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Global proteomic responses of *Escherichia coli* and evolution of biomarkers under tetracycline stress at acid and alkaline conditions

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HIGHLIGHTS

- The global proteomic responses to tetracycline in extreme ecosystems were elucidated.
- Energy and carbohydrate metabolism were promoted under tetracycline stress.
- TolC and Pck in *E. coli* could be biomarkers to reflect tetracycline stress.
- *E. coli* at alkaline group strongly impaired the protection mechanism.
- Omics approach was developed to perfect the antibiotic usage limitation.

GRAPHICAL ABSTRACT

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ABSTRACT

The global proteomic regulation and the mechanism of biomolecule evolution in acid and alkaline ecosystems triggered by tetracycline, a representative of antibiotics, are not clear. To reveal the related mechanisms, the global responses of *Escherichia* (*E.*) *coli* to tetracycline in acid and alkaline conditions were analyzed using a proteomic approach. The specific phospholipid C16:1ω9c showed a significant decrease between the treatment and control groups. The 77 and 111 upregulated proteins in *E. coli* in acid and alkaline groups were mainly involved in carbohydrate transport and metabolism and energy metabolism, whereas, the 78 downregulated proteins were related to ribosome and bacterial chemotaxis in the acid group. The 110 downregulated proteins involved in carbon, glycine, serine, threonine, glyoxylate, and dicarboxylate metabolism, biosynthesis of antibiotics, fatty acids, and secondary metabolites in the alkaline group. Protein sequence analysis showed that the respective distribution of phosphorylation, glycosylation, and methylation sites among stable-expressed, upregulated, and downregulated proteins all showed a significant difference. TolC and phosphoenolpyruvate carboxykinase (Pck) in *E. coli* could be biomarkers to reflect tetracycline stress under extreme conditions with high sequence homology in *Homo sapiens*, implying the potential impact of tetracycline on humans at the network level. Generally, *E. coli* in the acid group accelerated the highly efficient protection mechanism to defend against tetracycline stress, while *E. coli* in the alkaline group strongly impaired the protection mechanism. These findings provide important clues to reveal the microbial antibiotic resistance mechanism in *E. coli* under extreme conditions and perfect the antibiotic usage.

1. Introduction

Tetracyclines are broad spectrum antibiotics that are particularly useful in curing human and animal infections due to their high

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bacteriostatic activity (Chopra and Roberts, 2001; Dutta and Basu, 2011; Landers et al., 2012). It is well established that tetracyclines inhibit bacterial protein synthesis by preventing the association of aminoacyl-tRNA with the ribosome (Brodersen et al., 2000). Although the altered ribosomal protection proteins and outer membrane proteins in *E. coli* have been studied for the resistance of tetracycline (Connell et al., 2003; Xu et al., 2006; Zhang et al., 2008), the global proteomic responses and metabolic networks of this chemical remain unclear. Recently, comparative proteomic analysis was considered as an insightful tool to reveal the substantial changes of the proteome under different conditions (Casado Munoz et al., 2016; Schultrich et al., 2017). For example, proteomic approaches were used to determine that *E. coli* O157:H7 and O104:H4 would induce a global response to nutrient-poor environments to survive outside intestines by up- or downregulated enzymes for substrate uptake, transport, degradation, and product export (Islam et al., 2016). Utilizing iTRAQ-based quantitative proteomic technology, the global response to 17 estradiols in estrogen-degradation strain *Pseudomonas putida* SJTE-1 was studied. Some proteins mainly involved in the processes of stress response, energy metabolism, transportation, carbon metabolism, chemotaxis, and cell motility displayed significant changes in expression (Xu et al., 2017). For the influence of perfluorooctanoic acid on proteomic expression and phospholipid metabolism of *E. coli*, 69 down- and 63 upregulated proteins triggered the membrane homeostasis to resist perfluorooctanoic acid stress (Yang et al., 2017). Sulfur-34S stable isotope labeling of amino acids for quantification was used to investigate the proteomic changes related to naphthalene degradation in *Pseudomonas fluorescens* ATCC 17483 and uncovered a specific oxidative-stress-like response (Herbst et al., 2013). These findings enriched our knowledge of microbial adaptation mechanisms and fastened the biochemical pathway identification. Moreover, to elucidate the resistance mechanism of tetracycline when exposed to human stomach and insect guts, and the mechanism of biomolecule evolution in extreme ecosystems, the global responses of *E. coli* to tetracycline in acid and alkaline conditions were analyzed using the proteomic approach.

Amino acids are the elementary units of proteins. Their components and sequences directly determine the unique structures and molecular functions of proteins. Sequence similarity is even used as direct evidence of the evolutionary history of species. Under various exposures and stimuli, the sequences of proteins will be altered, triggering their evolution. That is why alignments of multiple sequences can be used to investigate protein homology and evolution.

Here, an iTRAQ-based proteomic analysis technique was utilized to characterize the proteomic profiles of *E. coli* in a tetracycline environment in acid and alkaline conditions, compared to those in neutral conditions. Bioinformatics analysis was used to analyze the key pathways and metabolic processes catalyzed by the differentially expressed proteins. Amino acid contents and sequences of these proteins were insightfully inspected to uncover the evolution of different species in extreme environments. The findings provide important clues to predict species evolution based on the amino acid sequence similarity, to reveal the proteomic mechanism of antibiotic resistance in *E. coli* under extreme conditions and ecological environments.

2. Materials and methods

2.1. Reagents and media

E. coli ATCC 8739 was purchased from a microbial culture collection center in Guangdong Province, China. The composition of enrichment medium for strain culture was as follows (g L^{-1}): beef extract (3), peptone (10), and NaCl (5), respectively. Mineral salt medium (MSM) for tetracycline treatment contained (in g L^{-1}) 3 KH_2PO_4, 2 NaCl, 3 NH_4Cl, and 1 $MgSO_4$. Tetracycline was purchased from Macklin Biochemical Co., Ltd. (Shanghai, China).

2.2. Microbial culture and tetracycline treatment

Cultures of *E. coli*, at an optical density of 0.6 at 600 nm for 6 to 8 h, were separated from the medium by centrifugation at 6000 *g* for 10 min and were then washed three times with sterile phosphate buffer to prepare the bacterial suspension of 20 g L^{-1} to achieve the initial biomass dosage at 1.0 g L^{-1} in a 20 mL of MSM. Then, they were incubated with tetracycline at the dose of 1.0 μM at pH = 5, 7, and 9 for 1 d, respectively. Next, the above samples were inoculated in the dark at 160 rpm as the treated group. Medium without tetracycline was used to perform the control experiment. Finally, they were harvested by centrifugation at 6000 *g* for 10 min at 4 °C for phospholipids, protein, and RNA extraction. Each treatment has three biological replicates.

2.3. Extraction and determination of phospholipids

Extraction of phospholipids was conducted according to the Schutter method (Schutter and Dick, 2000). Quantification of phospholipids was performed using the peak area and internal standard curve method, and the qualitative method was used to compare the retention time with the standard compound and according to the spectral library search. Contents of phospholipids were expressed with nmol g^{-1}.

2.4. Extraction, quantification, and digestion of cellular proteins

The extraction, quantification, and digestion of cellular proteins were based on the published method (Ou et al., 2017). Briefly, the cells before and after exposure to tetracycline were suspended in 1 mL of lysis buffer (15 mM Tris-HCl, 7 M urea, 2 M thiourea, 1% w/v DTT, 4% w/v 3-[(3-Cholamidopropyl) dimethylammonio] propanesulfonate) added with 0.2 g L^{-1} of phenylmethylsulfonyl fluoride, 2% v/v immobilized pH gradient (IPG) buffer, 0.6 g L^{-1} of DDT, and vibrated for 30 s. The samples were frozen in liquid nitrogen three times for 15 min each time and subsequently treated by ultrasonication for 20 min. Nuclease was added to the lysate at a final concentration of 1%. After incubation at 4 °C for 30 min, the cell debris was removed at 4 °C by centrifugation at 16,700 *g* for 1 h. The concentration of proteins was measured using the Bradford method (Bradford, 1976). Each treatment has three replicates.

The obtained 100 μg of proteins from each sample were reduced by 2 μL of 54.4 mM tris (2-carboxyethyl) phosphine (AB Sciex, Framingham, USA) at 37 °C for 1 h. The cysteines were blocked with 1 μL of cysteine-blocking reagent for 10 min at room temperature. The protein samples were then centrifuged using 10 KD Amicon Ultra-0.5 filters at 13,200 *g* for 20 min and washed three times with 100 μL of dissolution buffer (pH 8.5) (AB Sciex, Framingham, USA), which mainly consisted of 1 M of tetraethylammonium bromide. The samples in filter devices were digested overnight using 50 μL of dissolution buffer containing 2 μg of trypsin (Promega, V5280, USA) at 37 °C after removal of the liquid collected in the tube. Subsequently, the released peptides were collected by centrifugation at 13,200 *g* for 20 min, followed by trypsin digestion in a washing ultrafiltration tube with 50 μL of dissolution buffer for 2 h.

2.5. Protein labeling and SCX fractionation

The detailed description of the protein labeling and SCX fractionation is supplied in Text S1.

2.6. Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) analysis

The samples were resolved with solution (2% v/v ACN, 0.1% v/v formic acid), centrifuged at 12,000 *g* for 20 min, and detected by an AB Sciex Triple TOF 5600 mass spectrometer (AB SCIEX, Framingham, MA, USA) equipped with a Nanospray III source (AB SCIEX). Detailed analysis is summarized in Text S2.

2.7. Protein bioinformatics and statistical analyses

Up- and downregulated proteins in the 114/115 (post-exposure MSM with tetracycline group at pH = 5 to post-exposure MSM group with tetracycline at pH = 7), and 116/115 (post-exposure MSM with tetracycline group at pH = 9 to post-exposure MSM group with tetracycline at pH = 7) were subjected to functional pathway analyses using the database for annotation, visualization, and integrated discovery (DAVID, http://david.abcc.ncifcrf.gov/). An enhanced score of ≥1.3, set as the threshold, was considered significant when pathway enrichment was analyzed in the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.kegg.jp/) (Huang et al., 2009a; Huang et al., 2009b). Search Tool for the Retrieval of Interacting Genes/Proteins (STRING, https://string-db.org/) analysis was performed to construct the protein-protein interaction networks. Clusters of orthologous groups of proteins (COG) classifications were obtained according to *E. coli* K12 MG 1655 in IMG database (https://img.jgi.doe. gov/cgibin/er/main.cgi). PRIMER 7 was used to launch multivariate statistical analyses. MEGA 7 aimed to construct phylogenetic trees. Data are expressed as the mean ± standard deviation. Regarding the comparisons among multiple values, a one-way analysis of variance with Duncan's post-hoc test was used. Spearman's correlation was used to assess associations of the pH value with phospholipids in *E. coli*. All of the statistical analyses were performed with SPSS 23.0 software. The level of significance for all of the statistical analyses was set to $p < 0.05$. Each treatment has three biological replicates.

2.8. Protein validation by quantitative polymerase chain reaction (qPCR)

For total RNA extraction, the cells cultured for 24 h according to the above conditions were collected with centrifugation at 8000 *g* for 2 min at 4 °C and washed three times by sterile phosphate buffer. Immediately, the collected cells were suspended in 1 mL TransZol Up reagent (TRANS®) for lysis. Next, the target total RNA was extracted according to the instructions of the TransZol Up Plus RNA kit (TRANS®). In addition, the purity of RNA was detected by measuring the optical density (OD) value on a nucleic acid detector. For determining the integrity of the total RNA extraction, a 1-μL RNA sample was used to run gels through 1% agarose gel electrophoresis, followed by gel imaging.

The 5-fold All-in-One RT MasterMix (with AccuRT Genomic DNA Removal Kit) (abm®) was used for genomic DNA removal and first-strand cDNA synthesis. In brief, 2 μL of 4-fold AccuRT reaction mix (abm®) and nuclease-free water were added to a PCR tube containing 2 μg of extracted RNA followed by incubation at 25 °C for 5 min. After the addition of 2 μL 5-fold AccuRT Reaction stopper (abm®) and a reverse transcription reaction reagent (4 μL 5-fold All-in-One RT MasterMix and 6 μL nuclease-free water), the mixture was subsequently incubated at 25 °C for 10 min, and further incubated at 42 °C for 15 min.

The cDNA product was applied as a template in a standard qPCR. The primer sequence of the target protein was designed online on NCBI (https://www.ncbi.nlm.nih.gov/) and indicated in Table 1. The qPCR amplification was performed at 95 °C for 10 min, followed by 35 cycles at 95 °C for 15 s, and 35 cycles at 60 °C for 1 min by using a CFX96™ Real-time PCR system (BIO-RAD).

3. Results

3.1. Phospholipid synthesis under tetracycline stress

Bacteria have evolved mechanisms to regulate the biosynthesis of phospholipids, allowing bacteria to adjust membrane viscosity to match environmental requirements (Sen et al., 2015; Yang et al., 2011). The de novo fatty acid biosynthetic pathway is a major focal point for the regulatory events that control membrane homeostasis (Parsons and Rock, 2013). Lauric acid (C12:0), tridecanoic acid (C13:0), myristic acid (C14:0), pentadecanoic acid (C15:0), palmitoleic acid (C16:1ω9c), palmitic acid (C16:0), heptadecanoic acid (C17:0), linoleic acid (C18:2ω9,12c), elaidic acid (C18:1ω9t), stearic acid (C18:0), and eicosanoic acid (C20:0) were detected in *E. coli* under tetracycline stress at different pH conditions. Most of them did not show significant differences under three different pH conditions (Fig. 1A). The ratios of unsaturated phospholipids remained stable in the three different treatments (Fig. 1B), while the content of the specific phospholipid C16:1ω9c showed significant differences (Fig. 1C) ($p < 0.05$).

3.2. Proteins with significantly altered expression induced by tetracycline

The expression levels of 77 proteins were upregulated and 78 were downregulated in the acid group (114/115), while 111 were upregulated and 110 were downregulated in the alkaline group (116/115) among the 932 identified proteins (>1.2-fold change, $p < 0.05$). They were plotted in Table S1 and Figs. S1 and S2, which represented the most creditable tetracycline-regulated proteins involved in membrane homeostasis and the altered metabolic network in the conditions of acid and alkaline treatments. Additionally, in order to clarify whether the different proteome expression was triggered by tetracycline or pH

Table 1
qPCR profiling for verifying differentially expressed protein in *E. coli* after the exposure of 1.0 μM tetracycline when MSM was treated as control group (A) and tetracycline was treated as control group (B), at pH = 5, 7 and 9 for 1 d, respectively.

A

Protein	Forward prime sequence	Reverse prime sequence	qPCR (MSM, control*)		
			Tetracycline $_{pH=5}$/control $_{pH=5}$	Tetracycline $_{pH=7}$/control $_{pH=7}$	Tetracycline $_{pH=9}$/control $_{pH=9}$
TolC	TCGCTGTTACAGGCACGCTTG	TCCAGAGTCGGTAAGTGACCATCC	1.449	1.225	0.594
FadE	CTCGGCGTTGCTGGTCTGTG	GACATCGGCGGCATCACCTTAC	1.222	0.893	0.467
Pck	CGAACCGACGCCAACCTTCTC	CAGTGCCGTTCCAGCCAGTG	1.538	0.748	0.488
AceF	AGATGCCTCGCTTCAATAGTTCGC	CCACCGCCACACCGATGTTG	1.348	0.586	0.605
AtpE	CGCGTCAACCTGATCTGATTCCTC	GACAGCGAACATCACGTACAGACC	1.539	0.508	0.879

B

Protein	Forward prime sequence	Reverse prime sequence	qPCR (tetracycline, control*)	
			Tetracycline $_{pH=5}$/tetracycline $_{pH=7}$	Tetracycline $_{pH=9}$/tetracycline $_{pH=7}$
TolC	TCGCTGTTACAGGCACGCTTG	TCCAGAGTCGGTAAGTGACCATCC	1.723	1.849
FadE	CTCGGCGTTGCTGGTCTGTG	GACATCGGCGGCATCACCTTAC	0.874	0.788
Pck	CGAACCGACGCCAACCTTCTC	CAGTGCCGTTCCAGCCAGTG	1.213	0.915
AceF	AGATGCCTCGCTTCAATAGTTCGC	CCACCGCCACACCGATGTTG	1.406	1.802
AtpE	CGCGTCAACCTGATCTGATTCCTC	GACAGCGAACATCACGTACAGACC	1.666	1.873

* The fold change of the five genes including tolC, fadE, pck, aceF and atpE, was at least a 1.2-fold increase or decrease, which was considered as significant.

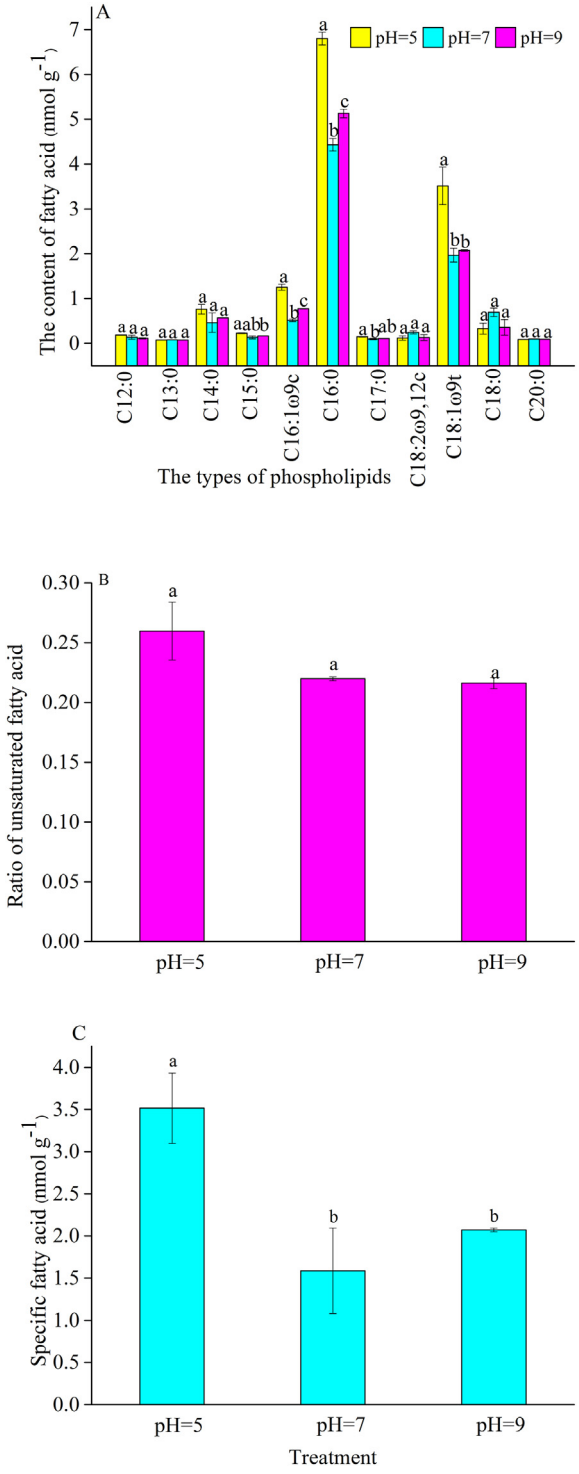


Fig. 1. Phospholipid concentrations of *E. coli* during the exposure process to tetracycline at pH = 5, 7, and 9, respectively. (A) Content of all detected phospholipids, (B) ratio of unsaturated phospholipids to total phospholipids, and (C) content of specific phospholipids, the results expressed as nmol g^{-1}.

values, a proteomic profiling in *E. coli* after the exposure of 1.0 μM of tetracycline compared to the control experiment without tetracycline was also identified at pH = 5, 7, and 9 for 1 d, respectively. Tetracycline in the acid condition caused 222 proteins to be significantly differential expressed, whereas tetracycline in the neutral condition triggered the synthesis of 347 up- and downregulated proteins. Also, tetracycline in the alkaline condition generated 285 up- and downregulated proteins among the 963 identified proteins (Table S2) (>1.2-fold change, $p <$ 0.05) for *E. coli* compared to the initial untreated state. Among these proteins, the 79 were upregulated, and 143 were downregulated after tetracycline treatment at pH = 5 (115/114) (Table S3), while the 135 were upregulated and 212 were downregulated after tetracycline treatment at pH = 7 (116/114) (Table S4). One hundred thirty-two were upregulated and 153 were downregulated after tetracycline treatment at pH = 9 (117/114) (Table S5).

3.3. Functional categories of the differentially expressed proteins

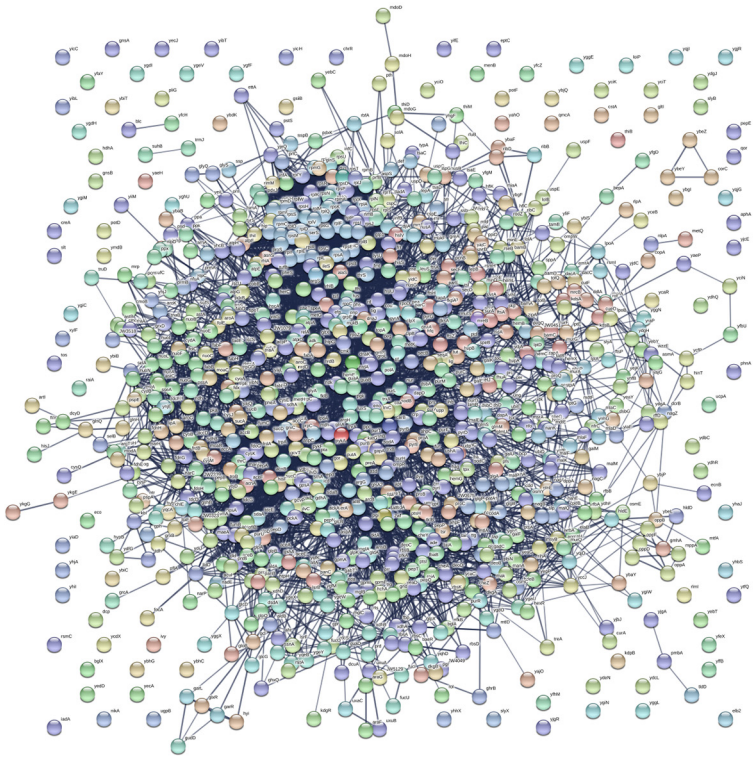
The up- and downregulated proteins were classified into eleven categories by their functions on the basis of COG functional classification (Fig. S3). Most differentially altered proteins were clustered into the carbohydrate metabolism process. Proteins in the amino acid transport and metabolism, energy production and conversion, translation, and nucleotide transport and metabolism also accounted for large portions of the differentially expressed proteins, indicating that some new special proteins were synthesized to resist tetracycline stress in extreme conditions.

GO enrichment analysis of the differentially expressed proteins was performed to classify the cellular components, molecular functions, and biological processes in Fig. S4. The molecular functions of the 77 upregulated proteins in the acid group are mainly associated with identical protein binding, peroxidase activity, porin activity, and lipopolysaccharide binding, while the 78 downregulated proteins are mainly related to structural constituents of ribosome, tRNA binding, translation elongation factor activity, and glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) activity. In the alkaline group, the 111 upregulated proteins are mainly involved in polyamine binding, porin activity, and protein binding, but they are not at the enrichment level. The 110 downregulated proteins have different molecular function, such as zinc ion, pyridoxal phosphate, tRAN and magnesium ion binding, hydroquinone: oxygen oxidoreductase activity, dihydrouracil dehydrogenase activity, oxidoreductase activity (Fig. S4B).

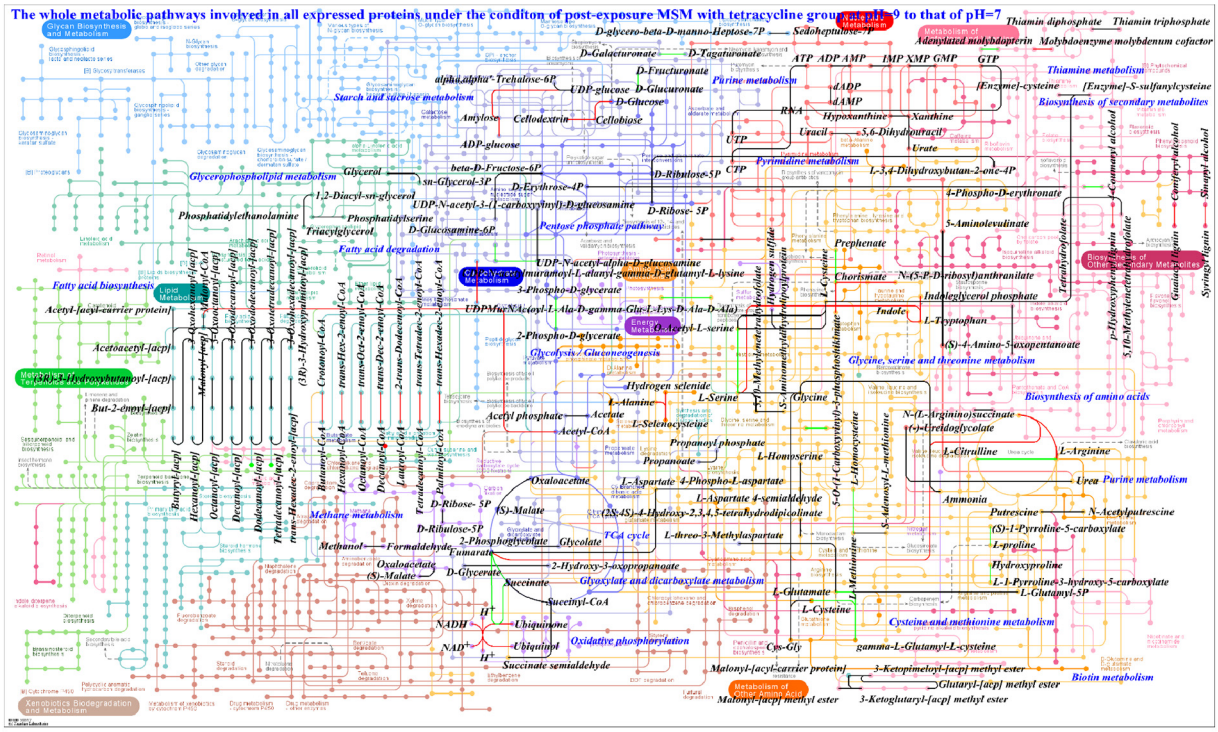
KEGG pathways showed that glycolysis/gluconeogenesis, biosynthesis of amino acids, and ribosome and bacterial chemotaxis at significant enrichment level were the important pathways in response to the stress of tetracycline at 1.0 μM with acid treatment, which were only found in the acid-treated group (Fig. S5). However, some of these pathways could be found in the alkaline-treated group and were significantly enriched compared to the initial untreated state in three different pH conditions (Fig. S6). Almost all tested proteins have certain direct or indirect interactions with other proteins (Fig. 2A). Several ribosomal proteins were found to be especially highly clustered (Fig. S7). Moreover, some proteins associated with protein biosynthesis and carbohydrate metabolism were also among the intensive interaction networks and metabolism networks (Figs. 2B, S8, S9).

3.4. Hierarchical clustering of differentially expressed protein sequences

Fig. 3 directly displayed that the differentially expressed proteins were almost regulated by alanine, asparagine, glycine, isoleucine, lysine, leucine and valine with high content in extreme conditions. Meanwhile, they could also be regulated by tryptophan and cysteine with the lowest content. Furthermore, it has been reported that serine and threonine were used to predict the phosphorylation and glycosylation sites in a protein sequence, while lysine and arginine were the predicted methylation sites of proteins (Gupta et al., 1999; Ingrell et al., 2007; Shi et al., 2012). Analyzing all expressed protein sequences between two groups showed that the respective distribution of phosphorylation, glycosylation, and methylation sites among stable-, up- and down-regulated proteins all showed a significant difference ($p < 0.05$) (Fig. 4(A and B)). Additionally, the amino acid profiles analyzed in this study were transformed into Bray-Curtis similarity matrices and subjected to multivariate analyses. The cluster analyses shown in Fig. 5A identified five clusters. The unique clusters for tryptophan and cysteine, plus four



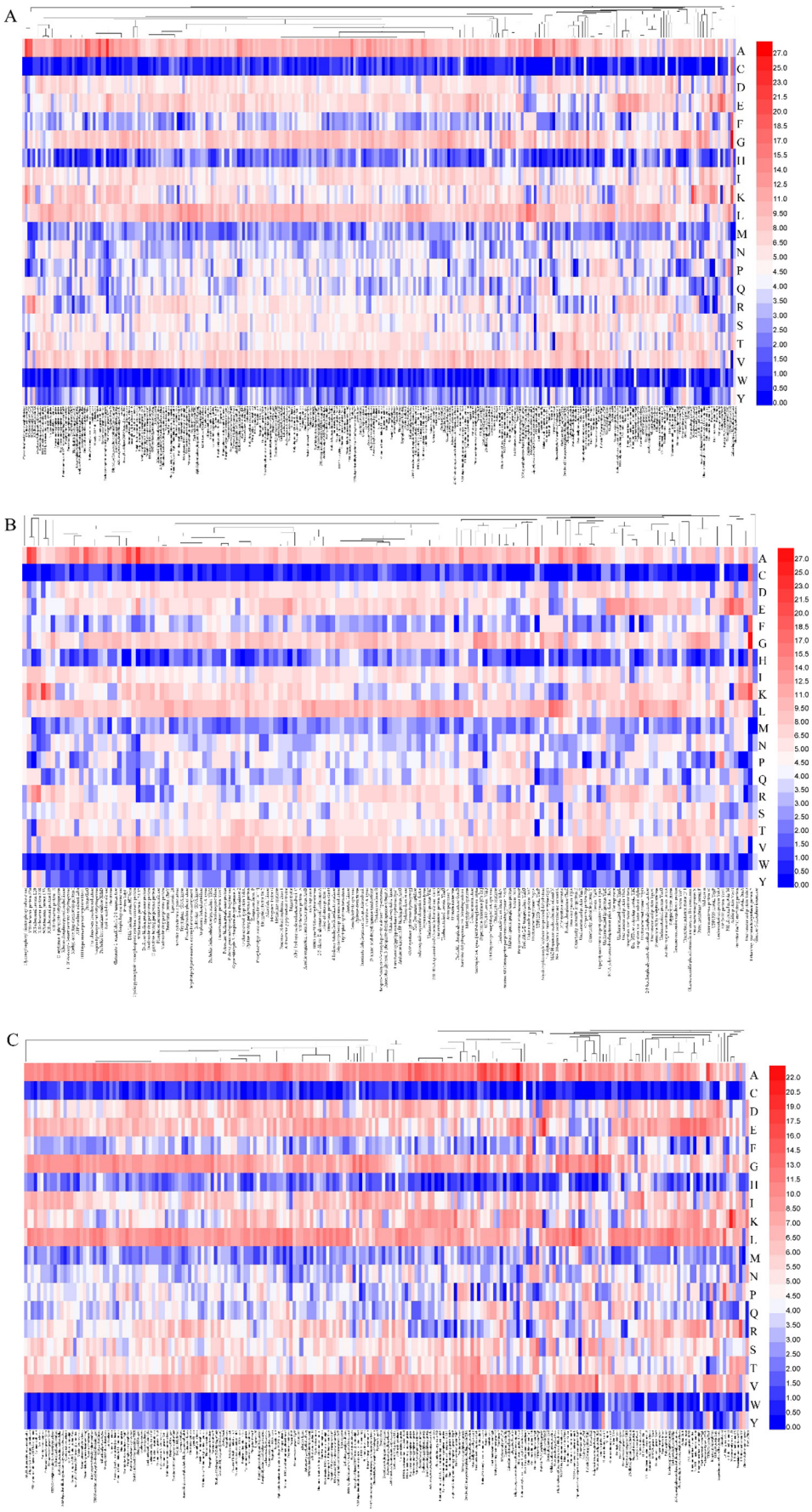
(A)



(B)

Fig. 2. The protein and protein interaction networks of all differentially expressed proteins. The protein-protein interaction networks were built using STRING 10.5 with a high confidence level (0.7) and all available prediction methods (A). The whole metabolic pathways involved in all expressed proteins under alkaline compound treatment in the tetracycline environment (B).

separate amino acid clusters could be distinguished. Multidimensional scaling (Fig. 5B) confirms this clustering. However, the acid-regulated amino acid profiles with seven clusters showed a different cluster ranking selection (Fig. S10A), which was confirmed by multidimensional scaling (Fig. S10B). To resist the acid stress, cells modified the component of amino acid sequence to keep normal metabolism, such as cluster tyrosine and methionine and cluster glutamic acid and arginine. Simultaneously, the alkaline-regulated amino acid profiles showed the same


A
B
C

cluster ranking selection as that of all differentially expressed proteins (Fig. S11(A and B)).

3.5. Phylogenetic evolution of functional proteins TolC and phosphoenolpyruvate carboxykinase (Pck)

For protein evolution, a phylogeny of bacterial groups that encoded outer membrane protein TolC, which was largely distributed among the bacterial species, was analyzed based on their amino acid sequences (Fig. 6). The result also revealed that the evolution distance among various species was very close. These species, including *E. coli*, *Salmonella typhimurium*, *Shigella flexneri*, *and Klebsiella aerogenes*, were screened for homology analysis based on the similarity of amino acid sequences (Fig. 7) to confirm this inference. The identification scores for all target species exceeded 90%, which validated the high homologous sequence of the protein. The functional domains of these target species were quite conserved during their evolutionary process. Thus, TolC might be a biomarker in different bacterial species to reflect the tetracycline stress under extreme conditions. This finding clarified that tetracycline could be a widespread antibiotic (Landers et al., 2012) and provide ribosomal protection (Connell et al., 2003) against various bacterial species.

Pck in *E. coli* and *Homo sapiens* sharing high sequence homology illustrated that this protein could be a wide spectrum biomarker in different species to resist tetracycline stress. To testify this inference, the phylogenetic relationship of Pck in prokaryotic and eukaryotic organisms was analyzed based on the amino acid sequences (Fig. S12). The results revealed the homologous correlation among various species, indicating the impact degree of tetracycline on humans and different target species. These species, including *E. coli*, *Zea mays*, and *Saccharomyces cerevisiae*, were screened for homology analysis based on the similarity of amino acid sequences (Fig. S13). The identification scores for all target species exceeded 40%, which validated the high homologous sequence of the protein Pck.

4. Discussion

Several proteins involved in energy production and conversion were significantly up-expressed in the tetracycline environment at alkaline conditions (Table S6, Fig. 2(A and B)). Compared to those of the acid condition, the up-regulated NADH-quinone oxidoreductase subunit L and subunit N, which is one of the three proton-translocating enzymes of the electron transport chain, generating proton motive force (Hirst and Roessler, 2016), jointly catalyzed ubiquinone and nicotinamide adenine dinucleotide (NADH) to form the ubiquinol and NAD^+. In the meantime, the expression of adenosine triphosphate (ATP) synthase subunit c and alpha increased 1.66-fold and 1.31-fold in the alkaline group, which promoted the oxidative phosphorylation pathway and synthesized more ATP to support the energy requirement in the tetracycline environment, whereas, only ATP synthase subunit c was up-expressed by 2.07-fold in the acid group. Overall, the overexpression of all these proteins could produce highly efficient electron transfer, increase the energy supply to overcome the tetracycline stress and ensure cellular metabolism.

For carbohydrate transport and metabolism, 37 related enzymes were found to be differentially expressed in the tetracycline environment (Table S6, Fig. 2(A and B)). Seven of these enzymes catalyzed starch and sucrose metabolism, and five proteins in the pentose phosphate pathway were all upregulated in the tetracycline environment in the acid group, while there were also seven proteins involved in the glycolysis/gluconeogenesis pathway which was upregulated in the alkaline group. The slight up-expression of the PTS system glucose-specific EIIA component (Crr) produced important precursor metabolite

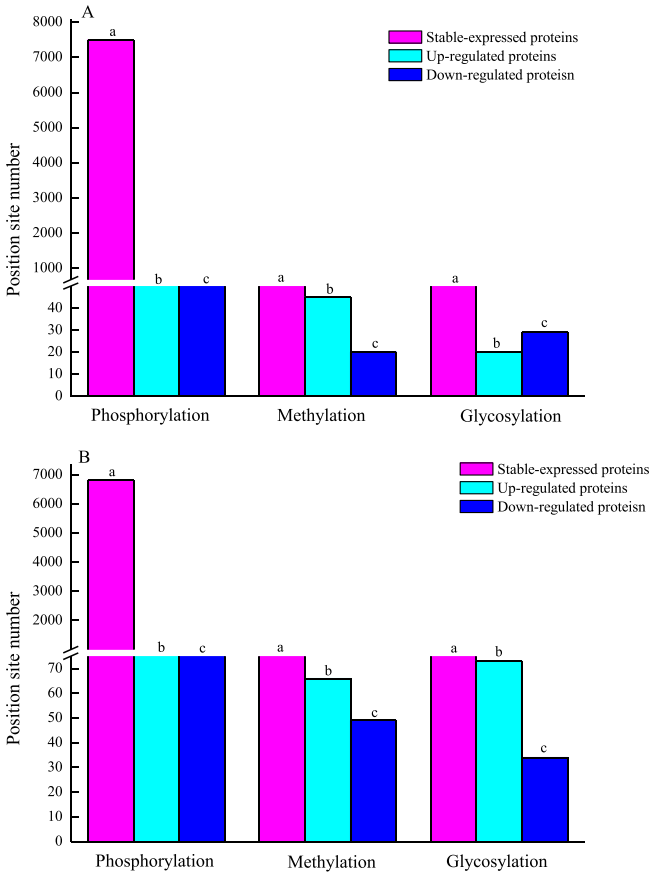


Fig. 4. The distribution of all expressed protein modification. The distribution of the phosphorylation, methylation, and glycosylation position sites among the stable-expressed, upregulated and downregulated proteins in acid treatment in the tetracycline environment (A), and in alkaline treatment in the tetracycline environment (B).

alpha-D-glucose 6-phosphate by catalyzing the extracellular alpha-D-glucose to accelerate the starch and sucrose metabolism. The up-expression of glucose-6-phosphate isomerase (Pgi) and ribose-phosphate pyrophosphokinase (Prs) are the key enzymes in the pentose phosphate pathway in the acid group. Especially, the most significant step is that the down-expression of Prs slowed down the transformation of D-ribose to phosphoribosyl pyrophosphate which is an activated compound used in the biosynthesis of histidine and purine/pyrimidine nucleotides. Under alkaline condition, the two pathways may be weakened. Additionally, up-expressed dihydrolipoamide acetyltransferase (AceF) was also a significant enzyme in the glycolysis/gluconeogenesis pathway in the alkaline group. It catalyzed pyruvate to form acetyl-CoA, another important precursor metabolite connected to glycolysis, amino acid metabolism, fatty acid metabolism, and the citrate cycle, by oxidative decarboxylation of pyruvate. The up-regulation of AceF in qPCR validation was consistent with the proteomic results, which confirmed that the proteomic approach used in the current study is reliable (Table 1). This finding provided strong evidence that tetracycline promotes the process of glycolysis/gluconeogenesis metabolism in *E. coli* under alkaline conditions.

It was noteworthy that *E. coli* in either the alkaline- or acid-regulated group was less susceptible to the tetracycline environment compared to the neutral condition. The possible reason was that over-expression of outer membrane proteins (TolC, OmpF, OmpW, OmpA, OmpX) had

Fig. 3. Hierarchical clustering of amino acid components of each differentially expressed protein. (A) Amino acid components of all differentially expressed proteins from two different treatments; (B) amino acid component of differentially expressed proteins from the acid treatment group; (C) amino acid component of differentially expressed proteins from the alkaline treatment group.

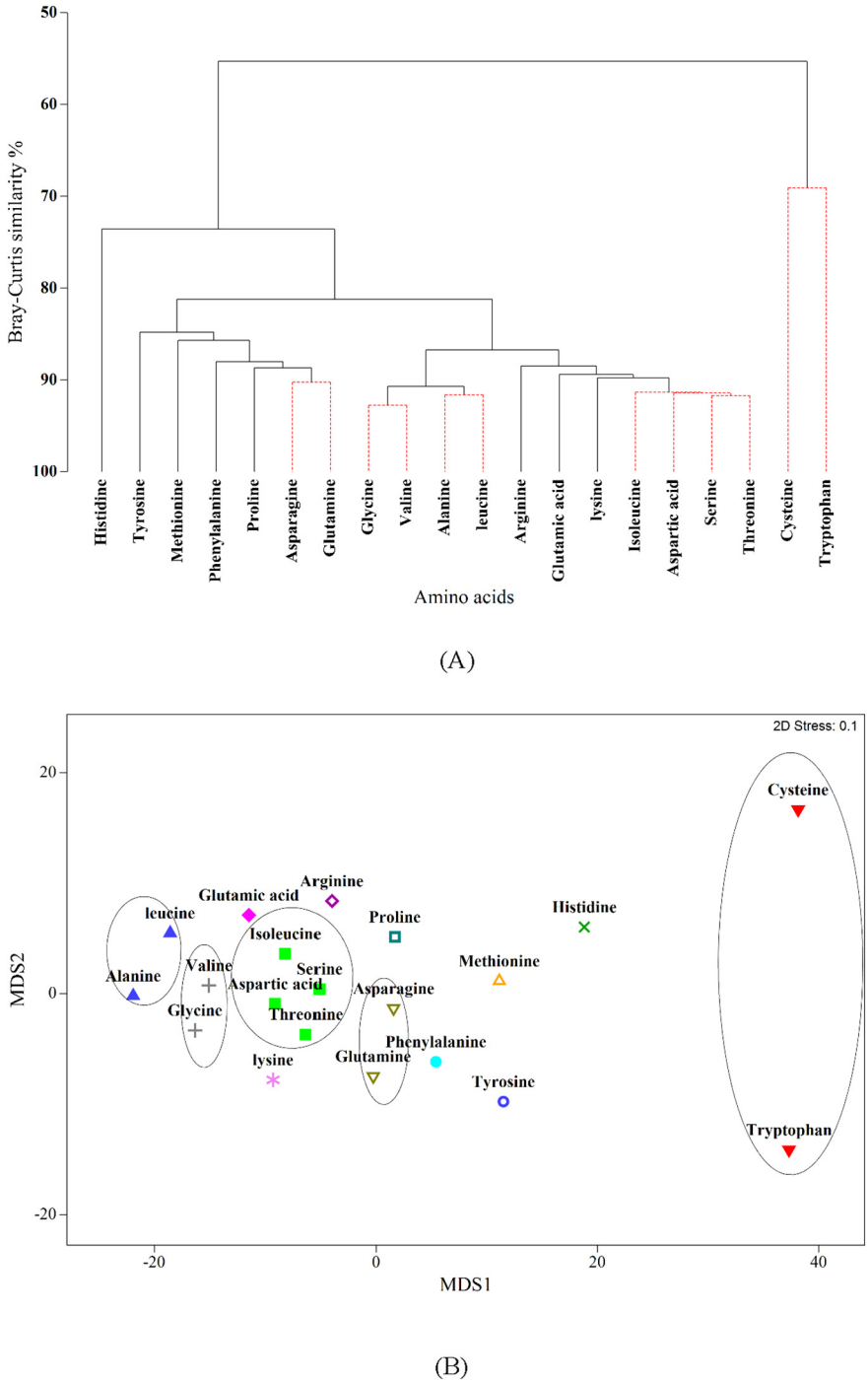


Fig. 5. Multivariate statistical analyses of all differentially expressed proteins. The input of the cluster analyses were Bray-Curtis similarity matrices of square-root-transformed amino acid sequences (% of every single amino acid content). The clustering was made using the group average linking cluster analyses (A), and the two-dimensional arrangement of datapoints represents relative differences in similarity and does not display the absolute differences (B).

shown a critical role in tetracycline resistance response. Specially, TolC plays an active part in the efflux of a wide range of substances, including antibacterial drugs, detergents, organic solvents, and hemolysis (Gilardi et al., 2017; Sulavik et al., 2001). This protein acts with AcrAB to form an efflux pump and allows direct passage of proteins and drugs from the cytosol out of the cell (Bergmiller et al., 2017), the up-expression of TolC (1.79- and 1.74-fold in acid and alkaline groups, respectively) increases the ability of efflux pumps to export tetracycline from cells. The upregulation of TolC in qPCR validation was also consistent with the proteomic results (Table 1). The large channel porin OmpF, along with another small channel porin OmpC was transcriptionally regulated by the EnvZ/OmpR two-component signal transduction regulatory system (Cai and Inouye, 2002). OmpF is always highly expressed in neutral conditions, while OmpC is upregulated in hostile circumstances, including a medium containing toxic agents, antibiotics, or detergents, in order to decrease membrane permeability (Villarreal et al., 2014). Thus, up-expression of OmpF (1.29- and 1.10-fold in acid and alkaline groups, respectively) resulted in reduced outer membrane permeability, thus blocking tetracycline entry into cells. *E. coli* was found to be less susceptible to the tetracycline environment in the acid-regulated group than that in the alkaline-regulated group.

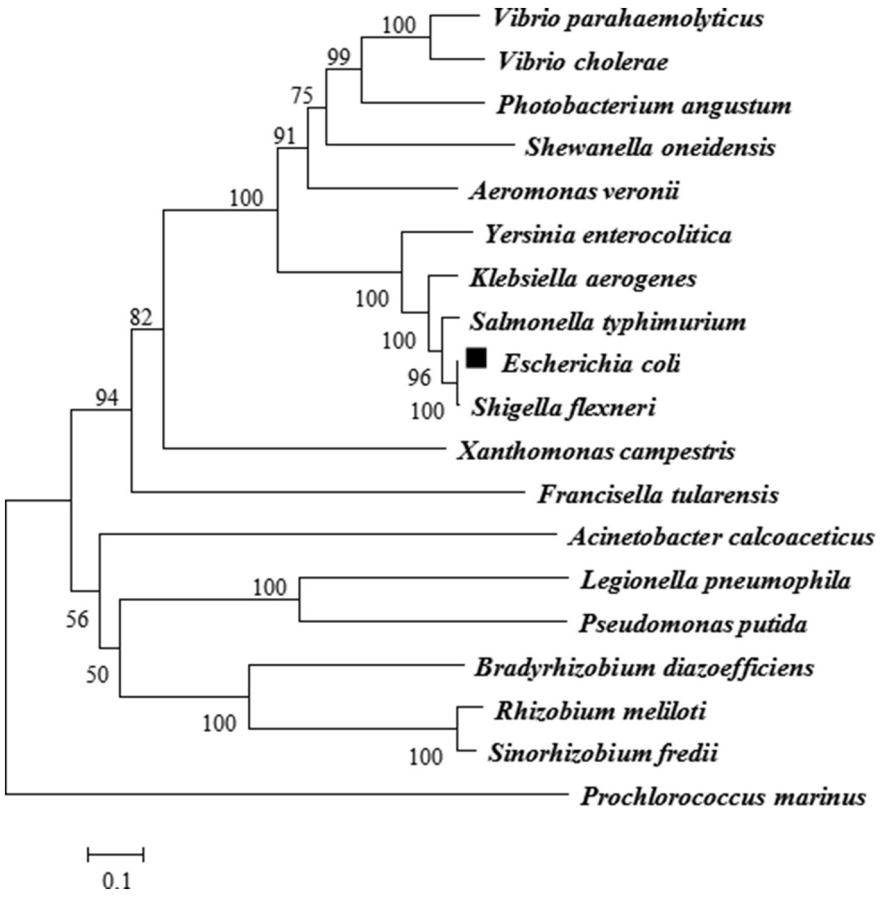


Fig. 6. Phylogenetic relationships of different species contained out membrane protein TolC. It was conducted by using the MEGA7.0 software according to the amino acid sequences of TolC from various bacteria. The numerical values represent the evolutionary distance of various species.

Tetracycline brought potential stress to bacterial growth regarding stress response, chemotaxis, and motility. According to our data, 21 proteins involved in this pathway were found to be significantly differentially expressed in the alkaline-regulation group, and most of them were responsible for the protection of protein activities (Table S6) and metabolism in acid or alkaline conditions. Lipopolysaccharide (LPS) export system protein LptA is a soluble periplasmic component of the LPS transport system of Gram-negative bacteria that transports newly synthesized LPS from the cytoplasm to the outer leaflet of the outer membrane (OM). It could bridge the periplasm to form a large, connected protein complex (Freinkman et al., 2012; Okuda et al., 2012) to bind to the LPS, which was responsible for permeability of the OM (Sperandeo et al., 2008; Werneburg et al., 2012). The up-expression of LptA promoted the synthesis of OM to support a protection for cells in this condition and decrease the toxic effect caused by tetracycline stress in the acid-regulation group. However, the downregulated LptA slowed down the synthesis of OM to force the cells to be vulnerable and susceptible to the alkaline-regulated tetracycline environment.

Another damaging effect of environmental stress to cells was the generation of a hyperosmotic and oxidative cellular microenvironment, which resulted in the aggregation of a large number of denatured proteins. The heat-shock protein DegP was a protein quality control factor in the bacterial envelope that was involved in eliminating misfolded or damaged proteins. It combined digestive and remodeling activities on a single polypeptide and could switch between these dual functions in a tightly regulated manner (Iwanczyk et al., 2007; Meltzer et al., 2008). DegP was also a member of the widely conserved HtrA family of serine proteases that were crucial to maintain protein homeostasis in cytoplasmic compartments (Clausen et al., 2002). The overexpression of DegP in the alkaline group (1.33-fold) was more active to clear out the misfolded and damaged proteins than in the acid group (1.26) in tetracycline environments. In addition, the LPS export system permease LptG, methyl-accepting chemotaxis protein I Tsr, and chemotaxis protein CheZ playing roles in cell motility were all found to be downregulated in tetracycline environments in both the acid group and the alkaline group. When under starvation or stress environments, bacterial cells will take advantage of chemotaxis and motility systems to avoid the hostile environment and sustain their growth.

Related to lipid transport and metabolism, there were eight enzymes involved in this pathway (Table S6). Acetyl-CoA was produced through oxidative decarboxylation of pyruvate catalyzed by up-expression of dihydrolipoamide acetyltransferase (AceF). It was formed into malonyl-CoA by the up-regulation of acetyl-CoA carboxylase (AccB). Acetyl-CoA carboxylase was the point of regulation in saturated straight-chain fatty acid synthesis, and was subject to both phosphorylation and allosteric regulation. The down-expression of [acyl-carrier-protein] s-malonyltransferase (FabD) slowed down the catalysis of malonyl-CoA into malonyl-[acyl-carrier protein], which joined in the formation of hexadecanoic acid. The downregulated FabB was a β-ketoacyl-ACP synthase that elongated and channeled intermediates into the mainstream fatty acid synthesis pathway. When FabB reacted with the cis-decenoyl intermediate, the final product after elongation was an unsaturated fatty acid (Feng and Cronan, 2011). The downregulated FabA was specific for the synthesis of 10-carbon saturated fatty acid intermediate (β-hydroxydecanoyl-ACP). Furthermore, FabA catalyzed the dehydration of β-hydroxydecanoyl-ACP, creating the trans-2-decenoyl intermediate. Either the trans-2-decenoyl intermediate can be shunted to the normal saturated fatty acid synthesis pathway by FabB, where the double bond will be hydrolyzed and the final product will be a saturated fatty acid, or FabA will catalyze the isomerization

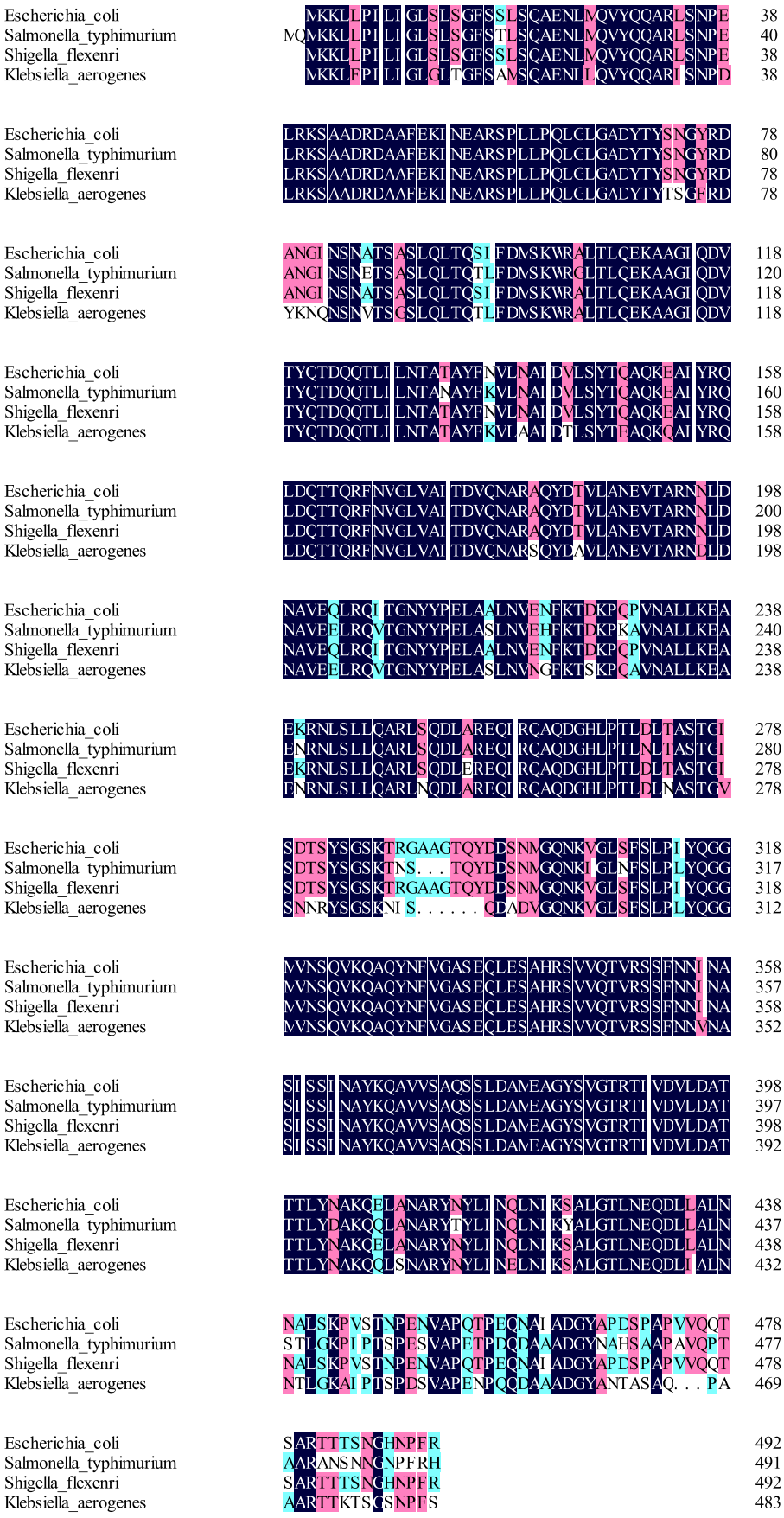


Fig. 7. The amino acid sequences of TolC from *E. coli*, *Salmonella typhimurium*, *Shigella flexneri*, and *Klebsiella aerogenes*. Black indicates the consistency of the same sites between amino acid sequences. Pink indicates that the similarity between the same sites in the amino acid sequence is high, and the cyan indicates that the similarity between the same sites of the amino acid sequence is low. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

into the cis-3-decenoyl intermediate. Most bacteria that undergo anaerobic desaturation contain homologues of FabA and FabB (Wang and Cronan, 2004). According to Fig. 1A, only straight-chain palmitic acid showed significant differences under three different pH conditions. Specifically, *E. coli* in the acid group generated more palmitic acid than that in the alkaline group. The ratio of unsaturated fatty acid still remained stable in the three different treatments (Fig. 1B), while the content of the specific palmitoleic acid showed significant differences among three different pH treatments ($p < 0.05$) (Fig. 1C). These may be related to the regulation of uncharacterized lipoprotein YdcL (1.41-fold and 2.11-fold at acid and alkaline groups, respectively), outer membrane lipoprotein SlyB (1.44-fold and 0.77-fold), uncharacterized lipoprotein YgdI (1.25-fold and 1.30-fold), osmotically-inducible lipoprotein OsmE (0.55-fold and 0.93-fold), and lipoprotein NlpA (0.66-fold and 0.56-fold). In the alkaline group, these upregulated lipoproteins may have stronger force to transport more fatty acids out of the cell, resulting in a decrease of fatty acids on cellular membranes, which was consistent with our findings (Fig. 1(A–C)).

Thirty-three proteins were involved in amino acid transport and metabolism. Through the catalysis of citrate synthase (PrpC), succinyl-coA ligase [ADP-forming] subunit alpha and beta (SucD and SucC) and aspartate ammonia-lyase (AspA), some organic acids such as 2-oxoglutarate and oxaloacetate were derived from acetyl-CoA, providing precursors of alanine, aspartate, glutamate, valine, isoleucine, and other kinds of amino acids for protein synthesis. In the glycine, serine, and threonine metabolism process, several groups of enzymes, phosphoglycerate mutase (GpmA and GpmB), dehydrogenase (GcvP and ThrA), dehydratase (DsdA), aminomethyltransferase (GcvT), hydroxymethyltransferase (GlyA), and aminotransferase (SerC), were responsible for the conversion of substrates to products via dehydration and dehydrogenation. In cysteine and methionine metabolism, three groups of enzymes, including cysteine and methionine synthase (CysK, CysM, and MetH), serine acetyltransferase (CysE) methionine adenosyltransferase (MetK), malate, and homoserine dehydrogenase (Mdh and ThrA) were involved in regulating the conversion between substrates and products. Among the generated amino acids in the current study, aspartate and glutamate were combined with carbon and nitrogen atoms from other intermediates to form the purines.

Associated with transcription, translation, and nucleotide metabolism, 11 ribosomal proteins and other proteins showed significant changes (Fig. S7). The enhanced expressed mRNA carried genetic information from DNA to the small ribosomal subunits RpsA, RpsF, RpsR, RpsS, and RpsT, transferring the information to the large subunits RplB, RplE, RplL, RplR, RplT, and RpmG, which were responsible for linking amino acids delivered by tRNA to form proteins. *E. coli* in the acid or alkaline groups responding to tetracycline modified the expression levels of different ribosomal proteins, which could help cells adapt to the tetracycline environment. Additionally, proteins related to fatty acid metabolism, amino acid metabolism, cell division, and cell envelope biosynthesis worked with the transcription and translation process and assisted cellular adaption in tetracycline environments.

5. Conclusion

The specific phospholipid C16:1ω9c showed a significant decrease between the treatment group and control group. The 77 and 111 upregulated proteins in the acid and alkaline groups, respectively, were mainly involved in biosynthesis of secondary metabolites, glycolysis/gluconeogenesis, biosynthesis of amino acids, biosynthesis of antibiotics, carbon metabolism. Whereas, the 79 downregulated proteins in acid conditions were mainly involved in ribosome and bacterial chemotaxis, the 111 down-regulated proteins in alkaline conditions were primarily involved in metabolic pathways, carbon metabolism, glyoxylate, and dicarboxylate metabolism, fatty acid biosynthesis, biosynthesis of secondary metabolites, biosynthesis of antibiotics, and glycine, serine, and threonine metabolism. The amino acid components of all differentially expressed proteins from the two treatments were almost regulated by alanine, asparagine, glycine, isoleucine, lysine, leucine, and valine with high content, and by tryptophan and cysteine with the lowest content in extreme conditions. Furthermore, the respective distribution of phosphorylation, glycosylation, and methylation sites among stable-expressed, upregulated, and downregulated proteins all showed a significant difference ($p < 0.05$). Besides, TolC and Pck could be biomarkers to reflect tetracycline stress under extreme conditions with high sequence homology in *Homo sapiens* implying the potential impact of tetracycline on humans at the network level. Generally, *E. coli* in the acid group accelerated the highly efficient protection mechanism to defend against tetracycline assault, while *E. coli* in the alkaline group strongly impaired the protection mechanism and was more susceptible to the tetracycline environment than in the acid group. This work can give an insight into the antibiotic resistance mechanism and perfect the antibiotic usage in different environments.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.01.342.

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