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RESEARCH ARTICLE OPEN ACCESS

High-Throughput Tn-Seq Screens Identify Both Known and Novel *Pseudomonas putida* KT2440 Genes Involved in Metal Tolerance

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ABSTRACT

Industrial and urban activities release toxic chemical waste into the environment. *Pseudomonas putida*, a soil bacterium, is known to degrade hydrocarbons and xenobiotics, and possesses numerous genes associated with heavy metal tolerance. Most studies on metal tolerance in *P. putida* focus solely on over- or underexpressed genes, potentially overlooking important genes with unchanged expression. This study employed a Tn-seq approach to identify the essential genes required for *P. putida* growth under metal stress. This method enables the identification of mutants with altered fitness in the presence of excess metals. The screen successfully identified a number of known genes implicated in metal resistance, including *czcA-1*, *cadA-3*, *cadR*, and *pcoA2*, thereby validating the approach. Further analyses using targeted mutagenesis and complementation assays revealed *PP_5337* as a putative transcriptional regulator involved in copper tolerance and the two-component system *RoxSR* (*PP_0887/PP_0888*) as a key determinant of cadmium tolerance. Additionally, *PP_1663* and *PP_5002* were identified as contributing to cadmium and cobalt tolerance, respectively. This study provides the first evidence linking these genes to metal tolerance, highlighting gaps in our understanding of metal tolerance mechanisms in *P. putida* and demonstrating the utility of Tn-seq for identifying novel tolerance determinants.

1 | Introduction

Pseudomonas putida is a ubiquitous saprophytic bacterium that can utilise various sources of carbon and energy. This soil microorganism has been widely used as an experimental model to study the biodegradation of aromatic compounds or hydrocarbons (Nelson et al. 2002; Wu et al. 2011). It can colonise

various habitats and has been isolated from water, soil and the plant rhizosphere, sometimes polluted by various compounds (Clarke 1982; Nelson et al. 2002). The analysis of its complete genome revealed that *P. putida* possesses a wide range of genes that are involved in metal homeostasis or tolerance. This suggests that the bacteria can survive in metal-polluted environments (Cánovas et al. 2003).

Laura Kergoat, Stefanie Lutz, Charlotte Oriol, Nicolas Parisot and Christian Schori contributed equally.

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Metals play a crucial role in several cellular processes of microorganisms. Certain metals, such as nickel, cobalt, copper and zinc, are essential nutrients. They function as stabilisers of protein structures or bacterial cell walls, as catalysts for biochemical reactions, and help maintain osmotic balance (Bruins et al. 2000; Chandrangsu et al. 2017). However, some metals can be highly toxic to cells. Metal toxicity can occur in various ways, such as oxidative damage caused by the production of reactive oxygen species, DNA damage, and protein damage due to the displacement of essential metals from their native binding sites or binding to respiratory enzymes (Bruins et al. 2000). While some metals are essential for cellular function, they can also be toxic when present in excess. Therefore, it is crucial to tightly regulate the concentration of metal ions in cells to maintain optimal cellular activity. To maintain metal homeostasis, bacteria use various systems, such as metal uptake/efflux, chelation, or sequestration (Bruins et al. 2000; Chandrangsu et al. 2017). Bacteria have developed sophisticated mechanisms to survive in environments contaminated with heavy metals, which can be toxic at high concentrations. Two key strategies—metal resistance and metal tolerance—allow bacteria to either neutralise or endure these toxic substances. Metal resistance involves active processes that bacteria use to detoxify or expel metals, enabling them to thrive in environments with high metal concentrations. For example, several metal resistance systems have been identified in *P. putida* that confer resistance to heavy metals such as cadmium, zinc, and cobalt, such as P-type ATPases (e.g., CadA for cadmium resistance or CzcA for cadmium, zinc, and cobalt resistance) and sequestration proteins (e.g., CopA for copper resistance) (Cánovas et al. 2003; Miller et al. 2009; Manara et al. 2012). On the other hand, metal tolerance refers to the ability of bacteria to withstand metal exposure without actively neutralising or removing the metals, often by entering a dormant state or employing passive mechanisms to mitigate their toxicity. For instance, the production of siderophores by a bacterium can be indirectly linked to tolerance to a toxic metal. Siderophores, although primarily designed to chelate iron, can also interact with other toxic metals such as copper, cadmium and zinc. Pyoverdine produced by *Pseudomonas aeruginosa* has been shown to chelate various toxic metals, thereby reducing their toxicity to the bacteria (Braud et al. 2010; Schalk et al. 2011; Schalk and Guillon 2013). Understanding these adaptations is essential for applications such as bioremediation, where bacteria can be harnessed to clean up metal-polluted environments.

Regarding metal stresses, a complete genome analysis has shown that the *P. putida* genome contains 61 open reading frames that are probably involved in metal tolerance or homeostasis, and seven more that are possibly involved in metal resistance (Cánovas et al. 2003). Proteomic or transcriptomic technologies have been used to investigate the response of *P. putida* to inhibitory concentrations of various metals (Cheng et al. 2009; Miller et al. 2009; Manara et al. 2012; Ray et al. 2013; Peng et al. 2018). These studies indicated that a significant number of genes in *P. putida* are responsible for maintaining homeostasis, as well as tolerating and resisting various metals.

Although omics approaches (proteomics and transcriptomics) are considered powerful, they have limitations in terms of

detectability and typically only detect genes whose expression levels significantly change between two conditions. These analyses may thus miss important factors not affected by gene/protein level changes. Therefore, more comprehensive screens are needed to identify new factors and ideally complete sets of genes involved in metal tolerance in *P. putida*. The screening of *P. putida* CD2 mutants obtained by Tn5 insertions was also conducted in the presence of cadmium, which confirmed and completed the overview of the *P. putida* stress responses (Hu and Zhao 2007; Molina-Henares et al. 2010). However, the low saturation levels of the Tn5 libraries suggest that some genes may have been missed during these analyses. Additionally, the number of tested mutants was limited by the need to test each individual mutant in every condition.

To gain a more comprehensive understanding of the genes necessary for metal tolerance in *P. putida*, we utilised a high-throughput sequencing of a saturated transposon library (Tn-seq) (van Opijnen et al. 2009; van Opijnen and Levin 2020) in this study to screen tens of thousands of random insertion mutants of *P. putida* in the presence of excess amounts of metal ions. Tn-seq is a powerful method that has successfully been used to characterise essential genes in various conditions and many different species. For example, it has been used to identify essential genes for human gut colonisation of *Bacteroides thetaiotaomicron* (Goodman et al. 2009), mouse colonisation by human pathogens such as *Vibrio cholerae*, *P. aeruginosa*, or *Streptococcus pneumoniae* (van Opijnen et al. 2009; Fu et al. 2013; Skurnik et al. 2013), plant colonisation by phytopathogens (Helmann et al. 2019; Royet et al. 2019; Morinière et al. 2022), tobramycin resistance genes of *P. aeruginosa* (Gallagher et al. 2011), toxic compound resistance genes in *P. putida* (Calero et al. 2017) and identification of genetic targets for improved tolerance of *P. putida* towards compounds relevant to lignin conversion (Borchert et al. 2023). This technique has also been employed to identify gold, silver, and copper tolerance genes in *Burkholderia cenocepacia* (Higgins et al. 2020; Gualdi et al. 2022). However, this technology has not yet been employed to discover genes involved in metal homeostasis and tolerance in *P. putida* KT2440. By applying Tn-seq to screen a *P. putida* KT2440 mutant library in the presence of metals in the culture medium, we identified numerous genes required for growth in a culture medium rich in cobalt, copper, zinc (essential metals), or cadmium (a non-essential metal). Among them were *czcA-1* (PP_0043), *cadA-3* (PP_5139), *cadR* (PP_5140) and *pcoA2* (PP_5380), which are already known to be involved in zinc, cadmium, and copper resistance, respectively, thereby validating the approach employed. In addition, we discovered several genes that were previously not associated with metal homeostasis and validated them through in-frame deletion and complementation assays. Our findings demonstrate that PP_1663 (Cd²⁺), *roxSR* (Cd²⁺), PP_5337 (Cu²⁺), and PP_5002 (Co²⁺) all are involved in metal tolerance in *P. putida*.

2 | Experimental Procedures

2.1 | Bacterial Strains and Growth Conditions

Bacterial strains, plasmids and oligonucleotides used in this study are described in Table S1 and Table S2. During the course of the project, we decided to re-sequence the genome of

our *P. putida* KT2440 strain present in our collection, referred to as PP1 (see Supporting Information S1). The genome is registered under Genbank accession CP036494. The Average Nucleotide Identity between this strain and *P. putida* KT2440 (Genbank AE015451.2) is 100% (<http://enve-omics.ce.gatech.edu/ani/>). The PP1 is thus referred to as the KT2440 strain in the article.

P. putida and *Escherichia coli* cells were grown at 28°C and 37°C respectively in LB medium or 2YT medium. When required, antibiotics were added at the following concentrations: ampicillin, 100 µg/L; gentamicin, 30 µg/L for *P. putida* and 7 µg/L for *E. coli*; streptomycin, 100 µg/L. Media were solidified with 1.5 g/L agar. During Tn-seq experiments, metals were used at a subinhibitory concentration: CoCl₂ 10 µM, ZnCl₂ 125 µM, CuCl₂ 2.5 mM, CdCl₂ 12.5 µM.

2.2 | Construction of the Transposon Library

P. putida strain KT2440 and *E. coli* MFDpir/pEGL55 were grown overnight in 2YT medium. pEGL55 is a R6K suicide plasmid carrying the mariner transposon. 100 OD_{600nm} units of each strain were mixed and centrifuged at 5000 × g for 10 min. The bacteria were resuspended in 1.2 mL of 2YT medium supplemented with diamminopimelic acid (300 µM) and plated on an over-dried LB agar plate containing twice the normal concentration of agar. After 3 h at 28°C, bacteria were collected and resuspended in 4 mL LB medium. A 20 µL aliquot was diluted and plated on LB agar with gentamicin to estimate the efficiency of mutagenesis. The other part was spread on 50 plates of LB agar with gentamicin and grown for 24 h at 28°C. To confirm that the *P. putida* mutants had lost the plasmid, we performed colony PCR with primers annealing to the *bla* gene of pEGL55. None of the 100 colonies tested produced a PCR fragment, indicating loss of the plasmid in the bacteria tested. 800,000 mutants were harvested in LB supplemented with 40% glycerol at -80°C. This library was directly sequenced and represents the mutant pool in LB agar (see Table S3).

2.3 | DNA Preparation for High-Throughput Sequencing

To identify essential genes in LB or LB with metal, ~10⁷ mutants were inoculated in 25 mL LB. The culture was then incubated at 28°C with shaking at 180 rpm. At OD₆₀₀ of 0.2, metals were added independently at the following subinhibitory concentrations: cobalt 10 µM, copper 2.5 mM, zinc 125 µM, and cadmium 12.5 µM. When OD₆₀₀ was 1.6, the culture medium was diluted in the same medium with OD₆₀₀ of 0.03. This procedure was carried out for 12 generations. The final pools of mutants were harvested by centrifugation of the culture medium and stored at -80°C. DNA was extracted from aliquots of the bacterial suspension using the Promega Wizard Genomic DNA Purification Kit. The next steps of the DNA preparation methods were performed as described previously (Royet et al. 2019). Quality control of Tn-seq DNA libraries (fragment size and concentration) and high-throughput sequencing on HiSeq 2500 (Illumina) were performed by MGX (CNRS sequencing service, Montpellier). 6 DNA libraries were multiplexed on a flow cell.

After demultiplexing, the total number of reads ranged from 19 to 35 million (Table S3).

2.4 | Bioinformatics Analysis

Raw reads from the fastQ files were first filtered using cutadapt v1.11 (Martin 2011) and only reads containing the mariner inverted left repeat (ACAGGTTGGATGATAAGTCCCCGGTCTT) were trimmed and considered bona fide transposon disrupted genes. The trimmed reads were then analysed using a modified version of the TPP script available in the TRANSIT software v2.0.2 (26447887). The mapping step was modified to select only reads that mapped uniquely and without mismatch in the *P. putida* KT2440 genome. The counting step was then modified to accurately count reads mapping to each TA site in the reference genome according to the Tn-seq protocol used in this study. Read counts per insertion were normalised using the LOESS method as described in Zomer et al. (Zomer et al. 2012). Next, we used the TRANSIT software (version 2.0) to compare the Tn-seq datasets (DeJesus et al. 2015). Gene states obtained by TRANSIT after growth of the mutant bank of *P. putida* KT2440 in LB agar and LB are presented in Table S4. Raw data of all datasets analysed by TRANSIT are presented in Table S5.

2.5 | Construction of the pKNG101 Plasmids Used for in-Frame Deletion in *P. putida*

The 500 bp of DNA upstream and downstream of a target gene were amplified by PCR (Primestar Max DNA Polymerase, Takara). The two 500 bp fragments were then fused by overlapping PCR. The resulting 1 kbp DNA fragment was inserted between the BamHI/SpeI restriction sites of pKNG101 by SLIC (Jeong et al. 2012). Finally, the construct was transformed into DH5α λpir and verified by colony PCR and sequencing (Table S1).

2.6 | Construction of the pJN105 Plasmids Used for Complementation

The target gene with native RBS was amplified by PCR (Primestar Max DNA Polymerase) from gDNA of *P. putida* KT2440. The amplified fragment was inserted by SLIC between the SpeI and SacI restriction sites of pJN105 and then transformed into DH5α. The resulting plasmids were validated by restriction mapping and sequencing (Table S1).

2.7 | In-Frame Deletion Mutant Construction

To construct the in-frame deletion mutants of the genes underlined in Figure 1, the counter selection method using the *sacB* gene was used (Kaniga et al. 1991). The suicide pKNG101 plasmid was transferred from MFDpir (Ferrières et al. 2010) to *P. putida* KT2440. The first recombination event was selected on LB agar supplemented with streptomycin. Transconjugants were then plated on LB agar without NaCl supplemented with 5% sucrose to allow the second recombination event. In-frame deletions were then verified by PCR (Dreamtaq polymerase, Thermofisher).

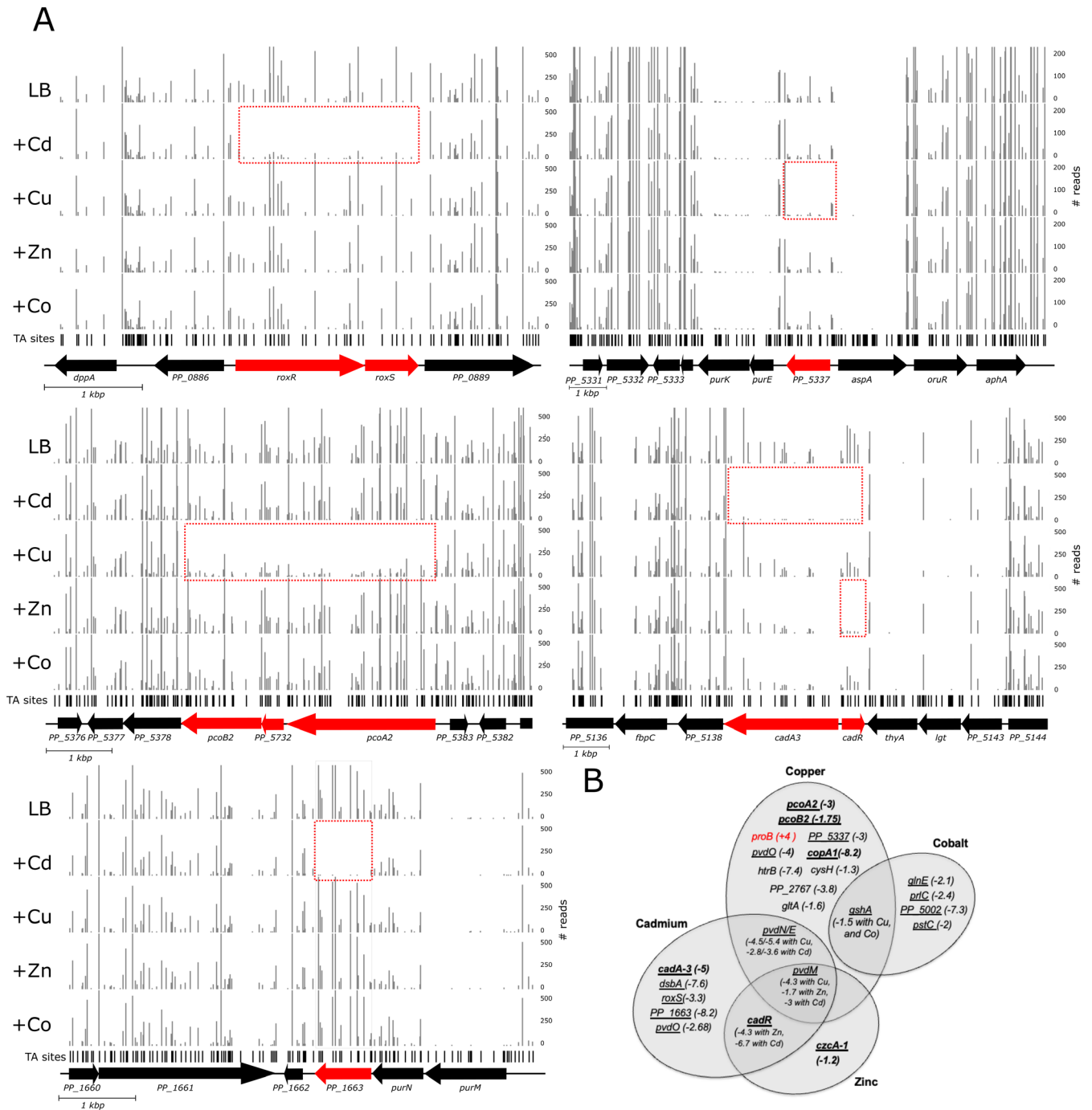


FIGURE 1 | Genes involved in metal tolerance according to the Tn-seq experiment. (A) Examples of negative selection revealed by Tn-seq. The graphs show the number of Tn-seq reads at each location aligned to TA sites on the *P. putida* KT2440 genome. Results are shown in LB only or LB with either Cu²⁺, Cd²⁺, Zn²⁺ or Co²⁺. The regions with significantly fewer reads are framed in red and the genes corresponding to these regions are indicated in red. Data are averaged from biological replicates and normalised as described in section 2.4. (B) Venn diagram of the genes with a positive or negative log₂FC, indicating the fitness difference between the test condition (LB with an excess of Cu²⁺, Cd²⁺, Co²⁺ or Zn²⁺) and the LB condition. Genes already known to be involved in metal tolerance in *P. putida* are in bold. The underlined genes were selected for in-frame deletion and further analysis.

2.8 | 1×1 Competition Assays

To compare the metal sensitivity of the mutants with the wild-type strain, 1×1 competition experiments were performed as

follows. First, to distinguish the mutants from the wild strain, a GFP⁺ WT strain was constructed by inserting the constitutively expressed *gfp* gene into the attTn7 site of the *P. putida* KT2440 chromosome using the pUC18-miniTn7-gfpmut3 plasmid (Choi

and Schweizer 2006). The GFP⁺ strain grows as well as the WT (Figure S1). Mutant and GFP⁺ WT strains were grown separately in LB medium from an overnight culture in LB to OD₆₀₀ of 0.8. Bacteria were then mixed in a 1:1 ratio at an initial OD₆₀₀ of 0.0125 in a 96-well plate containing 200 µL LB or LB with metal at a sub-inhibitory concentration. After 24 h of growth at 28°C in the Tecan M200 Pro with shaking, 5 µL of the cultures were used to inoculate a new 96-well plate and placed under the same conditions. After a total of 48 h of growth (approximately 10 divisions), the bacteria were diluted and plated onto LB agar plates. After 48 h at 28°C, GFP⁺ wild-type and mutant colonies were counted under blue light to detect colony fluorescence. A ratio was then calculated by dividing the number of mutant colonies by the number of wild-type colonies in each condition.

2.9 | Individual Growth in Presence of Metals

Single strain growth was performed in LB medium from an overnight culture in LB to an OD₆₀₀ of 0.8. Bacteria were then inoculated at an initial OD₆₀₀ of 0.006 into a 96-well plate containing 200 µL of LB or LB with metal at a sub-inhibitory concentration and placed at 28°C in the Tecan M200 Pro. OD₆₀₀ measurements were taken every 10 min after shaking. Complementation assays were performed using the same protocol but with 0.2% arabinose. Data are presented after 6.5 h of growth.

2.10 | Tn-Seq Data Availability

The transposon sequence reads obtained have been deposited in the SRA under Bioproject # PRJNA1175559. The supplemental data is in figshare. Here is the link:

<https://doi.org/10.6084/m9.figshare.28676777>.

3 | Results and Discussion

3.1 | Characterisation of *P. putida* KT2440 Himar1 Transposon Library

Tn-seq screening has been performed numerous times with the opportunistic pathogen *P. aeruginosa*. An elegant strategy that has been developed involves the use of a modified *Himar9* mariner transposon derivative carrying MmeI restriction sites in the inverted repeats (IR) and a gentamicin resistance cassette between the IRs (Skurnik et al. 2013). The Mariner transposon can specifically insert itself into the genome at TA sites. In *P. putida* KT2440, 129,002 TA sites can be targeted by this transposon. To generate a pool of approximately 1,000,000 colonies, we introduced by conjugation from *E. coli* the plasposon pSam_D-Gm into *P. putida* KT2440 (Skurnik et al. 2013). Two technical replicates of the DNA libraries were created from this pool and were subjected to high-throughput sequencing. The TPP software (DeJesus et al. 2015) was used to determine the number of reads at each TA site. The sequencing of the two samples detected 91,882 and 93,147 unique insertions into TA sites, with an average of 91 and 96 reads per TA, respectively (Table S3). The preparation of the Tn-seq library was highly reproducible, with a Pearson correlation coefficient of 98%. The density of Tn

insertions was approximately 70% from our initial pool of mutants (Table S3), and the unique insertions were distributed all around the chromosome (Figure S2). These results indicate high quality and coverage of our *P. putida* Tn-seq libraries.

The gene essentiality of the Tn-seq input libraries was next determined using the TRANSIT software (DeJesus et al. 2015), which employs a Hidden Markov Model (HMM) method to predict essentiality and non-essentiality for individual insertion sites (DeJesus and Ioerger 2013). The HMM analysis identified 600 genes essential for growth on LB agar, representing 10.8% of the genes of *P. putida* KT2440. 4458 genes were identified as non-essential genes (NE) (Table S4).

3.2 | Screening of Genes Important for Metal Tolerance

Since the term ‘metal tolerance’ generally encompasses ‘metal resistance’, we will primarily use the term metal tolerance in the manuscript to refer to the genes whose absence leads to a decrease in growth in the presence of excess metal. As some of these genes do not have a defined function, it is challenging to ascertain whether they are involved in a genuine mechanism of metal resistance. To identify new genes responsible for metal tolerance in *P. putida* KT2440, we tested the effects of copper, zinc, cobalt and cadmium chloride on the growth of *P. putida*. We used LB rich medium in our screens instead of minimal medium to prevent the loss of auxotrophic mutants or biosynthesis pathways that could be important for metal tolerance during growth. To determine the optimal metal concentration, we grew *P. putida* KT2440 in LB rich medium supplemented with varying concentrations of a metal ion solution. We compared the growth of the WT strain with and without excess Cu²⁺, Zn²⁺, Cd²⁺, or Co²⁺ under the same conditions used for screening, i.e., in an Erlenmeyer flask with shaking at 30°C (Figure S3). Under our laboratory conditions, we found that a concentration of 10 µM cobalt chloride, 2.5 mM copper chloride, 125 µM zinc chloride, and 12.5 µM cadmium chloride did not affect the growth of the cultures during the exponential phase, compared to the growth of the WT strain grown without an excess of these metals (Figure S3). We hypothesised that under these conditions, only homeostatic mechanisms will be selectively activated, rather than pleiotropic responses to metal toxicity.

For the Tn-seq screening, biological replicates were performed to ensure the reproducibility of the method. The cultures were inoculated with 10⁷ bacteria from the mutant pool. After 12 divisions in the presence of metals in the culture medium, at 28°C, the final pools of mutants were collected. Sequencing of transposon insertion sites of the final pools, followed by the TPP analysis, indicated highly reproducible results with a Pearson correlation coefficient > 90% for each dataset (Table S3).

To test the statistical significance of the genes that contribute to *P. putida*'s loss or gain of fitness in a metal-rich medium, we conducted a RESAMPLING (permutation test) analysis using the TRANSIT software. We compared the results obtained from culture in LB to those obtained from culture in LB with an excess of metal (Table S5). After applying Tn-seq to our datasets and selecting only genes with an FDR adjusted *p*-value (*q*-value) ≤ 0.05,

we identified 9 genes involved in cobalt tolerance, 14 in copper tolerance, 3 in zinc tolerance, and 8 in cadmium tolerance. From these 28 genes, we applied an additional cutoff by removing 3 genes with a mean read count in LB below 2 (less than 2 reads on average per TA) and that are classified as essential or causing growth defects in LB. Finally, we retained 25 genes (Table 1). 23 genes were classified as non-essential in LB, while the remaining 2 were identified as causing growth defects and growth advantages. Some of these genes, highlighted in bold, were previously known to be involved in metal tolerance in *P. putida*, thus confirming the validity of the Tn-seq approach. In the following sections, we discuss the function of some of the genes we consider most important in relation to metal tolerance. Examples of negative selection revealed by Tn-seq are shown in Figure 1A.

3.3 | Analysis of the Genes of *P. putida* Required for Metal Tolerance

3.3.1 | Copper Tolerance

Genes required for copper tolerance were identified using a sub-inhibitory concentration of copper (see Figure S3). Copper is an essential metal required as a cofactor for electron transport and redox enzyme systems in aerobic bacteria. In bacteria, copper exists in two different states: the less toxic oxidised Cu(II) state, which can be transformed into the more toxic reduced Cu(I) state under redox systems. Copper toxicity arises from its ability to displace essential metals from biomolecules, bind nonspecifically to cellular components, and generate reactive oxygen species (ROS) via Fenton-like reactions, leading to DNA, protein, and lipid damage. Fourteen candidate genes potentially contributing to copper tolerance were identified (Table 1), and their functions are discussed below.

3.3.1.1 | Inner Membrane Protein Cu⁺-ATPase. The role of *P_{IB}*-type ATPases in copper resistance has been extensively studied in *P. aeruginosa* PAO1. The transmembrane inner membrane protein *P_{IB}*-type ATPase, CopA, is responsible for cytoplasmic Cu⁺ efflux. *P. aeruginosa* PAO1 has two homologous Cu⁺-ATPases, CopA_{PAO1} (PA3920) and CopA_{2PAO1} (PA1549). CopA_{PAO1} was expressed in response to high Cu⁺ (Teitzel et al. 2006; Thaden et al. 2010; Quintana et al. 2017), and its deletion induced copper sensitivity (González-Guerrero et al. 2010). However, while CopA_{2PAO1} does not directly contribute to copper resistance, it does play a crucial role in loading copper into cytochrome c oxidase subunits. Both enzymes export cytoplasmic Cu⁺ into the periplasm (González-Guerrero et al. 2010). In *P. putida* KT2440, only one copper Cu⁺-ATPase is present, which is encoded by *PP_0586*. The protein is commonly referred to as CadA2 in *P. putida* KT2440. However, we will use the name CopA_{KT2440} in the further section in order to align with naming and homologies, since *copA_{KT2440}* is the orthologous gene of *copA_{PAO1}*. To ensure that the two proteins were the same, we compared their 3D structures predicted by AlphaFold (Varadi et al. 2022) using the TM-Align algorithm (Zhang and Skolnick 2005). The structures were found to be superimposed (Figure S4). Previous research has demonstrated that CopA_{KT2440} is highly produced in the presence of copper in minimal salt media (Miller et al. 2009). Our Tn-seq

screen in the presence of copper revealed that *copA_{KT2440}* mutants have a strong growth disadvantage (Table 1). In *P. aeruginosa*, *copA_{PAO1}* is positively regulated by CueR (PA4778) (Teitzel et al. 2006; Thaden et al. 2010; Quintana et al. 2017). The transcriptional regulation of *copA_{KT2440}* by CueR_{KT2440} has not been verified in *P. putida*. However, a putative CueR binding site (ACCTTGCTGCGTGGCAAGGT) is in the promoter region of *copA_{KT2440}* as indicated in the RegPrecise database (https://regprecise.lbl.gov/sites.jsp?regulog_id=5159; (Novichkov et al. 2013), suggesting a direct regulation like in *P. aeruginosa*.

3.3.1.2 | Outer Membrane Protein PcoB and Putative Periplasmic Multi-Copper Oxidase PcoA.

In certain bacterial species, an outer membrane porin called PcoB appears to contribute to periplasmic Cu⁺ efflux. *pcoB* is often co-localised with *pcoA* encoding a putative periplasmic multi-copper oxidase (Lee et al. 2002). *pcoAB* were first identified as part of a copper resistance Cop operon in the pPT23D plasmid of *Pseudomonas syringae* (Cha and Cooksey 1991). For this reason, PcoA proteins were sometimes mistakenly named CopA, despite being functionally distinct from the previously described CopA proteins which are ATPases of the inner membrane (Cha and Cooksey 1991; Miller et al. 2009). In *P. syringae*, PcoA and PcoB bind copper (Cha and Cooksey 1991). In *P. aeruginosa*, the orthologous system named *pcoA/B* (PA2065 and PA2064) is induced up to 70-fold in the presence of a high copper sulphate concentration (Teitzel et al. 2006; Quintana et al. 2017). In *P. putida* KT2440, the orthologous genes of PA2065 and PA2064 are *PP_5379* (*copB-2*) and *PP_5380* (*copA2*) respectively. The AlphaFold predicted 3D structure alignment of the orthologous proteins using the TM-Align algorithm confirms that these proteins adopt the same conformation (Figure S4). For clarity, we thus decided to rename these *P. putida* KT2440 genes *pcoB-2* and *pcoA-2* respectively. Mutants of these genes exhibit growth-defect phenotypes in the presence of copper (Table 1 and Figure 1). *P. putida* KT2440 also has a second PcoAB system (*pcoA-1/pcoB-1*) encoded by *PP_2204/PP_2205*, but mutants of these genes do not exhibit any growth-defects in the presence of copper. As only the PcoA-2/B-2 system appears to be essential for copper resistance, it is possible that the PcoA-1/B-1 system is not expressed under our laboratory conditions or is less efficient than the PcoA-2/B-2 system.

After copper treatment, *pcoA-2* and *pcoB-2* are highly transcribed in *P. putida* KT2440, and PcoB-2 accumulates in cells (Miller et al. 2009). In some strains of *E. coli* that harbour an episomal gene cluster *pcoABCDRSE*, the Pco operon transcription is mediated by the PcoRS two-component system. In *P. aeruginosa* PAO1, PcoA/B expression is suppressed in the $\Delta copR$ strain (Teitzel et al. 2006; Miller et al. 2009; Quintana et al. 2017). In *P. putida* KT2440, we searched for the consensus CopR binding site TGACANNNTGTNAT (Quintana et al. 2017) and found it in the intergenic region upstream of *copRI/CopS1* (*PP_2158/PP_2157*) and *pcoA-1/pcoB-1* (*PP_2205/PP_2204*). We did not detect any putative CopR binding site within the promoter region of *pcoA2/B2*, suggesting that these genes may be activated differently under copper stress. *P. putida* has two CopR regulators, CopR1 (*PP_2158*) and CopR2 (*PP_5383*). The proximity of *copR2* to *pcoA2/B2* suggests that CopR2 might regulate *pcoA2/B2*. However, this hypothesis has not yet been investigated.

TABLE 1 | Metal tolerance genes of *P. putida* KT2440 discovered by Tn-seq.

Metal ^a	Locus	Gene ^b	Function	State in LB ^c	No. of TAs	Mean read ^d		Log ₂ FC ^e	q-Value ^f	Orthologs in PAO1 ^g
						LB	LB + metal			
Co	PP_0096	prlC	Oligopeptidase A	NE	30	126.0	23.4	-2.43	0.00000	
	PP_0243	gshA	Glutamate-cysteine ligase	NE	28	16.5	5.6	-1.56	0.00000	
	PP_0340	glnE	Glutamate-ammonia-ligase adenylyltransferase	NE	37	28.1	6.5	-2.12	0.00000	
	PP_2645	mgtA	Magnesium transporter ATP-dependent	GD	24	6.1	78.9	3.70	0.00000	
Cu	PP_5002	PP_5002	Hypothetical protein	NE	5	38.1	0.2	-7.31	0.00000	PA5055
	PP_5328	pstC	Phosphate ABC transporter permease	NE	32	33.6	8.7	-1.95	0.00000	
	PP_0243	gshA	Glutamate-cysteine ligase	NE	28	16.5	5.9	-1.50	0.00000	
	PP_0586	cadA2 → copA1	Copper translocating P-type ATPase	NE	22	197.3	0.7	-8.19	0.00000	PA3920 = copA1
	PP_0691	proB	Glutamate 5-kinase	NE	7	3.8	55.8	3.89	0.00000	
	PP_1735	htrB	Lipid A biosynthesis lauroyl acyltransferase	NE	10	23.6	0.1	-7.39	0.00000	
	PP_2328	cysH	Phosphoadenosine phosphosulfate reductase	GA	11	390.5	158.9	-1.30	0.00000	
	PP_2767	PP_2767	ABC transporter ATP-binding protein	NE	5	22.1	1.6	-3.81	0.00000	
	PP_4194	gltA	Citrate synthase	NE	21	30.8	10.0	-1.62	0.00000	
	PP_4213	pvdM	Dipeptidase	NE	15	58.7	2.9	-4.35	0.00000	
PP_4214	pvdN	Pyoverdine biosynthesis-like protein	NE	15	62.4	2.8	-4.49	0.00000		
PP_4215	pvdO	Pyoverdine biosynthesis-like protein	NE	18	72.6	4.0	-4.18	0.00000		
PP_4216	pvdE	Pyoverdine ABC transporter ATP-binding protein/permease	NE	18	49.0	1.1	-5.45	0.00000		
PP_5337	PP_5337	LysR family transcriptional regulator	NE	11	19.5	2.4	-3.04	0.00000	PA5428	
PP_5379	copB-2 → pcoB2	Copper resistance protein B	NE	17	113.1	33.5	-1.75	0.00000	PA2064 = pcoB	
PP_5380	copA-2 → pcoA2	Copper resistance protein A	NE	36	259.6	32.3	-3.01	0.00000	PA2065 = pcoA	

(Continues)

TABLE 1 | (Continued)

Metal ^a	Locus	Gene ^b	Function	State in LB ^c	No. of TAs	Mean read ^d		Log ₂ FC ^e	q-Value ^f	Orthologs in PAO1 ^g
						LB	LB + metal			
Zn	PP_0043	<i>czcA-1</i>	Cation efflux system protein	NE	51	211.3	90.6	-1.22	0.00000	PA2520 = czcA
	PP_4213	<i>pvdM</i>	Dipeptidase	NE	15	58.7	18.4	-1.67	0.00000	
Cd	PP_5140	PP_5140 → <i>cadR</i>	MerR family transcriptional regulator	NE	9	181.6	9.1	-4.31	0.00000	PA3689
	PP_0127	<i>dsbA</i>	Thiol: disulphide interchange protein	NE	8	32.2	0.2	-7.59	0.00000	
	PP_0887	PP_0887 → <i>roxS</i>	Sensor histidine kinase	NE	19	193.2	19.5	-3.31	0.00000	PA4494 = <i>roxS</i>
	PP_1663	PP_1663	Hypothetical protein	NE	10	364.6	1.2	-8.22	0.00000	PA0943
	PP_4213	<i>pvdM</i>	Dipeptidase	NE	15	58.7	7.4	-2.98	0.00000	
	PP_4214	<i>pvdN</i>	Pyoverdine biosynthesis-like protein	NE	15	62.4	8.6	-2.86	0.00000	
	PP_4216	<i>pvdE</i>	Pyoverdine ABC transporter ATP-binding protein/permease	NE	18	49.0	4.1	-3.59	0.00000	
	PP_5139	<i>cadA-3</i>	Cadmium translocating P-type ATPase	NE	18	110.4	3.5	-4.99	0.00000	
	PP_5140	PP_5140 → <i>cadR</i>	MerR family transcriptional regulator	NE	9	181.6	1.7	-6.74	0.00000	

^aMetal tested for which a significant log₂FC has been calculated.

^bName of the gene in the TIGR KT2440 genome of the *Pseudomonas* database (version 22.1, date: 2023-10-06). When named, it has evolved in other *Pseudomonas* species; new names of the genes have been indicated in order to align with homologues.

^cState of each gene in LB defined by the TRANSIT software using a Hidden Markov Model: E, essential; GA, growth-Advantage; GD, growth-defect; NE, non-essential.

^dMean reads per TA site for a gene in each growth condition.

^eRatio of reads between the two conditions expressed in log₂.

^fp-Values adjusted for multiple comparisons using the Benjamini-Hochberg procedure (see Transit manual).

^gOrthologous gene in *Pseudomonas aeruginosa* PAO1. Only the numbers indicated in the manuscript are shown.

3.3.1.3 | The Cus System. In *P. putida* KT2440, the genes *PP_5379* (*pcoB-2*) and *PP_5380* (*pcoA-2*) are located adjacent to a cluster of five genes, *copR1/S1* and *cusCBAF*. The *cusCBA* genes encode an RND efflux pump that spans both the outer and inner membranes and has been proposed to be involved in copper and silver efflux (Cánovas et al. 2003). The two-component system CusR/S senses periplasmic copper and regulates the Cus RND-type transport system in *E. coli* (Outten et al. 2001; Gudipaty et al. 2012). In *P. aeruginosa*, upon sensing elevated copper levels, CusS activates CusR through phosphorylation. This activation leads to the transcriptional regulation of genes involved in copper resistance, including the *cusCBA* operon (Elsen et al. 2024). However, our Tn-seq datasets did not reveal any potential role of the CusCBAF system and CopRS two-component regulatory system in copper resistance at the copper concentration we used. This finding could align with previous observations indicating that *cusC* expression is not induced in the presence of copper (Miller et al. 2009). The *cusCBAF* operon, previously suggested to be involved in copper resistance, may respond to a different concentration of copper. Another possibility is that the inactivation of *cusCBA* could be compensated by the activation of the alternative copper resistance systems present in *P. putida* KT2440.

3.3.2 | Cadmium Tolerance

Cadmium commonly forms cations with an oxidation state of II. Unlike zinc, cadmium has a preference for binding to sulphur ligands, which can be problematic for proteins with disulphide bonds. Zinc homeostasis and cadmium resistance mechanisms often overlap due to their similarities. They share uptake and efflux transporters, as well as metal-responsive regulatory proteins (Cánovas et al. 2003; Leedjårv et al. 2008; Miller et al. 2009). Cadmium can be removed from the cytoplasm of bacterial cells through various systems, including the P-type ATPase CadA-3 (PP_5139). CadA-3 is a homologue of ZntA in *E. coli* (Nucifora et al. 1989; Rensing et al. 1997a, 1997b; Cánovas et al. 2003). Our Tn-seq screening indicates that CadA-3 is involved in cadmium resistance, with a strong negative \log_2FC of -4.99 (Table 1 and Figure 1). It confirms previous observations that CadA-3 confers resistance to Cd^{2+} , while CadA1 plays no role in resistance to Cd^{2+} , Zn^{2+} , Cu^{2+} , or Co^{2+} in *P. putida* (Leedjårv et al. 2008). The orthologous gene of *cadA-3* in *P. putida* CD2 was previously shown to be a major determinant of cadmium resistance (Hu and Zhao 2007). In addition, the transcription of PP_5139 was shown to be positively controlled by CadR in the presence of Cd^{2+} in the growth medium (Cayron et al. 2020).

The *dsbA* gene, which is essential for cadmium tolerance, was discovered with a negative \log_2FC of -7.59 (Table 1 and Figure 1). DsbA catalyses the oxidation of disulphide bonds of periplasmic proteins. As a result, the cysteine residues of DsbA become reduced, and the protein must be oxidised by DsbB to be regenerated (Ito and Inaba 2008). Notably, a *dsbA* mutant was found to be sensitive to cadmium and even zinc in both *E. coli* (Rensing et al. 1997a, 1997b) and *Burkholderia cepacia* (Hayashi et al. 2000). In contrast, a *dsbB* mutant did not appear to be essential for cadmium tolerance under the test conditions.

Our Tn-seq analysis showed that the gene encoding the RoxS sensor (PP_0887), which is part of the RoxS-RoxR two-component system (PP_0887-PP_0888), is essential for cadmium tolerance. The two genes coding this two-component system are transcribed in a single unit (Fernández-Piñar et al. 2008). The system belongs to the RegA/RegB family, where RegA functions as an integral membrane sensor histidine kinase, and RegB is a sigma 54-dependent regulator. A whole-genome transcriptional analysis was conducted to define the *P. putida* RoxS/RoxR regulon in LB. The regulon includes genes involved in amino acid and sugar metabolism, the sulphur starvation response, elements of the respiratory chain, and genes that participate in maintaining the redox balance (Fernández-Piñar et al. 2008). Although a putative RoxR recognition element has been identified in the promoters of genes regulated by this system (Fernández-Piñar et al. 2008), the specific genes that are up or down regulated by RoxS/RoxR in response to cadmium are still unknown.

3.3.3 | Cobalt Tolerance

Cobalt is a transition metal with an oxidation state of II. It plays an essential role for microorganisms as cofactors for diverse metalloenzymes. Cobalt toxicity is related to its potential interference with iron and possibly manganese homeostasis. Bacteria typically use efflux systems to survive in an environment with an excess of Co^{2+} . The cobalt tolerance system was poorly described in *Pseudomonas*, but it was studied in greater detail in other organisms (Nies 2003).

The genes of *P. putida* KT2440 involved in cobalt tolerance were determined using a sub-inhibitory concentration of cobalt. Our screen did not reveal any systems that cause cobalt resistance in other bacteria and that exist in *P. putida*. The *czcCBA* RND system in *Cupriavidus metallidurans* confers resistance to Cd^{2+} , Zn^{2+} and Co^{2+} (Nies et al. 2006). Although the CzcCBA system exists in KT2440, it was only reported to confer Zn^{2+} and Cd^{2+} resistance (Leedjårv et al. 2008). Additionally, it was discovered that CzcD, a member of the CDF family, confers cobalt resistance in *Ralstonia* sp. Strain CH34, although to a lesser extent than the CzcCBA system (Anton et al. 1999). A homologue of *czcD*, *PP_0026*, exists in *P. putida* KT2440, but it does not confer cobalt resistance in our Tn-seq screening. Interestingly, our screen did not reveal a role of the MrdH efflux pump (PP_2968), which is homologous to the RcnA efflux pump from *E. coli*. Although cobalt induces *mrdH* activity, the efflux pump does not confer resistance to cobalt (Haritha et al. 2009).

Although our screening did not identify any genes encoding efflux pumps, we did identify some genes that were not previously known to be involved in cobalt homeostasis. One of these genes is *mgtA*, which encodes an ATP-dependent magnesium transporter responsible for the active transport of magnesium into cells. This gene exhibits a positive \log_2FC value, indicating that the corresponding mutant confers a growth advantage in the presence of cobalt in the culture medium. MgtA has been experimentally characterised in *E. coli* and *Salmonella typhimurium*, where it facilitates magnesium uptake (Snively et al. 1989; Smith and Maguire 1998). MgtA can also mediate the influx of Ni^{2+} (reviewed in Smith and Maguire 1998); however, the transport of Co^{2+} has not been

formally demonstrated. Nevertheless, in vitro experiments have shown that Co^{2+} ions influence the activity of MgtA in *E. coli* by acting as an inhibitor of its ATPase function. While cobalt is not as potent as zinc (Zn^{2+}) in this role, it still significantly interferes with MgtA activity, likely by competing with magnesium (Mg^{2+}) for binding sites (Subramani et al. 2016). The connection between cobalt and magnesium transport is more evident in the case of the CorA transporter. Bacterial systems typically include redundant magnesium transport mechanisms, including CorA and MgtA. When CorA is rendered non-functional, MgtA can partially compensate for its magnesium transport role. It has been demonstrated that CorA can also transport cobalt ions in *E. coli* (Park et al. 1976). *corA* mutants in *E. coli* exhibit increased tolerance to high cobalt concentrations in the medium due to their ability to prevent cobalt accumulation. This finding underscores CorA's dual function as both an essential magnesium transporter and a potential carrier of toxic ions (Nelson and Kennedy 1971, 1972). Based on these data, we would have expected to observe a fitness advantage for *corA* mutants in our Tn-seq screening under cobalt exposure. However, no significant fitness difference was observed between the conditions with and without cobalt. In contrast, the observed fitness advantage for *mgtA* mutants suggests that the absence of this protein allows for greater tolerance to cobalt in the medium. This finding contradicts previous observations in *E. coli*. Nevertheless, a study investigating interactions between *P. aeruginosa* and *Candida albicans* demonstrated that MgtA is the primary Mg^{2+} transporter induced in co-culture, playing a crucial role in bacterial fitness and Mg^{2+} uptake under these conditions (Hsieh et al. 2024). This result suggests that magnesium transport in *Pseudomonas* may not operate in the same manner as in *E. coli* or *Salmonella*. Further investigations are needed to elucidate the relationship between cobalt and the MgtA and CorA proteins in *P. putida* and *P. aeruginosa*.

3.3.4 | Zinc Tolerance

Zinc has an affinity for ligands containing oxygen, nitrogen, or sulphur and is often used as an enzyme cofactor in the cell. As mentioned for copper, zinc toxicity occurs with its ability to replace another metal from enzymes or by forming complexes with other biomolecules. It exists in cells mainly in the oxidised state Zn^{2+} . Zinc homeostasis is well documented and is regulated by several processes: Zn^{2+} uptake regulation, sequestration by metallothioneins (MT) and efflux system (Blencowe and Morby 2003; Hantke 2005). Our screen revealed three genes involved in zinc tolerance: *czcA-1* (PP_0043), *cadR* (PP_5140) and *pvdM* (PP_4213). The CzcCBA system has been fully described in bacteria. It has been reported to confer Cd^{2+} , Zn^{2+} and Co^{2+} resistance in *C. metallidurans* and Cd^{2+} and Zn^{2+} resistance in *P. putida* KT2440 (Nies 2003; Leedj arv et al. 2008). The identification of the main component of the CzcCBA system, CzcA-1, confirms the validity of our screening in the presence of zinc. The CzcCBA system may be predominant at the zinc concentration used. At least five *czcA* genes have been described in *P. putida* KT2440 (C anovas et al. 2003). Our screen confirms a previous result showing that CzcCBA1 is the predominant CzcCBA system in *P. putida* under laboratory conditions (Leedj arv et al. 2008).

3.3.5 | Cross Metal Tolerance

3.3.5.1 | Pyoverdine. Pyoverdine is the major siderophore in fluorescent *Pseudomonads*, with 20 documented proteins involved in its regulation, synthesis, maturation, transport and uptake (Schalk and Guillon 2013). Maturation begins with the transport of a precursor (PVDIq) from the cytoplasm to the periplasm via the ABC transporter PvdE. PvdN and PvdO contribute to pyoverdine precursor maturation (Ringel et al. 2016, 2018), while PvdM is required for the oxidation of ferribactin by PvdP during periplasmic pyoverdine maturation (Sugue et al. 2022). The *pvdM,N,O,E* genes belong to an operon in *P. putida* KT2440 but not in *P. aeruginosa* PAO1. Mature pyoverdine chelates various metals, though with lower affinity than iron, potentially protecting cells from metal toxicity when it accumulates in the periplasm before secretion (Braud et al. 2010; Schalk and Guillon 2013). In our screen, since all mutants grow at the same time in the same culture medium, a pyoverdine-deficient mutant can acquire pyoverdine produced by the other mutants that are not defective in pyoverdine production and secretion. However, at least one gene of the *pvdMNOE* operon appeared to be involved in copper, cadmium, or zinc tolerance (Figure 1 and Table 1). Although not statistically significant for cadmium tolerance, the *pvdO* gene has a $\log_2\text{FC}$ of -2.68 , a value similar to the other genes in the operon. Similarly, the *pvdN,O,E* genes have a $\log_2\text{FC}$ of -1.56 , -1.26 and -2.01 , respectively, in the presence of zinc. These findings suggest that the *pvdMNOE* operon plays a role in metal resistance (Figure 1, Table 1). Since *pvdM*, *pvdN*, *pvdO* and *pvdE* belong to an operon and a transposition cassette may have a polar effect, determining which genes contribute to copper, zinc and cadmium tolerance requires further experimentation. According to the literature, PvdM is not a functional dipeptidase due to its lack of active zinc-binding sites, and no zinc site has been identified in its known structure (Sugue et al. 2022). Therefore, it is unlikely that PvdM binds metals or contributes to heavy metal tolerance. Instead, its role is to facilitate pyoverdine maturation by transferring the intermediate ferribactin to PvdP in the periplasm, preventing its loss and ensuring proper siderophore biosynthesis (Sugue et al. 2022). Disrupting *pvdM*, *pvdN*, *pvdO*, or *pvdE* should impair pyoverdine biosynthesis, as seen in *P. aeruginosa* (Yeterian et al. 2010; Ringel and Br user 2018). A high periplasmic pyoverdine concentration may (i) prevent unwanted metal diffusion into the cytoplasm, (ii) regulate intracellular metal levels and (iii) prevent non-specific protein–metal interactions (Schalk and Guillon 2013). One hypothesis for our results could be that newly synthesised siderophores are stored in the periplasm, as observed in *P. aeruginosa* PAO1 (Yeterian et al. 2010), and that this storage leads to metal tolerance.

However, although compelling, this hypothesis contradicts our Tn-seq data, which indicates that other genes in the pyoverdine biosynthesis pathway (such as *pvdJ*, *pvdP* and *pvdQ*) do not influence heavy metal tolerance. This suggests that the absence of pyoverdine in the periplasm may not explain the reduced fitness of *pvdM*, *pvdN*, *pvdO* and *pvdE* mutants in the presence of metals. Further experiments are necessary to clarify the role of these genes in metal tolerance.

3.3.5.2 | gshA. Among the genes involved in copper and cobalt resistance, *gshA* (PP_0243) was identified in our Tn-seq screen. *gshA* encodes the glutamate cysteine ligase

GshA, which catalyses the formation of glutamyl-cysteine from L-glutamate and is essential for copper and cobalt resistance, exhibiting a \log_2FC of -1.5 under both conditions (Figure 1 and Table 1). Glutamyl-cysteine is subsequently utilised by GshB to synthesise glutathione, a key player in metal homeostasis in *E. coli* (Helbig et al. 2008). Glutathione plays a crucial role in buffering an excess of intracellular copper in *Streptococcus pyogenes* (Stewart et al. 2020). The thiol group and cysteine residues in glutathione can directly chelate metal ions, thereby protecting cells from their deleterious effects. *gshB* (PP_4993) also exhibited a negative \log_2FC ; however, it did not pass the statistical threshold for copper tolerance (\log_2FC of -1.04) (Table S5). Notably, a mutant of the *proB* gene (PP_0691), which is involved in proline biosynthesis, conferred a growth advantage in the presence of copper. *proB* encodes glutamate 5-kinase, an enzyme that converts L-glutamate into L-proline. Since L-glutamate is also a substrate for glutamate cysteine ligase, which is encoded by *gshA*, this suggests a possible interaction between the two pathways. Given that *gshA* is essential for copper resistance, the growth advantage observed in the *proB* mutant may result from increased intracellular L-glutamate levels, which could be utilised by GshA for glutathione biosynthesis. However, this hypothesis remains speculative and requires further validation, particularly through quantification of glutathione levels in a $\Delta proB$ mutant. Moreover, the involvement of *gshA* in cross resistance is not unexpected, as its role in resistance to copper (II), zinc (II), and cadmium (II) in *E. coli* has already been established (Helbig et al. 2008). Additionally, a *gshA* mutant of *Salmonella enterica* demonstrated increased sensitivity to cobalt compared to the wild-type strain (Thorgersen and Downs 2007).

3.3.5.3 | CadR. We also highlight the importance in metal tolerance of the merR regulator CadR (PP_5140), which has a strong negative \log_2FC of -6.74 for cadmium tolerance and of -4.31 for zinc tolerance (Table 1 and Figure 1). *cadR* is adjacent to *cadA-3* (PP_5139), which our screen implicated in cobalt tolerance but not zinc tolerance (see upper section). CadR regulates its own transcription and responds to cadmium (Lee et al. 2001). While Canovas et al. identified CadR as the putative regulator of *cadA-3* (PP_5139), it does not regulate *cadA* in *P. putida* 06909 (Lee et al. 2001). Previous mutational analysis indicated that *cadA-3* and *cadR* contribute partially to zinc tolerance in *P. putida* 06909 (Lee et al. 2001). Although CadR preferentially binds cadmium, it can also weakly bind zinc, leading to lower transcription activation compared to when it is complexed with cadmium (Liu et al. 2019). This was confirmed using transcriptional fusions in *E. coli* (Cayron et al. 2020). In *P. aeruginosa*, CadR is constitutively bound to its promoter and promptly activating *cadA* gene expression upon Zn^{2+} binding. CadA is essential for a timely induction of the CzcCBA efflux system (Ducret et al. 2020). In our condition of an excess of zinc ions, *cadA-3* was not required for zinc resistance.

4 | In-Frame Deletion Mutants and Complementation Assays Confirmed That PP_1663 and roxSR Are Required for Cd^{2+} , PP_5337 for Cu^{2+} , and PP_5002 for Co^{2+} Metal Tolerance

Our Tn-seq screen also identified several genes that were not previously known to be associated with metal tolerance.

Among them, PP_1663, a gene encoding a putative periplasmic protein of unknown function, was found to be involved in cadmium tolerance with the strongest \log_2FC of -8.22 . To gain insight into the function of PP_1663, we conducted a structural similarity analysis using its predicted 3D structure, generated with AlphaFold from the AlphaFold Protein Structure Database (Jumper et al. 2021; Varadi et al. 2022, 2024). We compared this structure using the Dali server (Holm 2022) with proteins in the Protein Data Bank (PDB) and with the predicted 3D structure of its ortholog, PA0943, in *P. aeruginosa*. PP_1663 and PA0943 share 65% sequence identity and have a Z-score of 35.4, indicating a highly similar structure. Both proteins adopt a β -spiral fold composed of 14 antiparallel β -strands and are localised in the periplasm. Mutation of PA0943 renders *P. aeruginosa* hypersensitive to the production of the secretin XcpQ and disrupts the normal function of the Xcp protein export system (Seo et al. 2009). In summary, the absence of PA0943 compromises cell envelope integrity in *P. aeruginosa*. It is therefore reasonable to speculate that the absence of PP_1663 in *P. putida* may lead to a similar defect, potentially affecting membrane integrity and resulting in increased sensitivity to certain metals. Further experiments are required to confirm this hypothesis.

The Tn-seq screen in the presence of cobalt also identified the PP_5002 gene with unknown function. The putative protein encoded by PP_5002 contains a DUF971 domain (81/125 amino acids). Insights into the function of proteins containing this domain have been derived from large-scale RB-Tn-seq screenings, which integrate phenotypic data with comparative genomics to predict the functions of unannotated genes (Price et al. 2018). By analysing the conservation of phenotypes and cofitness patterns across different bacterial species, researchers have inferred the functions of previously uncharacterized genes. In these RB-Tn-seq screenings, several *Shewanella* and *Pseudomonas* proteins containing DUF971 exhibit cofitness with Mrp, BolA and YggX proteins, which are involved in various aspects of FeS cluster maintenance and iron regulation—critical processes for cellular function and stress resistance (Price et al. 2018). Although the precise biochemical function of DUF971 remains unknown, in eukaryotes, this domain is present in gamma-butyrobetaine hydroxylase and trimethyllysine dioxygenase proteins. It is also found in the chloroplast 4Fe-4S cluster scaffold protein HCF101 (Price et al. 2018). These findings suggest that DUF971 may play a role in FeS cluster maintenance, potentially elucidating its function under metal stress conditions. Consequently, the PP_5002 gene product could be integral to iron homeostasis, mitigating the detrimental effects of cobalt on this equilibrium. As Fe-S are one of the main targets of Co toxicity (Barras and Fontecave 2011), the PP_5002 product could play a major role in counteracting the deleterious effect of cobalt.

Finally, we identified the RoxS sensor (PP_0887), which is part of the RoxS-RoxR two-component system (PP_0887-PP_0888), as essential for cadmium tolerance. We could also identify a new transcriptional regulator of the LysR family, PP_5337, probably involved in copper tolerance (\log_2FC of -3.04). Analysis of its regulon has not been performed yet.

To confirm the role of these genes in metal tolerance, we decided to go a step further by performing in-frame deletions of these genes and selecting other genes identified in our Tn-seq screening. We

also made mutants of *pcoA-2/B-2* for copper resistance, *czcA-1* for zinc resistance and *cadA-3* for cadmium resistance because they can be used as positive controls. Since *roxS* (*PP_0887*) is in an operon with *roxR* (*PP_0888*), a double mutant was constructed. As several genes of the *pvdMNOE* operon were identified in our screening, we decided to make the $\Delta pvdMNOE$ mutant. The genes that were deleted are underlined in Figure 1B.

First, to validate the Tn-seq results, we performed a competition experiment with the WT strain and the mutants in a 1:1 ratio to calculate the fitness of the respective mutants compared to the WT strain. We calculated a ratio in \log_{10} by dividing the number of colony-forming units (cfu) of the mutant by the number of cfu of the WT strain after a co-culture of the two strains. The experiments were performed in LB only or LB with a respective metal at the identical concentration that was used in the Tn-seq screen (Figure 2). First, in LB only, all tested mutants grew as well as the WT strain, except for the $\Delta dsbA$ and $\Delta gshA$ mutants, which showed reduced growth fitness. In the presence of cadmium, we confirmed that the $\Delta roxSR$, $\Delta pvdMNOE$, $\Delta cadR$, $\Delta dsbA$, ΔPP_1663 and $\Delta cadA-3$ mutants had a lower fitness than the WT strain

(Figure 2A). The growth of the $\Delta dsbA$ and $\Delta cadA-3$ mutants in the presence of cadmium was so low that the fitness could not be calculated. In the presence of copper, the $\Delta pvdMNOE$, $\Delta copA1$, $\Delta gshA$, $\Delta pcoA2$, $\Delta pcoB2$ and ΔPP_5337 mutants had a lower fitness (Figure 2B). When exposed to cobalt, only the ΔPP_5002 mutant showed a significant fitness defect. We were unable to confirm the sensitivity towards cobalt for the $\Delta pstC$, $\Delta gshA$, $\Delta glnE$ and $\Delta prlC$ mutants (Figure 2C). Finally, the mutants $\Delta cadR$, $\Delta pvdMNOE$ and $\Delta czcA-1$ exhibited lower fitness levels in the presence of zinc. However, the *cadA-3* mutant showed no sensitivity to zinc (Figure 2D). This confirms our Tn-seq results. In conclusion, we also showed that the $\Delta cadR$ mutant is sensitive to both cadmium and zinc and that the $\Delta pvdMNOE$ strain is sensitive to both cadmium, zinc and copper. In general, all results confirm our Tn-seq results, except for cobalt where only one gene (ΔPP_5002) could be validated.

Next, we focused our work on the four genes *PP_5337*, *roxSR*, *PP_1663* and *PP_5002* because they had not been shown to be involved in metal tolerance prior to our work. The growth of these mutants was measured individually in LB liquid culture over

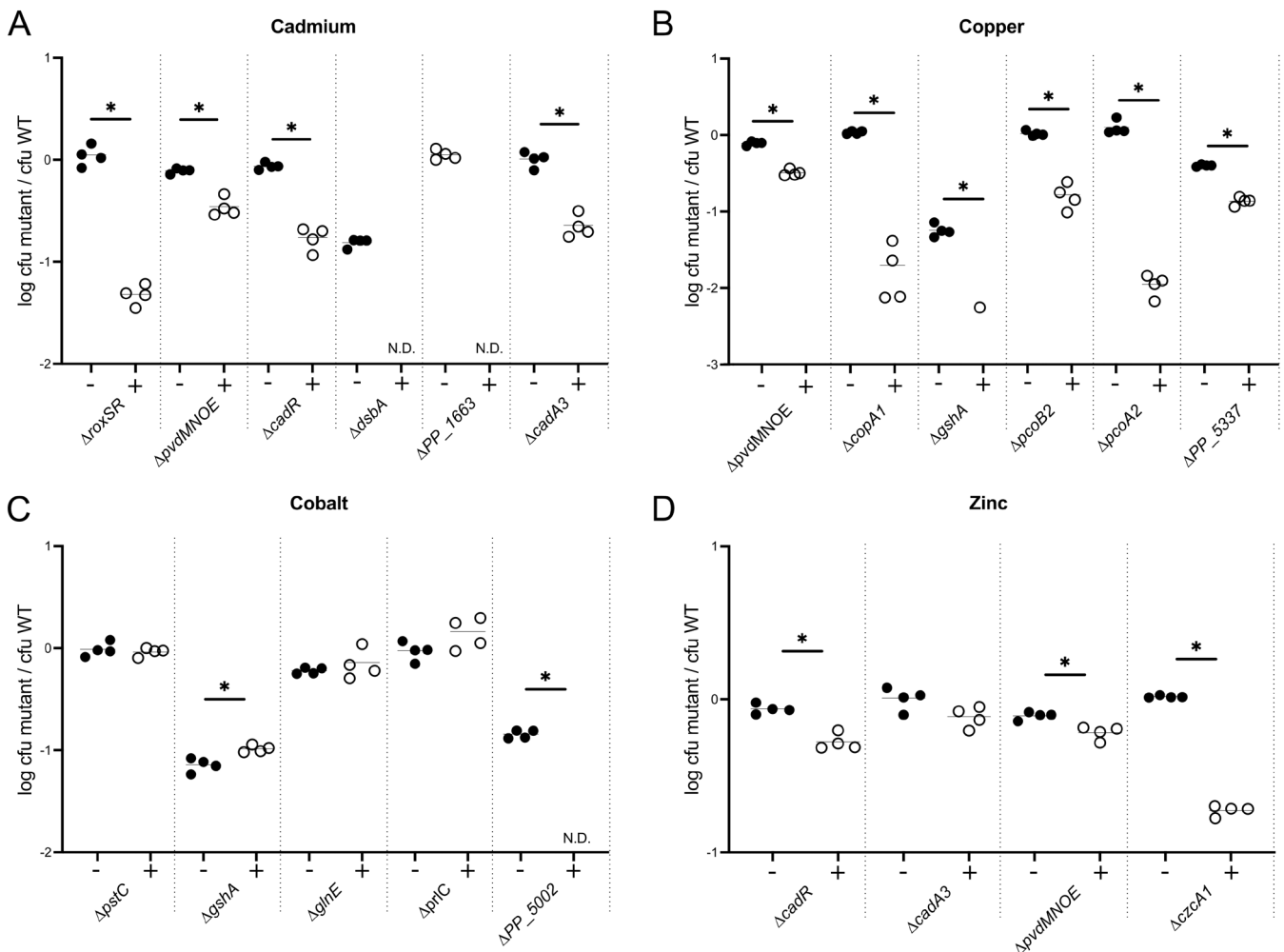


FIGURE 2 | Competition between the wild type and the mutant strains of *P. putida* KT2440 in the presence or absence of a metal in excess. Competitions were realised with an initial ratio of 1:1 in LB supplemented or not with a sub-inhibitory concentration of metals (cobalt $10\ \mu\text{M}$, zinc $125\ \mu\text{M}$, copper $2.5\ \text{mM}$, cadmium $12.5\ \mu\text{M}$). The respective final ratio was determined as described in section 2.8 and presented in Log_{10} . The experiment was realised four times. * indicates a statistically significant difference relative to the absence of the metal condition ($p < 0.05$, Mann-Whitney *U* test).

time and compared to the growth of the WT strain (*ure3A-G*). No statistical difference in growth was observed between the mutants and the WT strains. In contrast, in the presence of metal ions, the mutants ΔPP_5337 , $\Delta roxSR$, ΔPP_1663 and ΔPP_5002 showed a growth defect in LB supplemented with Cu^{2+} , Cd^{2+} , Cd^{2+} and Co^{2+} , respectively (Figure 3A,C,E,G).

To prove that the phenotypes of the mutants were in fact related to the deletion of the respective target gene, we cloned the genes into the pJN105 plasmid under the control of the arabinose-inducible promoter pBAD to perform a complementation assay. The WT and mutants were grown in LB with arabinose to induce gene expression from the pJN105 plasmid.

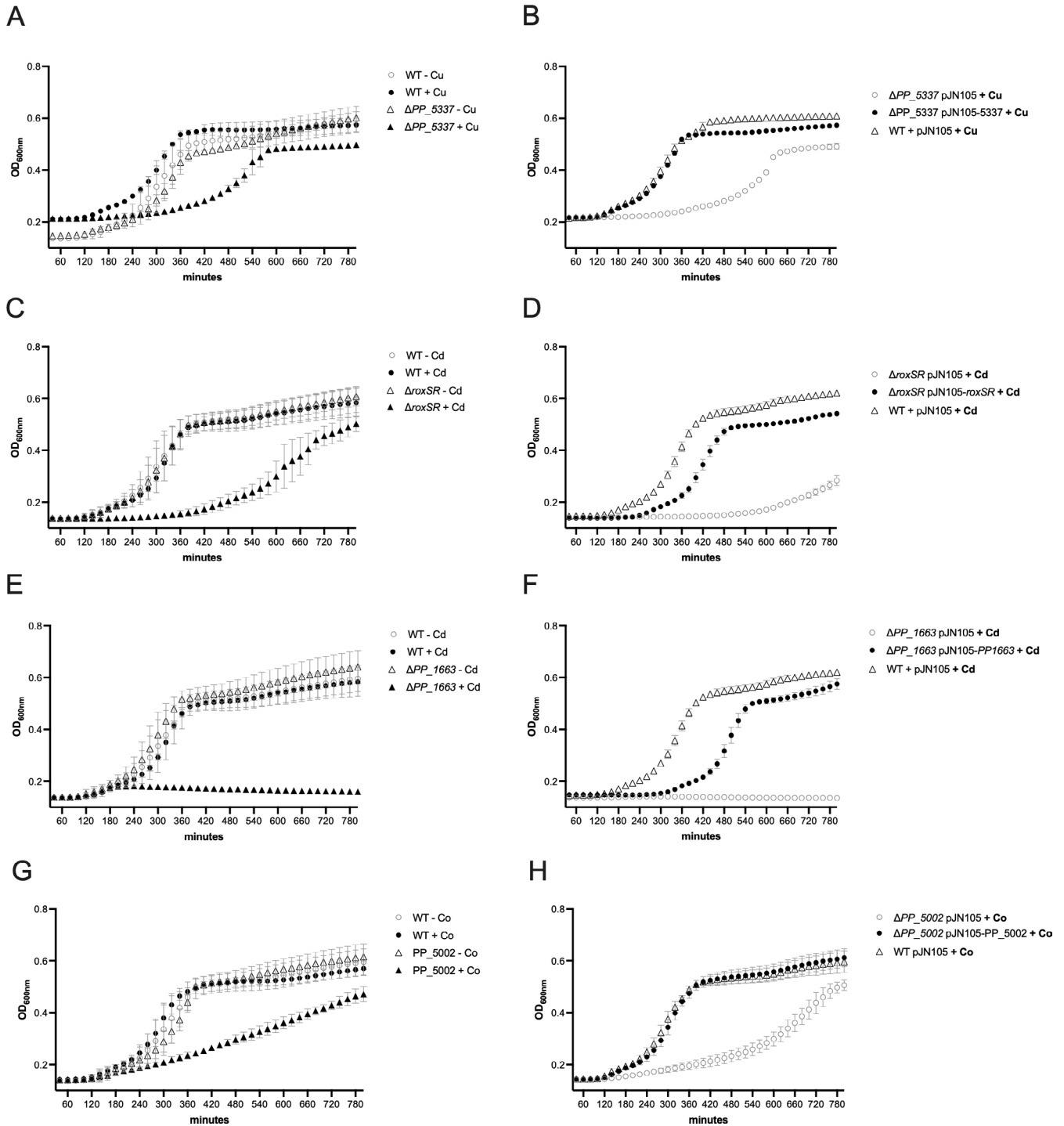


FIGURE 3 | Individual growth cultures of the mutants and complementation assay. Individual growth of each mutant strain was performed in LB medium supplemented or not with a sub-inhibitory concentration of metals (cobalt $10\mu M$, zinc $125\mu M$, copper $2.5\mu M$, cadmium $12.5\mu M$) in a 96-well plate. OD_{600nm} was measured over time. Panels A, C, E, and G show growth of the WT and the mutants in both conditions. Panels B, D, F, and H show the functional complementation assay in presence of metal. The data represent the mean of 4 replicates. The growth difference between the mutant and the WT (panels A, C, E and F) or the complemented mutant and the WT in the presence of a metal (panels B, D, F and H) is always statistically significantly different during the exponential phase ($p < 0.05$, Mann-Whitney U test).

Cultures were performed in the presence of Cu²⁺, Cd²⁺, or Co²⁺ (Figure 3B,D,F,H). Expression of the *PP_5337*, *roxSR*, *PP_1663* and *PP_5002* genes in the corresponding mutant could at least partially suppress the growth defect caused by the metals. In particular, these data demonstrated that *PP_1663* and *roxSR* are novel genetic factors required for Cd²⁺, *PP_5337* for Cu²⁺ and *PP_5002* for Co²⁺ metal tolerance in *P. putida* KT2440. Taken together, these results confirm that Tn-seq is (i) a reliable technique for identifying genes involved in metal tolerance in *P. putida* and (ii) is able to confirm known genes and also identify novel genes relevant for metal tolerance.

5 | Concluding Remarks

Tn-seq has previously been used to comprehensively study the essential genomes of several bacteria, sometimes in response to drugs (Gallagher et al. 2011; Barquist et al. 2013). However, there has been no Tn-seq genome-wide study of factors necessary for Cu, Cd, Co and Zn tolerance in *P. putida*. Motivated by previous studies, we ensured that we could rely on a completely de novo assembled, full genome sequence of our *P. putida* KT2440 strain in order to minimise the risk of missing relevant genes (Varadarajan et al. 2020). Overall, our approach was risky because *P. putida* has multiple genetic determinants that affect its tolerance to these metals. Functional redundancy can be a challenge in this type of experimental approach. The absence of a genetic determinant for metal tolerance may be compensated for by the expression of other metal-resistance genes. We chose to work with sub-inhibitory concentrations of Cu²⁺, Cd²⁺, Co²⁺ and Zn²⁺. Our approach led to the identification of genes already known to be implicated in metal tolerance or homeostasis. The study has identified key genes involved in resistance, such as *copA-1*, *pcoA-2* and *pcoB-2* for copper, *cadA-3* and *cadR* for cadmium, and *czcA-1* for zinc. This finding is also significant because it indicates that these genes do not have functional redundancy. Miller et al. (2009) have demonstrated the response of *P. putida* to the presence of cadmium and copper. Numerous transcriptional regulators, outer and inner membrane proteins that form efflux channels and pumps, periplasmic proteins, and stress-related proteins are involved. It is plausible that the genes identified in our screens are part of the initial defence against these metals. If higher concentrations occur, other genes not identified in our screens will come into play. To exemplify this vision, it is worth mentioning the research conducted by Peng et al. (2018). They performed RNA-seq on *P. putida* KT2440 grown under varying zinc concentrations. The authors found that the transcriptome of *P. putida* was dependent on the concentration of zinc in the medium. Specifically, at the lowest concentration tested (200 µM in a semi-synthetic medium), only *PP_5139* and *PP_0043* were overexpressed. These results support our observation that the two genes are necessary for tolerance to a zinc concentration of 125 µM in LB.

Another example is the RND complex CzcCBA (*PP_0043-PP_0045*), known for the resistance to zinc and cadmium (Cánovas et al. 2003). It appears that this system is dispensable for cadmium resistance in our screen. It is possible that the *czcABC* system responds to a higher quantity of metals and was therefore inactive in our conditions. This hypothesis is supported by the identification of the *czCBA1* genes involved in resistance to

3 mM cadmium during the screening of a Tn5 mutant library in *P. putida* CD2 (Hu and Zhao 2007). The difference in the experiments in the two studies could explain this phenomenon. It would thus be interesting to carry out a Tn-seq screen at higher metal concentrations. This would aid in identifying additional genetic factors required for survival in environments with high levels of heavy metals. Tn-seq has the added advantage of being able to identify genes that are not induced in the presence of metal, making its results different from those obtained by transcriptomics or proteomics.

Finally, our study allowed the identification of new important factors for metal tolerance in *P. putida*. Targeted in-frame mutagenesis and functional complementation prove that *PP_1663* encoding a periplasmic protein and *roxSR* encoding a two-component system are required for cadmium tolerance. *PP_5337* is a new putative transcriptional regulator required for copper tolerance, and *PP_5002* is a hypothetical protein required for cobalt tolerance. To better understand how these genes induce tolerance to metals, further characterisation is necessary. For *RoxR* and *PP_5337*, a transcriptomic study should be conducted to identify the genes that are regulated by these transcriptional regulators. In conclusion, our study shows that there are still many studies to be carried out to fully understand the *P. putida* “resistome” to metals.

Author Contributions

Kevin Royet: validation, formal analysis, investigation, visualization, writing – review and editing. **Laura Kergoat:** investigation. **Stefanie Lutz:** investigation. **Charlotte Oriol:** investigation. **Nicolas Parisot:** software. **Christian Schori:** investigation. **Christian H. Ahrens:** writing – review and editing, supervision, resources, methodology, funding acquisition, writing – original draft. **Agnes Rodrigue:** writing – review and editing, funding acquisition, writing – original draft, resources, project administration, supervision. **Erwan Gueguen:** supervision, writing – original draft, funding acquisition, writing – review and editing, methodology, data curation, validation, visualization, formal analysis, project administration, conceptualization, resources.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are openly available in SRA at <https://www.ncbi.nlm.nih.gov/bioproject/1175559>, reference number PRJNA1175559. The supplemental data in figshare. Here is the link: <https://doi.org/10.6084/m9.figshare.28676777>.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.