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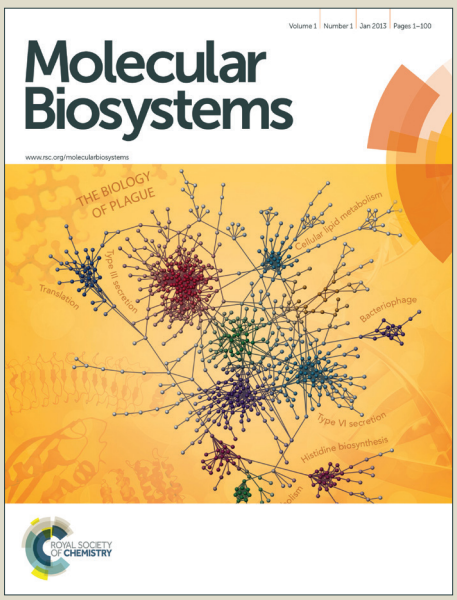

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Fluctuation of Multiple Metabolic Pathways is Required for *Escherichia coli* in Response to Chlortetracycline Stress

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Running title: fluctuated metabolic pathways against CTC

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Abstract: Bacterial antibiotic resistance has become a worldwide challenge with the overuse and misuse of drugs. Several mechanisms for the resistance are revealed, but information regarding to the bacterial global response to antibiotics is largely absent. In this study, we characterized the differential proteome of *Escherichia coli* K12 BW25113 in response to chlortetracycline stress using isobaric tags for relative and absolute quantitation labeling quantitative proteomics technology. Total 723 proteins including 10,763 peptides were identified with 184 decreasing and 147 increasing at abundance by Liquid chromatography matrix assisted laser desorption ionization mass spectrometry. Most interestingly, crucial metabolic pathways such as the tricarboxylic acid cycle, pyruvate metabolism and glycolysis/gluconeogenesis sharply fluctuated, while the ribosome proteins complexes contributing to the translation process generally elevated in chlortetracycline stress, which was known for a compensative tactic due to the action of chlortetracycline to ribosome. The further antimicrobial susceptibility assays validated the role of differential proteins in metabolic pathways using genetically mutants of gene deletion of these differential proteins. Our study demonstrated that the down-regulation of metabolic pathways was a part of the global response and played an important role in the antibiotics resistance. These results indicate that reverting of these fluctuated pathways may become a novel strategy to combat antibiotic-resistant bacteria.

Keywords: Antibiotics resistance; iTRAQ; quantitative proteomics; *Escherichia coli*

Introduction

The extensive use and misuse of antibiotics have led to the emergence of bacterial pathogens capable to resist the commonly used antibiotics, which have become a considerable threat to public health and agriculture. Bacteria have developed several resistant mechanisms to evade the action of antibiotics including drug inactivation or modification, alteration of target site, reduced drug accumulation by decreasing drug permeability and/or increasing active efflux[1]. However, information regarding to the bacterial global response to antibiotics is largely absent.

In this regard，increasing reports have explored the global response in mRNAs and proteins level using transcriptomics and proteomics technologies, respectively. For example, Lin et al., reported that genes, which encoding transport/binding proteins and protein synthesis and carbohydrate metabolism, were affected by chloramphenicol, erythromycin, and gentamicin in *Bacillus subtilis* [2]. Using 2-DE based proteomics, Bandow et al., compared the differential expression of cytoplasmic proteins in *B. subtilis* which were treated with 30 antimicrobial compounds and then predicted the modes of action of the novel compounds[3]. In our previous study, the role of outer membrane proteins in response to different classes of antibiotics was identified and the antibiotic-resistant network based on outer membrane protein was revealed in *Escherichia coli* [4-7]. Besides these, there have been others reports about functional proteins in

resistance to antibiotics in *Pseudomonas putida, Coxiella burneti*, *Fusobacterium nucleatumi* and *Acinetobacter baumanni* [8-11].

Recently, multiplexed labeling methods, such as isotope-coded affinity tags (ICAT), iTRAQ and stable isotope labeling by/with amino acids in cell culture (SILAC), have widely been applied to biological research as an alternative to two-dimensional gel electrophoresis (**2-DE**) [12, 13]. These increasing popularity methods for quantitative proteomics applications make it convenient to analysis of antibiotics-resistant-related proteins in a high-throughput level [14, 15]. In this study, combing isobaric tags for relative and absolute quantitation (iTRAQ) labeling and SCX fractions and then Liquid chromatography matrix assisted laser desorption ionization mass spectrometry/mass spectrometry (LC-MALDI MS/MS), we compared the differential protein expression of *E. coli* K12 BW25113 in the presence and absence of chlortetracycline stress. Then, the key pathways involved were identified by bioinformatics. Finally, the role of the differential proteins obtained from the key pathways was validated by genetically gene-deleted mutants of these proteins. These data provide novel insights into the resistant mechanisms when bacteria expose to antibiotics in a high-throughput proteomic perspective.

Results and discussion

Protein Identification and Quantification Comparisons of *E. coli* K12 BW25113 in the Presence and Absence of Chlortetracycline Stress.

Chlortetracycline is one of typical tetracyclines which bind to ribosome and prevent proteins from assembly[16]. A line of evidences has been demonstrated that ribosomal protection proteins (RPPs) play important roles in resistance to tetracyclines [17, 18]. Besides this, reports also indicated the roles of outer membrane proteins in the resistance[19, 20] . However, whether and what other pathways play in crucial role in the responses are largely unknown. In this study, iTRAQ based labeling combining with multi-dimensional separation was performed before LC-MALDI MS/MS to compare differential expression of *E. coli* in response to chlortetracycline stress. As showed in results, total 723 proteins including 10,763 peptides were identified with Proteinpilot software 4.0 confidence ≥ 95% and FDR<5% as cut off (Supplementary Tables 1 and 2). Compared with *E. coli* K12 BW25113 cultured in medium without chlortetracycline, total 331 differential proteins including 184 with decreasing abundance and 147 with increasing abundance were identified by iTRAQ quantification in medium with the drug (Supplementary Table 3).

Out of the increasing abundance of proteins, 50 (34.0%) were ribosome subunits, which validated the known chlortetracycline-resistant strategy to overcome the pressure caused by chlortetracycline since this drug inhibits protein synthesis. Of the decreasing abundance of

proteins, six, SiP, Lpp, OmpA, OmpX, OmpC and OmpF, are outer membrane proteins, in which the importance role of OmpA, OmpX, OmpC and OmpF in resistance to chlortetracycline has been reported [7]; whereas the other proteins, especially in metabolic pathways, were largely unknown for this response. Most importantly, we were interesting to find these crucial metabolic proteins, pyruvate dehydrogenase E1 component (Odp1), maltose-binding periplasmic protein (MalE) and ATP energy system related proteins, such as AtpB, AtpG, were down-regulated. These results indicate that *E. coli* responses to chlortetracycline are related to not only protein translation, but also metabolic regulation. In addition, our study shows that more ribosomal subunits than previous reports will be potential candidate drug targets for drug development.

Functional Classification Annotation of Differential Proteins

To understand the differential response to chlortetracycline, we investigated the GO terms of these differential proteins in response to chlortetracycline by bioinformatics. Functional annotation showed that 43.4% and 35.9% increasing abundance of proteins were categorized into translation in biological process and RNA binding in molecular function, respectively, and 24.5% and 27.2% decreasing abundance of proteins were classified into generation of precursor metabolites and energy in biological process and metal ion binding in molecular function, respectively. Furthermore, increasing abundance of proteins were enriched in translation, such

as RNA biosynthetic process, protein complex disassembly and ribosome biogenesis except for some stress stimulation, including response to antibiotic, response to temperature stimulus, whereas decreasing abundance of proteins were enriched in metabolism, such as the tricarboxylic acid (TCA) cycle, pyruvate metabolism, hexose, glucose and coenzyme metabolic process and some energy-related process, oxidation and anaerobic respiration (Figure 1). Our analysis indicates that chlortetracycline triggers a global response which is involved in known ribosome subunit biosynthesis and unknown energy metabolism.

Pathway Analysis and Protein-protein Interaction Prediction of Differential Proteins Responsible for Chlortetracycline Stress

Using DAVID online resource, we analyzed the pathways of these differential proteins in *E. coli* K12 BW25113 in response to chlortetracycline treatment. 195 proteins from total 331 differential proteins were matched the KEGG_PATHWAY database including 82 proteins with increasing abundance and 113 proteins with decreasing abundance. We did not surprise that the most affected pathway was ribosome, which contained 50 differential proteins and accounted for 61% of total increasing abundance proteins. In consistent with the previous reports that gentamicin promoted four ribosomal subunits, L1, L9, L10 and S2, in aerobic and oxygen-limited *E. coli*, our results further indicated that more ribosomal subunits were involved in antibiotics stress including

30S and 50S ribosomal subunits. Thus, our results confirm the conclusion that the promotion of ribosomal subunits may be a compensative tactic for bacterial defense to antibiotics, and further indicate that more ribosomal subunits responsible for antibiotic resistance may be revealed by high throughput proteomics.

To our surprise, 100 differential proteins (accounted for 51.3%) were involved in metabolic pathways such as pyruvate metabolism, pyrimidine metabolism, the citrate cycle and butanoate metabolism. Out of them, 82% decrease and only 18% increase in abundance. Very interestingly, 36 proteins and only 3 proteins decreased and increased, respectively, in the three most affected metabolism pathways (the citrate cycle, glycolysis/gluconeogenesis and pyruvate metabolism, Table1 and Figure 2). Besides these, many other metabolic pathways, such as purine metabolism, alanine, aspartate and glutamate metabolism and pentose phosphate pathway, were involved in the response to chlortetracycline as well. Thus, our results indicate that most of metabolic pathways downregulate while translation proteins upregulate in the resistance to the drug. These metabolic pathways contribute to a complex biological process that reaches a steady-state condition after series of related and interconvertible pathways. For example, glucose and pyruvate could convert into each other in glycolysis/gluconeogenesis pathway. The pyruvate metabolism is the last step of glycolysis to the TCA cycle and produces acetyl-CoA for

energy system. It is a common mechanism that the stimulation of the TCA cycle would result in increase of NADH level and finally led to the increase of proton-motive force (PMF) [21]. PMF may promote some of antibiotics uptake such as aminoglycoside antibiotics[39]. Based on these facts, our results provide a clue that the fluctuation of metabolic pathways including the TCA cycle may be an antibiotic-resistant mechanism while it is not an antibiotic-mediated cell death process. Those involved proteins would be potential drug targets for the development of synergistic antibiotics in the future and worth further investigation. Additionally, 11 differential proteins, PhoP, OmpC, OmpF, DegP, NarL, NarH, FdnG, FrdA, FrdB, NarG and GlnA, were involved in two-component system (TCS), which are well known as a part of innate resistance and play important roles in bacterial responses to a variety of environmental stresses[22].

We further predicted the protein-protein network constructed by differential increasing and decreasing proteins in chlortetracycline stress. The STRING software showed that the translation process was located in the center of up-regulated protein networks, whereas the metabolism pathways functioned in the center of down-regulated protein networks (Figure 3). Our quantitative proteomic analysis showed the comprehensive protein-protein network of *E. coli* in chlortetracycline stress that extended our knowledge on the intrinsic drug-resistant mechanisms.

Functional Characterization of Differential Proteins using Genetically Modified Strains of Gene Deletion.

Although these differential proteins were related with chlortetracycline response in the network, only some of key proteins played important roles while others might only contribute few for drug resistance, which were called "consequence proteins"[23, 24]. In this regard, we tested 41 genetically gene-deleted mutants in the presence and absence of chlortetracycline using survival capability assay including 17 genetically gene-deleted mutants related with metabolism pathways (*⊿aceE, ⊿glgA, ⊿luxS, ⊿polA, ⊿btuE, ⊿narG, ⊿sucA, ⊿sucB*, *⊿sucC, ⊿sucD, ⊿fumA, ⊿frdA, ⊿frdB, ⊿pflB, ⊿guaA, ⊿talD* and *⊿bglX,* accounted for 42.5%) (Figure 4). Meanwhile, 24 mutants of differential proteins in the other pathways were used in the same experiment. Out of the 24 mutants, 5 contribute to two-component system (*⊿narL, ⊿ompF, ⊿ompC, ⊿degP* and *phoP,* accounted for 12.5%), and 19 (*⊿malE, ⊿uuP, ⊿slP, ⊿paL, ⊿metQ, ⊿groL, ⊿ompX, ⊿secG, ⊿dnaJ, ⊿ybjB*, *⊿tsX, ⊿ompA, ⊿lpp, ⊿zwf, ⊿nlpB, ⊿osmC, ⊿clpX, ⊿mdtE*, and *⊿crP*, accounted for 47.5%) belong to other pathways. We showed that 51.2% (21/41) mutants grew differentially between the presence and absence of the drug. Specifically, *⊿glgA, ⊿luxS, ⊿sucD, ⊿mdtE, ⊿sucC* and *⊿talD* grew slower, while *⊿ompF, ⊿ompC, ⊿groL, ⊿ompX, ⊿secG, ⊿dnaJ, ⊿btuE, ⊿bglX, ⊿phoP, ⊿clpX, ⊿degP, ⊿ompA, ⊿aceE, ⊿frdA* and *⊿sucB* grew faster. Of the 17, 5 and 19 mutants related to metabolism pathways, two-component system and other pathways, 10

(58.8%), 4 (80%) and 7 (36.8%) were validated using the functional assay, respectively. Some of these proteins were reported to differentially expressed in medium with different classes of antibiotics in bacteria, but few was confirmed using functional assay[17,25-33]. Our results verify them by function validation and thereby further support our finding on bacterial metabolic strategy against chlortetracycline.

Experimental

Bacterial Strains and Culture

The bacterial strains used in the current study were *Escherichia coli* K12 BW25113 and its mutants, which were kindly provided by NBRP (NIG, Japan): *E. coli* [34]. After routinely grown overnight in Luria Bertani (LB) medium at 200 rpm, 37°C, the strains were diluted at 1:100 to fresh LB medium with chlortetracycline, which was equal to ¼ minimum inhibitory concentration (MIC) of wild type strain (1.56 μg/mL chlortetracycline) and then harvested at OD1.0 by centrifugation at 5,000 X g for 10 min and washed by saline twice. The bacteria were resuspended with 5 mL PBS with PMSF and broken by supersonic. After removed unbroken debris by centrifugation at 15,000 X g for 30 min, the supernatants were precipitated by 20%TCA in ice for 1 h and washed twice by cold acetone at 1, 5000 X g, 10 min at 4 °C. The precipitated pellets were resuspended in 200 μL dissolution buffer (8M urea, 0.1% SDS and 2% Triton X-100

in 0.5 M triethyl ammonium bicarbonate buffer (TEAB) pH 8.5). The protein concentrations were measured by BCA assay and aliquot to 100 µg per tube and then immediately use or stored at -80 °C before use[35].

iTRAQ Labeling and SCX fractions

Samples were labeled by iTRAQ and separated by SCX according to a procedure described previously [36, 37]. Briefly, proteins from each sample (100 µg) were reduced by final 5 mM Tris (2-carboxyethyl) phosphine (TCEP) at 37 °C for 1 h, alkylated with final 10 mM S-Methyl methanethiosulfonate (MMTS) for 10 min at room temperature as described previously[38]. After diluted 8 times with 0.5 M TEAB, the samples were digested with trypsin at 1:20 ratio at 37°C overnight. Digested samples were labeled with respective isobaric tags using two labeling from 8-plex iTRAQ reagent multiplex kit (Applied Biosystems Inc., CA, USA) following manufacturer's protocol. The labeling scheme was *E. coli* K12 BW25113 in LB medium without chlortetracycline as 118 and in LB medium with chlortetracycline as 116. The two labeled samples were pooled and fractionated usingÄKTA Purifier UPC-900 (GE Healthcare, Upssala, Sweden) with a PolySulfoethyl A ™ SCX column (150 × 2.1 mm, 5 µm, 300 Å, Poly LC Inc., USA). The peptides were reconstituted in SCX buffer A (5 mM KH2PO4, 25% ACN, pH 2.7-3.0) to make sure pH was lower than 3 and then loaded on SCX column using 100% buffer A for 5 min at a flow rate of

0.2ml/min. Peptides were eluted using serial gradients with 0%-25% in 30min, 25%-60% in 20min, and 60%-100% in 15min buffer B (600mM KCl in buffer A). Pooling of SCX fractions were monitored by UV 214 nm trace and a total of 8 fractions were collected and then dried down using a CentriVap Concentrator (Labconco Inc, Kansas City, MO, USA) and resuspended in 0.1%TFA to make sure pH was lower than 3.

LC-MALDI TOF/TOF and Data Analysis

After desalted by Sep-Pak® Vac C18 cartridge 1cc/100 mg (Waters Inc., Milford, MA, USA), the peptides were dried down and resuspended in 0.5%TFA and then separated on Thermo Scientific Surveyor LC nanoflow with BioBasic C18 column (100 x 0.18 mm, particle size: 5 μm) using serial linear gradients with 5%-35% in 90min and 35%-95% in 30min buffer C (100% ACN in 0.1% FA) at a flow rate of 160μl/min. Online MALDI spotting of the LC fractions was spotted on 384 Opti-TOF^{TM} plate and then analyzed with a 4800 Plus MALDI TOF/TOF^{TM} (Applied Biosystems, Foster City, CA, USA). The mass spectrometer was set to perform data acquisition with a selected mass range of 800–3500 m/z and signal to noise (S/N) ratio was set to greater or equal to 50 MS/MS spectra were processed by the software Proteinpilot version 4.0 (Applied Biosystems, Foster City, CA, USA) with the Paragon algorithm as previous described with modified [39] . Briefly, data were searched against a complete proteome from Uniprot *E. coli* K12

database (updated to Apr. 1st. 2013) ; cysteine alkylation setting was MMTS and digested by typsin; ID focused on biological modification and amino acid and substitutions; instrument was MALDI TOF/TOF; protein confidence was performed using a confidence threshold of 95% which matched with unused/total protein score of 1.3 cut off; the corresponding false discovery rate (FDR) of the identified protein should be lower than 5%; base correction, background correction was taken into consideration to normalize the data; the iTRAQ average reporter ion ratio must to be ≥1.5 (increase) or ≤0.67 (decrease); at less two peptides matched identified protein were considered for further analysis.

Gene Ontology Categories and Bioinformatics Analysis

The Gene Ontology (GO) terms of the increase or decrease degree of differential proteins between different conditions in this study were analyzed through the online DAVID 6.7 bioinformatics resource (http://david.abcc.ncifcrf.gov/), which is a powerful enrichment tool for gene ontology enrichment analysis with reducing groups of gene lists [40]. We selected all GO terms which p-value is ≤ 0.05 with the Bonferroni correction. The pathway analysis in this study was extracted through the online DAVID using KEGG pathway which ease is ≤ 0.05[41]. We also predicted protein-protein interactions by STRING 9.05 which could show the direct (physical) and indirect (functional) associations from database (http://string-db.org/).

Validation for Functions of Differentially Expression of Proteins Using Bacterial Survival Capability Assay.

Investigation of survival capability assay was performed as described previously with a modification[6] . Briefly, total 41 mutant strains with their wild-type strains as control were incubated in a serial concentration of chlortetracycline for 8 h and then measured OD at 600 nm. Survival capability was characterized by comparison between experimental and control groups, and was termed by the survival rate. These experiments were repeated at least three times and then analyzed by SPSS v.12.0

Conclusion

Our finding reveals a comprehensive global response to chlortetracycline stress, which is characterized by upregulation of ribosomal subunits and downregulation of major metabolic pathways. Since the downregulation of global metabolic pathways is largely known, they would be potential candidates of drug targets for novel antibiotics design. Our study demonstrates a global response to chlortetracycline stress through a complex regulation network based on key pathways including metabolic pathways, which may be a novel antibiotic-resistant mechanism.

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Table 1 Identification of differential proteins involved in the three most affected metabolic pathways in *Escherichia coli* K12 BW25113 in response to chlortetracycline using iTRAQ labeling Analysis

Accession	Name	%Cov(95)	Peptides	With/without chlortetracycline
K6PF1_ECOLI	6-phosphofructokinase isozyme 1	8.7	2	***3.05***
MAO1_ECOLI	NAD-dependent malic enzyme	8.7	5	***2.54***
MASY_ECOLI	Malate synthase A	35.1	27	***1.84***
FRDA_ECOLI	Fumarate reductase flavoprotein subunit	17.1	16	***0.66***
ALDA_ECOLI	Lactaldehyde dehydrogenase	31.3	15	***0.65***
GPMA_ECOLI	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	33.2	11	***0.65***
TDCE_ECOLI	Keto-acid formate acetyltransferase	11.3	10	***0.64***
KPYK1_ECOLI	Pyruvate kinase I	25.1	10	***0.64***
ALF1_ECOLI	Fructose-bisphosphate aldolase class 1	21.4	10	***0.56***
MAO2_ECOLI	NADP-dependent malic enzyme	5.5	3	***0.55***
DLDH_ECOLI	Dihydrolipoyl dehydrogenase	48.9	31	***0.53***
ODP1_ECOLI	Pyruvate dehydrogenase E1 component	44.5	63	***0.49***
SUCD_ECOLI	Succinyl-CoA ligase [ADP-forming] subunit alpha	19.7	5	***0.44***
CAPP_ECOLI	Phosphoenolpyruvate carboxylase	11.6	9	***0.35***
G3P1_ECOLI	Glyceraldehyde-3-phosphate dehydrogenase A	57.7	30	***0.34***
ACON2_ECOLI	Aconitate hydratase 2	41.2	38	***0.34***
CISY_ECOLI	Citrate synthase	19.4	9	***0.3***
IDH_ECOLI	Isocitrate dehydrogenase [NADP]	56	28	***0.27***
PCKA_ECOLI	Phosphoenolpyruvate carboxykinase [ATP]	25.4	15	***0.26***
KPYK2_ECOLI	Pyruvate kinase II	18.5	8	***0.26***
FRDB_ECOLI	Fumarate reductase iron-sulfur subunit	15.2	5	***0.24***
ADHE_ECOLI	Aldehyde-alcohol dehydrogenase	27.6	19	***0.22***
K6PF2_ECOLI	6-phosphofructokinase isozyme 2	10	2	***0.19***
YIDA_ECOLI	Phosphatase YidA	12.6	2	***0.19***
ALF_ECOLI	Fructose-bisphosphate aldolase class 2	41.2	20	***0.18***
DHSB_ECOLI	Succinate dehydrogenase iron-sulfur subunit	27.3	8	***0.18***

PGK_ECOLI	Phosphoglycerate kinase	61	33	***0.15***
PGM_ECOLI	Phosphoglucomutase	9	3	***0.14***
FUMA_ECOLI	Fumarate hydratase class I, aerobic	14.4	9	***0.13***
ODO2_ECOLI	Dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex	25.4	22	***0.13***
PTGA_ECOLI	Glucose-specific phosphotransferase enzyme IIA component	53.3	12	***0.1***
ENO_ECOLI	Enolase	64.3	27	***0.08***
PFLB_ECOLI	Formate acetyltransferase 1	45.7	51	***0.07***
MDH_ECOLI	Malate dehydrogenase	53.2	27	***0.05***

Note: The altered abundance of proteins were highlighted by italic and bold font

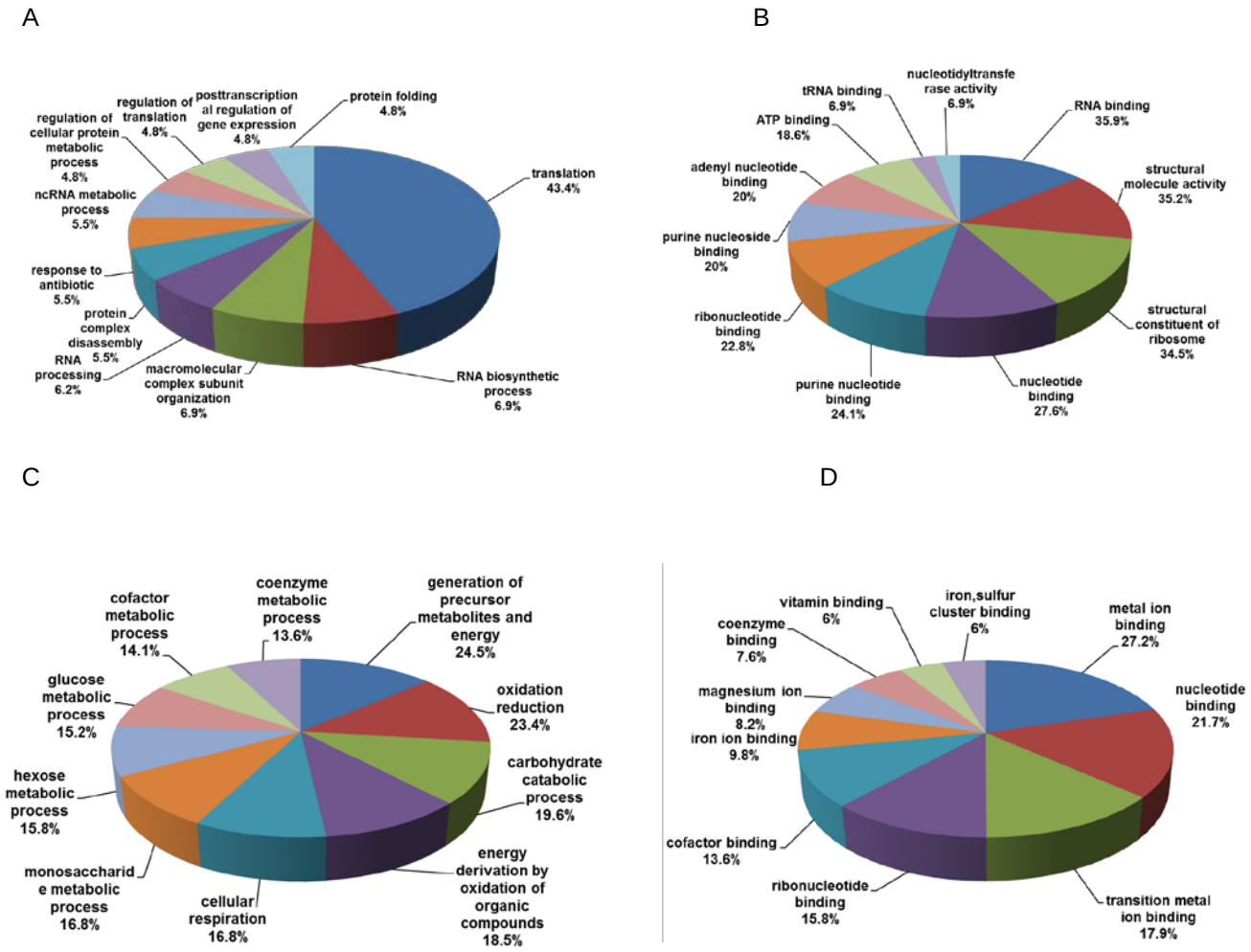


Figure 1 Gene Ontology categories for the differentially expressed proteins of *E. coli* K12 BW25113 in response to chlortetracycline according to DAVID Gene Ontology analysis. These proteins were classified into molecular functions and biological processes. A) and B) Functional classification of the chlortetracycline-related differentially increasing proteins in biological processes and molecular functions, respectively; C) and D) Functional classification of the chlortetracycline-related differentially decreasing proteins in biological processes and molecular functions, respectively.

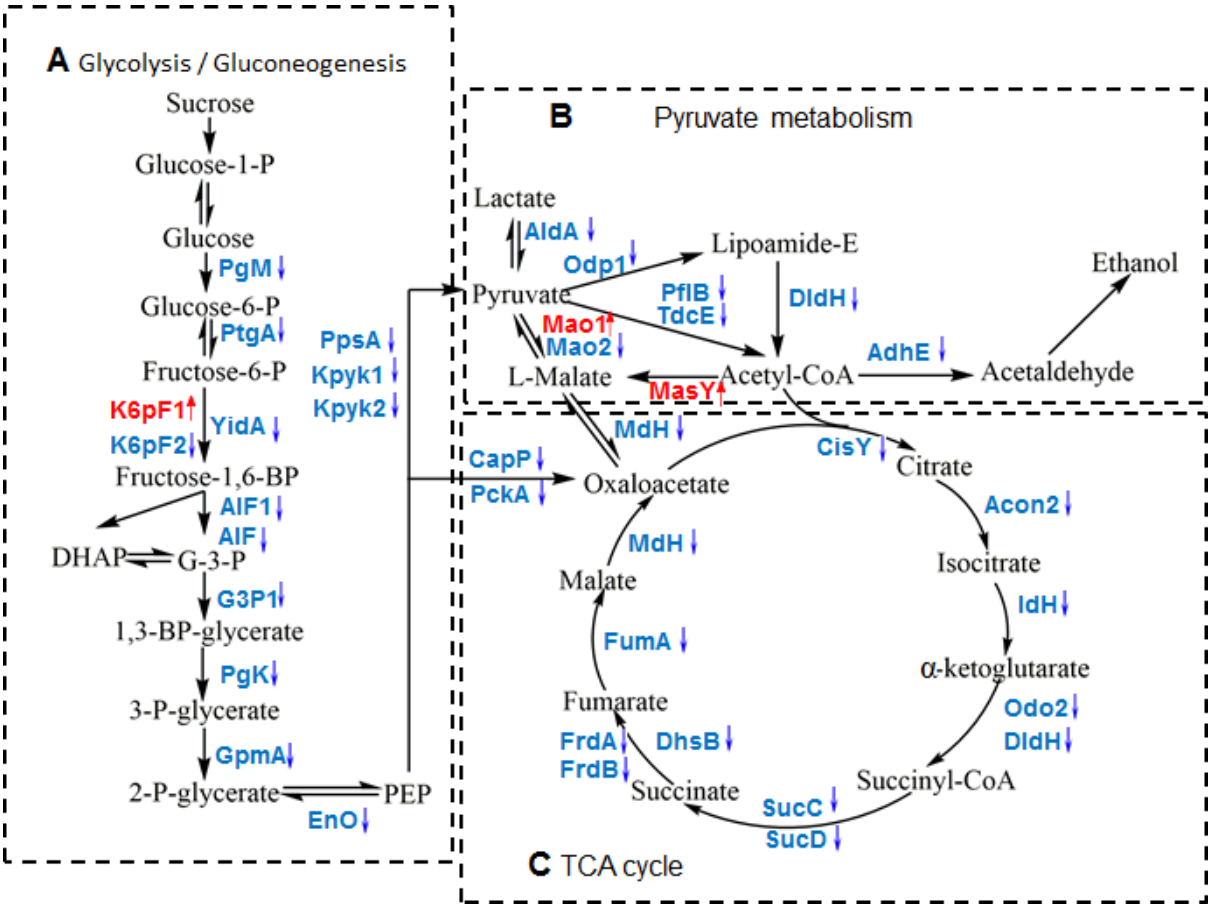
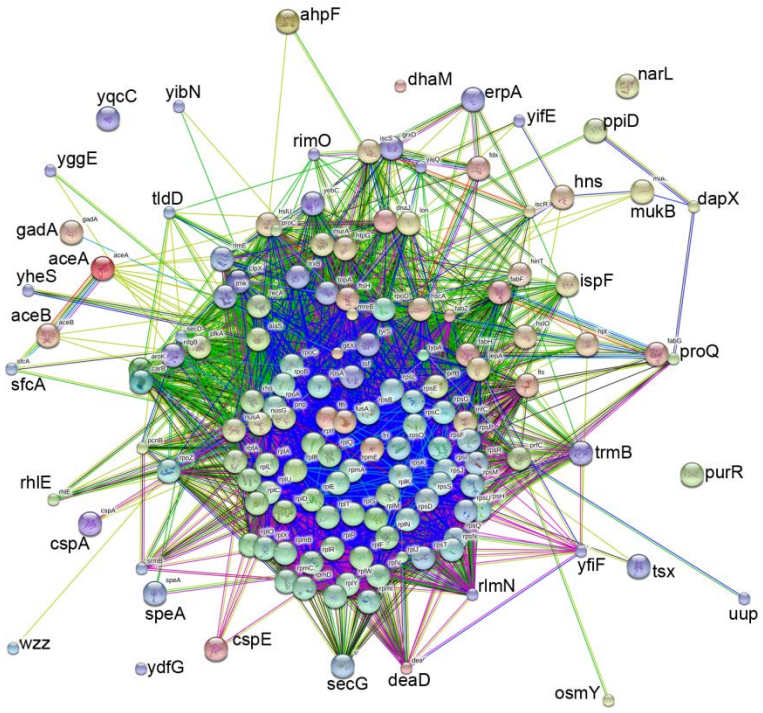


Figure 2 Three most affected metabolic pathways with involved differential proteins obtained from *E. coli* K12 BW25113 in response to chlortetracycline. The three most affected metabolic pathways were glycolysis/gluconeogenesis (A), pyruvate metabolism (B) and the TCA cycle (C). The decreasing and increasing abundances of proteins were highlighted by blue and red, respectively. These results show the downregulation of these metabolic pathways in response to chlortetracycline. Glucose-1-P, glucose 1-phosphate; Glucose-6-P, glucose 6-phosphate; Fructose-6-P, fructose 6-phosphate; G-3-P, glyceraldehyde 3-phosphate; DHAP,

dihydroxyacetone phosphate; 1,3-BP-glycerate, 1,3-bisphospho-D-glycerate; 3-P-glycerate, 3-Phospho-D-glycerate; 2-P-glycerate, 2-Phospho-D-glycerate; PEP, phosphoenolpyruvate.

A



B

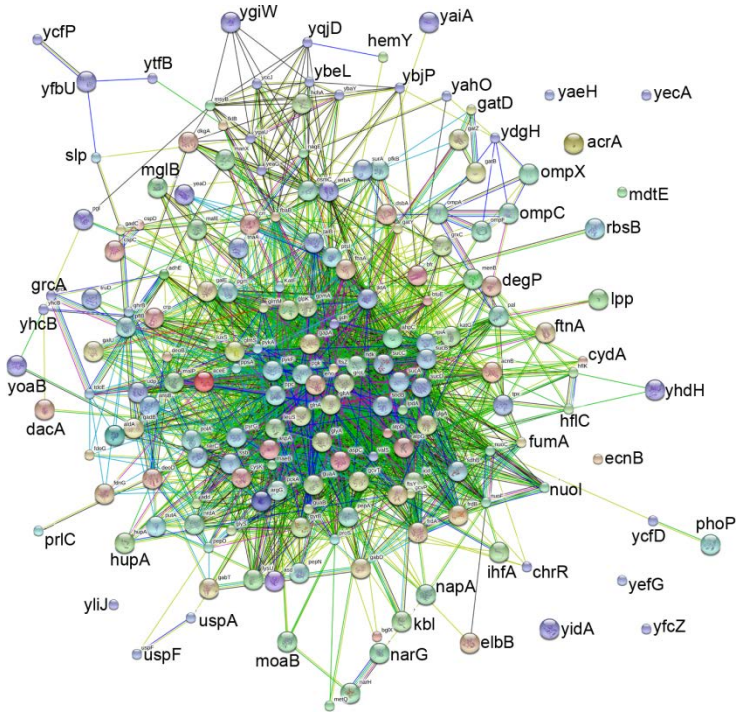


Figure 3 The STRING software predicted protein-protein interaction of related differential proteins in the presence and absence of chlortetracycline stress. A) The predicted protein-protein interaction network of increasing abundance of proteins in chlortetracycline stress. The red circle highlighted the differential translation proteins, 50 of them were ribosome subunits. B) The predicted protein-protein interaction network of increasing abundance of proteins in chlortetracycline stress. Many of them were involved in metabolic pathways.

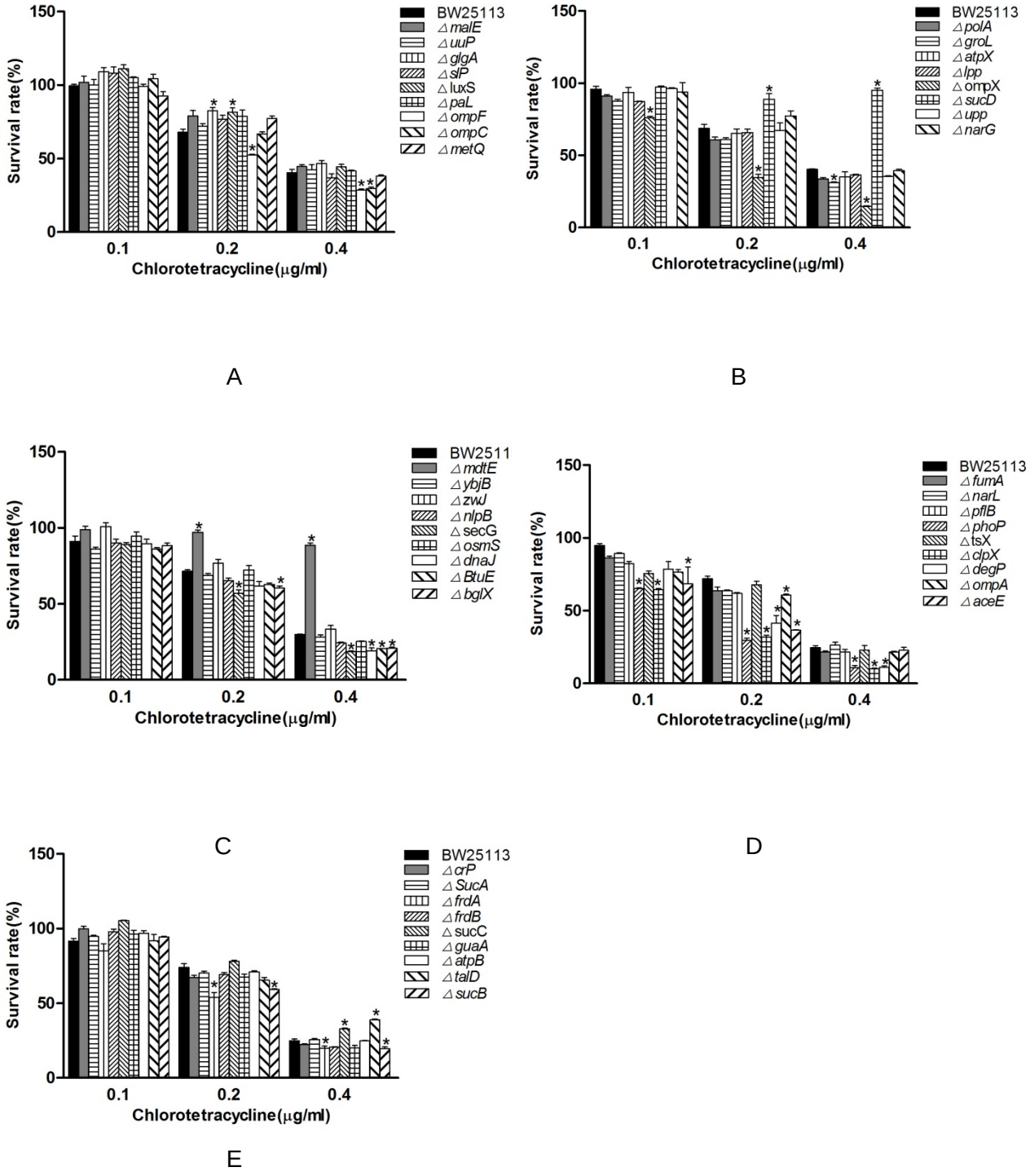


Figure 4 Histogram displayed survival capabilities of total 41 genetically mutants of gene deletion of the differential proteins in the presence and absence of chlortetracycline.

These mutants are indicated in these figures (A-E). The concentrations of chlortetracycline range from 0.1, 0.2 and 0.4 μg/mL. All the data were collected from three independent experiments and the asterisks denoting the datum points represented the experimental group has a significant difference ($P<0.05$) from the control group.

Supplementary Table 1 Proteins and peptides identification list by LC-MALDI MS/MS

Supplementary Table 2 False discovery rate FDR analysis of proteins and peptides identification by Proteinpilot V4.0

Supplementary Table 3. Identification of Significantly Differential Proteins of *Escherichia coli* K12 BW25113 in response to chlortetracycline using iTRAQ labeling Analysis