

A Genome-Scale Modeling Approach to Investigate the Antibiotics-Triggered Perturbation in the Metabolism of *Pseudomonas aeruginosa*

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Abstract: Recent studies indicate that pretreating microorganisms with ribosome-targeting antibiotics may promote a transition in the microbial phenotype such as the formation of persister cells; i.e., those cells that survive antibiotic treatment by becoming metabolically dormant. In this work, we developed the first genome-scale modeling approach to systematically investigate the influence of ribosome-targeting antibiotics on the metabolism of *Pseudomonas aeruginosa*. An approach for integrating gene expression data with metabolic networks was first developed to identify the metabolic reactions whose fluxes were positively correlated with gene activation levels. The fluxes of these reactions were further constrained via a flux balance analysis to mimic the inhibition of antibiotics on microbial metabolism. It was found that some of metabolic reactions with large flux change, including metabolic reactions for homoserine metabolism, the production of 2-heptyl-4-quinolone (HHQ) and isocitrate lyase, were confirmed by existing experimental data for their important role in promoting persister cell formation. Metabolites with large exchange-rate change, such as acetate, agmatine and oxoglutarate, were found important for persister cell formation in previous experiments. The predicted results on the flux change triggered by ribosome-targeting antibiotics can be used to generate hypotheses for future experimental design to combat antibiotic-resistant pathogens.

Index term: Computational modeling and simulations in biology; Systems biology; Flux balance analysis; Persister cells

1. Introduction:

Although antibiotics are known for eliminating pathogens through a variety of mechanisms [1, 2], antibiotics may also promote the formation of persisters, which are dormant variants of regular cells that are highly tolerant to antibiotics [3]. For example, antibiotic pretreatment to *E. coli* including rifampicin, tetracycline, and carbonyl cyanide *m*-chlorophenyl hydrazine increased the microbial persistence dramatically by halting protein synthesis [4]. The presence of persister-specific tolerance is suggested to account for the recalcitrance of infectious diseases [5, 6]. It is thus important to study the mechanisms via which antibiotics cause pathogens to change their metabolism and form persister cells. In experimental studies, screening knockout libraries has not produced mutants that lack persisters, indicating that dormancy is not regulated by one single gene or enzyme [3, 7]. Although research has been conducted on the reaction-flux distribution upon the antibiotic treatment [8], no genome-scale modeling approach has been published to incorporate the interaction of multiple genes, enzymes and metabolites to investigate potential persister-forming mechanisms triggered by antibiotics. This motivates us to develop the first genome-scale modeling approach to quantify the effect of antibiotics on the metabolic flux redistribution, and thus investigate potential mechanisms for the persister formation that is triggered by antibiotics.

The enzyme-dependent reactions can be generally identified from the metabolic pathways according to the up-regulated genes in the experimental data. However, existing studies show that the correlation between mRNA or protein levels and metabolic fluxes levels is not necessarily high for all metabolic reactions [9, 10]. Kim et al., 2013 developed the first *in silico* approach to capture the relationship between mRNA levels and metabolic fluxes [9]. This approach, called flux-coupled genes, requires transcriptome and fluxome data under different conditions. Since fluxome data are mostly limited to the central carbon metabolism, this approach cannot be applied to metabolic pathways for other metabolisms in genome-scale models. The flux balance analysis (FBA) is one of the most commonly used approaches to quantify microbial growth under specific nutrient conditions from genome-scale models. On the basis of the FBA platform, several approaches, including GIMME[11], iMAT[12], MADE[13], E-FLUX[8], Lee-12[14], RELATCH[15], and GX-FBA[16], have been developed to integrate gene expression data with genome-scale models to predict metabolism. Most of these methods place tight constraints on metabolic fluxes from mRNA data so that the change in metabolic fluxes matches the change in

mRNA levels. However, the performances of these methods are not as good as traditional FBA and Probabilistic Flux Balance Analysis (pFBA) [10, 17] in which no constraints from gene expression data were imposed to metabolic fluxes. One potential reason for this is that not all of reactions rates are enzyme-dependent, as some of them depend on the availability of reactants instead (i.e., substrate-dependent). In many cases, mRNA or protein levels in the same metabolic branch might engage alteration in opposite directions. Therefore, assigning a tight constraint or objective function to fit the fluxes to microarray data, as shown in the aforementioned approaches, might not exhibit good prediction capability. We hypothesize that adding a loose constraint to metabolic fluxes based upon gene expression data may return a better correlation between the metabolic fluxes and mRNA levels than FBA and pFBA. Hence, the reactions identified as mRNA/enzyme dependent were constrained here in a way that mimics the stress imposed by antibiotics to the bacteria. The reactions with large flux change and the metabolites with large exchange rates are determined to provide a systems-level investigation of potential persister formation mechanisms of *P. aeruginosa*. This pathogen was selected in this work, as it is one of the leading causes of nosocomial infections in hospitalized patients and it displays resistance to a wide array of antibiotics by forming a biofilm in chronic infectious processes [18, 19].

2. METHOD

2.1 An illustrative example of overall approach

Gene expression data of microorganisms under the control condition and other experimental conditions were first obtained (Figure 1A). The changes in gene expression levels were used to constrain metabolic fluxes, which were then used to identify the metabolic reactions that mainly depend on the enzyme levels instead of the reactant/substrate levels. For example, it can be concluded from Figure 1A that the fluxes of Reaction 1 are positively correlated with the expression levels of Gene 1, while the fluxes of Reaction 2 are not correlated with Gene 2 expression levels. Reaction 2 may be substrate-dependent instead of enzyme-dependent. Since substrates are constrained by mass balance, the fluxes of substrate-dependent reactions may be negatively correlative with gene express levels.

The reactions whose fluxes are positively associated with gene expression levels were then further constrained with limited metabolic fluxes as shown in the RED color reactions in Figure 1B. This can mimic the inhibition of the antibiotics on the protein

synthesis, which can be reflected by the low activity levels of the metabolic reactions associated with these proteins/genes. For example, the flux through Reaction 1 is limited to a small value. On the other hand, the flux of Reaction 2 was not constrained as it is not positively correlated with the expression levels of Gene 2, that is, it may not be influenced by the antibiotics that inhibit the protein synthesis.

The flux change of each metabolic reaction was further investigated. In particular, the flux distributions of each metabolic reaction before and after the treatment with antibiotics (i.e., the BLACK and RED curves shown in Figure 1B respectively) were compared to identify the reactions with the largest flux changes. On the basis of these reactions, we can further study how the antibiotic may change the microbial metabolism and possibly induce the persister pathogen formation.

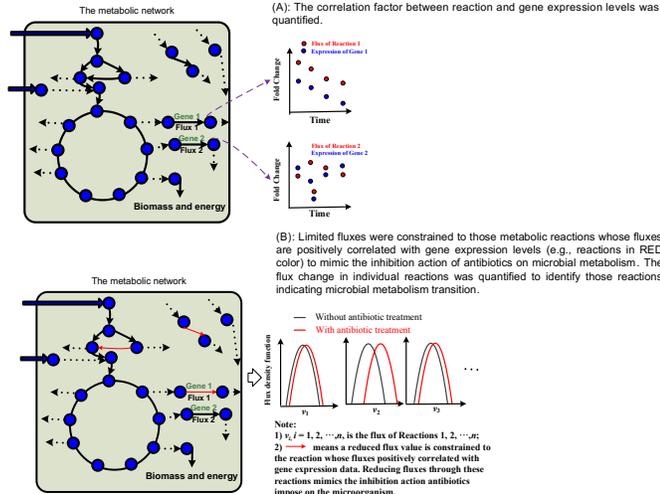


Figure 1, a schematic description of the proposed approach to quantify the metabolism alteration imposed by the treatment of antibiotics on pathogens

2. 2 Detail of the developed approach

The metabolic model for *P. aeruginosa* used in this work consists of 1056 metabolic genes, 1030 enzymes, and 1111 metabolic reactions (Oberhardt et al., 2008 [20]). The COBRA toolbox developed by Dr. Palsson's group at UCSD was used in this work to perform the flux balance analysis and sample feasible flux solutions [21, 22]. In order to mimic the stress imposed by ribosome-targeting antibiotics on microbial metabolism, the gene expression data was used to identify metabolic reactions whose fluxes are highly correlated with gene expression levels via the following steps:

Step 1: The lower bound of the Reaction i flux was constrained as shown in Equation (1) in the flux balance analysis if the gene associated with Reaction i was up-regulated. On the contrary, the upper bound was constrained (shown in Equation (2)) if the gene was down-regulated.

$$lb_i = v_{\text{mean}} \times C_i \times N \quad (1)$$

$$ub_i = v_{\text{mean}} \times C_i \times N \quad (2)$$

where v_{mean} is the mean value of reaction fluxes sampled for the control condition (e.g., antibiotics were not used). C_i is the ratio of the gene expression levels between the new experimental condition and the control condition. If multiple genes are involved in one metabolic reaction, C_i is set to the geometric mean of the expression-level ratios for those genes. N is the minimal integer that guarantees the model-predicted growth rate is not less than 90% of the experimental growth rate.

Step 2: The flux change of Reaction i was defined by the difference between the fluxes of new and control conditions. The flux change was finally normalized by the mean flux value for the

control condition, represented by ΔF_i in Equation (3). Similarly, the normalized gene expression change ΔG_i was calculated. The correlation factor θ_i between the metabolic flux and the gene expression level was defined by the ratio of ΔF_i over ΔG_i as shown in Equation (3). If gene expression data was obtained for multiple time points, the final θ_i represents the average value. Since some reaction rates were more substrate dependent (i.e., constrained by up/down stream reactions) than enzyme dependent, the correlation factor θ_i for these reactions might be negative. In this case, a zero value was set to θ_i to waive any constraint on the flux of Reaction i . On the other hand, if θ_i was larger than one, θ_i was set to one so that a maximum constraint was applied to the flux of Reaction i .

$$\theta_i = \frac{\Delta F_i}{\Delta G_i} \quad (3)$$

The correlation factor θ_i was then integrated into flux balance analysis to constrain the upper bounds of metabolic reactions to mimic the inhibition effect from the antibiotics on microbial metabolism (as shown in Equation (4)). For antibiotics that halt protein synthesis, it was assumed that the synthesis of all enzymes was inhibited equally 90%, as antibiotics generally are not able to completely inhibit enzyme levels. Additionally, other inhibition percentages were also tested, and the results were similar in general.

$$ub_i = v_{\text{mean}} - 90\% \times v_{\text{mean}} \times \theta_i \quad (4)$$

3. RESULTS

3.1 Determination of correlation factor between gene expression levels and metabolic fluxes by integrating gene expression data into the metabolic network

In order to test the performance of our approach for integrating gene expression data into metabolic models, the fluxomics dataset obtained by C^{13} -labeling in Ishii et al., 2007 [23] was utilized as the experimental data. In addition, our approach was compared with pFBA and FBA (Figure 2), as these two methods have been approved to predict higher correlation between metabolic fluxes and gene expression levels than other aforementioned existing approaches. The experimental data was obtained with different dilution rates: 0.1 h^{-1} , 0.4 h^{-1} , 0.5 h^{-1} and 0.7 h^{-1} . The 0.1 h^{-1} condition was treated as the control. As shown in Figure 2(a), the prediction by our approach for the 0.4 h^{-1} dilution rate generated a correlation factor R of 0.81, which is higher than 0.75 and 0.76 predicted from FBA and pFBA, respectively. For the data obtained with the 0.5 h^{-1} dilution rate, our approach generated R equal to 0.75, while R for pFBA and FBA are 0.71 and 0.70, respectively. Our approach remained the best for the 0.7 h^{-1} dilution rate (R equal to 0.75), while pFBA outperformed FBA again (R equal to 0.73 versus 0.72).

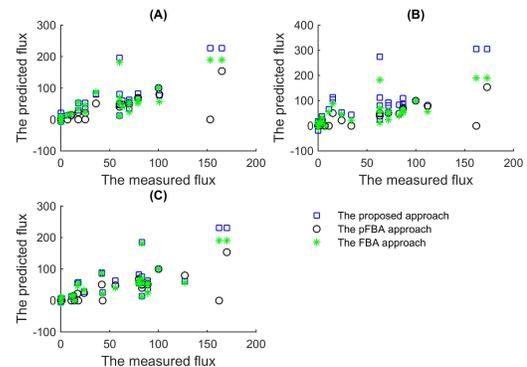


Figure 2, Prediction of the intracellular fluxes from the gene expression data measured at (A) 0.4 h^{-1} dilution rate, (B) 0.5 h^{-1} dilution rate, (C) 0.7 h^{-1} dilution rate. The X axis shows the measured fluxes, while the Y axis represents the predicted fluxes through individual reactions of the central carbon metabolism.

Among the 28 intracellular reactions in central carbon metabolism, 18 reactions demonstrated a positive correlation in their fluxes to the expression levels of their corresponding genes. Meanwhile, the rate limiting steps in central carbon metabolism have large positive correlation with their genes. For example, the reaction in which isocitrate is converted to 2-oxoglutarate is the rate limiting step in the TCA cycle. Its correlation factor was predicted to be 0.95 according to our approach, and 0.79 according to fluxomics data. Other rate limiting enzymes such as pyruvate kinase and α -ketoglutarate dehydrogenase were inferred to have a strong correlation between reaction rate and gene expression level by both our approach and the fluxomics data. This also indicates a good prediction ability of our approach.

3.2 Investigating the metabolic alteration of *P. aeruginosa* upon the treatment of ribosome targeted antibiotics

Our approach was applied to the microarray data presented in Dötsch et al., 2012 [24] to determine the metabolic reactions that are positively correlated with the gene expression levels in *P. aeruginosa*. In particular, gene expression data of *P. aeruginosa* at multiple time points (4h, 12h, 24h and 48h, 4h as control) in planktonic growth and biofilm formation was used to determine the correlation factors between gene expression levels and reaction rates. Expression patterns of metabolic genes were extracted and then integrated with the metabolic model for *P. aeruginosa*. Results revealed that 112 reactions out of all 1110 reactions are associated with positive correlation factors. As mentioned above, the activities of reactions depend on both the enzyme levels and the reactant (or substrate) availability. The latter is constrained by the topology of the metabolic network. The reactions with higher correlation factors are more enzyme-dependent, while the reactions with lower correlation factors are more substrate-dependent or network-dependent. Thus the reactions with higher correlation factors should be more sensitive to the ribosome-targeted antibiotics.

3.2.1 Investigation of the metabolic flux alteration after the treatment of antibiotics

The obtained correlation factors were applied to constrain metabolic fluxes as shown in Figure 1B to predict the metabolism variation. It turned out that 10 reversible reactions changed their flux directions, 171 reactions increased their fluxes, 372 reactions had decreased flux values, and 558 reactions did not change their fluxes. Figure 3 shows the distribution of flux variation among different metabolic pathways. The categories of metabolic pathways were defined by Oberhardt et al., 2008 [20]. Some of the metabolic pathways with large flux change have been found important by experiment for the persister cell formation of *P. aeruginosa*. These metabolic pathways will be discussed in detail in next subsections.

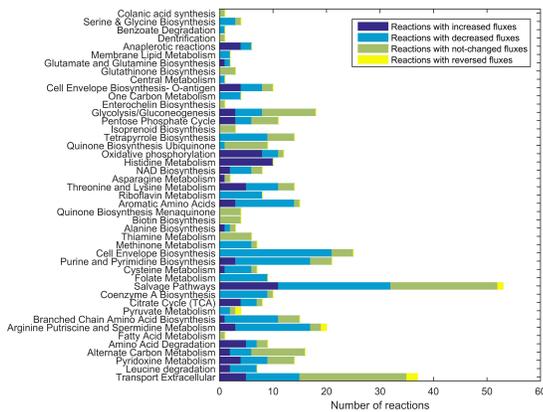


Figure 3, the flux change of metabolic reactions for metabolisms of *P. aeruginosa* upon the treatment of antibiotics.

3.2.2 Some metabolic pathways with large flux change were found to be important for persister cell formation in experiment

Some of the reactions with large flux change were found to be involved in persister cell formation and the antibiotic resistance of *P. aeruginosa*. These reactions were mainly related to the homoserine metabolism, the production of 2-heptyl-4-quinolone (HHQ), isocitrate lyase and indole derivatives, and exchange reactions of some extracellular components. The detail of these reactions was given in this section.

3.2.2.1 Homoserine metabolism engages in the metabolism adjustment upon the treatment of antibiotics

Acyl-homoserine-lactone behaves as a quorum-sensing molecule and triggers persister cell formation in *P. aeruginosa* [25]. Our approach predicted the flux change in the homoserine metabolism that was consistent with this observation. Figure 4 illustrated the change of fluxes in the metabolic reactions for the synthesis of L-Homocysteine. Specifically, the fluxes of the synthesis reactions for L-Threonine and L-Homocysteine were enhanced, while the synthesis rate of L-Cystathionine was decreased. L-Homocysteine is a precursor of acyl-homoserine-lactone, as it leads to the synthesis of S-adenosylmethionine, a reactant for acyl-homoserine-lactone synthesis. According to the FBA results, the enhanced L-Homocysteine synthesis results in the enhancement of the acyl-homoserine-lactone. This provides a possible explanation for the presence of antibiotics triggering the formation of *P. aeruginosa* persister cells.

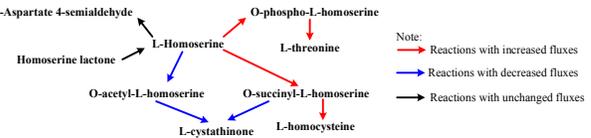


Figure 4, Flux adjustment of the homoserine metabolism upon the treatment of antibiotics

3.2.2.2 Alteration of the production rate of 2-heptyl-4-quinolone (HHQ), isocitrate lyase, and indole derivatives upon the treatment of antibiotics

Wei et al., 2011, reported that 2-heptyl-3-hydroxy-4-quinolone (PQS) production was strongly reduced in persister strains PAO-SCV [26]. The production rates of HHQ and PQS were predicted to be down-regulated to 0.0559 of their nominal values, which is consistent with the data shown in Wei et al., 2011.

It has been suggested that the persistence of *Mycobacterium tuberculosis*, which shares orthologous genes for energy production and conversion with *P. aeruginosa* [27], requires the elevation of the intracellular level of isocitrate lyase, which is a key anaplerotic enzyme for the glyoxylate bypass [28]. Our results showed that the flux of the reaction catalyzed by isocitrate lyase was enhanced to 5.89 fold of its nominal value upon the treatment of ribosome-targeting antibiotics such as rifampicin. In addition to this enzyme, enzymes acetyl-CoA C-acetyltransferase and putrescine aminotransferase were found to be positively related to the persister cell formation of *P. aeruginosa* [29]. Our results indicated that the fluxes of the reactions catalyzed by these enzymes were increased by 1.26 and 1.28 folds of their nominal values.

Vega et al., 2012, showed that the indole signaling inoculated *E. coli* population against antibiotics by activating the stress response and leading to the persister formation [30]. Although indole is not within the network of *P. aeruginosa*, its derivatives are incorporated into the tryptophan side chain pathway of the model. Our results showed that the flux of tryptophan-synthase reaction in tryptophan metabolism increased 7.1 fold upon the treatment of antibiotics.

3.2.2.3 Change of exchange reaction fluxes for extracellular components

The flux change in the exchange reactions was further analyzed, and Figure 5 showed the metabolites whose exchange rates were significantly increased compared to the control condition. Some of them are related to microbial persister formation. For instance, acetate enhances protein aggregation and the generation of persisters [31]. The data of Figure 5 indicate that the exchange rate of acetate increased more than 9 fold in *P. aeruginosa* upon addition of antibiotics. Arginine was reported to induce antibiotic resistance [32], and our results showed an increase in production of arginine after antibiotics treatment. Ma et al., 2010, demonstrated that oxoglutarate metabolism was important for antibiotic tolerance in *E. coli* showing that a *sucB* mutant deficient in 2-oxoglutarate dehydrogenase complex had decreased viability when exposed to antibiotics [33]. Our results support this result since the exchange rate of oxoglutarate was significantly enhanced. Amato et al., 2013, demonstrated that the switch from glucose to fumarate promoted persister cell formation of *E. coli* [34]. This is shown in our results as there was an increased in fumarate exchange rate. Cava et al., 2011, demonstrated that D-alanine was able to inhibit the spore germination [35]. Our results about D-alanine support the finding.

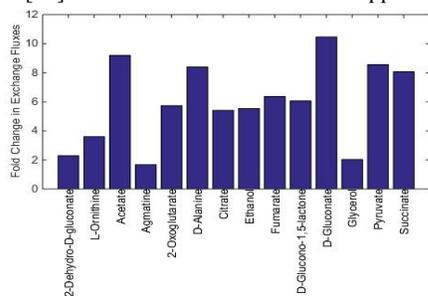


Figure 5, metabolites which the exchange rates are significantly increased after antibiotic treatment

4. Conclusions

In this paper we developed a systems biology approach to investigate the perturbation of microbial metabolism of *P. aeruginosa* upon the treatment of ribosome-targeting antibiotics. Metabolic fluxes depend on both enzyme activation level and reactant availability. The core idea of mimicking the stress imposed by ribosome-targeting antibiotics was to constrain the fluxes of enzyme-dependent reactions. This novel approach is capable of uncovering the correlation factors between metabolic fluxes with gene expression levels. The results indicate that our approach outperformed FBA and pFBA by obtaining higher correlation factors with the experimental data. Additionally, fluxes of the reactions that were highly correlated with gene expression levels and were constrained to predict the perturbation of microbial metabolism upon the treatment of antibiotics. Different change regimes were discovered in which some were already validated by experimental results on their important role in persister cell formation. The exchange rates of several metabolites with significant alteration were also studied, which provides candidate metabolites in further controlling persister formation via nutrition supplement. This approach can be applied to study the metabolism adjustments in phenotype transition of other microorganism given metabolic network information.

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