Original Article

ABC Transporters are Hub Genes in Response of Resistant E. Coli ST131 to Ciprofloxacin

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Abstract

The worldwide spread of bacterial infection and the emergence of resistance to antibiotics have become two major problems in the field of medical sciences. As a serious threat, antibiotic resistance genes, in addition to pathogenic bacteria, have been found in bacteria that are generally recognized as safe. One of the common mechanisms of multidrug resistance (MDR) is due to overexpression of efflux pumps. ATP-binding cassette (ABC) transporters are one type of efflux pumps which have an important role in bacterial MDR. ABC transporters mediate the antibiotic expel from the bacterial cell by hydrolyzing ATP. In order to study antibiotic resistance caused by ABC transporters and to capture molecular/gene networks activated/inactivated by any antibiotic, a RNA-seq analysis is performed on *Escherichia coli ST131* which is treated by ciprofloxacin. Based on gene expression analysis, 589 genes expressed differentially (FDR p-value < 0.05). Totally 22 significant networks were extracted from differentially expressed genes (PPI < 0.05) which 3 of them have ABC transporters as enriched function including *malEFG*, *lolCDE,* and *glnHPQ* and the genes *malG*, *lolE,* and *glnP* are their hubs respectively. Among them, *malEFG* has two distinct enriched functions, ABC transporters and two-component system coincidently which means it is more likely to actively cooperate in antibiotic resistance. Since *malEFG* is up and two other networks are down regulated, ciprofloxacin can play the role of an activator for the first network and an inactivator for the others.

Keywords: Molecular network, ABC transporter, Two-component system, Efflux pump

Introduction

The spread of infectious bacteria worldwide on one side and the quick emergence of antimicrobial resistance on the other side are a global dilemma [1]. The prevalence of antibiotic resistance is not limited to pathogenic bacteria, but also in bacteria that are generally recognized as safe (GRAS), e.g. *Bacillus subtilis* [2, 3]. As a worldwide pandemic clone, *Escherichia coli* ST131 caused predominantly community-onset microbial infection [4]. Almost all E. coli ST131 isolates are resistant to a special class of antibiotics named fluoroquinolones, e.g. Ciprofloxacin [5]. Ciprofloxacin (CIP) is a fully synthetic analog of nalidixic acid which is the first quinolone discovered in 1962. CIP inhibits topoisomerase II (DNA gyrase) and IV and ultimately inhibits DNA synthesis which leads to bacterial death [6]. Among different mechanisms of resistance to antibiotics, e.g. CIP, one of the mechanisms is mediated by ATP-Binding Cassette (ABC) transporters which are one out of five classes of resistant efflux pumps [7]. ABC transporters are integral membrane proteins that pass various molecules through the cell membrane by the active transport mechanism [8] and in prokaryotes usually have three components, two integrated membrane proteins, two peripheral proteins that bind to ATP and hydrolyze it, and finally a periplasmic (or lipoprotein) substrate-binding protein. Based on the evidences in bacterial genomes, the majority of the genes involved in these three components form operons [9]. While some efflux pumps are selective for a particular substrate, many transporters show that they have certain characteristics that enable them to eject a set of structurally unrelated drugs [10]. Since the bacteria that has acquired ABC transporters can simultaneously diminish or even suppress the susceptibility to a wide range of antimicrobials, these microorganisms are called multidrug resistant (MDR) bacteria [11]. The study of the coding genes of ABC proteins in Escherichia coli showed that there are 79 ABC proteins in the genome and this amount makes it the largest paralogous family of proteins in E. coli [12]. In 1996, the researchers found that bacterial MDR transporter, LmrA which is a multidrug resistance ATP in Lactococcus and

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expressed in *Escherichia coli*, can hydrolyze ATP and use its free energy to extrude drugs out of the cell. LmrA is structurally and functionally similar to MDR1 which is a human multidrug resistance P-glycoprotein [13]. In addition to multidrug resistance in bacteria, neoplastic cells are refractory to a different drugs because of ABC transporters [14].

Despite the importance of deliberations on antibiotic resistance from the perspective of the gene networks, not much research has been done in this regard. In this study, to address the molecular networks activated/inactivated by ciprofloxacin, we have analysed up and down regulated genes using Cytoscape stringApp and CentiScaPe to explore the significant networks and finally, corresponding genes to each network are analysed functionally using KEGG and GO databases.

Materials and Methods

Study Design and Data Collection

Study design, data preparation, data processing, and analysis are shown in a flowchart **(Figure 1)**. RNA-seq data were obtained from the NCBI Gene Expression Omnibus (GEO). Datasets are GSM2374959 (control) and GSM2374960 (ciprofloxacin-treated) [15], and RNA-seq was performed on the MDR E. coli strain ST131, treated with a clinically relevant concentration of ciprofloxacin (2 μg/mL). In this study, two samples from the time point 30 min were used. One sample is treated (CIP) and the other is not treated with ciprofloxacin (control). Because of the exploration of the biological significance of the differentially expressed genes between treated and control groups, it is necessary to perform network and functional enrichment analysis. To facilitate the interpretations, each network is called based on its corresponding ABC transporter name.

Network Analysis and Identification of Hub Genes

Hub genes are those nodes that are highly connected. In this study a protein-protein interaction (PPI) network was constructed to identify hub nodes using Cytoscape stringApp [16] and all differentially expressed genes, down and up regulated, are imported to extract the significant networks. To have a comprehensive analysis on the relationships between nodes, Cytoscape CentiScaPe is used [17]. The nodes that have a high degree of connectivity in the network are treated as hub [18].

Gene Set Enrichment Analysis (GSEA)

Functional enrichment analysis was conducted on each network separately using Cytoscape stringApp [16]. To conduct enrichment analysis, two different databases are used including KEGG-pathway DB and Gene Ontology (GO) DB [19, 20]. The former is to find the related pathways for each network and the latter is to classify the genes based on their biological processes, cellular components, and molecular functions.

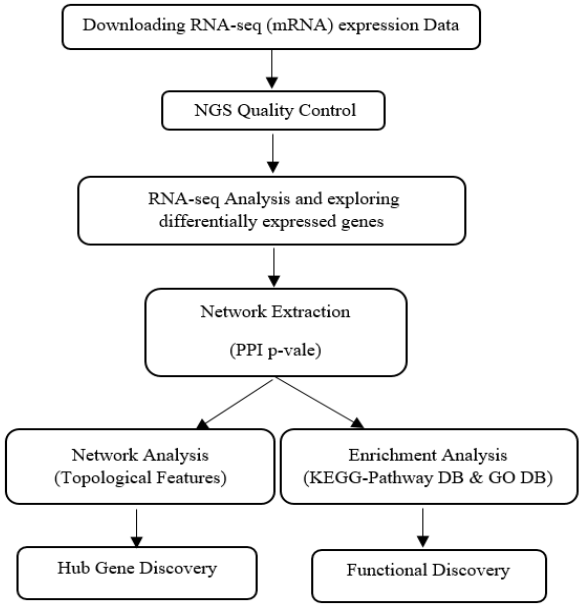


Figure 1. The Flowchart of data preparation and analysis used in this study.

Results and Discussion

After RNA-seq and differential gene expression analysis, 589 genes have FDR p-values less than 0.05. All of the differentially expressed genes including up and down regulated genes are imported to Cytoscape stringApp (protein query) (confidence cutoff score was=0.9). Totally 22 networks were found which 3 of them have the function of ABC transporter **(Figure 3)**. The heat map for 29 genes related to the ABC transporter function are shown in **Figure 2**.

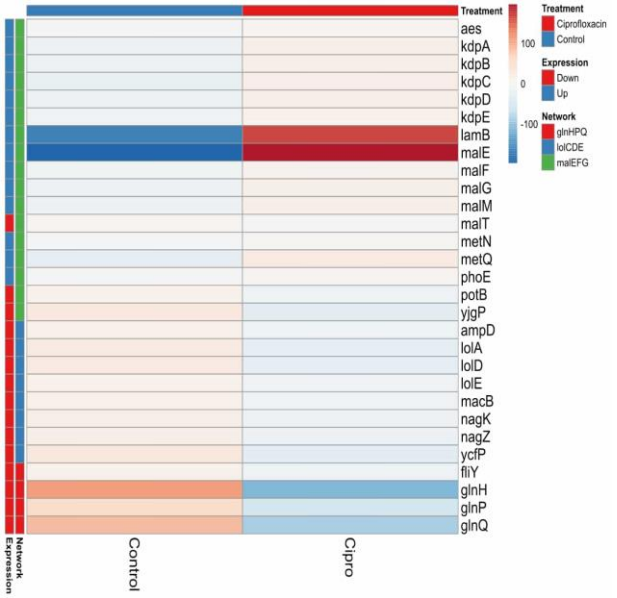
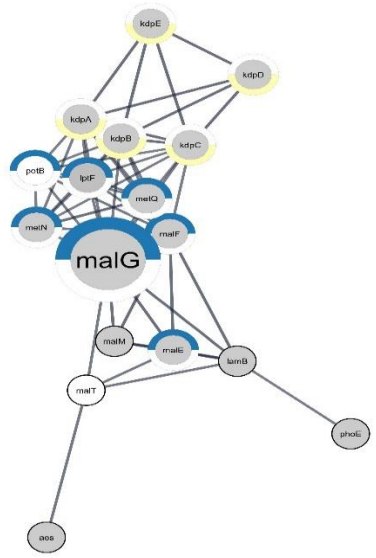
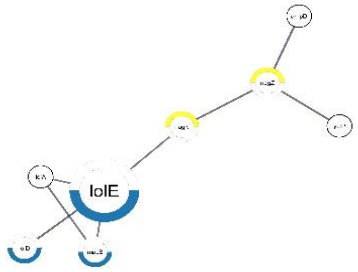


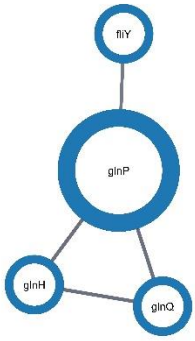
Figure 2. Heat map for 29 genes related to ABC transporter pathway



a) *malEFG*, PPI p-value: 1.0E-16



b) *lolCDE*, PPI p-value: 6.66E-16



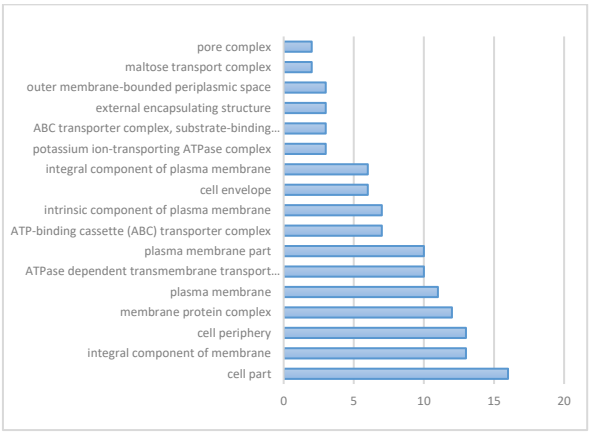
c) *glnHPQ*, PPI p-value: 1.09E-12

Figure 3. a) *malEFG*, b) *lolCDE* and c) *glnHPQ* networks consist of 17, 8, and 4 genes, respectively. The grey and white circles are up and down regulated genes, respectively. The big node in each network is the hub gene. The nodes with a dark belt are specifically involved in the ABC transporter pathway.

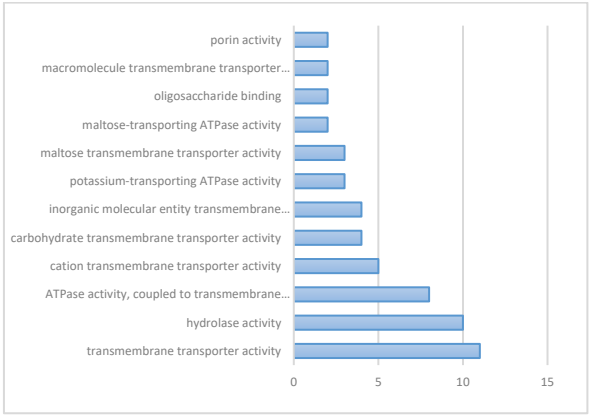
Network analysis on node parameters shows that the highest degree belongs to the nodes *malG*, *lolE*, and *glnP* in *malEFG*, *lolCDE*, and *glnHPQ* networks, respectively, so they can be treated as hub genes. The hub genes *malG*, *lolE*, and *glnP* have 12, 4, and 4 direct relationships, respectively, so they can be considered as essential genes [21]. The product of gene *malG* is a protein with 296 amino acid residues. Due to the presence of six hydrophobic segments, this protein is considered as highly hydrophobic. This protein is an integral inner membrane protein and as the sequence of *malG* gene is highly conserved, it can be found in any integral membrane protein of binding protein-dependent transport system [22]. *lolE* gene produces a protein of the same name which along with *lolC* are integral membrane proteins [23]. The product of gene glnP along with the products of glnH, glnQ are the main players of the glutamine transport system and the presence of them gives the ability to Escherichia coli to utilize glutamine as a sole carbon source [22].

GO analysis in different classes including molecular function, cellular component, and biological process has valuable results. The enriched GO terms and their charts are shown in **Figures 4, 5, and 6**. The length of each term is based on the number of genes in each term. To answer this question that each network by which molecular function and where in the cell can do what kind of biological process can say as the following:

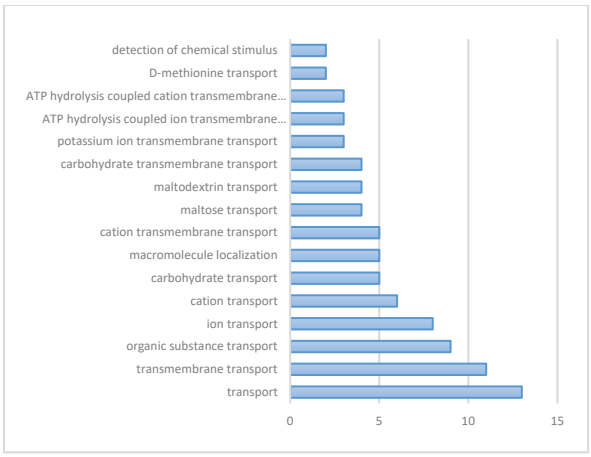
- *malEFG* mostly as a transporter at cell membrane transports ions and organic substrates **(Figure 4)**.
- *lolCDE* as a transporter moves lipoproteins to outer membrane **(Figure 5)**.
- *glnHPQ* as a transmembrane transporter translocate amino acids **(Figure 6)**.



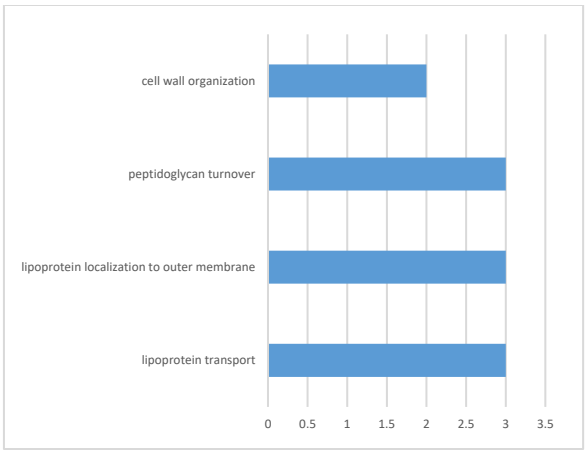
a) Cellular Component



b) Molecular Function



c) Biological Process



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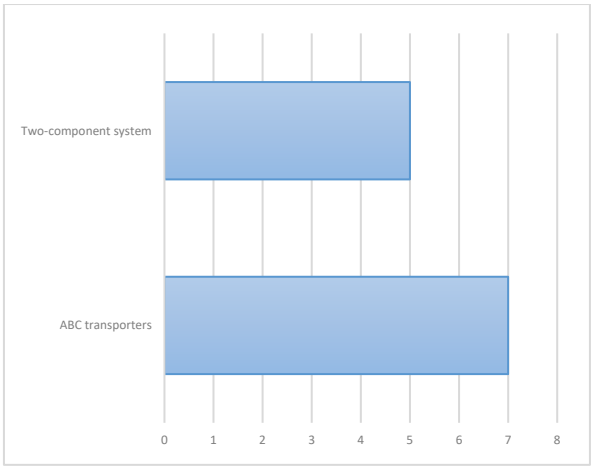
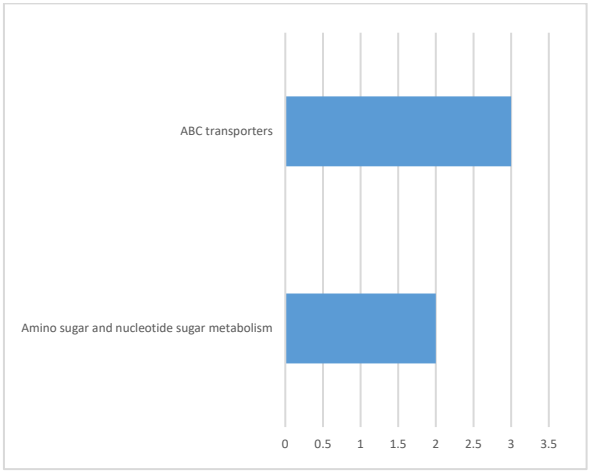


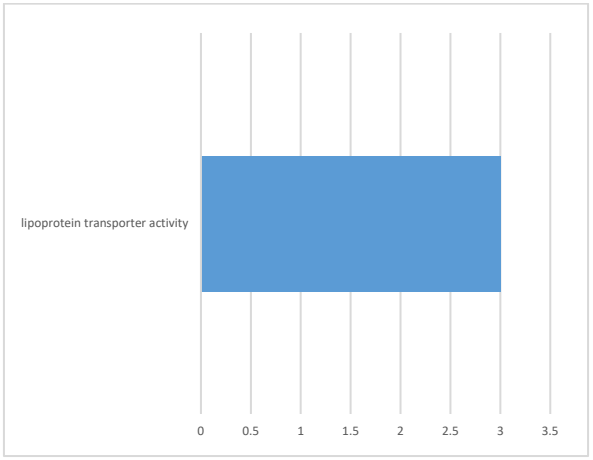
d) KEGG Pathways

Figure 4. Functional analysis of genes in the *malEFG* network. GO DB: a) Cellular component b) Molecular function c) Biological process. KEGG DB: d) Pathway analysis

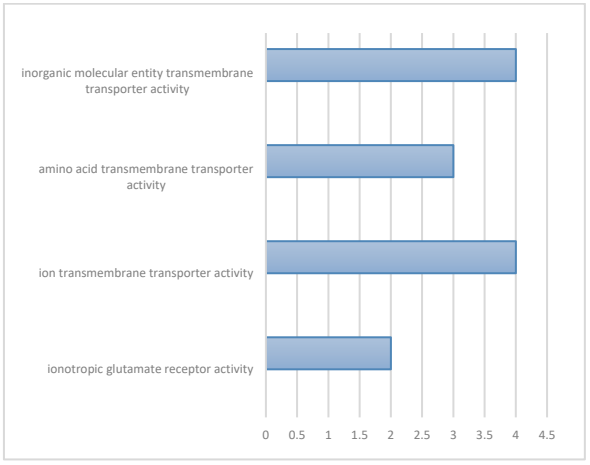


c) KEGG Pathways

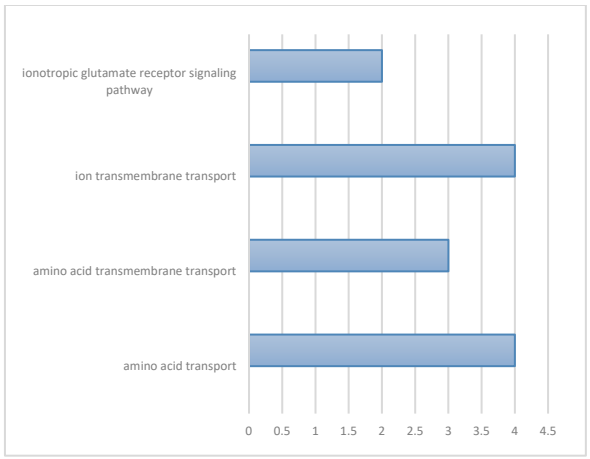
Figure 5. Functional analysis of genes in *lolCDE* Network. GO DB: a) Molecular function. b) Biological process. KEGG DB: c) Pathway analysis



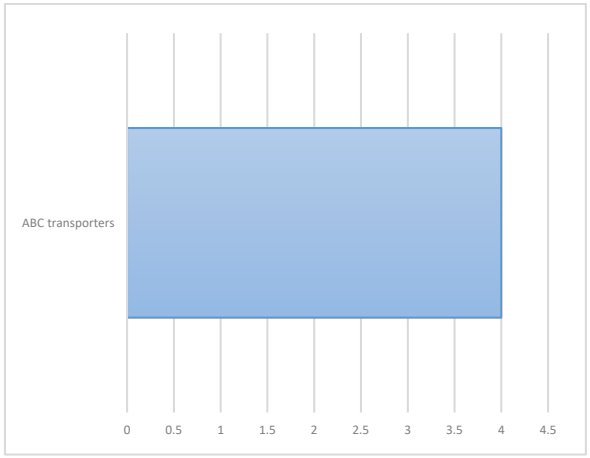
a) Molecular Function



a) Molecular Functions



b) Biological Processes



c) KEGG Pathway

Figure 6. Functional analysis of genes in the *glnHPQ* network. GO DB: a) Molecular function. b) Biological process. KEGG DB: c) Pathway analysis

Based on KEGG-pathway enrichment analysis, two pathways are enriched for the *malEFG* network in **Figure 1**. Seven genes (circles with dark belt) are enriched for ABC transporters and 5 genes (Circles with yellow belt) are involved in two-component systems (TCSs). Based on the first neighbors of the hubs, although the hub gene does not involve in TCS directly all the genes in the so-called pathways have a relationship with the hub. As these two pathways have no genes in common, therefore the main driver of them is the hub gene. In both pathways, all genes except, *potB* and *lptF* are up-regulated.

malEFG is one of two operons in the *malB* region. *malA* and *malB* regions of *E. coli* main genome are involved in maltose system. *malA* region is composed of *malT* gene and the *malPQ* operon. In *malB* region, *malK-lamB* operon is next to the *malEFG* operon which both are transcribed divergently from promoters located between *malE* and *malK* (the first gene in each operon) [24]. *malT* is the transcription factor (TF) of the maltose system and its function is tightly related to other parts of the system [25]. Among 7 genes in the *malEFG* network enriched as ABC transporter, 3 genes including *malE*, *malF*, and *malG* are involved in maltose system. Besides, *metN* (*abc*) and *metQ* (*yaeC*) are parts of *metD* locus which encodes methionine ABC transporter system [26]. Remained genes, *lptF*, and *potB* are parts of *lptFG* and *potABCD* operons. The *lptFG* operon produces proteins which are essential lipopolysaccharide transportations. The protein complex $lptB_2FG$ is an ABC transporter and has *lptF* and *lptG* as its main parts [27]. Depletion of *lptF* results in a filamenting phenotype increased sensitivity to hydrophobic antibiotics and an altered form of lipopolysaccharides [28]. The *potABCD* operon encodes a polyamine (putrescine/spermidine) transport system [29]. In both eukaryotes and prokaryotes, normal growth and multiplication are completely dependent on polyamines [30]. In *malEFG* network, there is another enriched function as two-component system (TCS). TCS is the dominant method by which bacteria respond to changing environments and plays a decisive role in the adaptation of bacteria to diverse niches [31, 32].

lolCDE makes an inner membrane ABC transporter that releases mature lipoproteins from the inner membrane to *lolA*. *lolA* passes lipoproteins across the periplasm to *lolB which* is an outer membrane lipoprotein [33]. *lolCDE* complex in *Escherichia coli* is a part of a bigger machinery named *lolABCDE* which sends lipoproteins to the outer membrane. Lipoprotein (*lol*) pathway is one out of three main pathways aiming at outer membrane assembly. The other two pathways are β-barrel assembly machine (*bam*) and lipopolysaccharide transport proteins (*lpt*). Their importance is so great that inhibition of any of them leads to *E. coli* death and subsequent lysis [34].

The glutamine permease operon, *glnHPQ*, is responsible for encoding a specified transport system of glutamine, a polar amino acid, in *Escherichia coli*. This operon contains three genes *glnH*, *glnP*, and *glnQ*. glnH encodes the periplasmic glutamine-binding protein (*glnH*). The other two genes *glnP* and *glnQ* which are located downstream of glnH have one promoter in common [35, 36].

Ciprofloxacin changed the expression of three ABC transporters including *malEFG*, *lolCDE*, and *glnNPQ* in the way that upregulates *malEFG* and downregulates the other two networks. When the bacteria is a MDR one and the engine behind this characteristic is ABC transporters, it does not mean all ABC transporters can pump all antibiotics. In the current study, among all, we found just one ABC transporter which is activated by ciprofloxacin. We have the same phenomenon in cancer cells. In blood, colon, and breast cancer the genes related to ABCB1/P-pg, ABCC1/MRP1, and BCRP are overexpressed respectively when they are prescribed with common medications [37].

The *malEFG* by itself is involved in 4 different ABC transporters and coordinates a TCS as well. Based on the findings by Ahmad *et al*., the majority of ABC transporters

can detect the presence of antibiotics in the external environment. But this detection ability is tightly regulated by TCSs. TCSs play a significant role in inducing a fast and specific response to antibiotics [38]. So coincidence of ABC transport system and TCS in a *malEFG* network did not happen by chance.

In order to increase efficacy and decrease resistance, there are techniques like drug/antibiotic combination [39, 40]. As mentioned above, *lolCDE* is downregulated by ciprofloxacin. One of downregulated genes in *lolCDE* network is *macB*. Although this gene is not enriched in a separate ABC transporter, genetically, this gene is located in an operon called *macAB*. This operon encodes two proteins in macAB ABC transporter which is involved in involved in resitance to macrolide antibiotics [41, 42]. Based on the evidence in the current study, ciprofloxacin by downregulating *lolCDE* network is going to increase sensitivity to other antibiotics which is the main framework of antibiotic resistance breaking (ARB) protocol [43-45].

Conclusion

According to the results of this study, ciprofloxacin has activated *malEFG* and inactivated *lolCDE* and *glnHPQ* ABC transporters. Since the activation of *malEFG* and TCS has taken place at the same network, it is possible that *malEFG* could be one of the causes of resistance to ciprofloxacin. *lolCDE* ABC transporter also causes resistance to macrolide antibiotics and its inactivation means the possibility of controlling its resistance. As a result, the antibiotic resistance can be controlled by drug combination. Of course, the final decisions need more wet lab validations.

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CONFLICT OF INTEREST: None
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ETHICS STATEMENT: None

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