the pBAD vector was verified through colony PCR using Taq polymerase (Invitrogen). This involved using a forward primer for the cloned gene paired with a reverse primer from the pBAD TOPO kit to perform the PCR reaction. The PCR product is then analyzed using gel electrophoresis on a 1% agarose gel. Colonies that produce a band at expected positions ~2700 bp and ~350 bp for *golT* and *golB* respectively, were positive clones with the gene ligated in the correct orientation.

The plasmids were extracted from these colonies for sequencing of the gene. The *golB* plasmid was sequenced using the pBAD forward primer, while *golT* required 4 primers to fully sequence. The primers used for *golT* sequencing are shown in Table 2.

Primer name	*golT* primer sequence
pBAD forward primer (Invitrogen)	5’-ATGCCATAGCATTTTTATCC-3’
pBAD reverse primer (Invitrogen)	5’-GATTTAATCTGTATCAGG-3’
golT primer 2	5'-CTTCGGTAAGCGTCCCGGTTTTG-3'
golT primer 3	5'-GTCAACGGTAGCGTATACG-3'

Table 2. *golT* sequencing primers.

Induction of *golB* and *golT* transfected *E. coli* and Protein Expression Analysis

To induce the synthesis of GolB and GolT, 0.15 mL of culture was used to inoculate 5 mL of LB amp media and incubated in a 37 ℃ shaker until a 0.5 OD600. L-arabinose was then added in different concentrations to create 0.1%, 0.01%, 0.005%, and 0.002% solutions of arabinose and then incubated for 3 hr. A 3 hr no arabinose and a 0 hr culture were also made as controls. 2 mL of the cultures were then centrifuged at 16,000 x g into a pellet, and the resulting supernatant was aspirated.

The pellets are resuspended in 200 µl Buffer B (25 mM Tris-HCl pH 8, 100 mM sucrose, 500 mM NaCl, 1 mM DTT, 1mM PMSF), and sonicated for a total of 3 min in 6 sets of 30 seconds to prevent overheating. The homogenate is centrifuged, and the supernatant and pellet are separated. The pellets are resuspended with 200 µl Buffer B. Samples are then serially diluted by a factor of 2, into the following concentrations: 1:1, 1:2, 1:4, 1:8, 1:16, and then blotted onto a nitrocellulose membrane in 1 µl volumes alongside a reference his-tagged protein of known concentration. The membrane is blocked for 40 min in 10 mL of 5% milk. The blocking solution is discarded, and 5mL of rabbit anti-his antibody (GenScript) is added and incubated for 1 hr. The primary antibody is removed and the membrane is washed 3 times for 5 min, two times with 1x PBS 0.1% tween and one time with 1x PBS. Goat anti-rabbit (GenScript) is added and incubated for 1 hr. The membrane is washed again 3 times for 5 min, two times with 1x PBS 0.1% tween and one time with 1x PBS. The membrane is then developed for 1 min, and then imaged.

Results

Cloning of *E. coli* with pBAD vector containing *golB* and *golT* inserts

To study the GolT and GolB proteins, two strains of *E. coli* were made to express GolT and GolB proteins in a controlled manner. This was done by transforming *E. coli* with a pBAD vector encoding GolT and GolB.

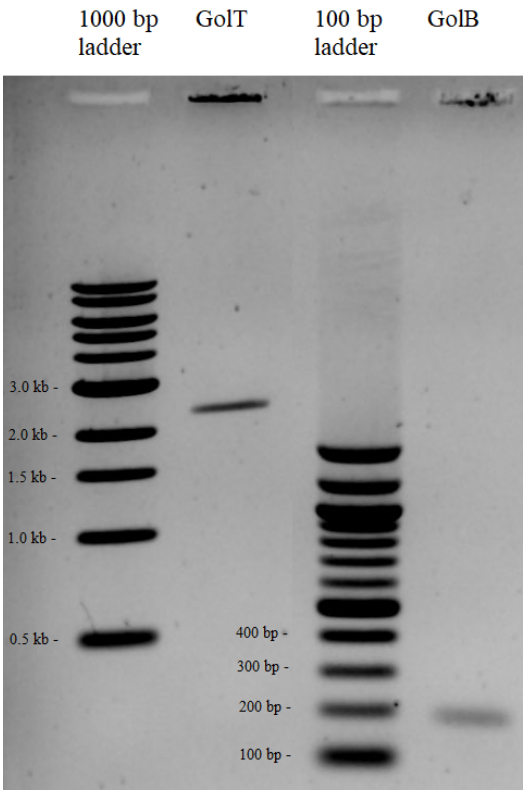

Figure 4. 1% agarose gel of PCR amplification of *golT* and *golB*. Bands can be observed at ~2500 bp and ~190 bp

Figure 4 shows the amplified *golT* and *golB* from Q5 polymerase PCR produced a gel with bands corresponding to the lengths of *golT* and *golB*, suggesting successful amplification. This DNA was purified and then used for ligation with pBAD. The ligated vectors were transformed into *E. coli*, and the resulting colonies were analyzed using colony PCR with a forward primer from *golT/golB*, and the reverse primer from the pBAD vector. The distance between the pBAD reverse primer and the gene insert is 148 bp, while the lengths of *golB* and *golT* are 192 bp and 4572 bp respectively. The band lengths expected for *golB* and *golT* are 340 bp and 4720 bp, respectively. These lengths are depicted in Figure 5, which displays the proper orientation of the insert found in the plasmid within the colony.

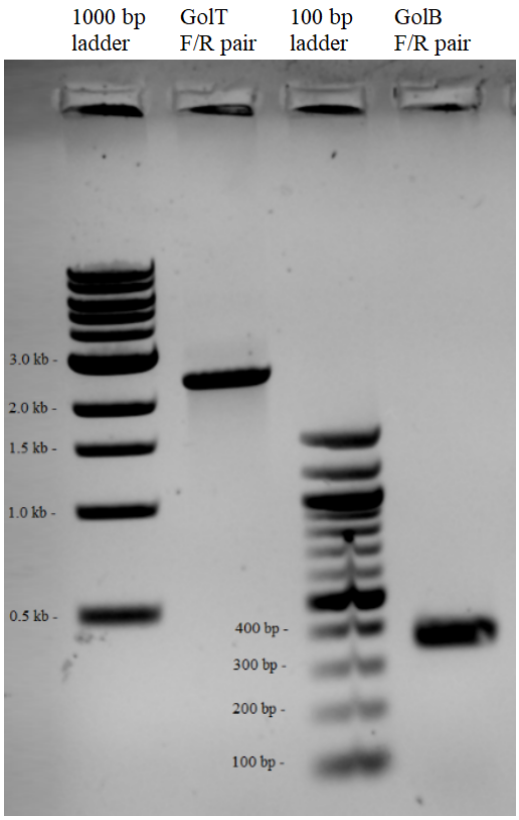

Figure 5 Orientation analysis of clone. Well 2 holds a *golT* colony PCR using a forward primer from *golT* and a reverse primer from pBAD, yielding a band at ~2700 bp. Well 4 holds the *golB* colony PCR using a forward primer from *golB* and a reverse primer from pBAD shows a band at ~350 bp.

The colony with *golB* plasmid was then isolated and sequenced using pBAD forward primer, yielding the expected sequence including the two T mutations shown in Figure 6. The two T mutations were included to reduce the high melting temperature of the reverse primer. The two T mutations are silent when translated into a protein. The pBAD TOPO with inserted *golB* is shown in Figure 8. The colony with *golT* plasmid was also isolated and sequenced, yielding a 100% identical result in Figure 7.

Score	Expect	Identities	Gaps	Strand
344 bits(186)	6e-100	190/192(99%)	0/192(0%)	Plus/Plus

```
Query  1    ATGCAGTTCCATATTGATGACATGACCTGCGGCGGCTGCGCCAGTACGGTAAAAAAGACG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    ATGCAGTTCCATATTGATGACATGACCTGCGGCGGCTGCGCCAGTACGGTAAAAAAGACG  60

Query  61   ATTCTGACTCTCGATGCTAATGCGACGGTGAGAACTGACCCGGCGACGCGTCTGGTTGAC  120
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   ATTCTGACTCTCGATGCTAATGCGACGGTGAGAACTGACCCGGCGACGCGTCTGGTTGAC  120

Query  121  GTTGAAACGTCGCTATCCGCGGAGCAGATTGCCGCCGCCCTGCAAAAGGCCGGTTTCCCG  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  GTTGAAACGTCGCTATCCGCGGAGCAGATTGCCGCCGCCCTGCAAAAGGCCGGTTTCCCT  180

Query  181  CCGCGCGAGAGG  192
            || |||||||||
Sbjct  181  CCTCGCGAGAGG  192
```

Figure 6. GolB sequence alignment with sequenced *golB* sample. Query represents the GolB sequence and Sbjct represents the sequenced sample. The sequenced sample contains no mutations other than the two T mutations.

Score	Expect	Identities	Gaps	Strand
4222 bits(2286)	0.0	2286/2286(100%)	0/2286(0%)	Plus/Plus

```
Query  1    ATGAGTCAGTCAGAAAATCGTCACGACACGATAAGCTTACTTATTGAAGGTATGACCTGC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    ATGAGTCAGTCAGAAAATCGTCACGACACGATAAGCTTACTTATTGAAGGTATGACCTGC  60
```

Figure 7. GolT sequence alignment with sequenced *golT* sample. Query represents the *golT* sequence and Sbjct represents the sequenced sample. Only the first 60 sequenced are shown in this figure.

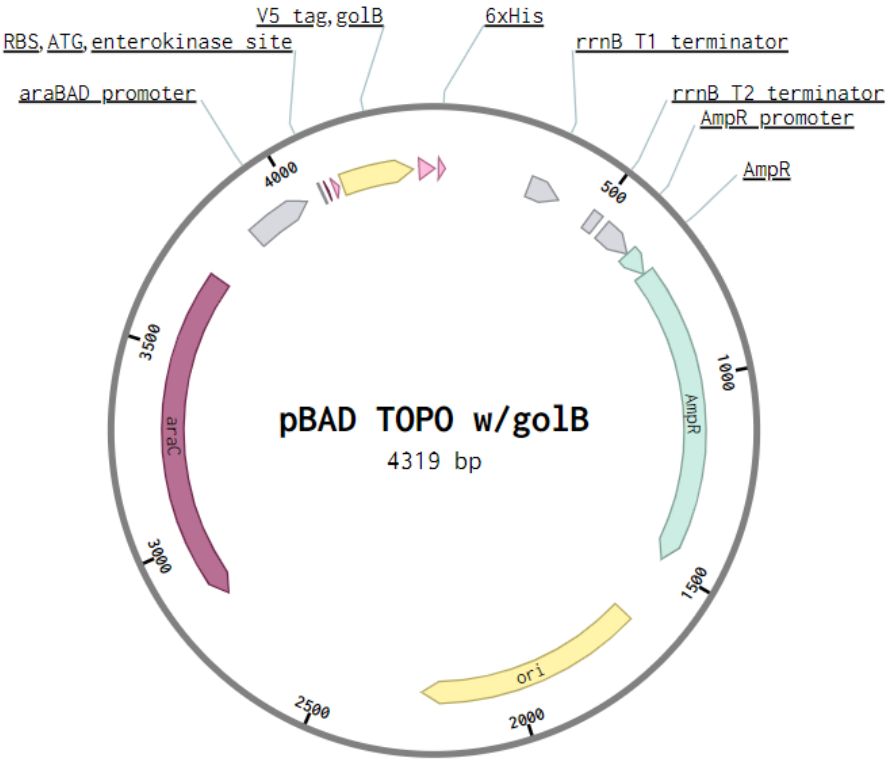

Figure 8. *golB*/pBAD plasmid. The inserted plasmid is shown, including araBAD promoter, *golB* sequence, 6x His tag, as well as the ampicillin resistance gene and promoter.

Effect of Arabinose Concentration on the Induction of GolT and GolB Expression.

GolB and GolT inoculations were grown to a log phase of OD600 = 0.5. The dot blots for GolB and GolT can be seen in Figures 9 and 10.

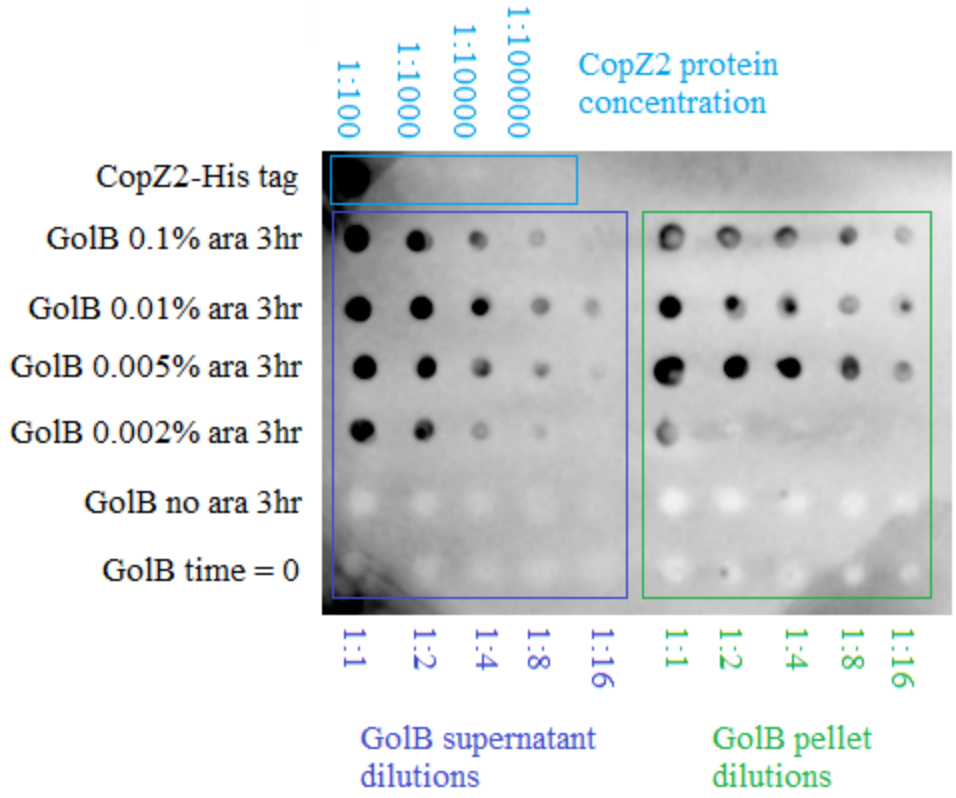

Figure 9. Dot blot of GolB Induction, Supernatant and Pellet. The first row shows the reference protein CopZ2 (Novoa-Aponte et al., 2019), a his-tagged protein of concentration 2.3 mg/ml. Different concentrations of arabinose inductions are shown on the left. Serial dilutions are marked on the bottom, at 1:1, 1:2, 1:4, 1:8, and 1:16 concentrations, all in 1 µl dots. The concentration of protein in the samples was determined to be 3.23 mg/ml and 2.28 mg/ml for the supernatant and pellet, respectively, using the Bradford assay.

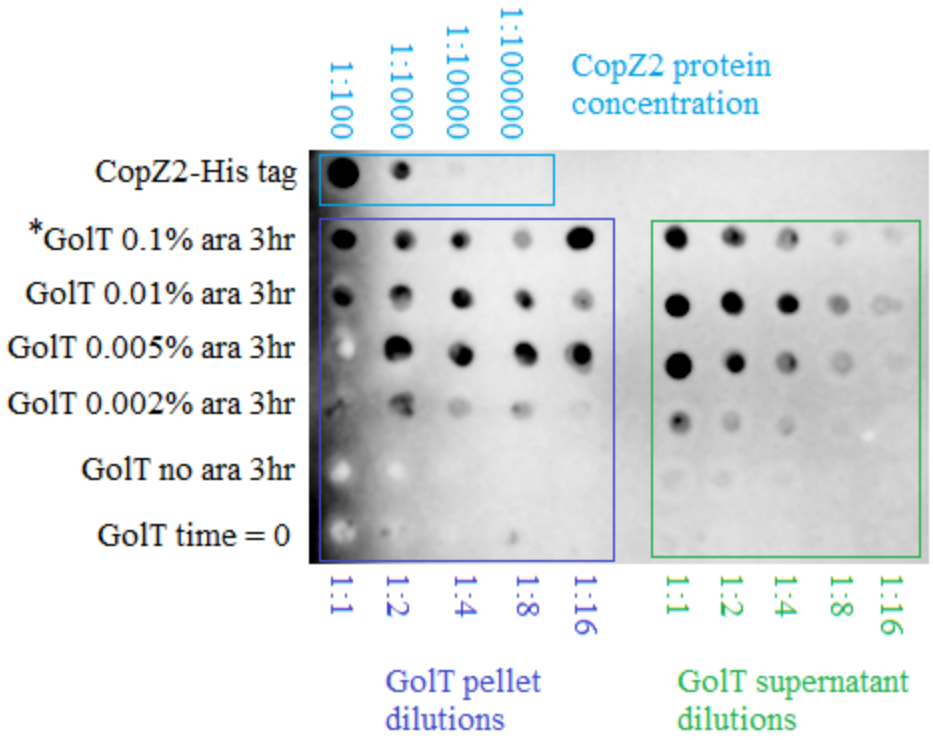

Figure 10. Dot Blot of *golT* Induction, Supernatant and Pellet. The first row shows the reference protein CopZ2 (Novoa-Aponte et al., 2019), a his-tagged protein of concentration 2.3 mg/ml. Different concentrations of arabinose inductions are shown on the left. Serial dilutions are marked on the bottom, at 1:1, 1:2, 1:4, 1:8, and 1:16 concentrations, all in 1 µl dots. The concentration of protein in the samples was determined to be 4.07 mg/ml and 2.38 mg/ml for the pellet and supernatant, respectively, using the Bradford assay.

* The GolT 0.1% pellet concentrations from left to right are actually 1:2, 1:4, 1:8, 1:16, 1:1.

The dot blot reveals the concentrations of arabinose at which the proteins can be best produced. For GolB, the highest concentration of protein in the supernatant can be found in the 0.01% arabinose induction, however, it leaves lots of protein in the pellet, suggesting insoluble protein aggregation. The 0.002% arabinose induction results in the expression of GolB in the

supernatant, while producing the smallest amount in the pellet. For the GolT membrane, 0.005% arabinose resulted in the highest protein concentration in the pellet while 0.002% resulted in the least expression in the supernatant. The GolT membrane protein should only appear in the pellet fraction, as it is not soluble. The presence of GolT in the supernatant suggests insufficient centrifugation of the sample. For both blots, the negative controls, time = 0 hrs and no arabinose samples yielded no dot at all. There is good control over the clone's expression of both GolT and GolB.

Discussion

The aim of this study was to clone the proteins GolB and GolT in expression vectors and express the proteins. The study generated *E. coli* clones for both *golT* and *golB* with controllable protein expression through the pBAD vector's arabinose induction route. The clones were sequenced and found to be free of mutations.

The study also determined the optimal concentrations of arabinose for inducing protein expression using dot blot analysis. For the *golB* clone, an arabinose concentration of approximately 0.002% was found to induce low levels of protein aggregation in the *golB* clone, which is desirable for studying protein function. The *golT* clone showed the highest expression in the pellet fraction of the sample when induced with 0.005% arabinose. The next steps in the study of the GolT and GolB proteins would be to purify and analyze the protein through western blot analysis to determine the size and stability of the protein, followed by functional studies on its impact on copper homeostasis.

In conclusion, this study generated *E. coli* clones for both GolT and GolB and determined the optimal arabinose concentrations for inducing protein expression. These findings provide a foundation for further investigations into the GolS system and its role in copper homeostasis.

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