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The novel potential multidrug-resistance biomarkers for *Pseudomonas aeruginosa* lung infections using transcriptomics data analysis

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ABSTRACT

Development of multidrug resistance is the main obstacle for treating infections of *Pseudomonas aeruginosa*, an opportunistic pathogen responsible for a wide range of persistent infections throughout the body. Hence, identifying the major genes contributing to the development of multidrug resistance in *Pseudomonas aeruginosa* could help to introduce new efficient drugs to prevent resistance development. Here we conducted a differential gene expression analysis and a series of systems biology investigations on a RNASeq data set of multidrug resistant *Pseudomonas aeruginosa* isolates obtained from the patients' airways and wild-type, drug-sensitive strains. Respectively 67 and 178 up- and down-regulated genes were detected, including several genes of unknown functions that their functional characterization will help to elucidate the hidden mechanisms for multidrug resistance development in *Pseudomonas aeruginosa*. By inspecting the constructed gene regulatory network, two cases of feed forward loops were identified which in the case of establishing the type of regulatory interactions between transcription factors and their target genes, as activatory or inhibitory, their true contribution to multidrug resistance will be better understood. Inspecting the constructed gene co-expression network revealed co-expression between four deregulated genes including *PA14_32830*, *PA14_03380*, *fpvA* and *PA14_15610* and four already known drug resistance biomarkers. Functional characterization of these four co-expressed genes, will elucidate their possible roles in the process of multidrug resistance development in *Pseudomonas aeruginosa.* These findings will suggest new potential multidrug resistance biomarkers that following confirmation by larger number of samples, can be considered as new promising drug targets.

1. Introduction

Multidrug resistant (MDR) and extensively resistant (XDR) *Pseudomonas aeruginosa* strains have recently become one of the major concerns of public health. This is of great importance because *Pseudomonas aeruginosa*, is an opportunistic pathogen able to cause underlying severe infections in immunocompromised patients, and also in the lung of patients with genetic disease cystic fibrosis (CF) [1]. The worldwide spread of resistant *Pseudomonas aeruginosa* clones poses a threat to public health and therefore new treatment alternatives are urgently required [2]. Several studies have also underscored the link between multidrug resistance and increased morbidity and mortality [3–5].

Pseudomonas aeruginosa is equipped with several mechanisms of antibiotic resistance including inducible *AmpC* cephalosporinase expression, *MexAB-OprM* and inducible *MexXY* efflux pumps, and low outer membrane permeability that helps to have lower intrinsic antibiotic susceptibilities compared to other gram-negative organisms [6]. Drug exposure can also promote acquisition of resistance in *Pseudomonas aeruginosa* through different mutational events that activate resistance genes, modify antimicrobial targets and up-regulate several multidrug efflux pumps. Horizontally acquired resistance is another mechanism of resistance acquisition in *Pseudomonas aeruginosa* [7]. For instance, there is a growing spread of B-lactamases, the extended-spectrum B-lactamases (ESBLs) and carbapenemases [8].

Most *Pseudomonas aeruginosa* treatment protocols lead to the insufficient immunity and therefore *P. aeruginosa* infections are commonly treated with a combination of antibiotics to overcome multidrug resistance [9]. However, resistance development has also been frequently reported following combination therapy in *Pseudomonas aeruginosa* infections.

Identification of deregulated genes and pathways associated with drug-resistance can help to understand underlying molecular mechanisms of resistance development. Construction of gene co-expression network for resistant samples, on the other hand, would help to

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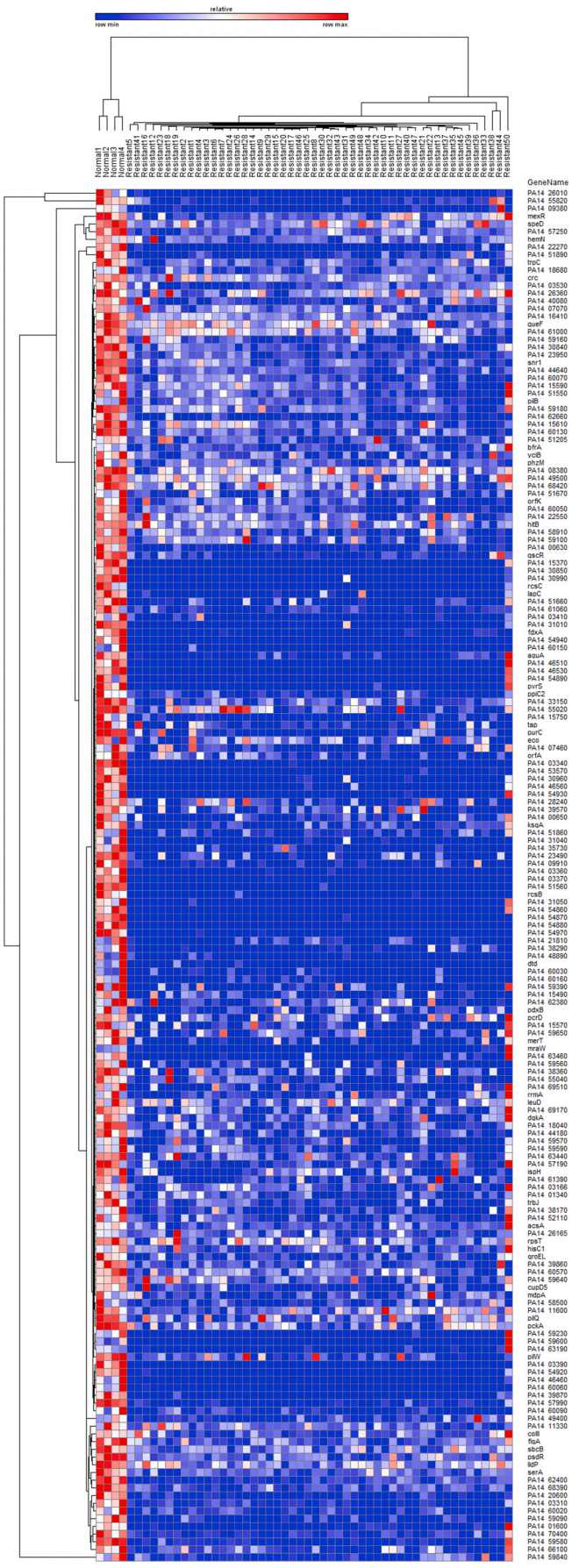

Fig. 1. Heatmap diagram of down- (a) and up-regulated (b) genes for 50 resistant and 4 wild-type(sensitive) samples.

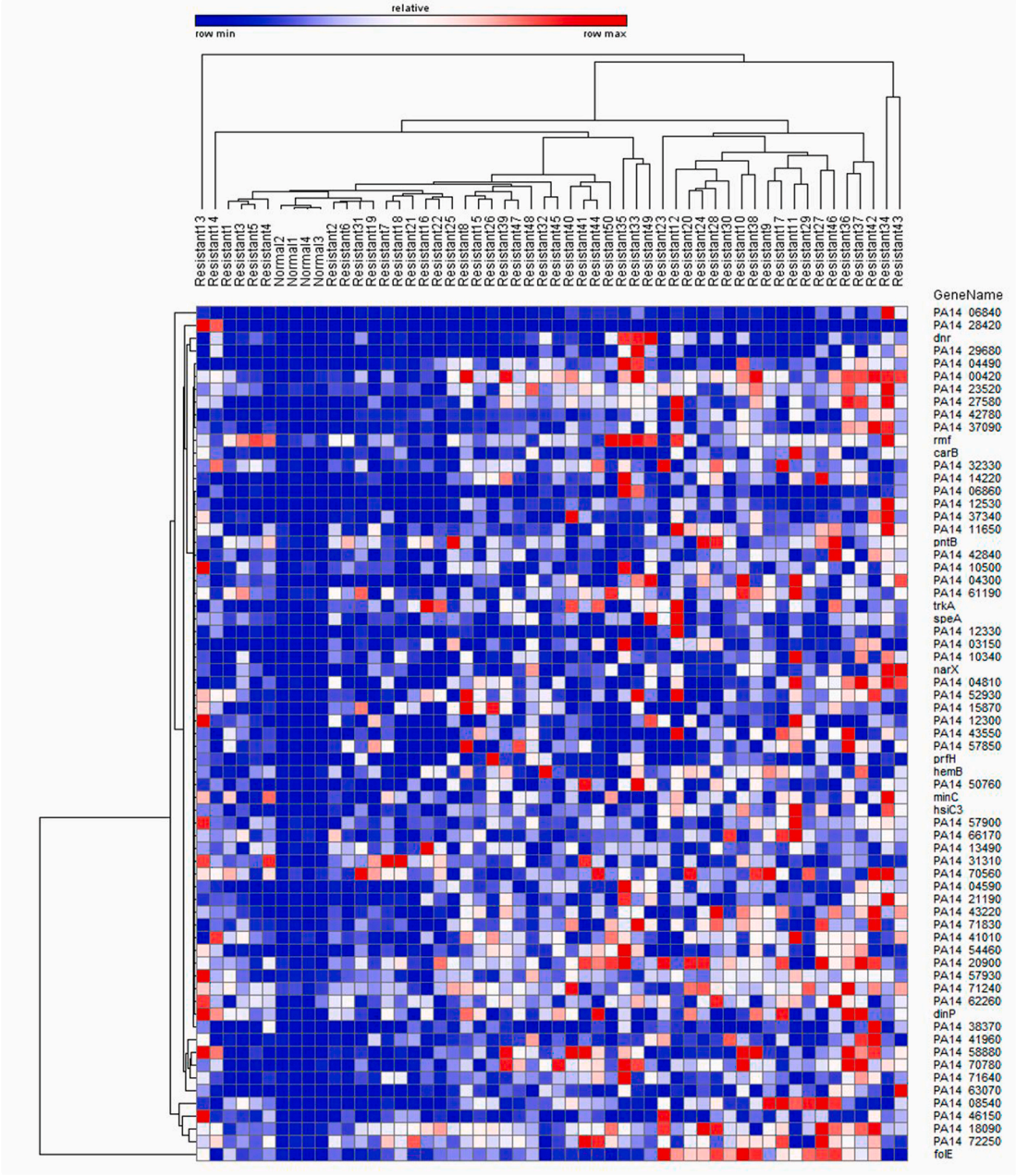

Fig. 1. (*continued*).

identify functional groups of co-expressed genes known as modules. Inspection of highly intra-connected modules within co-expression networks is an efficient way to identify new drug-resistance biomarkers.

In this study, our major goal was to identify novel biomarkers associated with multidrug resistance in *Pseudomonas aeruginosa.* Using a combination of differential gene expression and gene co-expression network analyses on a gene expression data set of multidrug resistant *Pseudomonas aeruginosa PA14* strains isolated from patients' respiratory tracts and wild-type (drug-sensitive) strains (SRP034661), we suggested new potential drug resistance-associated biomarkers which after further investigations could be considered as new drug targets.

2. Methods

2.1. Gene expression data set

A gene expression data set (SRP034661) including 50 RNA-seq samples of multidrug resistant *Pseudomonas aeruginosa PA14* isolated from human respiratory tract and 4 wild-type *P. aeruginosa* strains were retrieved from SRA-NCBI [10] (Table S1). *Pseudomonas aeruginosa* strains had previously been isolated and categorized as multidrug resistant (resistant to 2 or more drugs) by several research institutes in Germany that collaborated in this project. The antibiotic susceptibility tests had been performed by using the Vitek2 system (bioMérieux) or Etest strips (bioMérieux).

2.2. Differential gene expression analysis

108 *fastq* files (54 paired end samples, including resistant and wild-types), were subjected to trimming using "*Trimmomatic*" [11] pipeline applying "*the average quality*" threshold of 20 and the "*sliding window*" size of 4. The trimmed *fastq* files were then introduced to "*map with bowtie for illumina*" [12] program using *Pseudomonas aeruginosa UCBPP PA14* genome obtained from "*Ensembl*" database [13]. The counts

Table 1
Lists of up- and down-regulated transporters in resistant samples.

Up-regulated genes	Molecular Function	Down-regulated genes	Molecular function
PA14_32330	Transmembrane transport	***PA14_09380***	acetyl-CoA transmembrane transporter activity
PA14_18090	Putative major facilitator subfamily transporter protein	***PA14_55020***	transporter activity
PA14_23520	Putative MFS transporter	***PA14_16410***	transmembrane transporter activity
PA14_12300	Putative Mg^{2+} and Co^{2+} transporter CorC	***PA14_57990***	metal ion transmembrane transporter activity
PA14_10340	Putative toxin transporter	***PA14_31010***	cation transmembrane transporter activity
PA14_20900	Putative MFS transporter	***PA14_31040***	efflux transmembrane transporter activity
PA14_57930	Transmembrane transport	***PA14_26360***	transporter activity
trkA	Cell volume homeostasis -potassium ion transport	***PA14_11600***	Possible ABC transporter component
hsiC3	Metal ion binding -ubiquinol-cytochrome-c reductase activity -mitochondrial electron transport, ubiquinol to cytochrome c	***PA14_58500***	amino acid transport
PA14_12330	PhoH family protein	***PA14_46560***	efflux transmembrane transporter activity
		pilB	ATP binding - protein transporter activity
		hitB	Putative iron ABC transporter, permease protein
		lldP	lactate transmembrane transporter activity
		lapC	protein transporter activity
		merT	mercury ion transmembrane transporter activity
		pilQ	protein transporter activity

(number of fragments) of the aligned (mapped) sequences on each gene were subsequently determined using "*htseq-count*" program [14] employing the *P. aeruginosa PA14* GTF (Gene transfer format) file obtained from "*Ensembl*". 50 multidrug resistant and 4 wild-type "*htseq--count*" output files were then introduced to "*DeSeq2*" program as two separate groups (resistant vs sensitive). Applying adjusted *p-value* $\leq$0.05 and |logFC|$\geq$1 cutoffs, the list of statistically and biologically significant up- and down-regulated genes were identified respectively.

2.3. Gene and pathway enrichment analysis

The list of up- and down-regulated genes were separately introduced to "*DAVID*" [15] and "*PANTHER*" [16]programs and the molecular functions and associated pathways of deregulated genes were determined.

2.4. Inferring transcription factors-target genes regulatory network of the identified deregulated genes

The corresponding transcription factors of the identified deregulated genes were found from the Galan et al. study [17] -which is the only available source- and the TF-target regulatory relationships was visualized in "*Cytoscape*" *3.4* [18].

2.5. Gene co-expression network reconstruction for 50 multidrug resistant samples

The normalized expression values -in the form of *FPKM*-of 50 multidrug resistant samples were used to reconstruct gene co-expression network using "*Aracne*" algorithm [19]. *Aracne* calculates the pairwise expression correlation among all genes (here, around 6500 genes), using a correlation measure known as "*mutual information*" (*MI*) [20]. The weak correlations are then removed to leave only biological meaningful correlations. There is an option to introduce a list of markers -transcription factors or any gene set of interest-to "*Aracne*" that results in a co-expression network in which the expressional correlations between these markers and other genes are reported and so the resulted network is much smaller but more meaningful. We provided a list of 68 markers associated with antibiotic resistance in *Pseudomonas aeruginosa UCBPP PA14* retrieved from *PSEUDOMONAS* genome database [21] and introduced it to Aracne to get the drug resistance associated co-expression network. "*Aracne*" was run using the cutoff of 1e-7 for *p-value*. We removed pairwise correlations with *MI* $\leq$ 0.25 from the results and visualized drug resistance-associated co-expression network in "*Cytoscape*".

3. Results

3.1. Differential gene expression results

We identified 67 and 178 biologically significant up- and down-regulated genes respectively (with adjusted *p-value* $\leq$ 0.05 and |logFC| $\geq$1) using "*DeSeq2*" program (Fig. 1a and b). Among identified deregulated genes, 13 and 57 up- and down-regulated genes respectively, were of uncharacterized molecular functions (Bolds in Table S2).

3.2. Gene enrichment analysis results

We identified 10 up- and 16 down-regulated transporters in resistant samples (Table 1). *PhoH* is one of the up-regulated transporters that its up-regulation in the antimicrobial-resistant *Dickeya dadantii* (a plant pathogen) isolates has also been indicated [22]. We also identified the down-regulation of *PA14_18090*, *PA14_23520* and *PA14_20900*, and the up-regulation of *hitB* and *PA14_11600*, all are the members of MFS transporters -that provide energy by proton motive force.

3.3. Gene regulatory network

Two cases of coherent feed forward loops (FFLs) including *anr-dnr-narX* and *anr-dnr-hemN* were identified in the constructed gene regulatory network of deregulated genes in the resistant state (Fig. 2). In coherent FFls, a global TF activates a local TF and both activates a shared target gene.

3.4. Gene co-expression network inspection results

Construction of co-expression networks can significantly help to predict functions of already uncharacterized genes. Here, by constructing gene co-expression network among well-known antibiotic resistance biomarkers and other genes, we tried to introduce new potential antibiotic resistance biomarkers. Four highly connected resistance biomarkers including *PA14_48300*, *mexB*, *PA14_12820* and *PA14_33770* were detected in co-expression network which most of their co-expressed partners do not have known functions (Fig. 3).Three genes including *PA14_32830*, *PA14_03380*, and *fpvA* simultaneiusly showed co-expression with 4 highly connected resistance-associated biomarkers.

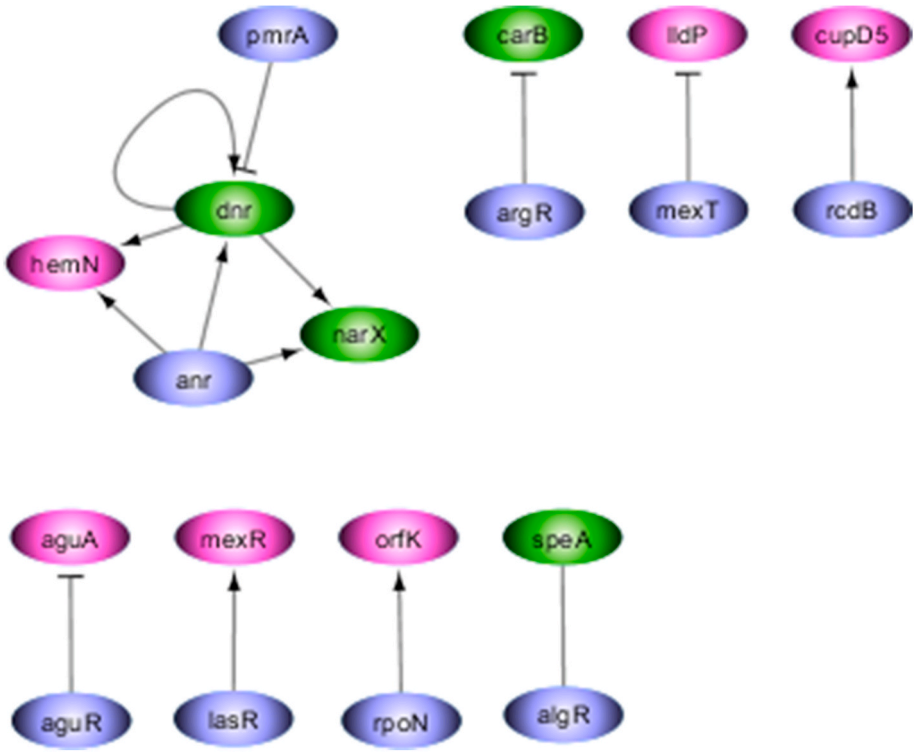

Fig. 2. Regulatory relationships among transcription factors (violet) and up- (green) and down-regulated (magenta) genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

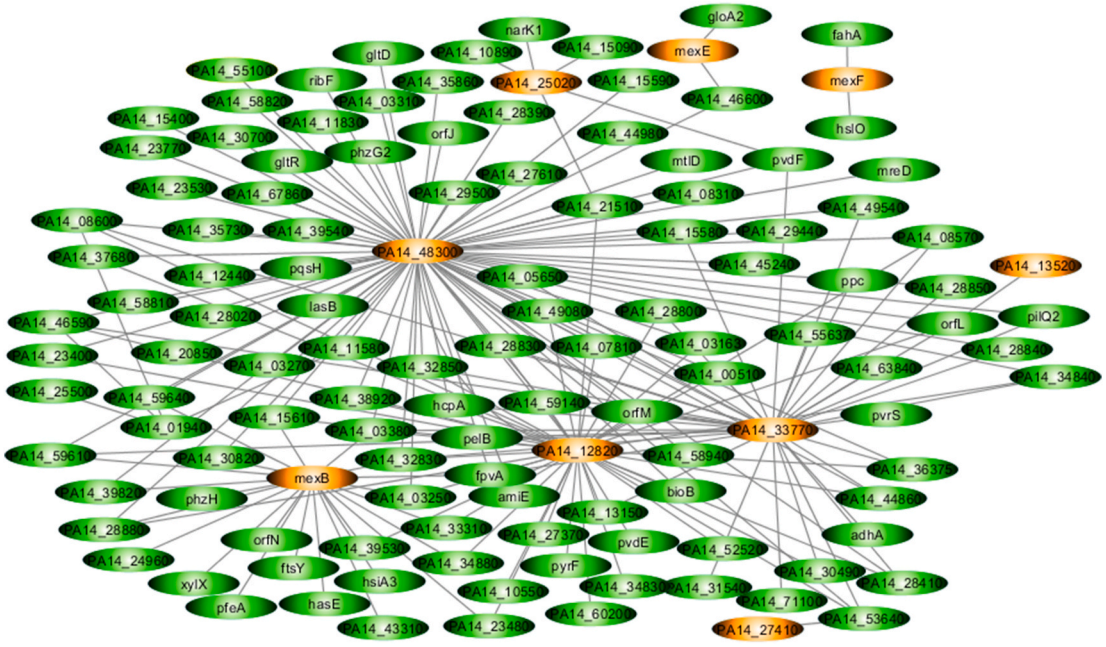

Fig. 3. Constructed co-expression network among known drug resistance genes (orange) and other genes (green) in 50 *Pseudomonas aeruginosa* resistant samples using "*Aracne*" algorithm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

Emergence of multidrug resistant gram negative bacteria including *Pseudomonas aeruginosa*, in lower respiratory tract infections, is one of the leading cause of global mortality [23]. Novel multidrug resistance-associated biomarkers can be used as predictive molecules that helps to select the most appropriate therapeutic strategies and in turn improves the survival rate [24]. In addition, these candidate biomarkers can be considered as new drug targets that may lead to the development of new drugs to combat antibiotic resistance [25].

In this systems biology study, respectively 67 and 178 up- and down-regulated genes in multidrug resistance *Pseudomonas aeruginosa* strains, which were isolated from different regions of respiratory tracts, were detected. We could not find similar studies on the transcriptional profile

Fig. 4. Co-expression between 5 down-regulated genes (magenta) and 4 known drug resistance biomarkers (violet). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

alterations in multidrug resistant *Pseudomonas aeruginosa* compared to wild-type strains for validation of identified deregulated genes.

The functional characterization of the large number of deregulated genes with still uncharacterized functions in this study will, at least to some extent, elucidates the molecular mechanisms of multidrug resistance in *Pseudomonas. aeruginosa*.

Since proteome profiling can uncover the alterations in the abundance of proteins between different physiological conditions, and therefore is a more real representative of cellular phenotypic alterations, we further searched for proteomics studies in which the proteome profiling of multidrug resistant *Pseudomonas aeruginosa* and wild-type strains, were compared. Although no proteomics study has previously been conducted to investigate proteome alterations of multidrug resistant *Pseudomonas aeruginosa*, we found few studies in which the resistance development against one single drug (Cirofloxacin, Tobramycin and silver compounds) had been the subject of study [26–30]. In addition, in all of these studies, the proteome alterations in *Pseudomonas aeruginosa PAO1* strain, which is moderately virulent, has been investigated and since SRP034661 data set consists of highly virulent *Pseudomonas aeruginosa PA14* isolates -with multidrug resistance properties-, their results was not completely comparable with this study. Nonetheless, the decreased abundance of *GroEL*, *PilQ* and *PhzM* and increased abundance of *PA14_04300*, confirmed their respectively down- and up-regulations in our results.

The presence of 10 membrane transporters in the list of up-regulated genes, confirms the role of transporters in antibiotic export. However, we also identified 16 down-regulated transporters in resistant samples. Decrease in the number of transporters is one the known resistance mechanisms that help to reduce antibiotic absorption. Delcour and colleagues demonstrated that down-regulation of outer membrane porin F (*ompF*) directly correlate with Betalactams and Quinolons absorption reduction in *Escherichia coli* that in turn leads to the multidrug resistance development [31]. Similarly, decreased ompF expression has been reported in resistant *Escherichia coli* isolates to a wide range of antibiotic including Norfloxacin, Tetracyclin, Cephalothin, Cefoxitin and Carbapenems [32,33].

We identified the expressional elevation of *dinP*, a member of SOS response which assists to tolerate against DNA damage. Cabot and colleagues showed that mutagenic effects of ciprofloxacin leads to the overexpression of DNA polymerase *dinP* [34]. Similarly, recent studies showed the up-regulation of *dinP* in *Staphylococcus aureus*, *Escherichia coli*, *Salmonella enterica* and *Yersinia pestis* strains in response to Quinolones [35,36]. Qin and colleagues also indicated that mutations in DNA

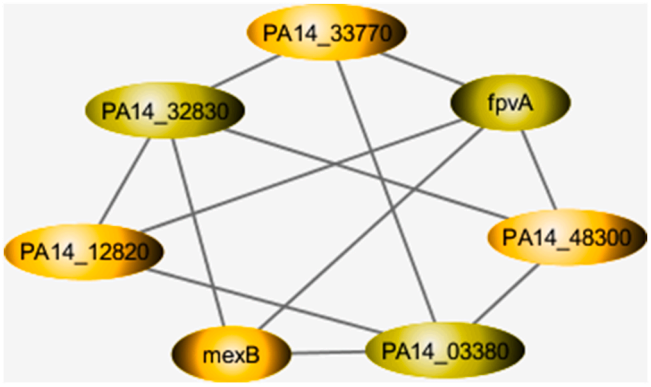

Fig. 5. Simultaneous co-expression of *PA14_32830*, *PA14_03380* and *fpvA* with four known drug resistance genes (Yellow). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

polymerase enzyme is increased following exposure to Ciprofloxacin leading to SOS response initiation. In the presence of Fluroquinolons (such as Ciprofloxacin), lexA-the inhibitor of Fluroquinolon resistance genes, is degraded and as a consequence the expression of resistance genes is elevated [37].

By inspecting the constructed regulatory nework of deregulated genes, 2 cases of coherent feed forward loops (FFLs) including *anr-dnr-narX* and *anr-dnr-hemN* were identified(Fig. 2). This kind of FFLs works as a delay element and prevents from rapid cellular responses to the transient changes in the surrounding environment. Both identified FFLs associate with nitrate respiration in anaerobic conditions in *Pseudomonas aeruginosa*. *Anr* is the transcription factor of anaerobic respiration genes. Due to the anaerobic conditions in biofilms, *Pseudomonas aeruginosa* is able to use nitrate and nitrite as the final electron acceptor [38]. Thus, by increasing the expression of *narX* through *anr* and *dnr*, which the latter is up-regulated here, *Pseudomonas aeruginosa* scapes from the antibiotic pressure and survives within biofilms. *HemN* (down-regulated here) is a dehydrogenase enzyme responsible for coproporphyrinogen III to coproporphyrinogen IX conversion in anaerobic conditions. Mutant *Escherichia coli* (with deletion of *hemN*) are highly resistant to tellurite compounds [39]. Considering the down-regulation of *hemN* despite the activatory effect of *anr* and *dnr*, reveals the possible role of other regulatory mechanisms such as genomic alterations in the reduction of *hemN*

expression.

As stated earlier, most of the co-expressed partners of 4 antibiotic resistance-associated biomarkers (*PA14_48300*, *mexB*, *PA14_12820* and *PA14_33770*) are proteins of unknown functions (Fig. 4). Functional characterization of these genes would probably shed more lights on the molecular mechanisms of resistance in *Pseudomonas aeruginosa.* Simultaneous co-expression of *PA14_32830*, *PA14_03380*, and *fpvA* with 4 highly connected resistance biomarkers (Fig. 5) can probably reveal their involvement in the process of resistance development. *PA14_32830* and *PA14_03380* are proteins of unknown functions. *fpvA*, is a siderofore receptor, responsible for collecting Fe ions from surrounding environment. The role of Fe in Tobramycin and Tigecycline resistance development in *Pseudomonas aeruginosa* has been verified [40].

In overall, in this study we introduced potential antibiotic resistance-associated biomarkers for *Pseudomonas aeruginosa* that after confirmation by qRT-PCR in multidrug resistant samples, can be considered as new targets to combat multidrug resistance. One of the main limitations of the present study is the imbalance between the number of samples in resistant and wild-type (antibiotic sensitive) groups (50 vs. only 4), that could have a significant impact of the results. We suggest that to confirm the gene expression alterations of the identified biomarkers, larger number of resistant and wild-type *Pseudomonas aeruginosa* isolates will be used to get more reliable results. In addition, it is well known that multiple antibiotic resistance genes have higher expression in the infections than in the laboratory conditions. Therefore, to get the true estimate of *Pseudomonas aeruginosa* transcriptional alterations in resistant state, the samples must be immediately subjected to RNA extraction and subsequent sequencing.

Authors' contribution

N·H designed the experiment, performed the data analysis, and wrote the paper, A.A helped to visualize the networks, F.A helped to write the introduction.

Availability of data and material

Data are available at (SRA, NCBI, SRP034661).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.imu.2020.100509.

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