



CuiT is a Cu importer required for metal homeostasis in *Salmonella enterica*

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ABSTRACT

Copper (Cu) is an essential micronutrient that serves as a cofactor for redox enzymes but becomes toxic when unregulated. In bacteria, while Cu efflux systems are well characterized, mechanisms of Cu import remain poorly understood. Here, we characterize the major facilitator superfamily transporter CuiT (STM1486) as a key Cu importer in *Salmonella enterica*. Comparative genomics revealed that *cuiT* is evolutionarily conserved across Enterobacteriaceae, and structural modeling predicts a 12-transmembrane-helix architecture with conserved His, Met, and Cys residues suitable for Cu⁺ coordination. Functional analyses demonstrated that deletion of *cuiT* reduces intracellular Cu accumulation, slows Cu uptake kinetics, and diminishes expression of Cu-responsive genes, including *copA*, *cueP*, *cueO*, and *golB*. Conversely, overexpression of CuiT increases intracellular Cu but sensitizes cells to Cu stress, highlighting the need for tight regulation. Kinetic modeling indicates that CuiT mediates rapid Cu import, supporting larger intracellular Cu pools compared to *Pseudomonas* influx transporters. These findings position CuiT as a central component of the *Salmonella* Cu homeostasis network, linking Cu import to transcriptional regulation, redox balance, and stress adaptation. Our work provides mechanistic insights into bacterial Cu acquisition and suggests CuiT and associated pathways as potential targets for antimicrobial strategies.

1. Significance

Copper (Cu) is essential for bacterial redox enzymes but toxic when dysregulated. While Cu efflux pathways are well studied, mechanisms of Cu import are poorly understood. We identify CuiT, a conserved major facilitator superfamily transporter, as a key Cu importer in *Salmonella enterica*. CuiT controls intracellular Cu levels, influences Cu-responsive gene expression, and maintains redox balance and stress adaptation. Disruption or overexpression of CuiT perturbs Cu homeostasis, highlighting its regulatory importance. These findings reveal a critical bacterial Cu acquisition pathway and suggest CuiT and its network as potential antimicrobial targets, advancing understanding of metal

homeostasis in pathogens.

2. Introduction

Copper (Cu) is an essential micronutrient that serves as a cofactor for electron transport proteins and redox enzymes, such as plastocyanin and cytochrome *c* oxidases [1–3]. To ensure proper incorporation into cuproproteins, organisms have evolved diverse Cu⁺ distribution mechanisms, including transporters for translocation across lipid bilayers, soluble cuprochaperones for intracellular distribution, and Cu⁺ sensing transcription regulators that control homeostasis-related gene expression [1–6]. These systems are crucial not only for delivering Cu⁺ to

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target proteins but also for preventing toxicity associated with free Cu^{+2+} ions, which can generate reactive oxygen and nitrogen species (ROS/RNS), oxidize thiol groups, and displace essential metals by binding to non-cognate sites [7,8].

Transmembrane Cu^+ -transporters play a central role in maintaining cellular Cu levels [1,4,9]. Cu^+ -ATPases, found across all domains of life, drive cytoplasmic efflux of the ion into extracellular or organellar compartments [9–11]. Many bacteria also employ the tripartite CusABC system for Cu^+ efflux [12,13]. Deletion of bacterial Cu^+ efflux systems result in easily noticeable metal sensitivity, slow growth and cell death [1,4]. However, identifying Cu^+ influx transporters has been more challenging, as their removal does not yield clear phenotypic changes. Nonetheless, multiple Cu^{+2+} import pathways have been proposed in both Gram-negative and Gram-positive bacteria [14]. For instance, *Rhodobacter capsulatus* CcoA, a transporter member of the major facilitator superfamily (MFS), functions as a Cu^+ importer required for cytochrome *c* oxidase activity, with conserved methionine (Met) and histidine (His) residues facilitating intracellular Cu^+ allocation [15]. In *Bacillus subtilis*, the YcnJ transporter, along with the putative Cu^+ -binding protein YcnI and the transcriptional repressor YcnK, are implicated in Cu^+ uptake [16–18]. Other proposed Cu^+ importers include *Enterococcus hirae* CtaA and *B. subtilis* ZosA, though further validation is needed [19,20]. Additionally, porins contribute to Cu^{+2+} influx, as observed in *Mycobacterium smegmatis*, where MspA and MspC mutants exhibit impaired Cu uptake [21–23]. In *Pseudomonas stutzeri*, the TonB-dependent NosA mediates Cu uptake for nitrous oxide reductase assembly [24,25], while its *P. aeruginosa* OprC, binds Cu^{2+} and its expression is repressed under Cu^{2+} stress [26,27]. In *Escherichia coli*, the outer membrane protein ComC was identified as a general stress response protein, although mutation of *comC* results in lower Cu^{2+} tolerance and reduced Cu^{+2+} import [28,29].

We have characterized the Cu^+ homeostasis mechanisms in the opportunistic pathogen *P. aeruginosa* [30–34]. This organism possesses well-defined efflux transporters (CopA1, CopA2, and CusABC), influx transporters (OprC and CcoA), and Cu^+ -sensing transcriptional regulators (CopRS and CueR). Transcriptome analyses following Cu^{2+} stress revealed upregulation of Cu^+ efflux transporters and downregulation of OprC [31]. Interestingly, expression of CcoA the Cu^+ importer involved in cytochrome *c* oxidase metalation remained unchanged, while the novel MFS transporter CuiT (PA5030) was repressed, hinting at a possible role in metal influx [31]. Noticeably, *cuiT* (PA5030) ortholog in *E. coli* *ynfM* was included in a respiratory screen with not observably phenotype reported, moreover that study did not measure the effect of *ynfM* deletion on Cu uptake, leaving open the possible participation of these transporters in Cu-homeostasis [35].

In this study, we investigate CuiT (STM1486) in *Salmonella enterica*, a major foodborne pathogen responsible for over 27 million infections and 200,000 deaths annually [36]. Transition metals, particularly Cu^{2+} , influence host-pathogen interactions, playing a central role in the oxidative burst as part of the innate immune response [6,37]. The Cu^+ homeostasis network in *Salmonella* includes the transcriptional regulator CueR controlling the expression of the Cu^+ -ATPase CopA, a periplasmic chaperone CueP, and the multicopper oxidase CueO [3]. Additionally, GolS, a CueR-like sensor, regulates GolB the sole apparent cytoplasmic Cu^+ chaperone, and the Cu^+ -ATPase GolT [3]. Unlike many other enteric species, the *Salmonella* core genome lacks the genes coding for the CusCFBA efflux system and seems to rely on CueP to compensate for the absence of the three partite efflux system [38,39]. CueP expression requires the joint activation of CueR and the envelope stress-induced two-component CpxRA system [40]. Also, CpxRA triggers the expression of the Cu-activated ScsABCD system, comprising thiol-oxidoreductases and membrane-linked proteins, linking Cu^+ homeostasis to redox stress resistance [6,41]. Despite these insights, Cu^{+2+} import mechanisms remain poorly understood [3,5].

To characterize *Salmonella* CuiT, we examined bacterial Cu^{2+} tolerance, gene expression, and Cu uptake in wild-type, CuiT-overexpressing,

and *cuiT* deletion mutant *Salmonella* strains. CuiT expression was reduced under Cu stress and CuiT overexpression reduced tolerance to this ion, while *cuiT* deletion impaired Cu^{+2+} uptake. Sequence analysis combined with structural modeling with Alpha Fold revealed conserved Met, His, and cysteine (Cys) residues in CuiT transmembrane fragments, congruent with Cu^+ transport function. These findings suggest CuiT facilitates periplasm-to-cytoplasm Cu^+ movement, broadening our understanding of bacterial Cu import systems.

3. Results

3.1. Evolutionary conservation and structural modeling insights into CuiT-mediated Cu-transport

Conservation of a gene and its genomic environment provide information on its likely functional importance. To this end, we performed a comparative genomic analysis of chromosomal regions harboring *cuiT* homologs (Fig. 1A). The analysis illustrates the synteny of *cuiT* and its neighboring genes across a diverse set of bacterial taxa [42]. The analysis revealed that *cuiT* is flanked by a highly conserved gene coding for a LysR family regulator across different genera, with a strong syntenic conservation in *S. enterica* and closely related *Enterobacteriaceae* species, such as *E. coli* and *Shigella flexneri*. Interestingly, *cuiT* and the *lysR*-like homolog are also present in more distantly related bacteria, including *P. aeruginosa*, *B. subtilis*, and *Burkholderia cenocepacia*. This widespread distribution across phylogenetically diverse bacterial species suggests that the role of CuiT in Cu transport may be evolutionarily conserved beyond *Salmonella*. Conversely, in more distantly related bacteria such as *Mycobacterium avium* and *Corynebacterium glutamicum*, the genomic context surrounding *cuiT* appears more divergent, possibly reflecting species-specific adaptations in Cu regulation. These findings support the hypothesis that CuiT is a key component of Cu homeostasis pathways across multiple bacterial lineages, with potential functional conservation in Cu uptake and stress response mechanisms.

The AlphaFold 3-predicted three-dimensional structure reveals that CuiT contains 12 transmembrane α -helices of archetypical MFS transporter (Fig. 1B) [43]. The hypothetical structure also shows a clear division between the outward-facing (extracellular/periplasmic) and inward-facing (cytoplasmic) regions, suggesting an alternating access mechanism where conformational changes allow Cu uptake and release into the cytoplasm. The structure contains a singular cytoplasmic transversal helix (orange in Fig. 1B) that resembles a similar structure of P-type Cu-ATPases that participates in Cu-chaperone docking [9,44]. This docking is key for the Cu transfer among transporters and chaperones [9]. Sequence conservation can be applied to identify residues that are crucial for protein functions such as catalytic activity, ligand binding, or maintaining the stability of the folded structure [45]. Analysis of the sequences of putative transmembrane segments shows highly conserved His, Met, and potentially Cys residues (Fig. 1C). These amino acids are known to participate in Cu binding and coordination in other transporters, facilitating the selective movement of the ion across the membrane [1,4,10,46–50]. In particular, His and Met residues are enriched in TM1, TM4, TM6, TM9, TM10, and TM11 (Fig. 1C). Notably, Met residues (MET104, MET146, MET162, MET174, MET251, MET314) and a His residue (HIS344) are positioned along a putative transport path, reinforcing the idea of their potential role in $\text{Cu}^+/\text{Cu}^{2+}$ coordination and translocation [1,4,49] (Fig. 1B).

3.2. CuiT enhances Cu tolerance in *Salmonella*

Bacterial Cu tolerance can be used as an early simple approach to explore the participation of a gene in Cu homeostasis. To this end, the growth rate of wild-type (WT), Δ *cuiT* mutant and complemented strains, and cells overexpressing *cuiT* under control of a *Plac* promoter were analyzed under increasing CuSO_4 concentrations. Growth curves in LB broth show that in the absence of CuSO_4 (0 mM), all strains exhibited

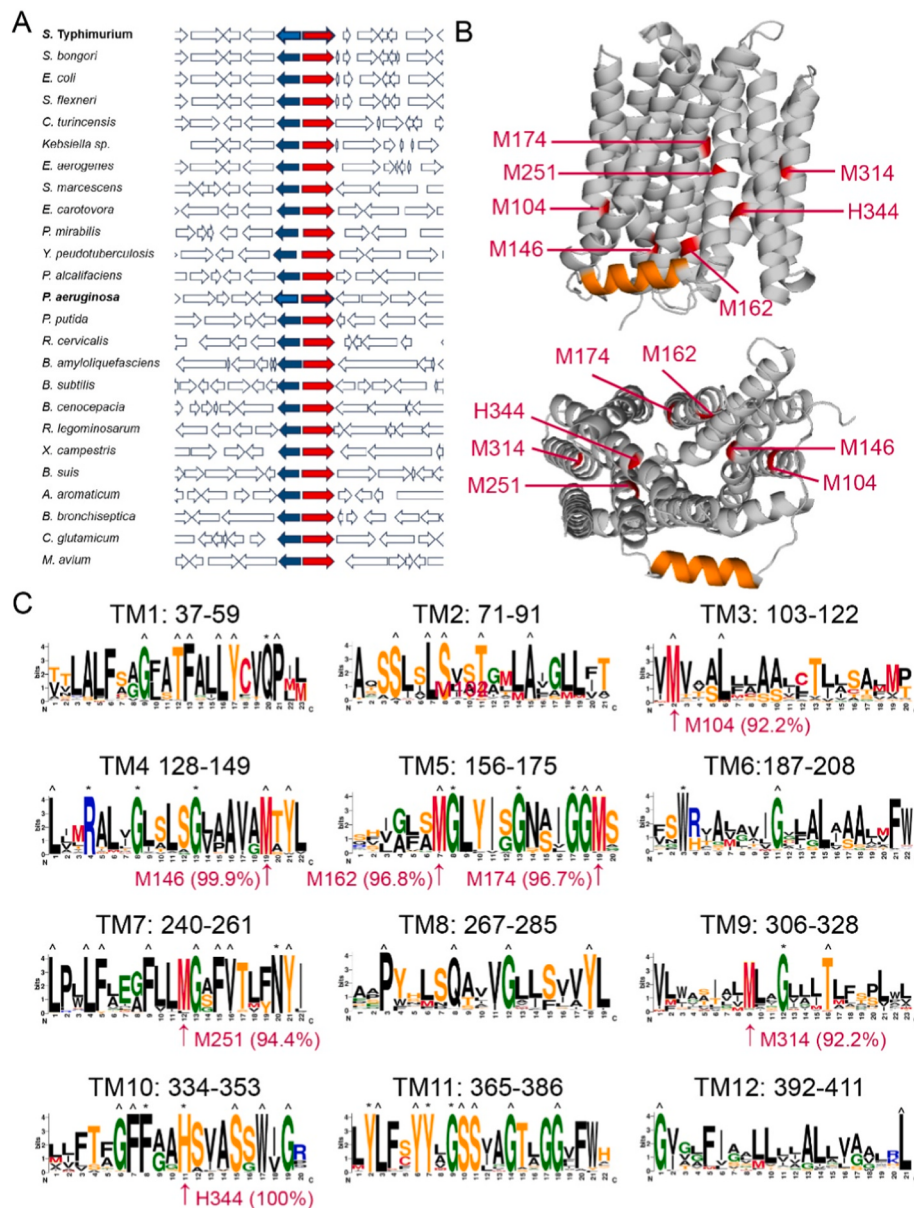


Fig. 1. Evolutionary Conservation and Structural Features of the Putative Bacterial Cu-Transport Protein CuiT. (A) Conservation of *cuiT* (red) and its putative regulatory gene (blue) across phylogenetically diverse bacterial species. Shown are representative chromosomal regions from bacteria harboring *cuiT* homologs, illustrating conserved gene synteny. Analysis was performed using the SEED Viewer platform. A single representative strain of *S. enterica* or *Salmonella bongori* is shown; all sequenced *Salmonella* genomes exhibit an identical chromosomal arrangement at this locus. (B) AlphaFold-predicted three-dimensional model of CuiT. Membrane and cytoplasmic side views. Conserved residues possibly participating of binding/transport are highlighted in red. The orange helix is a hypothetical chaperone docking site. (C) Predicted transmembrane segments of *Salmonella* CuiT. Sequences logos represent the conservation of individual residues in the 792 sequences analyzed. Annotations represent: ^ 95-99.9% and * 100% conservation and † points to conserved residues likely facing the cation transport path or Cu-binding sites.

comparable growth rates, indicating that the deletion of CuiT does not affect basal growth (Fig. 2A). However, complementation of $\Delta cuiT$ ($\Delta cuiT$ /CuiT) and overexpression of CuiT (WT/CuiT) resulted in slightly impaired growth compared to WT at 4 mM CuSO₄, suggesting that the transporter increases the entrance of Cu and therefore these strains showed a slightly lower metal tolerance (Fig. 2A). At 6 mM CuSO₄, all strains exhibited growth inhibition.

Tolerance in solid media was performed to further validate these observations (Fig. 2B). Interestingly, the overexpressing strain WT/CuiT and the complemented strain $\Delta cuiT$ /CuiT exhibited an even clearer phenotype with a markedly reduced viability on LB agar plates supplemented with 5 mM CuSO₄, whereas WT, $\Delta cuiT$ and their empty vector control (WT/pUH, $\Delta cuiT$ /pUH) showed similar growth even when

exposed to high CuSO₄. These Cu sensitivity phenotypes were consistent at both 25 °C and 37 °C. Western blot analyses of membrane and soluble cellular fractions confirm that IPTG induced CuiT expression resulted in the membrane-localized transporter, in a similar manner that a known membrane protein, the intracellular growth attenuator A (IgaA) (Fig. 2C). In summary, the data is consistent with CuiT enabling the entrance of Cu with a resulting loss of tolerance to the metal.

3.3. Expression of *cuiT* gene upon Cu stress

We reported that *cuiT* expression is repressed in *Pseudomonas* upon Cu stress [31]. A similar phenomenon was observed in *Salmonella* when the relative *cuiT* expression was assessed in cells exposed to increasing

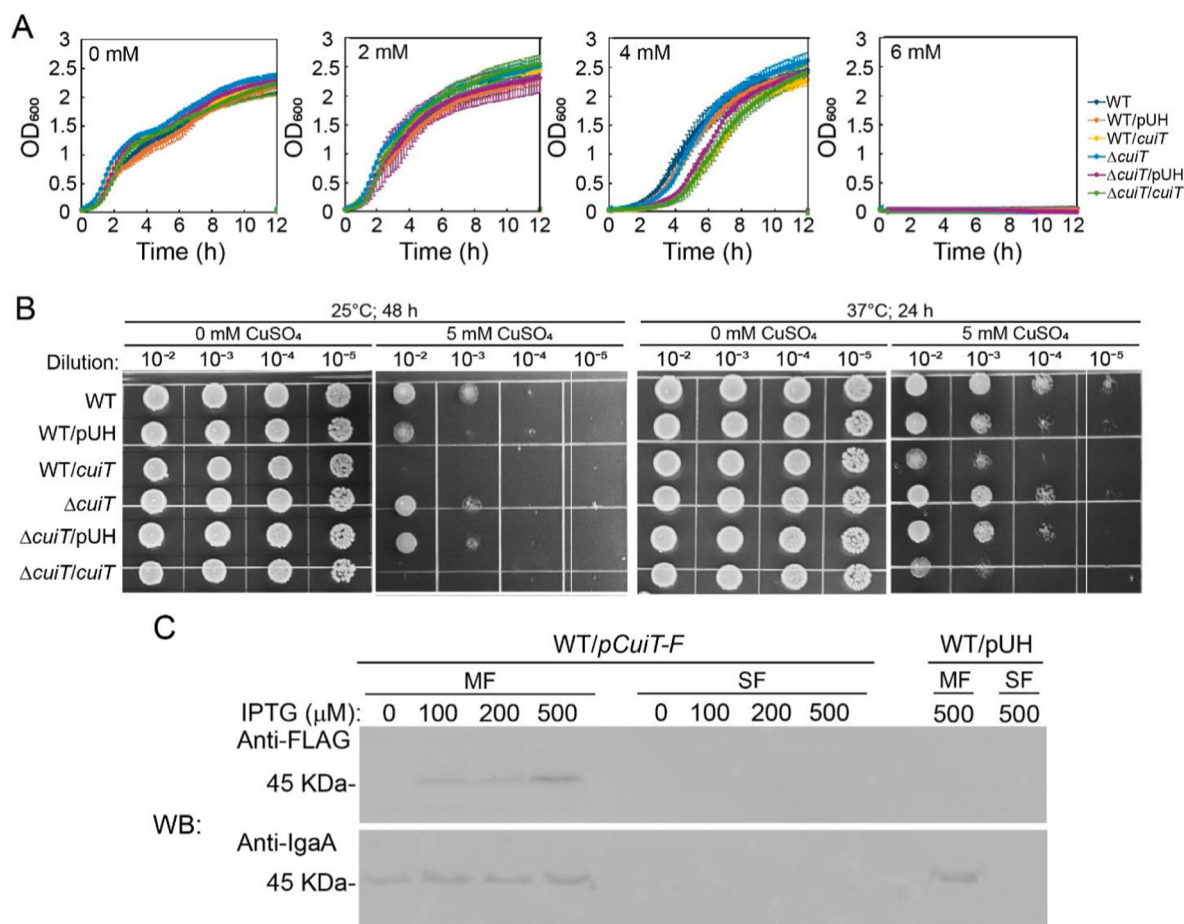


Fig. 2. CuiT confers Cu sensitivity in *Salmonella enterica* serovar Typhimurium. (A) Growth analysis of wild-type *S. enterica* WT), *cuiT* overexpressing WT (WT/CuiT), *cuiT* deletion mutant (Δ *cuiT*), complemented (Δ *cuiT*/CuiT) and empty vector controls (WT/pUH and Δ *cuiT*/pUH) strains, cultured in LB medium supplemented with increasing concentrations of CuSO_4 (0–6 mM). (B) Cu sensitivity of spot-plated serial dilutions of strains shown in (A) onto LB agar with or without 4 mM CuSO_4 . (C) Recombinant CuiT protein localizes to the membrane fraction. WT cells harboring CuiT-3 $\times$ FLAG (CuiT-F) or the empty vector (pUH) were grown, subjected to subcellular fractionation and analyzed by SDS-PAGE and immunoblotting using anti-FLAG (α -FLAG) or anti-IgaA (α -IgaA) antibodies, as indicated. The inner membrane protein IgaA, was included as a loading control and membrane marker.

concentrations of CuSO_4 for 5 min (Fig. 3A; black bars) and 60 min (Fig. 3A; gray bars). This is, *cuiT* expression was repressed by increased intracellular Cu. Testing the hypothesis that deletion of *cuiT* would reduce intracellular Cu, the expression of key Cu homeostasis genes (*copA*, *cueP*, *cueO*, *golB*, and *scsC*) in WT and Δ *cuiT* mutant strains was analyzed after 5 min of exposure to 4 mM CuSO_4 (Fig. 3B). As expected, the Δ *cuiT* mutant exhibited a marked decrease in expression across all

analyzed genes, with the most significant reduction observed for Cu chaperones *golB* and *cueP*. These findings are congruent with CuiT mediating cellular Cu influx.

3.4. CuiT modulates intracellular Cu and its uptake kinetics in *Salmonella*

Seeking a direct demonstration of the role of CuiT as a Cu importer,

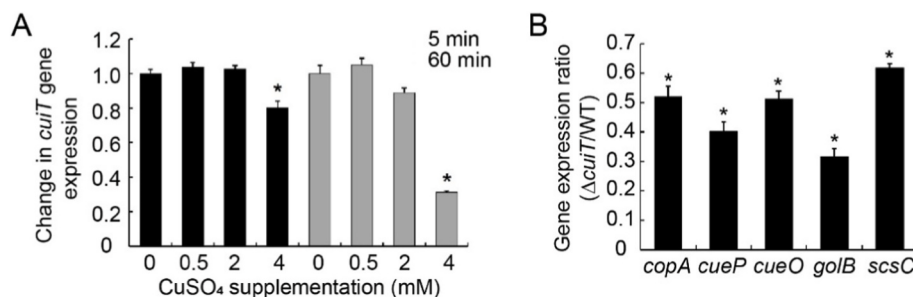


Fig. 3. CuiT expression is regulated by Cu and affects the expression of Cu-responsive genes in *Salmonella*. (A) Cu-responsive *cuiT* gene expression in wild-type (WT) cells following exposure to increasing concentrations of CuSO_4 for 5 min (black bars) or 60 min (gray bars). Data were normalized to the housekeeping gene *rnpB* and expressed relative to time 0. Data are the mean $\pm$ S.E. of three independent experiments ($n = 3$). Statistical significance was determined using an unpaired Student's *t*-test (* $p < 0.05$). (B) Cu-responsive expression of Cu homeostasis genes in Δ *cuiT* strains relative to that in WT, following treatment with 1 mM CuSO_4 for 5 min. Data were normalized to the housekeeping gene *rnpB* and expressed relative to time 0. Data are the mean $\pm$ S.E. of three independent experiments ($n = 3$). Statistical significance was determined using an unpaired Student's *t*-test ($p < 0.05$).

the Cu contents in WT and $\Delta cuiT$ mutant strains were measured. Both WT and $\Delta cuiT$ strains accumulated Cu in a dose-dependent manner under increasing CuSO_4 concentrations (0–4 mM). However, at 4 mM CuSO_4 , the $\Delta cuiT$ mutant exhibited significantly lower intracellular Cu levels compared to WT (Fig. 4A). Toward further confirming the role of CuiT, the Cu levels in complemented and CuiT-overexpressing strains were measured (Fig. 4B). As shown, $\Delta cuiT$ and $\Delta cuiT/pUH$ strains showed significantly lower Cu content. Interestingly, the complementation of $\Delta cuiT$ with a plasmid expressing CuiT ($\Delta cuiT/CuiT$) restored Cu accumulation to levels comparable to the WT, while CuiT overexpression (WT/CuiT) resulted in a marked increase in intracellular Cu. These results indicate that CuiT plays a critical role in Cu import.

To further examine the role of CuiT in Cu transport, we monitored the kinetic of Cu uptake in the WT and the $\Delta cuiT$ mutant strain exposed to 4 mM CuSO_4 . The WT cells rapidly accumulated Cu, reaching steady-state level within 15–20 min (Fig. 4C). In contrast, the $\Delta cuiT$ strain exhibited a slower Cu uptake rate with reduced overall accumulation once steady state is reached (Fig. 4C). Interestingly, the distinct steady state Cu levels can be maintained (Fig. 4D) and, as shown, it does not visibly affect the cell viability (Fig. 2A). We have previously

demonstrated that the whole cell Cu uptake kinetics and the steady state metals levels can be modeled using compartmental analysis (Fig. 5) [30]. In this approach, the transmembrane transporters are defined by Michaelis-Menten kinetics parameters ($V_{\max}$ and K_m), while Cu transfer among chaperones and cuproproteins are described by affinity (k_{ds}) and equilibrium (K_d) constant (Table 1). These parameters are integrated in a full equation that defines Cu content versus time. We initiate the fitting of our data using the model designed for *Pseudomonas aeruginosa*. However, this model performed poorly, i.e., the fitting curve did not match the experimental data. We hypothesize that the issue could be due to the inclusion of a direct transfer of Cu from the cytoplasm to the extracellular media via a CusABCF type system, since this transporter is absent in *Salmonella*. Removal of CusABCF as shown in Fig. 5 allowed fitting the curves, as well as providing support to the model strength. Further confirming the validity of the approach and the role of CuiT as the plasma membrane Cu influx transporter, the $\Delta cuiT$ strain data could be fitted by simply making the plasma membrane influx null ($v_{\text{CuiT}} = 0$).

Table 1 lists the fitting parameters for *Salmonella* and includes for reference the previously reported equivalent values observed in

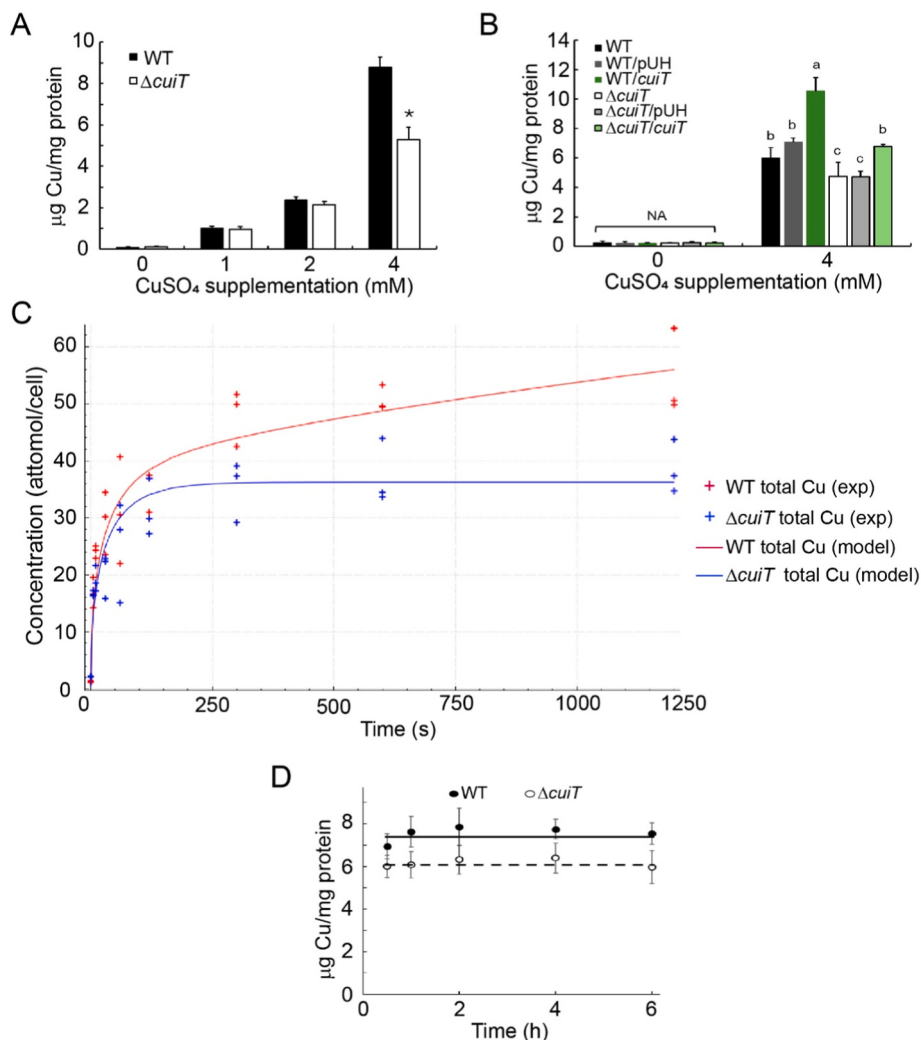


Fig. 4. CuiT regulates Cu uptake kinetics and intracellular Cu accumulation in *Salmonella*. (A) Intracellular Cu accumulation in WT and $\Delta cuiT$ mutant strains following exposure to increasing concentrations of CuSO_4 for 1 h. (B) Intracellular Cu levels in WT, $\Delta cuiT$, complemented ($\Delta cuiT/CuiT$), and *cuiT*-overexpressing strains grown in the absence or presence of 4 mM CuSO_4 for 1 h. Data are mean $\pm$ S.E. of three independent experiments. Statistical significance was determined using an unpaired two-tailed Student's *t*-test ($p < 0.05$). (C) Cu uptake kinetics in WT and $\Delta cuiT$ mutant strains following exposure to 4 mM CuSO_4 . Red and blue lines result of curve fitting using the computational compartmental distribution model described in Fig. 5. Fitting parameters are listed in Table 1. Cu-uptake into the $\Delta cuiT$ strain was modeled making the uptake through CuiT null ($v_{\text{CuiT}} = 0$). Data are the mean $\pm$ S.E. of three independent experiments ($n = 3$). (D) The intracellular Cu levels of WT and $\Delta cuiT$ mutant strain measured in an extended timeframe in the presence of 4 mM CuSO_4 . Data are the mean $\pm$ S.E. of two independent experiments.

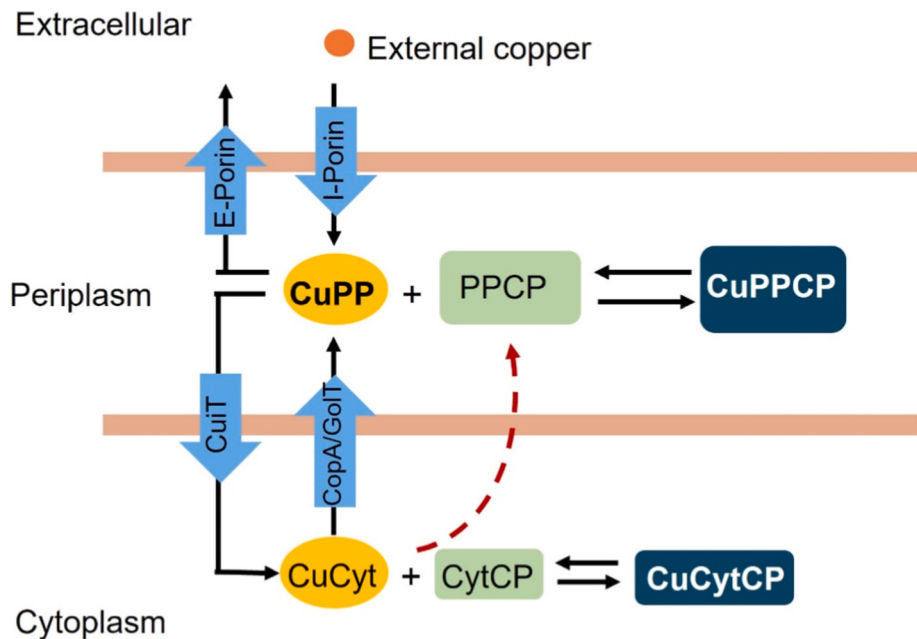


Fig. 5. Model of the Cu homeostasis network in *Salmonella*. Data of Cu uptake kinetics was analyzed with a computational compartmental distribution model [5, 30]. Our previously reported model describing the Cu uptake kinetics into *Pseudomonas aeruginosa* was modified by removing equations describing an ABCF transporter absent in *Salmonella* [50]. The model assumes the presence of outer membrane porins for influx and efflux. The plasma membrane contains influx (CuiT) and efflux (Cu-ATPases) systems. Various soluble Cu chaperones (CuPP and CuCyt) that carry Cu to target cuproproteins and membrane transporters. The model requires the upregulation (dotted line) of the periplasmic Cu pool. As a consequence of the predicted high affinity binding to all systems the model implies the absence of free Cu.

Table 1

The fit parameter values for Cu uptake kinetics. Comparison between *Pseudomonas* and *Salmonella* models.

Parameter	<i>Pseudomonas</i> *	Parameter	<i>Salmonella</i>	Unit
v_{CuiT}	4.43	v_{CuiT}	380	mM/min
K_m^{CuiT}	0.00950	K_m^{CuiT}	0.0149	mM
v_{OprC}	6.84	$v_{\text{I-Porin}}$	210	mM/min
K_m^{OprC}	1.66	$K_m^{\text{I-Porin}}$	0.252	mM
v_{CopA1}	0.713	$v_{\text{CopA/GolT}}$	100	mM/min
K_m^{CopA1}	0.000476	$K_m^{\text{CopA/GolT}}$	0.000354	mM
v_{PcoB}	3.68	$v_{\text{E-Porin}}$	200	mM
K_m^{PcoB}	0.390	$K_m^{\text{E-Porin}}$	0.605	mM
$k_{\text{ds}}^{\text{CuCytCP}}$	98.6	$k_{\text{ds}}^{\text{CuCytCP}}$	187	1/min
$k_{\text{ds}}^{\text{CuPPCP}}$	0.00126	$k_{\text{ds}}^{\text{CuPPCP}}$	0.264	1/min
K_d^{CuCytCP}	0.000218	K_d^{CuCytCP}	0.00220	mM
K_d^{CuPPCP}	2.13×10^{-7}	K_d^{CuPPCP}	0.00525	mM
$[\text{CuCytCP}]$	0.00892	$[\text{CuCytCP}]$	0.0178	mM
$[\text{CuPPCP}]$	0.419	$[\text{CuPPCP}]$	0.837	mM

* Previously reported values obtained for *Pseudomonas aeruginosa* [30].
 v are V_{max} and K_m the Michaelis constant for the corresponding transporter.
 k_{ds} are dissociation constants for Cu binding to the corresponding chaperone.
 K_d are the equilibrium constants for the metal transfer reaction between the chaperone and the corresponding cuproprotein.
 $[\text{CuCytPP}]$ and $[\text{CuPPCP}]$ are the size of the corresponding cuproprotein pools.

Pseudomonas. *Salmonella* showed a faster Cu uptake rate (v_{CuiT}) albeit with lower affinity compared to *Pseudomonas*. Additionally, *Salmonella* exhibited higher parameter values for Porin-mediated uptake. This was logically paralleled by an increase in the efflux rates by P-type ATPases and E-Porins. This means that the *Salmonella* Cu pool has a higher turnover rate. Also in agreement are the relatively higher Cu pool in *Salmonella* with and observed slightly higher K_{eq} for the metal transfer. In spite of these kinetics differences, the Cu chaperone affinities (k_{ds}) are similar in both systems, *Salmonella* and *Pseudomonas*, indicating that they operate in the absence of free/hydrated metal as expected.

4. Discussion

4.1. CuiT-dependent Cu stress phenotypes reveal a critical role in Cu acquisition

Cu homeostasis requires a precise balance between acquisition and detoxification, as both Cu deficiency and excess are detrimental to bacterial physiology [1,2,5,7]. In this study, we identify CuiT as a key determinant of Cu stress phenotypes in *S. enterica*. Unlike canonical Cu efflux systems, whose deletion leads to dramatic sensitivity to the ion and growth defects [1,4,5,12], loss of CuiT did not impair basal growth or gross Cu tolerance under moderate Cu stress. Instead, CuiT overexpression or complementation under Cu stress conditions led to heightened Cu sensitivity, as evidenced by impaired growth and reduced viability in spot assays. The relatively high Cu concentrations used here were selected to resolve the contribution of CuiT under defined acute Cu stress conditions, a common strategy to identify metal-homeostasis determinants. These conditions should therefore be interpreted as experimental stress tests rather than as a quantitative proxy for all Cu exposures experienced by *Salmonella* during infection. In any case, these phenotypes are consistent with CuiT functioning primarily in Cu acquisition rather than export. The observed sensitivity associated with CuiT overexpression likely reflects excessive intracellular Cu accumulation beyond the buffering capacity of the cellular Cu-handling network. This interpretation is supported by the elevated intracellular Cu levels detected in the CuiT-overexpressing strain compared to WT cells. Such Cu overload can exacerbate oxidative damage, thiol oxidation, and metal mismetallation, processes well known to underlie Cu toxicity [5,7,8]. Thus, while CuiT-mediated Cu import is beneficial under Cu-limiting or physiological conditions, its dysregulation becomes detrimental under high Cu stress, emphasizing the necessity for tight control of Cu influx pathways.

Notably, the Cu stress phenotypes associated with CuiT differ from those reported for *Pseudomonas*, where deletion of the CuiT homolog conferred enhanced Cu tolerance in solid media [31]. These differences

likely reflect species-specific adaptations in Cu handling networks, particularly given that *Salmonella* lacks the CusCFBA efflux system and relies heavily on CueP and other periplasmic mechanisms to buffer Cu stress [40,51]. Deletion of *cuiT* resulted in a broad attenuation of Cu-responsive gene expression, including *copA*, *cueP*, *cueO*, *golB*, and *scsC*, indicating that this transporter is required for full activation of the Cu homeostasis network under Cu stress. The most pronounced reductions were observed for the Cu chaperones *golB* and *cueP*, suggesting that impaired Cu import in the Δ *cuiT* mutant limits intracellular Cu availability necessary to activate CueR- and GolS-dependent transcriptional responses. These findings support a model in which CuiT-mediated Cu uptake functions upstream of Cu-sensing regulators, ensuring sufficient intracellular Cu to trigger protective and adaptive gene expression programs while coordinating metal export, chaperoning, and redox stress resistance pathways [5,38,52]. Thus, CuiT emerges as a contributor to Cu stress phenotypes in *Salmonella*, functioning at the intersection of Cu acquisition and toxicity.

4.2. Integration of CuiT into the *Salmonella* Cu homeostasis network

Multiple lines of evidence from this study establish CuiT as a central component of the *Salmonella* Cu homeostasis network. Comparative genomic analyses revealed that CuiT is highly conserved across *Enterobacteriaceae* and present in diverse bacterial taxa, often within conserved genomic neighborhoods [42]. AlphaFold 3 structural modeling predicts that CuiT presents a 12-transmembrane-helix topology, characteristic of major facilitator superfamily transporters, forming a central channel compatible with an alternating-access transport mechanism [43]. CuiT proposed topology closely resembles the established Cu importers CcoA in *R. capsulatus* and YcnJ in *B. subtilis* [15,16,53]. Consistently, conserved His, Met, and Cys residues are enriched within key transmembrane segments and are strategically positioned along the predicted transport pathway, foreseen from established principles of Cu⁺ coordination and translocation [46–49]. The conservation of both sequence and structural features across bacterial lineages indicates that CuiT represents an evolutionarily maintained solution for Cu acquisition, reinforcing its functional importance in bacterial Cu homeostasis. CuiT evolutionary conservation suggests a broadly preserved role in bacterial Cu physiology, analogous to other characterized Cu importers such as CcoA and YcnJ [15–17]. Nevertheless, our data do not exclude the possibility that CuiT may transport additional substrates or operate with broader selectivity under some conditions. Because the present work was motivated by Cu-responsive phenotypes and regulation, we focused specifically on copper; defining the full substrate range of CuiT will require direct testing with other metal ions or related ligands.

Functionally, deletion of CuiT resulted in significantly reduced intracellular Cu accumulation under Cu stress, while complementation restored Cu levels to those observed in WT cells. Moreover, *cuiT* overexpression led to Cu accumulation far exceeding control levels, directly implicating the protein in Cu import or retention. Kinetic analyses further demonstrated that CuiT is required for rapid Cu uptake during the early phases of Cu exposure, with the Δ *cuiT* mutant displaying delayed and diminished Cu accumulation. These findings indicate that CuiT contributes to both the rate and magnitude of intracellular Cu acquisition. Strikingly, kinetic modeling revealed that CuiT-mediated Cu uptake in *Salmonella* occurs at a substantially higher rate than CcoA-mediated uptake in *P. aeruginosa*, accompanied by a large intracellular Cu pool. This enhanced uptake capacity may reflect the adaptation capacity of *Salmonella* to host-associated environments, where Cu is deployed as an antimicrobial weapon during the oxidative burst [6,31, 51]. This divergence likely reflects adaptation to host-associated environments, where *Salmonella* encounters fluctuating and often elevated Cu concentrations as part of the innate immune response [6]. In contrast, Cu importers in environmental bacteria such as *R. capsulatus* and *B. subtilis* may be optimized for basal Cu acquisition to support cuproenzyme assembly rather than rapid Cu influx under stress. Thus,

CuiT appears to represent an evolutionarily conserved yet functionally specialized Cu importer, tuned to meet the distinct Cu demands imposed by pathogenic lifestyles.

Efficient Cu acquisition may be essential not only for cuproenzyme metalation but also for sustaining Cu-dependent defense systems, such as SodCI and SodCII, whose activity is supported by CueP-mediated Cu delivery [38,52]. Consistent with this model, loss of CuiT resulted in reduced expression of multiple Cu-responsive genes, including *copA*, *cueP*, *cueO*, *golB*, and *scsC*. This coordinated downregulation suggests that CuiT-dependent Cu import is necessary to activate Cu-sensing regulatory circuits, likely through CueR- and GolS-mediated transcriptional responses [38]. Thus, CuiT acts upstream of transcriptional and enzymatic components of Cu homeostasis, positioning it as a key gatekeeper of intracellular Cu availability in *Salmonella*.

This analysis of CuiT function is also compatible with comparisons to *E. coli* YnfM, the closest annotated homolog of STM1486. Khalfaoui-Hassani et al. (2023) included *ynfM* in a study of respiratory assembly but did not directly assay Cu uptake or intracellular Cu accumulation [15]. Therefore, the absence of a strong phenotype in that context should not be taken as evidence against a Cu-related function, but rather in a role in Cu oxidases assembly.

4.3. Cu-dependent control of CuiT expression and transport activity

The expression and activity of Cu import systems must be tightly regulated to prevent Cu toxicity, and our data indicate that CuiT is subject to Cu-responsive regulation. Exposure to high Cu concentrations led to a rapid and sustained decrease in *cuiT* transcript levels, suggesting that CuiT expression is actively repressed under Cu stress. This regulatory behavior mirrors that of other Cu importers, such as *oprC* in *Pseudomonas*, whose expression is downregulated in response to elevated Cu levels [26,27,31]. Such repression likely serves as a protective mechanism to limit further Cu influx when intracellular Cu is already abundant. The repression of Cu-responsive genes in the Δ *cuiT* mutant suggests that CuiT-mediated Cu import is required to reach intracellular Cu thresholds necessary for activation of Cu-sensing regulators such as CueR and GolS [38]. In this context, CuiT does not merely function as a passive transporter but plays an active role in shaping Cu-dependent regulatory responses. Downstream, CueP and the ScsABCD system may buffer and redistribute imported Cu, linking CuiT activity to periplasmic Cu handling, redox balance, and stress resistance in the absence of the CusCFBA efflux system [6,38,52]. Thus, the integration of CuiT-mediated import with transcriptional control ensures that Cu uptake is dynamically tuned to environmental availability and cellular demand.

Collectively, our findings identify CuiT as an evolutionarily conserved Cu importer that plays a critical role in *Salmonella* Cu homeostasis. CuiT facilitates rapid Cu uptake, supports activation of Cu-responsive gene expression, and must be tightly regulated to avoid Cu toxicity. Given the central role of Cu in host-pathogen interactions and innate immune defense [5,54], CuiT likely contributes to *Salmonella* fitness during infection. These results expand the current framework of bacterial Cu homeostasis by highlighting Cu import, rather than efflux alone, as a crucial determinant of Cu stress adaptation in pathogenic bacteria. CuiT and its associated regulatory networks represent promising antimicrobial targets that exploit vulnerabilities in bacterial Cu homeostasis while minimizing effects on host metal metabolism.

5. Materials and methods

5.1. Bacterial strains and culture conditions

Bacterial strains and plasmids applied in this study are listed in Supplemental Table 1. Bacterial cells were routinely cultured at 37 °C in Luria-Bertani (LB) broth or on LB-agar plates, except when indicated. When necessary, the culture medium was supplemented with

chloramphenicol at 34 µg/ml and/or ampicillin at 100 µg/ml. When indicated 0.5 µM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to express CuiT from a plasmid. Cu sulfate salt used were of ACS analytical grade ≥98.0% purity.

5.2. Mutant strain construct

The ATCC 14028s Δ cuiT derivative was generated by Lambda Red-mediated recombination using the primers listed in [Supplemental Table 2](#) and previously described protocols [41]. Then, the *cuiT::Cm^R* modification was moved to non-modified 14028s strain by P22-mediated transduction. DNA fragments and plasmids were introduced into bacterial cells by electroporation. All constructs were verified by DNA sequencing.

For generation of CuiT and CuiT-F plasmids, the *cuiT* (STM1486) open reading frame was amplified by PCR from the chromosome of the WT 14028s strain using the oligonucleotides listed in [Supplemental Table S2](#). The product containing *cuiT* was digested with *Bam*HI/*Hind*III and cloned into the pUH21-2lacI^q plasmid digested with these enzymes. The plasmids were initially introduced into competent *E. coli* Top10 cells. Positive clones were verified by colony PCR and DNA sequencing.

5.3. Western blot analysis

Western blot analysis of 3xFLAG-tagged CuiT was done as described previously [5]. Briefly, 14028s cells carrying either the empty vector or the CuiT-3xFlag plasmid were grown over-night at 37 °C in LB broth supplemented with 100 mg/ml ampicillin. When the cultures reached OD₆₀₀ ~0.5, 0.2 mM IPTG was added and cell incubated overnight. Cells were harvested by centrifugation at 6000 rpm for 10 min, washed and resuspended in 1/10 of the original volume of 50 mM Tris-HCl pH 8, 5 mM EDTA, 10% Glycerol solution supplemented with 1 mM phenyl-methylsulfonyl fluoride (PMSF) and 50 mM β-mercaptoethanol. Cell suspensions were sonicated on ice centrifuged at 6000 rpm for 10 min at 4 °C to eliminate non-disrupted bacteria. The supernatant was subjected to ultracentrifugation for 60 min at 45,000 rpm at 4 °C to obtain soluble and insoluble (membrane) fractions. The pellets were solubilized in buffer containing 50 mM Tris-HCl pH 8, 5 mM EDTA, 10% Glycerol and 2% SDS. Pierce BCA Protein Assay Kit (Thermo Scientific) was used to determine protein concentration in total, soluble or insoluble extracts following the manufacturer's protocols. Aliquots containing 20 µg of protein were analyzed in 15% (w/v) SDS polyacrylamide gels. Two gels processed in parallel were transferred to nitrocellulose membranes (Amersham Protran Premium 0.45 µm NC, Cytiva) for either CuiT-3xFLAG or IgaA immunodetection, using mouse anti-FLAG monoclonal (ab125243, Abcam) or rabbit anti-IgaA (ab97216, Abcam) polyclonal antibodies, respectively. Blotted membranes were incubated with the corresponding secondary antibodies conjugated with horseradish peroxidase (HRP; AP106P and 12-349, Sigma). Immunoreactive bands were revealed using the Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific) and registered in a ChemiDoc™ XRS Imaging System (Bio-Rad).

5.4. Cu sensitivity assay

Bacterial cultures at the mid-log phase (OD₆₀₀ = 0.5-0.6) were diluted in LB medium to achieve an initial OD₆₀₀ = 0.05. Following dilution, the cultures were supplemented with varying concentrations of CuSO₄, ranging from 0 to 6 mM. Cell growth in 0.2 ml liquid medium was tracked over 16 h at 37 °C with continuous agitation using an Epoch 2 microplate spectrophotometer (BioTek) [4]. OD₆₀₀ readings were taken regularly to evaluate bacterial growth dynamics. Cu tolerance in solid media was assessed using LB-agar plates supplemented with increasing concentrations of CuSO₄. Mid-log phase bacterial cultures (OD₆₀₀ = 0.5-0.6) were diluted to an OD₆₀₀ = 0.1 and further serially

diluted down to an OD₆₀₀ of 10⁻⁶ in LB. Subsequently, 10 µl of each dilution was spot plated onto the LB-agar plates. The plates were incubated at either 25 °C or 37 °C to allow colony growth [55].

5.5. Gene expression analysis

Salmonella WT and Δ cuiT strains in mid-log phase were grown during 5 or 60 min at 37 °C in LB medium supplemented with the indicated CuSO₄ with 0, 0.5, 2 and 4 mM of CuSO₄ at 37 °C. Aliquots of 0.5 mL were stabilized with RNA protect Bacteria Reagent (Qiagen), RNA was isolated with RNeasy Mini Kit (Qiagen), treated with DNase (Qiagen). RNA (500 ng) was used for cDNA synthesis using the iScript™ gDNA Clear cDNA Synthesis Kit (Bio-Rad). Quantitative PCR (qPCR) were carried out using 25 ng of cDNA in a LightCycler® 96 System (Roche) with AzuraView™ GreenFast qPCR Blue Mix LR (Azura Genomics) in a 20 µl final volume, using 0.8 µM of each primer. *rnpB* was used as the reference gene. Gene-specific primers are listed in [Supplementary Table 2](#) [55,56].

5.6. Whole-cell Cu uptake assays

Cu levels were determined by atomic absorption spectroscopy (AAS), as described [31]. Briefly, bacterial cells in mid-log phase were incubated in LB medium supplemented with varying concentrations of CuSO₄ during the times indicated in the figures. Subsequently, a fraction of the cell suspension (100 µl) was reserved for protein quantification using the Bradford method [57], while the remaining portion (1 ml) was passed through LabExact Mixed Cellulose Ester (MCE) membrane filters (0.45 µm pore size, 25 mm diameter, Global Industrial, Model #: WBB2684613) under vacuum conditions. The filters were subsequently washed twice with 150 mM NaCl (30 ml) under vacuum. Following filtration and washing, the filters were air-dried and transferred into cap crewed Micretubes (Simport #T339). The cells with the filters were mineralized using fuming HNO₃ (trace metal grade; 400 µl) at 80 °C for 60 min. After cooling, the mineralized samples were treated with 10 M H₂O₂ (200 µl) and ddH₂O (400 µl) for an additional 60 min at room temperature. Resulting samples were analyzed by AAS.

5.7. Kinetic analysisC

Kinetic analysis and parameter fitting were carried out with the software COPASI (v. 4.45; [58]), using a kinetic model based on that previously described for *Pseudomonas* [30] as described in the legend of [Fig. 5](#).

5.8. Bioinformatics analysis

NCBI Reference Sequence WP_000091796.1 was used to Blast the proteins with NCBI RefSeq Select proteins ([Supplemental Table S3](#)). The resulting 1000 sequences with higher similarity were screened removing redundant and incomplete sequences. The selected 792 sequences were aligned in MuscleWS. The alignment was visualized in Jalview Version 2.11.3.2 [59] and the sequence logos were generated in WebLogo [60, 61]. The structure of the protein was predicted in AlphaFold 3 [43]. The structure was visualized and modeled in ChimeraX-1.7.1 [62–64][65], [66]).

CRediT authorship contribution statement

Zhenzhen Zhao: Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – review & editing. **Karla F. Díaz Rodríguez:** Investigation, Methodology, Writing – review & editing. **Andrea A.E. Mendez:** Investigation, Methodology, Writing – review & editing. **Lisandro M. Sommer:** Investigation, Methodology, Writing – review & editing. **Pedro Mendes:** Formal analysis, Methodology, Validation, Visualization, Writing – review & editing. **Fernando C. Soncini:**

Data curation, Formal analysis, Investigation, Supervision, Visualization, Writing – review & editing. **Susana K. Checa:** Supervision, Writing – review & editing. **Teresita Padilla-Benavides:** Funding acquisition, Writing – original draft, Writing – review & editing. **José M. Argüello:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The author is an Associate Editor for Biochemistry and Biophysics Reports and was not involved in the editorial review or the decision to publish this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrep.2026.102581>.

Data availability

Data will be made available on request.

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