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Phenotypic changes associated with the fitness cost in antibiotic resistant *Escherichia coli* strains

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Abstract

Background

The acquisition of antibiotic resistance in bacterial cells is often accompanied with a reduction of fitness in the absence of antibiotics, known as the "fitness cost." The magnitude of this fitness cost is an important biological parameter that influences the degree to which antibiotic resistant strains become widespread. However, the relationship between the fitness cost and comprehensive phenotypic and genotypic changes remains unclear. Here, we quantified the fitness cost of resistant strains obtained by experimental evolution in the presence of various antibiotics, and analyzed how the cost correlated to phenotypic and genotypic changes in the resistant strains.

Results

We measured the specific growth rate of the resistant strains in the presence of various concentrations of drugs or in their absence. In the absence of drugs, the resistant strains showed reductions of approximately 20% to 50% in growth rate compared with the parent strain, which corresponded to the fitness cost. We found that the decrease of specific growth rate was correlated with overall expression changes between the parent and resistant strains, measured by Euclid distance between expression profiles. We also found that there are a number of genes whose changes in expression levels were significantly correlated with the growth rate, which may account for the observed correlation between the fitness cost and overall expression changes.

Conclusions

Our analysis provides a basis for quantitative understanding of the mechanism of the fitness cost. This understanding may provide clues on how to influence the fitness cost that accompanies resistance acquisition and consequently how to limit the spread of antibiotic resistant strains.

Introduction

The emergence of antibiotic resistant bacteria is a serious global problem for human health, which continues to worsen [1, 2]. The clinical dose of antibiotics typically used gives a selective advantage to naturally emerging resistant bacteria, leading to widespread resistant strains [3, 4]. A number of genotypic and phenotypic changes that contribute to antibiotic resistance have been identified, which shed light on how to control the emergence of antibiotic resistant strains [5-9].

The acquisition of resistance is often accompanied by a reduction in competitive ability against antibiotic susceptible strains in the absence of the antibiotics. This is known as the "fitness cost" [10-12]. Genetic alterations that cause antibiotic resistance can disrupt normal physiological processes in the cell, which result in detrimental side effects. For example, expression of plasmid encoded resistant genes require additional resources such as nucleic acids and proteins, interfering with cell growth [13]. Similarly, some mutations in the *rpsL* gene of *E. coli*, which cause resistance to streptomycin, bring a fitness cost most likely due to altered ribosomes with a reduced peptide-chain elongation rate [14]. The magnitude of the fitness cost is one essential parameter which governs i) the dynamics of resistance acquisition, ii) the stability of resistance, and iii) the decreasing rate of the resistant population to the total population in the absence of the antibiotics. Thus, the quantification of fitness cost is important for developing adequate treatment protocols to prevent antibiotic resistance. Various studies have shown quantitative evaluation of the fitness cost both in vitro and in vivo (e.g., Refs. in [11]). However, few studies link comprehensive phenotypic and genotypic characterization with the fitness cost.

In this study, we quantified the fitness cost of resistant *E. coli* strains to various antibiotics, by measuring the specific growth rates under varying antibiotic concentrations. These antibiotic resistant strains were obtained by laboratory evolution experiments in our group [9], in which genetic alterations were identified by next generation sequencing, and comprehensive gene expression changes were quantified by microarray. Here, we analyzed the relationship between the fitness cost and phenotypic and genotypic changes in antibiotic resistant strains.

Results

Decreased growth rates in antibiotic resistant strains

We selected nine antibiotics shown in Table 1 that exert their action using a wide range of mechanisms, including those that disrupt cell wall synthesis, protein synthesis, folic acid biosynthesis and DNA replication. After 90 days of experimental evolution under each of these antibiotics, we obtained 36 resistant strains (4 independently evolved strains for 9 antibiotics), which showed significant increases in minimum inhibitory concentrations (MICs) as described in [9]. We first quantified the specific growth rate of the resistant strains and their parent strains during the

exponential growth phase, in the absence or presence of the corresponding antibiotic at varying concentrations. As shown in Fig. 1, specific growth rates were almost unchanged when the drug concentration was low, but drastically decreased in concentrations close to their MICs. For the resistant strains of some drugs, the decrease of the specific growth rates was gradual with increasing drug concentrations, e.g., chloramphenicol (CP) and trimethoprim (TP), while for other resistant strains, the decreases in the specific growth rate showed threshold-like behaviors. Interestingly, the classification of the growth behavior corresponded to the categories of antibiotics, i.e., bacteriostatic drugs resulted in gradual decreases in the growth rate of the resistant strains while bactericidal drugs resulted in more drastic changes.

Notably, the specific growth rates of the resistant strains were generally lower than the parent strains in the absence of antibiotics. This means that the acquisition of antibiotic resistance imposed fitness costs for growth even under no-drug conditions. The decrease of the specific growth rate (Δ growth rate) in the resistant strains ranged from 20% to 50%. Interestingly, the decrease of the specific growth rate under no-drug conditions correlated with an increase in MIC, as shown in Fig. 2. Our result suggests that the larger the increase in resistance imposed, the larger the fitness cost, regardless of drug type.

Relationship between fitness cost and transcriptome change

The correlation between the resistance and the fitness cost shown in Fig. 2 might suggest that a larger acquisition of resistance results in greater phenotypic changes that lead to the larger fitness cost. To evaluate this possibility, we characterized the magnitude of the phenotypic change based on the transcriptome data of the resistant strains [9]. For the generation of the transcriptome data, all resistant strains were cultured in synthetic medium without drugs in order to standardize the culture conditions among the strains. The overall expression change between the resistant strain and the parent strains was estimated by Euclid distance between log-transformed expression profiles. Here, the distance D^i between i-th resistant strain and the parent strain was calculated by

$$D^i = \sqrt{\sum_{j=1}^{N} (X_j^i - X_j^P)^2}$$

where X_j^i and X_j^P show the log-transformed expression level of the j-th gene in the i-th resistant strain and the parent strain, respectively. *N* denotes the number of genes used for the calculation of distance. In this analysis, we removed genes with low expression levels since the quantification of expression changes were unreliable (see Materials and Methods for details). Fig. 3a shows the relationship between the decrease in specific growth rates and the distance D^i of the resistant strains. This clear correlation suggests that the magnitude of the fitness cost can be represented by overall expression changes. Our previous study [9] indicated that different types of expression

changes contribute to resistance to different antibiotics. For example, the down-regulation of genes related to electron transfer systems such as *cyo* genes, contributes to resistance to aminoglycoside drugs, while the up-regulation of *acrAB*, which encodes a well-characterized multidrug efflux pump, causes resistance to different drugs, including quinolone, beta-lactam, and chloramphenicol. Thus, the clear correlation in Fig. 3a suggested two possibilities: (i) even though the direction of expression changes were completely different among resistant strains to different antibiotics, the magnitude of the fitness cost can be scaled by the distance of expression profiles, or (ii) in addition to specific expression changes contributing to the resistance of each drug, there were common expression changes among the resistant strains which were correlated with the growth rate, and the distance was governed by common expression changes. To examine these possibilities further, we screened genes whose expression changes were correlated with the change of growth rate in the resistant strains.

Common gene expression changes correlated with fitness cost

To analyze the relationship between the fitness cost and changes in gene expression, we calculated the Pearson correlation coefficient between the change of the specific growth rate and log-transformed expression levels for each gene in the resistant and the parent strains. Fig. 4a shows the distribution of correlation coefficients for all the genes we inspected, in which the distribution of correlation coefficients obtained by randomized data is also plotted for reference. As shown, the distribution of experimentally obtained correlation coefficients was significantly wider than that of randomized data ($p < 10^{-15}$), indicating that the expression levels of a certain fraction of genes were highly correlated to the growth rate as observed for *LysA* and *RplY* (Figs. 4b and 4c). These results suggested that there were common expression changes correlated to the growth rate, which significantly contributed to the overall expression changes of the resistant strains, resulting in the correlation between the Euclid distance D^i and the growth rate as previously noted. To characterize the correlation between these expression changes and the fitness cost, we performed a gene set enrichment analysis (GSEA) [15] to identify gene functions that were significantly enriched in the genes whose expressions were correlated to the growth rate. Table 2 shows the functional categories screened by GSEA, which satisfied a false discovery rate (FDR) q-value < 0.1. Using this threshold, we identified only functional categories in which positively correlated genes were enriched, while no categories exhibited a significant enrichment of negatively correlated genes. As shown in Table 2, growth-correlated genes were enriched in the function of amino acid biosynthesis, translation and some metabolic pathways such as the tricarboxylic acid (TCA) cycle, all relating to generating the building blocks of cells. Our results suggested that even though these strains acquired resistance to various drugs in different ways, e.g., activation of efflux pump, changing metabolic fluxes, and so forth, the fitness cost measured by the change of specific growth rate was caused by common

expression changes. To further analyze the growth-related common expression changes, we quantified the relationship between the gene expression changes observed in the drug resistant strains and those in the previous study [16], in which expression changes by changing the growth rate were quantified by using a chemostat culture system. The results demonstrated that, the expression changes observed in the resistant strains generally exhibit significant correlation with the expression changes caused by growth rate changes as shown in supplementary Fig. S1, which also suggested that they shared the common growth-related expression changes.

In addition to the common growth-related expression changes, which were also observed in the case only changing the growth rate, there can be drug-resistant specific growth-related expression changes. To extract such expression changes, from the following analysis we removed genes which changed their expression levels when the growth rate was changed in the previous study [16]. Here, we removed all genes whose expression was increased (>2 fold) or decreased (<0.5 fold) when the specific growth rate was changed from 0.2 h^{-1} to 0.5 h^{-1} in the data of [16] (656 genes in total). As a result, we found that after this gene selection, still there was a significant correlation between the decrease in specific growth rates and the distance D^i of the resistant strains (Fig. 3b). Then, we screened gene functions that were significantly enriched in the drug-specific perturbed genes whose expressions were correlated to the growth rate. Table 3 shows the functional categories screened by GSEA, in which the screened functions were similar with those in Table 2. However, we found that the function "chromosome condensation" was significantly enriched only after the removal of the common growth-related genes. In fact, the expression levels of some genes related to maintenance of chromosome organization, such as *mukB* and *hupB*, showed significant correlations with the growth rate (Figs. 4d and 4e). This result might suggest that the change in chromosome organization contributes to the drug-resistant specific expression changes that were correlated to the growth rate.

Lastly, we considered the relationship between the fitness cost and fixed genomic mutations in the resistant strains. Our previous study [9] identified fixed mutations in the resistant strains. Although the fitness cost was commonly observed in all resistant strains, there were no mutations commonly fixed in these resistant strains. This fact indicated that the observed fitness costs cannot be explained by a single or a small number of mutations. Also for expression profile changes, the data of identified mutations and gene expression changes suggested that the relationship between fixed mutations and gene expression changes was not always a simple one-to-one correspondence; instead, multiple mutations were suggested to cause similar gene expression changes. For example, the expression levels of *acrB* encoding multidrug exporter was commonly up-regulated in multiple resistant strains including CP1–4, whereas there was no common mutation in CP1–4. In the CP1–3 strains, mutations were fixed in *acrR* encoding the repressor of *acrB*, which can contribute the up-regulation of *acrB*. However, there is no mutation that is known to regulate

directly the expression level of *acrB*, and thus there is an indirect interaction between the expression change of *acrB* and mutations or other factors in the CP4 strain. Such complex relationship between fixed mutations and expression changes were observed in other genes, including *cyo* genes related to electron transfer system and *omp* genes encoding porin proteins, as discussed in [9].

Discussion

In this study, we analyzed the fitness cost in bacterial strains that had acquired resistance to various antibiotics, by measuring the specific growth rate in the absence of antibiotic or in the presence of increasing concentrations. We demonstrated that resistant strains obtained by experimental evolution generally exhibited a reduced growth rate under no-drug conditions, suggesting an inherent fitness cost when microbial cells acquire antibiotic resistance. By integrating transcriptome data of the resistant strains, we found that the magnitude of the fitness cost was highly correlated with the magnitude of overall gene expression changes. We investigated if this correlation originated from common expression changes in growth related genes.

The quantification of fitness cost in the experimentally obtained antibiotic resistant strains provided fundamental information on the stability of antibiotic resistance. For example, several resistant strains such as those resistant to chloramphenicol showed a decrease in specific growth rate of approximately 40% compared with the parent strain in the no-drug condition. This means that replacement of 99.9% of the population by susceptible bacteria would only take approximately 24 hours. Of course, these results were obtained in laboratory settings (e.g., liquid medium with sufficient nutrients, exponential growth without tight cell-cell interactions), which are quite different from bacterial population in vivo. Furthermore, the effect of compensatory evolution [17, 18], which recovers the growth activity of resistant strains in the absence of drugs, is not included in this analysis. Further quantitative analysis including these factors will be important to understand population dynamics of antibiotic resistant strains in vivo.

Our results demonstrated there were a number of genes, whose expression levels were significantly altered correlating with changes in the growth rate of the resistant strains, which were involved in the molecular mechanisms of the fitness cost. Although to extract a causal relationship between resistance acquisition and these expression changes is difficult based only on the correlation between them, we expect that the common expression changes contributing to the fitness cost could provide clues on how to influence the fitness cost that accompanies resistance acquisition. Such information may be useful to design novel drugs that inhibit the emergence of antibiotic resistant strains.

Materials and Methods

Bacterial strain and culture conditions

The IS elements-free *E. coli* strain MDS4251 was purchased from Scarab Genomics and used throughout this study. Bacterial cells were cultured in 200 μL modified M9 medium [19] in 96-well microplates (Corning Inc. 3595) with shaking at 900 strokes min^{-1} on a microplate shaker (TITRAMAX1000, Heidolph Instruments) at 34°C. All antibiotics used in this study were purchased from Wako Pure Chemical Industries, Ltd. Antibiotic stock solutions were made by dissolving powder stocks in specified solvents according to the manufacturer's instructions. All antibiotic stocks dissolved in water were 0.2 μm filter-sterilized and stored at −80°C prior to use.

Measurement of specific growth rate and MIC

Serial dilutions of each antibiotic were made in 96-well microplates using modified M9 medium and stored at −80°C prior to use. For the measurement of specific growth rate, precultured cells calculated to yield an initial OD_{600} of 1×10^{-4} were inoculated into each well of freshly thawed 96-well plates with antibiotics to a final volume of 200 μL. After the inoculation, the cell density was quantified at OD_{600} using a 1420 ARVO (Perkin-Elmer) at one-hour time intervals. The specific growth rate was calculated based on three data points with minimum OD_{600} values under the condition OD_{600} >0.02 and the initial OD_{600} value using linear regression. When the OD_{600} value did not exceed 0.02 by 24 hours post inoculation, the specific growth rate was set to zero. MIC was defined as the minimum concentration of antibiotics whose addition to the culture reduced the specific growth rate to zero.

Data analysis

Transcriptome and genome resequencing data of the resistant and parent strains were obtained from our previous paper [9] (supplementary data 1 and 2; the data have been deposited in the GEO database under accession code GSE59408 and DDBJ Sequence Read Archive under accession code PRJDB2980). To ensure only quantitatively reliable data were used, genes with low expression levels (less than 300 a.u. in any strain) were excluded from the analysis. Gene set enrichment analysis (GSEA) [15] was performed using the online tools (www.broadinstitute.org/gsea/index.jsp). The functional categories of *E. coli* genes were obtained from gene ontology (http://geneontology.org/). For supplementary figure S1, the expression data in [16] were obtained from *Escherichia coli* Multi-omics Database (http://ecoli.iab.keio.ac.jp/) and were used after quantile normalization. All other statistical analyses were performed using R statistical language (http://www.r-project.org).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

S. S. and C.F. designed the experiments. S.S. and T. H. carried out the experimental studies, participated in the growth measurements of resistant strains. C. F. performed the statistical analysis and drafted the manuscript. All authors read and approved the final manuscript.

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Figure legends

Figure 1

The effect of antibiotics on the specific growth rate. The specific growth rates of the parent strain and four independently evolved resistant strains were quantified in the presence of various concentrations of corresponding drugs or in the absence of the drug. The plots are (a) CPZ, (b) CFIX, (c) AMK, (d) ENX, (e) CPFX, (f) DOXY, (g) CP, (h) AZM, and (i) TP resistant strains, respectively.

Figure 2

The relationship between the fitness cost and the increase of MIC in the resistant strains. The fitness cost was defined by the difference in the specific growth rate between resistant and parent strains in the absence of drug. The increase of MIC on the vertical axis was calculated by the log2-transformed ratio of the MIC compared to the parent strain. Throughout the paper, "CPZ/CFIX" and "ENX/CPFX" are grouped together because they share same action mechanisms, i.e., both of CPZ and CFIX are beta-lactam antibiotics, while both of ENX and CPFX are quinolone.

Figure 3

The relationship between the fitness cost and overall expression changes. (a) The relationship between the fitness const and the overall expression changes quantified by D^i, the Euclid distance between log-transformed expression profiles of the parent and resistant strain. (b) The relationship between the fitness cost and overall expression changes after removal of the common growth-related genes. The overall expression changes quantified by D^i was calculated after removing the genes which change their expression levels when the growth rate was changed in the previous study [16].

Figure 4

The correlation of gene expression and growth rate. (a) The distribution of correlation coefficients between log-transformed gene expression levels and the specific growth rates in the resistant strains and the parent strain. For reference, we show the distribution obtained by randomized data sets generated by randomly permuting the gene coordinates at each expression profile. (b,c) Examples of genes whose expression levels exhibited significant correlation with the growth rate, including (b) *lysA* encoding diaminopimelate decarboxylase in the lysine biosynthesis pathway, and (c) *rplY* encoding the 50S ribosomal subunit. (d, e) Examples of drug-specific responding genes whose expression levels exhibited significant correlation with the growth rate. (a) *mukB* whose product contributes to chromosome organization and (b) *hupB* encoding transcriptional regulator HU-β.

Supplementary figure S1

The growth-related common expression changes in the resistant strains. (a) The relationship between the expression changes in CPZ2 strain (Cefoperazone resistant strain No.2) and the expression changes in the previous study [16], in which expression changes by changing the growth rate were quantified by using a chemostat culture system. The x-axis shows the log-transformed expression changes between CPZ2 strain and the parent strain, while the y-axis shows the log-transformed expression changes between different growth rates (0.2 h^{-1} and 0.5 h^{-1}) obtained in the previous study. Each dot represents the expression change of each gene. (b) The correlation coefficients between expression changes in the resistant strains and the previous data. The correlation coefficients of expression changes in all resistant strains we used and expression changes between different growth rates (0.2 h^{-1} and 0.5 h^{-1}) in the previous study were calculated. The correlation coefficients were generally positive, suggesting that they shared the common growth-related expression changes.

Table 1. List of antibiotics used for experimental evolution

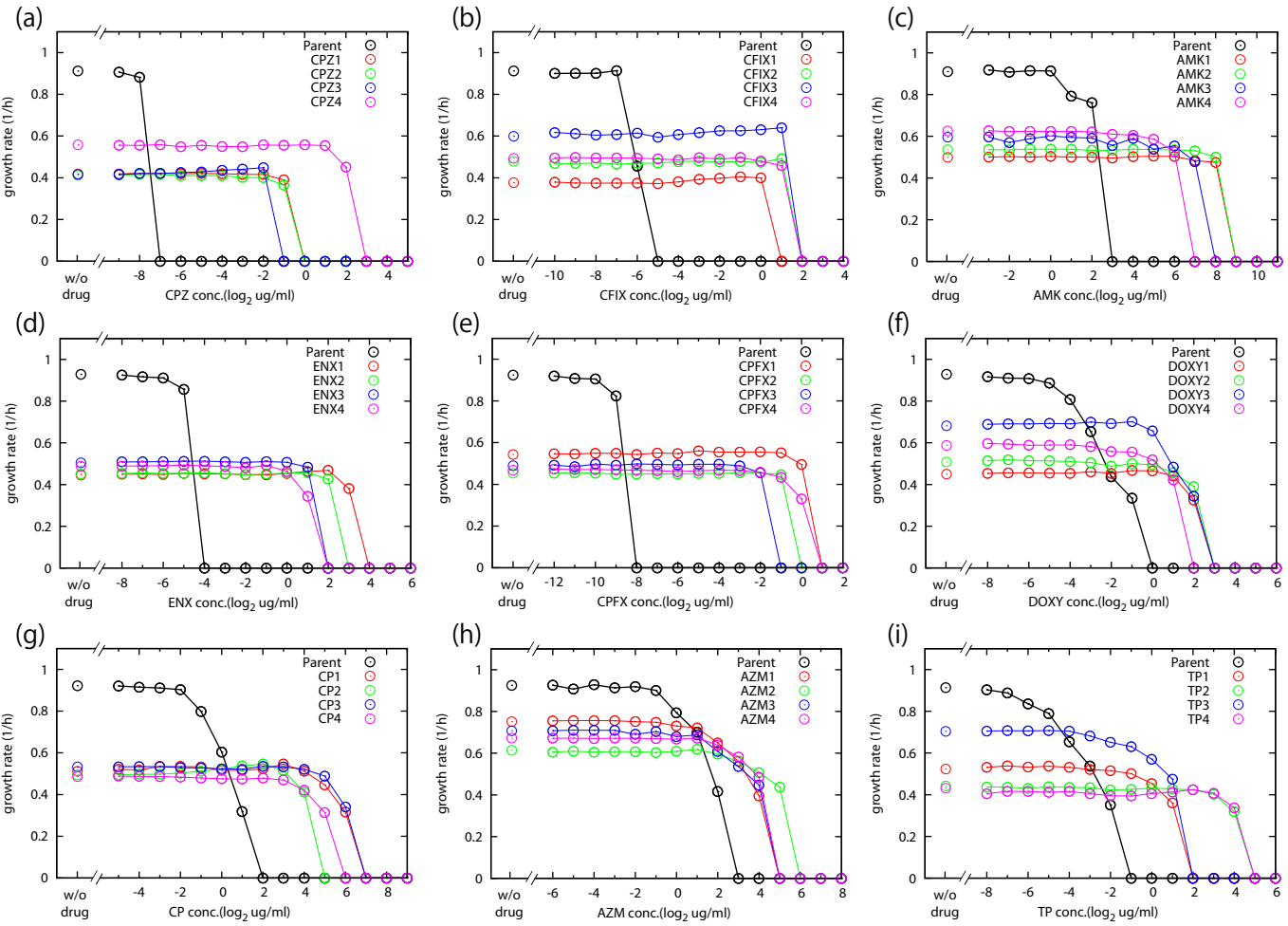
Name	Abbr.	Class	Cellular target	Type of function
Cefoperazone	CPZ	beta-lactam	Cell wall	bactericidal
Cefixime	CFIX	beta-lactam	Cell wall	bactericidal
Amikacin	AMK	Aminoglycoside	Protein synthesis, 30S	bactericidal
Doxycycline	DOXY	Tetracycline	Protein synthesis, 30S	bacteriostatic
Chloramphenicol	CP		Protein synthesis, 50S	bacteriostatic
Azithromycin	AZM	Azalide, Macrolide	Protein synthesis, 50S	bacteriostatic
Trimethoprim	TP		Folic acid synthesis	bacteriostatic
Enoxacin	ENX	Quinolone	DNA gyrase	bactericidal
Ciprofloxacin	CPFX	Quinolone	DNA gyrase	bactericidal

Table 2. Representative gene sets in which growth-correlated genes were enriched

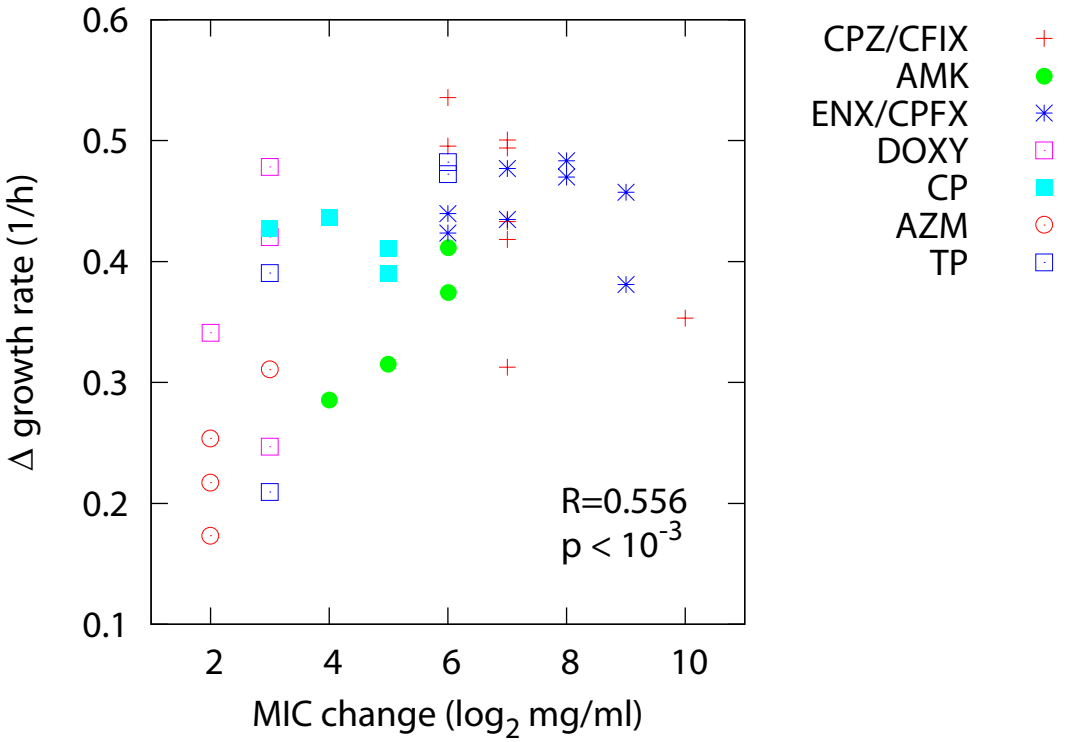
Name of functional category	Size (# genes)	p-value	FDR q-value
Arginine biosynthetic process	10	$<10^{-3}$	$<10^{-3}$
Translation	100	$<10^{-3}$	$<10^{-3}$
Cellular amino acid biosynthetic process	90	$<10^{-3}$	1.3×10^{-4}
Pyrimidine nucleotide biosynthetic process	11	$<10^{-3}$	1.5×10^{-4}
tRNA binding	23	1.3×10^{-3}	3.0×10^{-2}
Isoleucine biosynthetic process	10	7.6×10^{-3}	5.1×10^{-2}
Aromatic amino acid family biosynthetic process	17	9.4×10^{-3}	5.7×10^{-2}
Tricarboxylic acid cycle	17	1.9×10^{-2}	5.8×10^{-2}
Carbohydrate transport	13	2.2×10^{-3}	8.8×10^{-2}

Table 3. Representative gene sets in which drug-specific growth-correlated genes were enriched

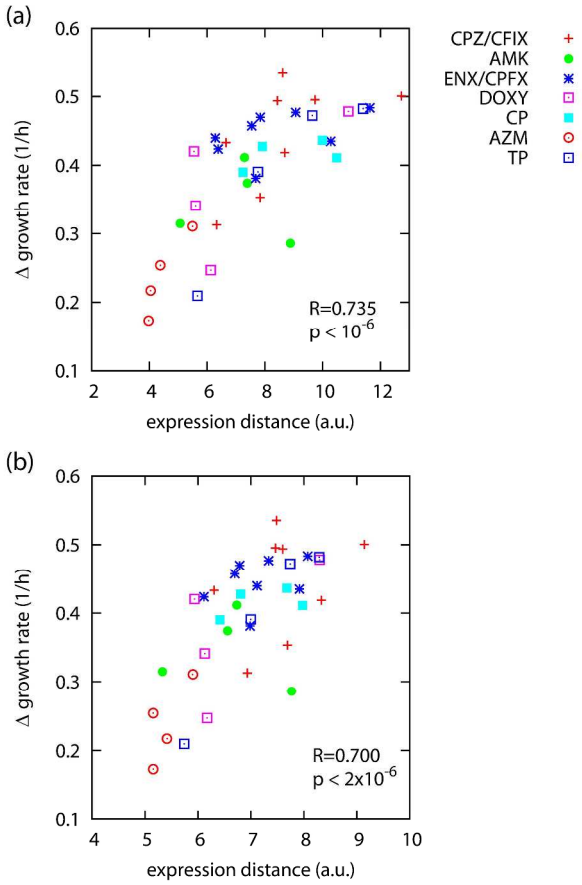
Name of functional category	Size (# genes)	p-value	FDR q-value
Translation	73	$<10^{-3}$	4.5×10^{-3}
Cellular amino acid biosynthetic process	67	$<10^{-3}$	1.8×10^{-2}
Arginine biosynthetic process	5	$<10^{-3}$	3.0×10^{-2}
Tricarboxylic acid cycle	13	4.4×10^{-3}	3.2×10^{-2}
Isoleucine biosynthetic process	8	9.3×10^{-3}	8.7×10^{-2}
Chromosome segregation	5	8.4×10^{-3}	1.2×10^{-1}



Suzuki et al., Figure 1



Suzuki et al., Figure 2



Suzuki et al., Figure 3

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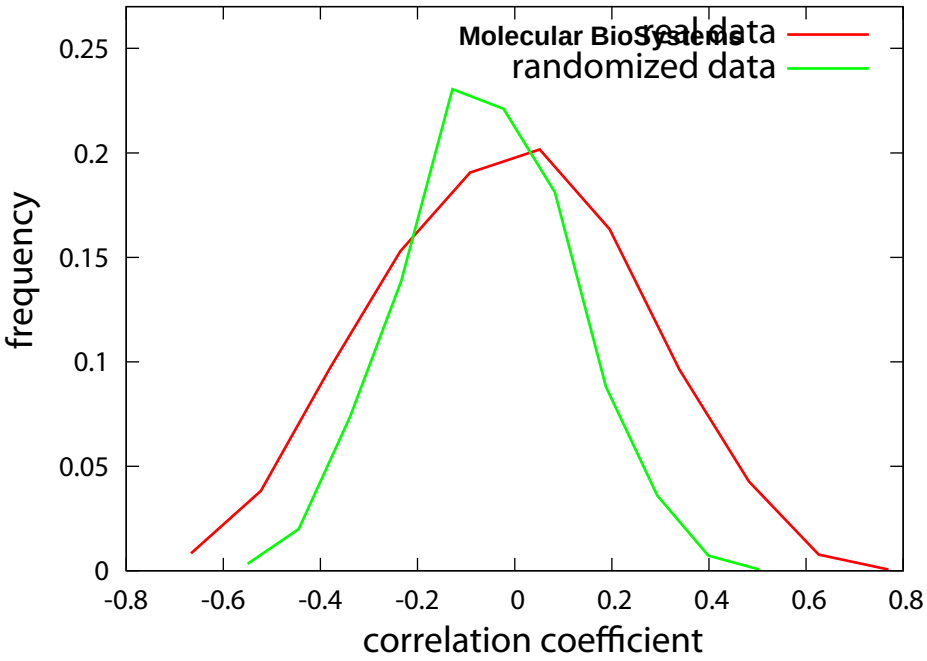

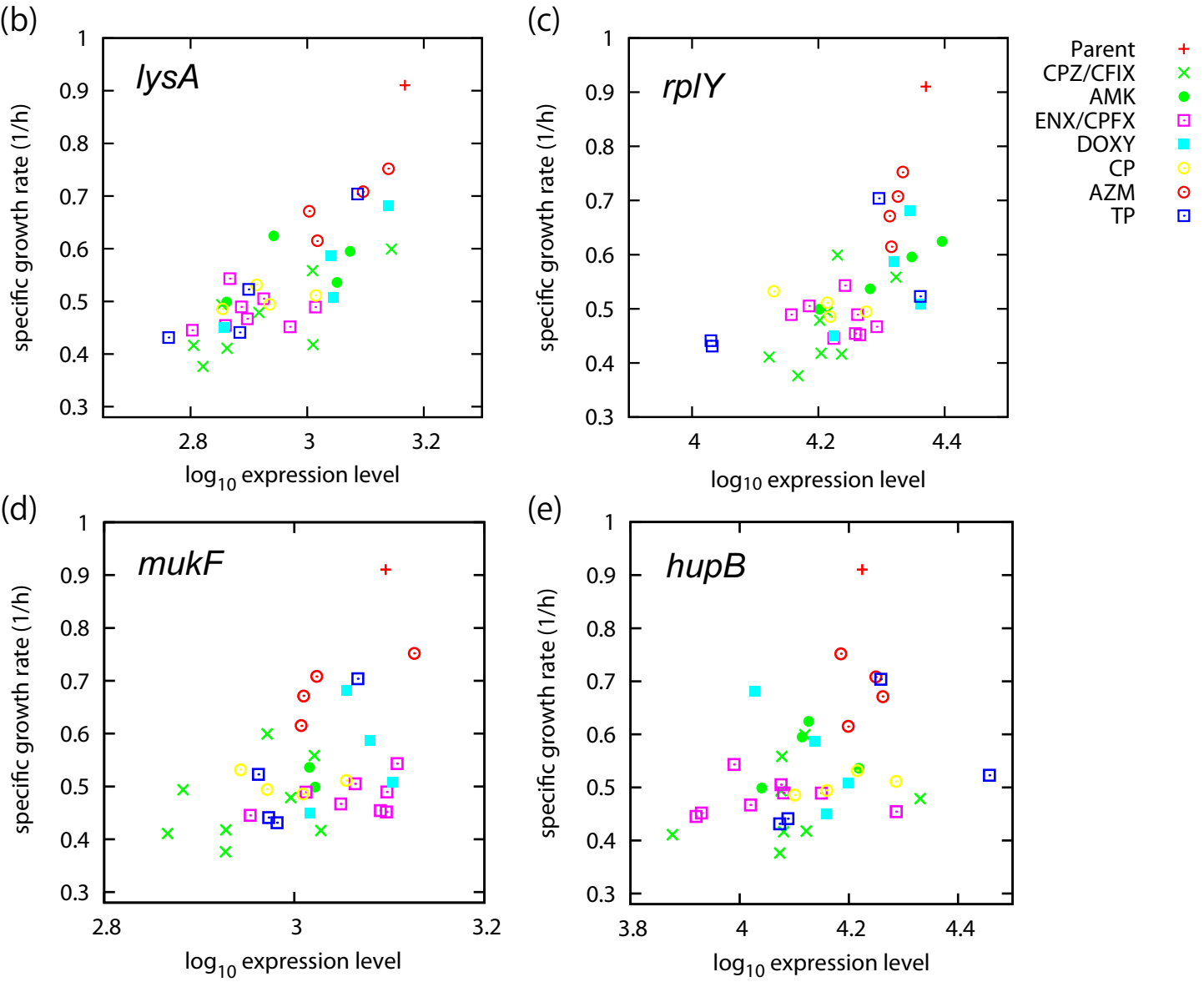

Suzuki et al., Figure 4