



Tobramycin Stress Induced Differential Gene Expression in *Acinetobacter baumannii*

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Abstract

Acinetobacter baumannii is a multidrug-resistant bacteria responsible for nosocomial infections with significant fatality rates globally. Therapeutic failure and relapse of infection has been associated with persister cells formation which can also lead to resistance in *A. baumannii*. In the present study, we observed that *A. baumannii* ATCC 17978 in exponential phase survived lethal concentrations of amikacin, rifampicin and ciprofloxacin by generating persister cells but was unable to survive tobramycin treatment. The transcriptome of *A. baumannii* ATCC 17978 was analyzed following exposure to a high concentration of tobramycin (10×MIC) for a short period of time to study the possible mechanisms responsible for lethality. Tobramycin reduced the expression of genes involved in energy production (*nuoH*, *nuoN*, *nuoM*, *cydA*, *sucC*), oxidative stress protection (*tauD*, *cysD*), and nutrition uptake (*ompW*) significantly. In addition, hemerythrin (non-heme di-iron oxygen-binding protein) was found to be the most downregulated gene in response to tobramycin which needs to be further studied for its role in susceptibility to antibiotics. Tobramycin upregulated the expression of genes that are mainly involved in stress response (leucine catabolism, DNA repair and HicAB toxin-antitoxin system). The differentially expressed genes highlighted in the study provided insight into the probable molecular mechanism of tobramycin-induced cell death and revealed some novel targets that can be explored further for their potential to control *A. baumannii*.

Introduction

Acinetobacter baumannii is a Gram-negative coccobacillus that causes ventilator-associated pneumonia (VAP), bacteraemia, meningitis and infections related to skin and soft tissue, central nervous system, and urinary tract in patients admitted to intensive care units [1]. It is one of the six superbugs in the “ESKAPE” group that are responsible for nosocomial infections [2]. *A. baumannii* has also been associated with secondary infections of lower respiratory tract in COVID-19 patients [3]. It has been designated as a “red alert” human pathogen due to its high level of antibiotic resistance [4]. The World Health Organization (WHO) has listed *A. baumannii* in the “critical” category of priority

pathogens against which new antibiotics are urgently needed [5].

The formation of persister cells is a key factor in the development of recalcitrant *A. baumannii* infections and antibiotic treatment failure [6, 7]. Persisters are a subset of antibiotic-sensitive bacterial cells that enter a slow or non-growing transient state to survive lethal dose of antibiotics. They can be distinguished from resistant cells as they do not divide actively in the presence of antibiotics and their MIC does not change after regrowth [8, 9]. *A. baumannii* has been shown to form persister cells in exponential phase against amikacin, carbenicillin, colistin, rifampicin, polymixin B, meropenem, ceftazidime, tetracycline, and imipenem [10–13]. However, tobramycin has been found to make *A. baumannii* cultures sterile [12].

In the present study, *A. baumannii* ATCC 17978 culture in exponential phase survived against amikacin, rifampicin, and ciprofloxacin by forming persister cells but could not survive tobramycin treatment as reported earlier. To gain insight into the reasons for eradication shown by tobramycin, transcriptome of *A. baumannii* ATCC 17978 cells in exponential phase exposed, for short duration, to lethal concentration of tobramycin was analyzed. Our results highlight

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the relevance of genes involved in energy production, sulphur metabolism, OmpW, and hemerythrin for tobramycin-induced cell death. These findings provide new target genes necessitating their further investigation as potential therapeutic targets.

Materials and Methods

Determination of Minimum Inhibitory Concentration (MIC)

A. baumannii ATCC 17978 was grown and maintained in Lysogeny Broth (LB) at 37 °C. MIC of ciprofloxacin, rifampicin, amikacin and tobramycin was determined by broth micro-dilution method as per the Clinical and Laboratory Standards Institute guidelines [14]. Overnight grown culture was diluted in Mueller Hinton (MH) broth to a turbidity equivalent to 0.5 McFarland standard. The culture was mixed with serial two-fold dilutions of antibiotics and incubated for 24 h at 37 °C, 180 rpm. The lowest antibiotic concentration that inhibited visible growth was defined as the MIC.

Persister Assays

A single colony of *A. baumannii* was inoculated in LB medium and cultured at 37 °C, 180 rpm for 16 h. 100 µl of overnight grown culture was transferred to 10 ml of LB and grown to exponential phase ($OD_{600} \sim 0.5$). Concentration-dependent persister assay was done using different antibiotic concentrations ($\times MIC$) for 3 h. The time-dependent persister assay was performed with constant concentrations of antibiotics as selected from the concentration-dependent assay for different (3–24 h) time intervals. After the addition of antibiotics, cells were separated by centrifugation at $8000 \times g$ for 10 min at different time points, washed twice with phosphate buffer saline (PBS), and diluted tenfold serially in PBS. 10 µl aliquot of each dilution was spot-plated on LB agar plates to determine colony-forming units per milliliter (CFU/mL) [15].

RNA Isolation, Sequencing and Transcriptome Analysis

Exponential phase *A. baumannii* cells were treated with $10 \times MIC$ of tobramycin for 60 min and total RNA was extracted using TRI Reagent (Sigma) and RNeasy Mini Kit (Qiagen, USA). Untreated cells were used as control. The quality and quantity of isolated RNA was assessed on 1%

agarose gels and NanoDrop (Eppendorf) respectively. DNA was removed from the samples using Turbo DNase I (Invitrogen) according to the manufacturer's instructions. RNA samples were evaluated for integrity using Agilent 2100 Bioanalyzer (Agilent Technologies), and samples with RNA integrity number (RIN) greater than 7 were used to build cDNA libraries. RNA sequencing was outsourced to SciGenom Labs Pvt. Ltd. (Kochi), India where Illumina HiSeq 2500 platform with paired-end sequencing (2×100 bp) was used. RNA-Seq reads were aligned with *A. baumannii* ATCC 17978 genome using Hisat2 [16], and differential gene expression analysis was done with Cuffdiff [17]. A gene was considered significantly differentially expressed when the $\log_2|FC| \geq 2$ with an adjusted P value ≤ 0.05 . RNA-Sequencing raw data have been deposited in NCBI BioProject database under Accession no. PRJNA787919.

Functional Annotation of Differentially Expressed Genes (DEGs)

To analyze the functional roles of the DEGs, OmicsBox (version 3.2) was used for Gene Ontology (GO) annotation [18]. The Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways database and Cluster of Orthologous Groups (COGs) protein database were used to further classify the genes.

Protein–Protein Interaction (PPI) Network Analysis

The PPI network of DEGs was constructed using the Search Tool for the Retrieval of Interacting Genes (STRING; <http://string-db.org>) and a combined score > 0.7 (high confidence) was set as the cut-off criterion. Nodes in the network that were not connected were excluded. Based on the results from STRING, the open source platform Cytoscape (version 3.8.2, <https://cytoscape.org/>) was used to visualize molecular interaction networks, and the degree centrality was determined using the plug-in CytoHubba.

Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) Validation

To verify transcriptome results, RNA was extracted as described above. The cDNA was synthesized by the Verso cDNA synthesis kit (Thermo Fisher, Co., USA) following the manufacturer's manual. Primer3 software was used to design specific primers for qRT-PCR (Supplementary Table 3). qRT-PCR was performed using iTaq Universal SYBR® Green Supermix (Biorad, USA). The *16S* rRNA gene was used as the reference gene, and the gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Results

Persister Cell Subpopulation of *A. baumannii* ATCC 17978 on Treatment with Different Classes of Antibiotics

A. baumannii ATCC 17978 culture in exponential phase formed varying percentage of persister cells in response to ciprofloxacin (MIC, 0.25 µg/ml), rifampicin (MIC, 4 µg/ml) and amikacin (MIC, 2 µg/ml) (Supplementary Fig. 1). The time-dependent assay revealed maximum persisters against 10× rifampicin (1.71%) followed by 50× ciprofloxacin (0.022%) and 10× amikacin (0.0065%) at 24 h (Fig. 1). However, increasing concentration of tobramycin led to a rapid decline in viable cells. Tobramycin at 1× MIC in a time-dependent assay did not affect the viability of cells, while treatment with 3× MIC led to an effective reduction in the number of viable cells, with only 0.005% cells surviving after 5 h. Treatment with 5× MIC resulted in 0.03% cell survival after 2 h but after 3 h, the viable cell count fell below detection limit. Treatment with 10× MIC for 1 h had 0.04% cell survival which dropped below the detection limit in 2 h (Supplementary Fig. 2). Hence to study the effect of tobramycin in *A. baumannii*, cells in exponential phase were treated with 10× MIC for 1 h.

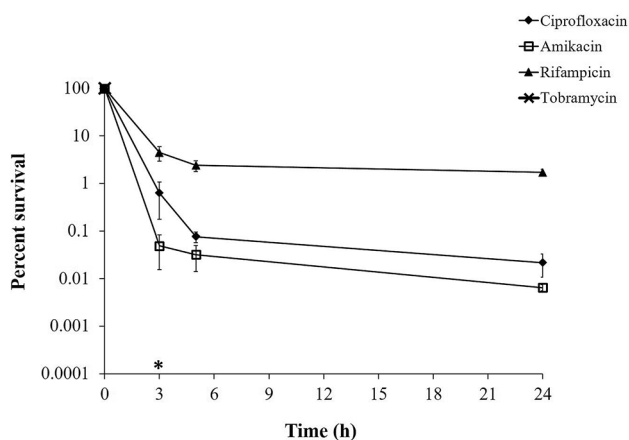


Fig. 1 Exponential phase cells of *A. baumannii* ATCC 17978 on treatment with ciprofloxacin (50×), rifampicin (10×), amikacin (10×) and tobramycin (10×) for different time intervals. Untreated cells at 0 h ($3.2 \pm 0.36 \times 10^8$ CFU/ml) were taken as 100% and percent survival was calculated accordingly. Data are represented as mean log percent survival \pm SD of three independent experiments. The asterisk indicates cell count below the detection limit

Identification of Differentially Expressed Genes in *A. baumannii* After Exposure to Lethal Concentration of Tobramycin

The transcriptome profiles of *A. baumannii* ATCC 17978 cells with (10× MIC for 1 h) and without tobramycin treatment were analyzed by high-throughput sequencing. The differential gene expression analysis showed that 97 genes were differentially expressed, including 62 genes that were upregulated and 35-genes that were downregulated significantly (Fig. 2, Supplementary Table 1). Among these, 25 genes (14 upregulated and 11 downregulated) encoded hypothetical proteins.

Functional Annotation of Differentially Expressed Genes

In GO classification, a total of 81 differentially expressed genes (DEGs) having annotated biological functions were assigned 188 GO terms based on their location in the cell, their biological and molecular functions. Two main enriched terms in biological process (BP) category were metabolic process and cellular process. In cellular component (CC) category, catalytic activity and binding were the most enriched GO terms. In molecular function (MF) category, the most enriched term was catalytic activity, followed by binding (Fig. 3A). In COG classification, DEGs were functionally classified into 17 COG categories (Fig. 3B). Among all COG categories, Transcription (K) occupied the largest proportion, followed by the Energy production and conversion (C), and Lipid transport and metabolism (I). Further, the KEGG pathway analysis revealed that the upregulated DEGs were linked to 40 pathways, with valine, leucine and isoleucine degradation and fatty acid degradation being the most dominant. The downregulated DEGs were linked to 22 KEGG pathways, with oxidative phosphorylation and sulphur metabolism being the most enriched (Fig. 3C).

PPI Network Analysis of Differentially Expressed Genes

PPI network analysis was performed to gain insight into the interaction among DEGs. The PPI network of DEGs contained 33 nodes (including 26 upregulated and 7 downregulated genes) and 27 interactions (Supplementary Fig. 3). Genes identified with the highest degree of connections (Supplementary Table 2) included putative ferredoxin (A1S_0945), putative vanillate o-demethylase

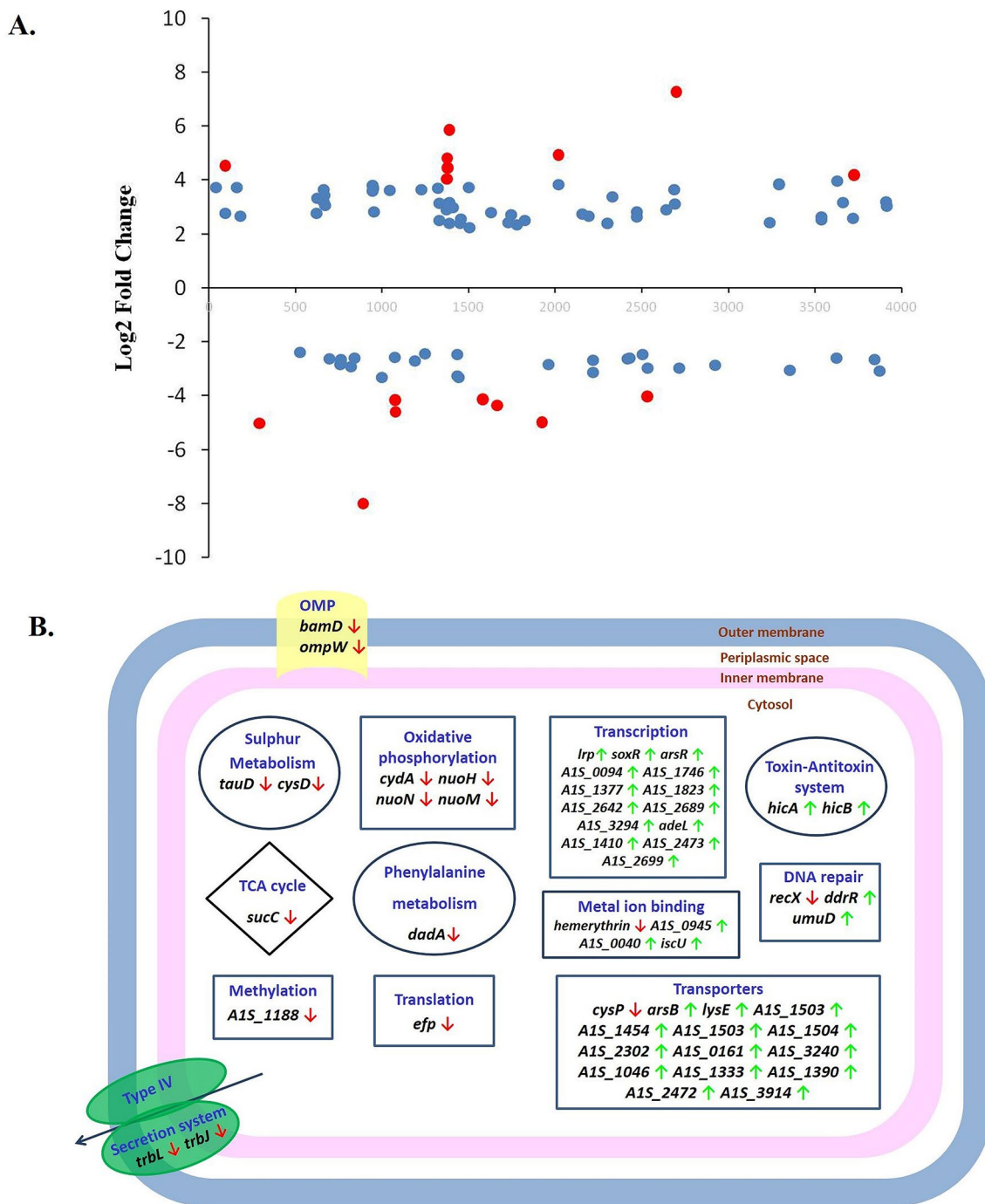


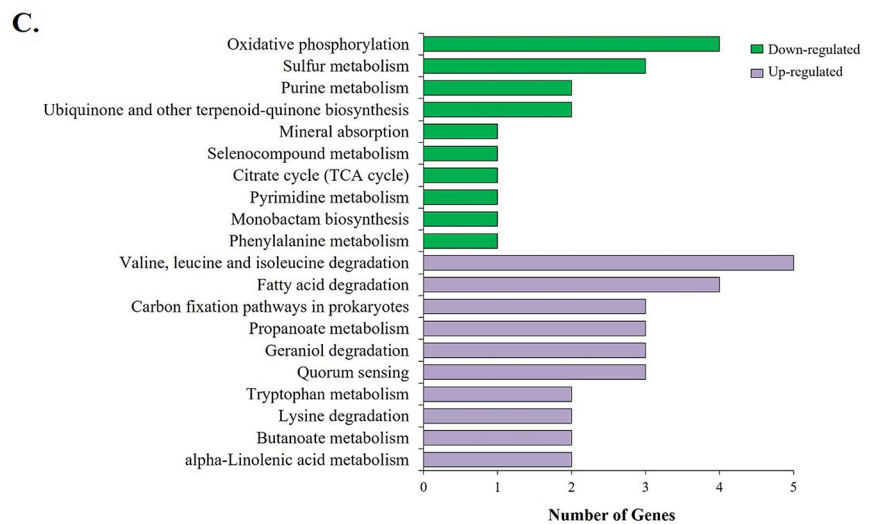
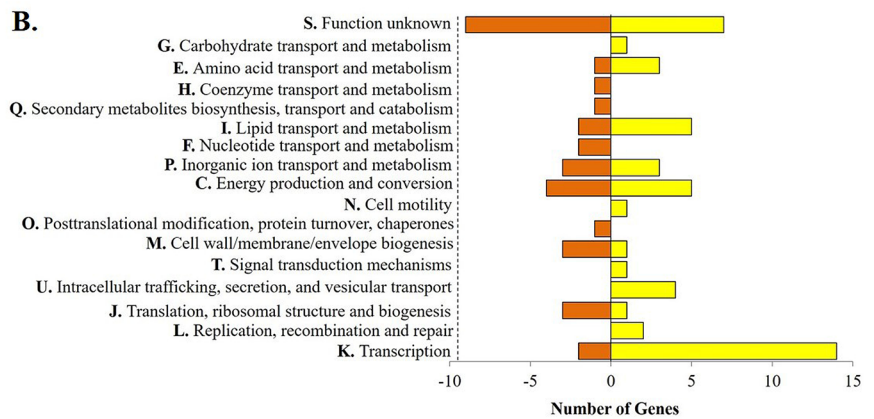
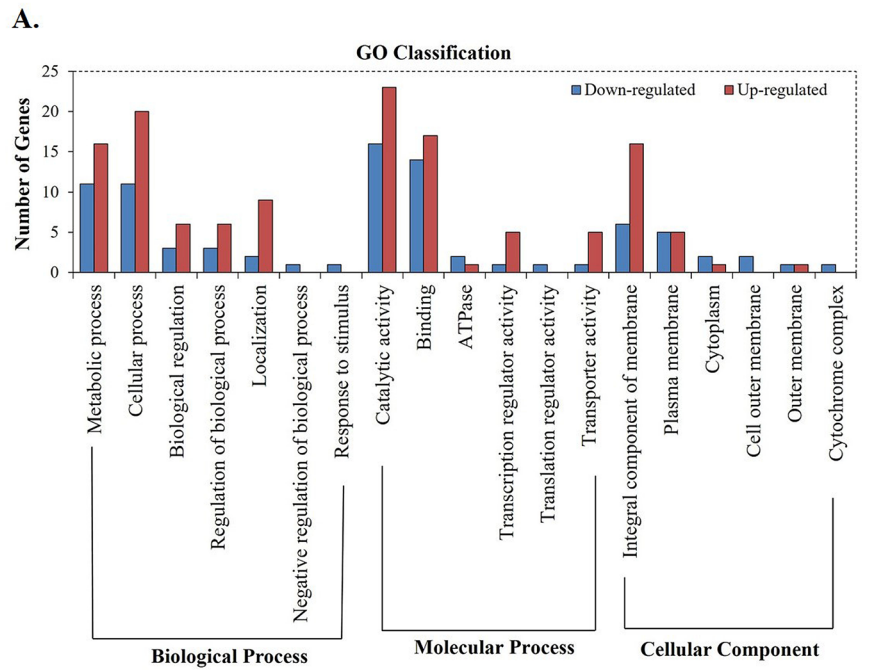
Fig. 2 **A** Plot showing log₂ fold change values of all significantly ($P \leq 0.05$) differentially expressed genes identified from transcriptome analysis of *A. baumannii* ATCC 17978 in response to tobramycin treatment. Log₂ fold change values were plotted with respect to gene locus tag number. Genes with log₂ fold change ≥ 4 and ≤ -4 but ≥ 2 are

shown in red and blue, respectively. **B** Depiction of genes that are differentially expressed in response to tobramycin treatment. Downregulated genes are highlighted with a red downward pointing arrows, and upregulated genes with green upward pointing arrows. (Color figure online)

oxygenase subunit (*A1S_0947*), NADH dehydrogenase I chain N membrane subunit (*nuoN*, *A1S_0764*), hypothetical protein (*A1S_0953*), putative dioxygenase

(*A1S_0949*), acyl-CoA dehydrogenase (*ivd*, *A1S_1376*), regulatory protein (*repA*, *A1S_0663*), type IV secretion system protein TrbJ (*A1S_0665*), type IV secretion system

Fig. 3 **A** Gene ontology (GO) classification of DEGs grouped into three categories: biological process (BP), molecular function (MF), and cellular component (CC). Blue and red bars indicate downregulated and upregulated genes, respectively. The Y-axis indicates number of genes. **B** Cluster of orthologous genes (COG) classification of DEGs. Yellow bars represent upregulated genes, and orange bars, downregulated genes in each COG category. **C** KEGG pathway classification of DEGs. Top 10 KEGG terms of downregulated and upregulated DEGs represented by green and purple bars, respectively. (Color figure online)



protein TrbL (A1S_0666), and NADH dehydrogenase I chain H membrane subunit (*nuoH*, A1S_0758).

Genes DownRegulated by Tobramycin Treatment

Genes encoding hemerythrin (A1S_0891), outer membrane protein *ompW* (A1S_0292), putative membrane protein (A1S_1665), transcriptional regulator Cro/CI family (A1S_1582) and six genes involved in metabolism (*cydA*, A1S_1924; *dadA*, A1S_1075; *sucC*, A1S_2718; *tauD*, A1S_1445; *cysD*, A1S_1000; *cysP*, A1S_2531) were among the top 10 downregulated genes. Other significantly downregulated genes were involved in oxidative phosphorylation (*nuoH*, A1S_0758; *nuoN*, A1S_0764; *nuoM*, A1S_0763), methylation (A1S_1188), translation (*efp*, A1S_2419) and transportation (*bamD*, A1S_0840).

Genes UpRegulated by Tobramycin Treatment

Two genes involved in leucine catabolism pathway (*mccB*, A1S_1375; *ivd*, A1S_1376), two genes encoding type II toxin-antitoxin system (*hicB*, A1S_2019; *hicA*, A1S_2020), four genes encoding transcriptional regulators (A1S_2699; A1S_0094; A1S_1377; A1S_3294), *ddrR* (A1S_1388) and putative NADPH:quinone reductase and related Zn-dependent oxidoreductase (A1S_3293) were among the top ten upregulated genes. Apart from these, genes involved in DNA repair (*umuD*, A1S_1389), type IV secretion system (*trbJ*, A1S_0665; *trbL*, A1S_0666), iron homeostasis (A1S_0040; A1S_0945; *iscU*, A1S_1631) and transporters (*arsB*, A1S_1454; A1S_1503; A1S_1504; A1S_2302; A1S_0161; A1S_3240; A1S_1046; A1S_1333;

A1S_1390; A1S_2472; A1S_3914) were also among the significantly overexpressed genes.

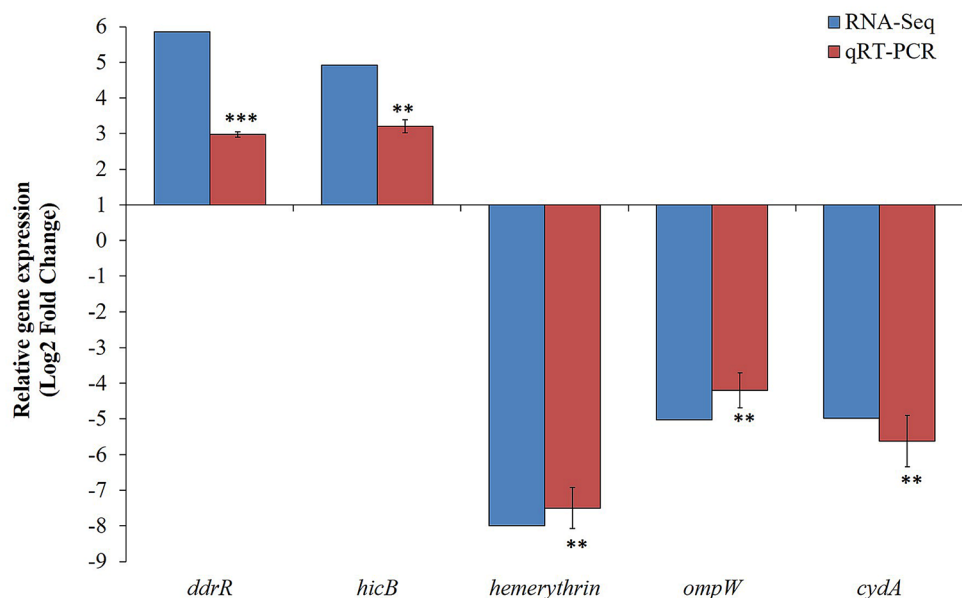
Validation of DEGs by qRT-PCR

Highly upregulated and the most downregulated genes from the RNA-Seq data were validated by qRT-PCR. *ddrR* (A1S_1388) and *hicB* (A1S_2019) genes upregulated with 5.86- and 4.92 log₂ fold change, respectively in RNA-Seq data, showed 2.97- and 3.2 log₂ fold upregulation respectively by qRT-PCR. RNA-Seq data showed hemerythrin (A1S_0891), *OmpW* family protein (A1S_0292) and cytochrome d ubiquinol oxidase subunit 1, *cydA* (A1S_1924) to be downregulated with -8.0 , -5.02 and -4.97 log₂ fold change, respectively, were also found to be -7.5 , -4.91 and -5.62 log₂ fold downregulated by qRT-PCR (Fig. 4). Hence, the expression pattern of five genes determined by qRT-PCR was found to be consistent with the transcriptome data.

Discussion

A. baumannii has become a major global public health concern [19]. Bacterial persistence allows antibiotic susceptible bacteria to survive lethal concentrations of antibiotics. Persistence is responsible for the recurrence of infections and is also considered an important contributor to the emergence of resistance [20]. The present study showed that *A. baumannii* ATCC 17978 in exponential phase formed persister cells against rifampicin (rifamycin), ciprofloxacin (fluoroquinolone) and amikacin (aminoglycoside) but could not survive lethal concentrations of another aminoglycoside

Fig. 4 Relative expression of five DEGs (*ddrR*, *hicB*, hemerythrin, *ompW* and *cydA*) in exponential phase cells of *A. baumannii* ATCC 17978 upon treatment with tobramycin (10×MIC for 1 h). Data are representative of three independent experiments. Bars represent the mean ± SD. ** $P \leq 0.01$; *** $P \leq 0.001$



tobramycin. Therefore, to explore genes and pathways that might be important in eradicating *A. baumannii*, the transcriptome of exponential phase *A. baumannii* cells after exposure to lethal concentration of tobramycin ($10\times$ MIC for 1 h) was evaluated.

Transcriptome analysis revealed that upregulated genes outnumbered the downregulated genes in response to tobramycin stress (Fig. 2). Aminoglycosides have been shown to inhibit protein synthesis in bacteria [21]. In accordance 23S rRNA methyltransferase and translation elongation factor (*efp*) was found to be downregulated in the present study. In *A. baumannii* ATCC 17978, there was significant downregulation of genes involved in energy production and conversion (*cydA*, *nuoH*, *nuoN*, *nuoM*, *sucC*) after tobramycin treatment. *cydA* encodes subunit I of cytochrome bd oxidase, which generates proton motive force across the membrane for ATP production [22]. Lack of a functional cytochrome severely compromised virulence and intracellular viability of *Staphylococcus aureus* [23], *Salmonella enterica* [24], *E. coli* [25], *Mycobacterium tuberculosis* [26] and *Mycobacterium smegmatis* [27]. The *cydA* deletion mutant of *M. tuberculosis* was found to be more susceptible to the FDA-approved drug bedaquiline and resulted in rapid killing of cells, underscoring the role of this respiratory protein in survival [28]. *nuoH*, *nuoM* and *nuoN* genes encode subunits of NADH-quinone oxidoreductase (complex I) that catalyzes the transfer of electrons from NADH to quinone in cytoplasmic membrane, forming a proton gradient required for energy production [29]. *sucC* encodes β subunit of succinyl-CoA synthetase that converts succinyl-CoA into succinate accompanied by the production of ATP. In *E. coli*, *ubiF* and *sucB* deletion mutants have been shown to reduce persister survival against ampicillin and gentamicin [30]. PPI network analysis of DEGs also highlighted *nuoN* and *nuoH* as the hub genes. The downregulation of these genes reflected decreased respiration, which probably lowered PMF and subsequently ATP production contributing to tobramycin-induced cell death.

Expression of genes involved in sulphur metabolism (*tauD* and *cysD*) was found to be significantly downregulated under tobramycin stress. *tauD* encodes taurine dioxygenase that is required for the utilization of taurine as a sulphur source. In *A. oleivorans* DR1, deletion of *tauD* exhibited increased sensitivity to H_2O_2 , highlighting its protective role in oxidative stress [31]. *cysD* encodes a subunit of sulfate adenylyltransferase that catalyzes the first step in sulfate assimilation pathway. Upregulation of *cysD* has been shown in *M. tuberculosis* in response to nutritional deprivation, hypoxia, hydrogen peroxide, and vancomycin treatment [32]. *cysD* deletion mutant of *Aeromonas hydrophila* showed decreased survival rate against furazolidone treatment [33]. Since aminoglycosides produce reactive oxygen

species [34], the downregulation of *tauD* and *cysD* genes in *A. baumannii* might have compromised the management of oxidative stress related to tobramycin.

Hemerythrin was also found to be downregulated by tobramycin. In *A. baumannii*, downregulation of hemerythrin has been reported under iron-limiting conditions [35]. Hemerythrin is non-heme, di-iron and O_2 -binding protein involved in iron and oxygen detoxification, transport, and storage [36]. Tobramycin may have disarmed the protective effect by reducing the expression of hemerythrin in *A. baumannii*. Downregulation of hemerythrin expression in *A. baumannii* was found to be concentration-dependent as $10\times$ MIC showed higher downregulation than $3\times$ MIC (Supplementary Fig. 4). Hemerythrin-like proteins affect oxidation–reduction regulation and antibiotic resistance [37]. The number of hemerythrin-like proteins varies in different organisms. This multiplicity makes it difficult to understand their role. However, *A. baumannii* ATCC 17978 has only one gene (A1S_0891) coding for hemerythrin. Since not much is known about hemerythrin-like proteins in *A. baumannii*, knockout of hemerythrin may help in providing insight into its function.

Expression of *ompW* was found to be significantly downregulated under tobramycin stress. OmpW is a β -barrel shaped outer membrane protein with a hydrophobic channel that is involved in the transport of hydrophobic molecules [38, 39]. In *A. baumannii*, OmpW is implicated in uptake of iron and its homeostasis, and binding of colistin to the membrane [40]. Downregulation of *ompW* might have reduced the uptake of iron by *A. baumannii* under tobramycin stress.

mccB, *ivd*, and *liuC* genes involved in leucine catabolism were upregulated on exposure to tobramycin. Leucine catabolism produces important intermediates of tricarboxylic acid (TCA) cycle (acetyl-CoA and acetoacetate) [41, 42], which are probably required to meet energy requirements under stress conditions. Genes related to leucine degradation and transport were found to be induced in *Acinetobacter harbiniensis* HITLi7^T under stress of low temperatures [43]. Tobramycin treatment upregulated the expression of *ddrD* and *umuD* genes involved in DNA repair [44]. Kanamycin, tobramycin, and gentamicin have been shown to induce the SOS response in *Vibrio cholera* as a result of drug-induced oxidative DNA damage [45]. Increased expression of these genes may be due to DNA damage at high concentrations of tobramycin.

Tobramycin also increased the expression of the *hicA* and *hicB* genes of the HicAB toxin-antitoxin system. Earlier, we had shown increased expression of *hicA* and *hicB* genes in *A. baumannii* in response to ciprofloxacin which may be related to persistence against it [46]. Since expression of these genes was significantly upregulated even under tobramycin stress, further studies are required to understand

their role in persistence. In *Burkholderia pseudomallaei*, the deletion of *hicAB* was found to reduce persistence against ciprofloxacin but not ceftazidime [47].

Conclusion

The present study shows that *A. baumannii* ATCC 17978 cannot survive lethal concentrations of tobramycin. Various genes and pathways were differentially expressed in *A. baumannii* as a response to tobramycin treatment. Significant downregulation of genes involved in energy production might have contributed to the lethality of tobramycin to *A. baumannii* cells. Downregulation of outer membrane protein W might have reduced the uptake of nutrients required for survival. Furthermore, decreased expression of hemerythrin and genes involved in sulphur metabolism (*tauD* and *cysD*) may have disarmed the protective response against tobramycin. Further work involving knockouts of these DEGs are required to confirm their involvement in tobramycin induced cell death.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s00284-022-02788-7>.

Author Contributions SK: Conceptualization, data curation, formal analysis, investigation, methodology, validation, visualization, writing—original draft, writing—review and editing. PS: Conceptualization, writing—review and editing. NC: Conceptualization, methodology, funding acquisition, resources, project administration, supervision, writing—review and editing. All authors have read and approved the final manuscript.

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Data Availability The data used to support the findings of this study are available from the corresponding author upon request.

Code Availability Not applicable.

Material Availability The data used to support the findings of this study are available from the corresponding author upon request.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethical Approval Not applicable.

Informed Consent Not applicable.

Consent to participate Not applicable.

Consent for publication Not applicable.

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