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ORIGINAL ARTICLE

Comparative Proteomic Analysis of *Escherichia coli* Under Ofloxacin Stress

Ofloksasin Stresi Altında *Escherichia coli*'nin Karşılaştırmalı Proteomik Analizi

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ABSTRACT

Objectives: In the present study, proteomics was utilized to evaluate changes in *Escherichia coli* proteins in response to ofloxacin to understand the mechanism of action of ofloxacin and the mechanisms of ofloxacin resistance in *E. coli*.

Materials and Methods: Proteomics analysis of *E. coli* was performed by using liquid chromatography quadrupole time-of-flight mass spectrometry followed by a data processing step using MaxQuant. Functional classification and pathway analysis showed a systematic effect of ofloxacin over *E. coli* proteome structure.

Results: In total, 649 common proteins were identified in the untreated and ofloxacin-treated groups, while 98 proteins were significantly different in the ofloxacin-treated group. Functional classification and pathway analysis showed that ofloxacin has a systematic effect over ribosomal processes, energy pathways (tricarboxylic acid cycle and glycolysis), membrane proteins, microbial targets, and biofilm formation.

Conclusion: The results showed that ofloxacin affected many cellular processes and pathways. In addition, proteomic analysis revealed that *E. coli* develops resistance mechanism with different biological processes.

Key words: Proteomics, LC/MS, antibiotic resistance, ofloxacin, *E. coli*

ÖZ

Amaç: Bu çalışmada ofloksasinin etki mekanizmasına karşı yanıt olarak *Escherichia coli* proteinlerinde görülen değikliklerin belirlenmesi ve *E. coli*'deki oflotoksin direnç mekanizmalarının değerlendirilmesi için proteomiks kullanılmıştır.

Gereç ve Yöntemler: *E. coli*'nin proteomik analizi sıvı kromatografisi/kuadrupol uçuş zamanlı kütle spektroskopisi kullanarak gerçekleştirilmiş ve sonrasında veri prosesleme aşaması MaxQuant kullanarak yapılmıştır. İşlevsel sınıflandırma ve yolak analizi oflotoksinin *E. coli*'nin proteome yapısına sistematik bir etkisinin olduğunu göstermiştir.

Bulgular: Çalışmada 649 tane sıklıkla rastlanan proteinin yapısı oflotoksin ile muamele edilen ve edilmeyen gruplarda belirlenirken, 98 proteinin oflotoksin ile muamele gören grupta belirgin derecede farklı olduğunu göstermiştir. İşlevsel sınıflandırma ve yolak analizi oflotoksinin ribosomal prosesler, enerji yolakları (trikarboksilik asit siklusu ve glikoliz), membran proteinleri, mikrobiyal hedefler ve biofilm oluşumu üzerine sistematik bir etkisinin olduğunu göstermiştir.

Sonuç: Bu sonuçlar ofloksasinin birçok hücresel prosesi ve yolağı etkilediğini göstermiştir. Ayrıca, proteomik analizler *E. coli*'nin farklı biyolojik prosesler ile direnç mekanizması geliştirdiğini göstermiştir.

Anahtar kelimeler: Proteomik, LC/MS, antibiyotik direnci, ofloksasin, *E. coli*

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INTRODUCTION

Antibiotic resistance is one of the most pressing health issues worldwide. Improper use of antibiotics has led to the emergence of resistant microorganisms.[1,2] A more detailed understanding of antibiotic-bacteria interactions is key to increasing the efficiency of antibiotic use as well as developing novel combination treatment strategies. In recent years, omics-based technologies have emerged in bacterial antibiotic resistance.[3,4] Mass spectrometry (MS)-based proteomics is a powerful method for the comprehensive characterization of proteome structure and to understand the cellular process at the protein level within an organism. In microbiology, proteomics is a promising approach to understanding the effect of antibiotics on bacteria and resistance mechanisms against antibiotics.[5-7]

Ofloxacin is one of the second-generation fluorinated quinolones with broad-spectrum antibacterial activity against most Gram-negative and, Gram-positive bacteria, including anaerobes.[8,9] Ofloxacin inhibits the activity of DNA gyrase and topoisomerase IV in DNA replication. Alterations in the target enzymes (DNA gyrase and topoisomerase IV) by chromosomal mutations is the main mechanism of resistance.[9] However, the contribution of proteins to resistance still requires explanation for a deeper understanding of bacteria-fluoroquinolone interactions.

The present study aimed to evaluate the effects of ofloxacin on *Escherichia coli* at the protein level. Apart from its broad-spectrum activity on clinically important infections, ofloxacin is also renowned for its wide application in livestock production and its high frequency in environmental sources such as surface water.[10,11] Livestock and environmental sources are emerging due to its high capacity for horizontal gene transfer of antimicrobial resistance genes. Thus, the present study is also important for elucidating the potential resistance mechanisms of bacteria against ofloxacin.

MATERIALS AND METHODS

Bacterial culture and minimum inhibitory concentration (MIC) experiments

E. coli ATCC 25922 was cultured on tryptic soy agar (TSA). In order to confirm the MIC value of ofloxacin against *E. coli*, standard broth microdilution was performed according to the method reported by the Clinical Laboratory Standards Institute.[12] The MIC value of ofloxacin was determined and compared with the latest breakpoint tables of European Committee on Antimicrobial Susceptibility Testing (EUCAST).[13] For proteomics experiments, the 0.5x MIC value for ofloxacin was used (0.01 mg/L). *E. coli* ATCC 25922 was cultured on Mueller Hinton Broth (MHB) and incubated at 37°C until the log phase was achieved. The bacterial suspension was prepared using MHB containing 0.01 mg/L ofloxacin to obtain a bacterial concentration of $5x10^5$ cfu/mL bacteria. For the control group, the same concentration of the bacterial culture was also prepared without adding any antibiotics. Flasks were incubated at 37°C for 20 h for proteomics experiments. A bacterial growth curve was constructed for 48 hours by plotting time against the log 10 cfu/mL values. Colony counting for each hour was performed by serial dilution and inoculation of the bacterial suspension into TSA.

Sample preparation for proteomics

After the incubation period, the cultures were centrifuged, and washed with phosphate buffered saline in triplicate. The bacterial cells were disrupted using lysis buffer containing lysozyme. Protein concentrations of ofloxacin in the treated and control groups were calculated by using the Bio-Rad DC assay. The chloroform (Sigma)/methanol (Sigma)/water co-solvent system was used for protein extraction.

Extracted proteins were suspended in 100 mM ammonium bicarbonate solution (Sigma). As a reducing agent, 200 mM dithiothreitol (DTT) (Sigma) was used. Proteins were incubated with DTT at 56°C for 20 min. After reduction, proteins were incubated with 100 mM iodoacetamide (Sigma) at room temperature. Proteins were digested with trypsin (1:100 (w/w) at 37°C for 16 hours. After desalting, tryptic peptides were dissolved in acetonitrile that contained 0.1% formic acid.

Liquid chromatography/quadrupole time-of-flight/MS (LC/QTOF) analysis

Peptides were analyzed in a LC/QTOF/MS platform. They were separated in C18 columns [Zorbax C18 column 150x0.05 mm, 5 column 1 Å (Agilent)] at 55°C performed with an Agilent 1290 high performance LC. A total volume of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) was used as the mobile phase, and the flow rate was adjusted to 0.070 mL/min. Peptides were eluted with a gradient of 1%-55% mobile phase B over 80 minutes, followed by 55%-85% mobile phase B over 5 minutes. The total gradient run time was 90 minutes. Post run was 25 minutes.

Peptides were analyzed in an Agilent 6530 QTOF/MS system in positive-ion mode of the electrospray ionization source. The capillary voltage was adjusted at 4000 V with a drying temperature of 350°C. Automated MS/MS data of peptides were recorded between 300 m/z and 1400 m/z above the 1500-count threshold.

The six most intense ions were selected for MS/MS analysis. The ion charge states were +2, +3, and +4. The fragmentation energy was adjusted to 45 V. Twenty micrograms of protein was loaded onto the LC/MS system for each run. Four technical replicates were analyzed for each of ofloxacin-treated and control groups.

Protein identification and quantification

The recorded MS/MS data were processed using Maxquant (Max Planck Institute, Germany, https://www.maxquant.org). The *E. coli* database was downloaded from UniprotKB. Peptides and proteins were identified by matching the recorded MS/MS data with *in silico* MS/MS data. In the matching process, 20 ppm mass tolerance was used for the first and main search. Carboamidomethylation on cysteine and oxidation on methionine were chosen as fixed and variable modifications, respectively. Two missed cleavages were allowed. In the identification process, the false discovery rate (FDR) value was selected as 0.01 for reliable identification.

Statistical analysis for comparative proteomics

The label-free quantification algorithm in Maxquant multilevel feedback queue (MLFQ) was used for semi-quantification between ofloxacin-treated and control groups.

Perseus software (https://www.biochem.mpg.de/5111810/perseus) was used for the statistical analysis. Principal component analysis (PCA) showed the overall effects of ofloxacin on *E. coli*. A two-sample t-test ($p<0.05$) was performed with 250 randomizations and a FDR of 1%, to identify the differentially expressed proteins between the control and ofloxacin-treated groups.

Gene list, protein-protein interaction, and pathway analysis

Panther, the gene list software (http://pantherdb.org), was used to classify differentially expressed proteins according to cellular component, molecular function, and biological process. We also investigated the relationships of downregulated and upregulated proteins in a protein map by using the String functional tool (https://string-db.org). In addition, differentially expressed proteins were evaluated in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (https://www.genome.jp/kegg/mapper.html) to observe the ofloxacin-induced metabolic pathways.

RESULTS

LC/QTOF/MS proteomics assay

The determined MIC value for *E. coli* ATCC 25922 was 0.02 mg/L, which was in accordance with the EUCAST breakpoints. Thus, the 0.5x MIC value of 0.01 mg/L was used for proteomics experiments. The growth curve of *E. coli* ATCC 25922 after treatment with 0.01 mg/L of ofloxacin was constructed over 48 hours. The colony counts for treated bacterial cells were significantly different from those of the control at 6, 20, and 48 hours (Figure 1).

Proteins were extracted and digested from the control and ofloxacin-treated groups and analyzed in four technical replicates by using the LC/QTOF/MS system. Chromatograms are shown in Figure 2. Proteins were identified with a conservative threshold (FDR ≤0.01 and matched peptide numbers ≥2). In total, 649 common proteins were identified in the control and ofloxacin-treated groups. In the present study, we used micro-LC-based proteomics analysis. Micro LC-based proteomics is less sensitive but more robust and reproducible than nano LC-based proteomics. Thus, we identified fewer proteins than we would have using nano LC based proteomics.

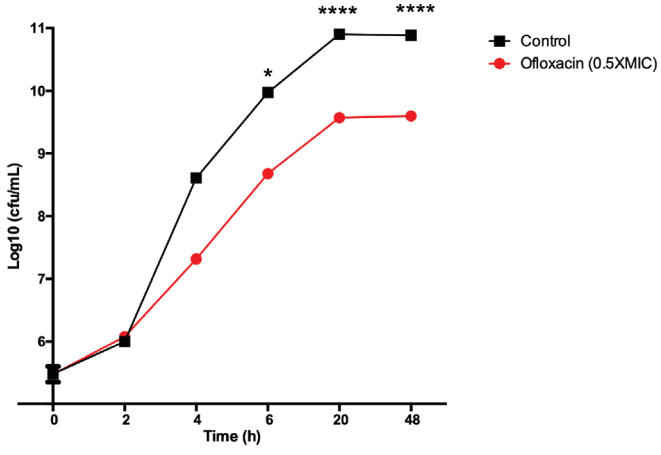

Figure 1. Growth curve of *Escherichia coli* ATCC 25922 with or without 0.02 mg/L ofloxacin treatment (0.5x MIC). Sampling for proteomic experiments was conducted at the 20th hour. Two-Way ANOVA was performed for multiple comparisons

*: $p<0.05$, ****: $p<0.0001$, MIC: Minimum inhibitory concentration

The label-free protein quantification algorithm of Maxquant MLFQ was used for semi-quantitative proteomics. The MLFQ intensities of proteins were evaluated statistically. First, treated and untreated groups were analyzed individually through a multi-scatter plot analysis to observe the reproducibility of technical replicates. The Pearson's correlation coefficients were between 0.885 and 0.968 for the untreated group. For the treated group, the correlation coefficients ranged from 0.871 to 0.946. These results indicated highly reproducible findings between technical replicates. PCA was used to observe the global effect of ofloxacin on the *E. coli* proteome. In addition, PCA analysis was used to observe the similarity between the technical replicates of ofloxacin-treated and control groups (Figure 3). PCA results showed that ofloxacin significantly impacts proteome structure in *E. coli* (PC1 50.8%, PC2 19.2%).

The MLFQ intensities of proteins were evaluated using the statistical software Perseus. The two-sample t-test showed alterations in the expression levels of 98 proteins. A total of 30 proteins showed increased and 68 proteins showed decreased expression (altered proteins and fold changes are given in the supplementary material).

Gene ontology (GO) analysis of differentially expressed proteins

Differentially expressed proteins were classified into GO categories. Proteins were first classified according to their location inside cells. Differentially expressed proteins were mostly located in the cytosol (34.88%), plasma membrane (23.26%), and ribosome (23.26%). Moreover, some of them were categorized as integral membrane (9.30%), mitochondrial (4.65%), adenosine triphosphate (ATP) synthase complex (2.33%), and nucleus-related proteins (2.33%) (Figure 4A).

The proteins were also evaluated to understand the effect of ofloxacin stress on biological processes. Proteins were mostly

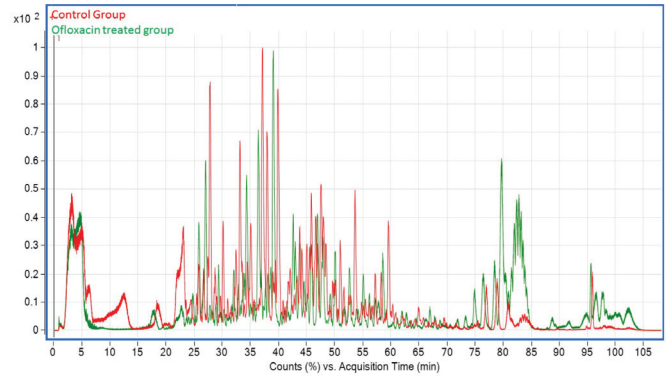

Figure 2. Chromatograms of control and ofloxacin-treated groups

involved in metabolic processes (47.4%), cellular processes (36.6%), biogenesis (7.3%), localization (6.1%), and biological regulation (2.1%) (Figure 4B). As expected, metabolic processes were mainly altered in *E. coli* under ofloxacin stress. Since primary metabolic processes are essential for cell viability, those processes were analyzed in detail (Figure 4C).

According to the molecular functions of proteins (Figure 4D), it was determined that differentially expressed proteins have catalytic (44.6%), binding (21.5%), structural molecular (18.5%), translation regulatory (3.1%), transporter (9.2%), antioxidant (1.5%), and receptor activities (1.5%).

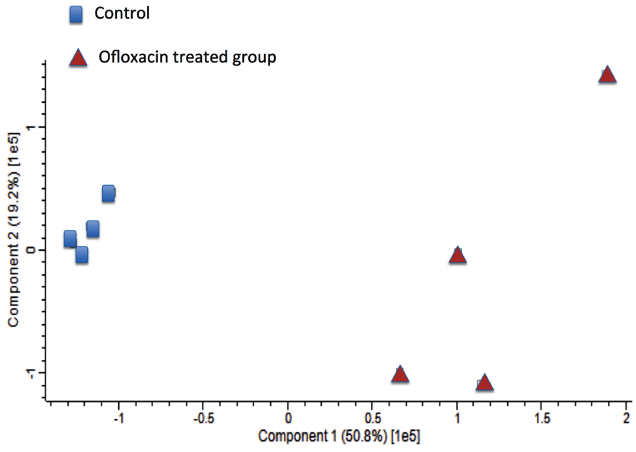

Figure 3. PCA analysis of treated and untreated groups. The blue zone shows the technical replicates of untreated groups, and the red zone shows the technical replicates of the treated group

PCA: Principal component analysis

Protein interaction analysis

The string protein interaction tool was used to observe interactions between differentially expressed proteins in a protein map. Up- and downregulated proteins were analyzed separately (Figure 5A, 5B).

KEGG pathway analysis

Next, we evaluated increasing and decreasing proteins together in the KEGG pathway (FDR <0.05). The ofloxacin-induced metabolic pathways are presented in Figure 6. KEGG pathway analysis showed that the ofloxacin treatment mainly affects cells' metabolic functions (especially energy metabolism) and ribosomes. Other affected ofloxacin pathways include the following: Biosynthesis of secondary metabolites, microbial metabolism in diverse environments, carbon metabolism, biosynthesis of amino acids, pyruvate metabolism, methane metabolism, and purine metabolism.

DISCUSSION

In the present study, changes in the proteome structure of *E. coli* were evaluated in response to ofloxacin exposure. PCA analysis showed that ofloxacin has a widespread effect on *E. coli*. Ninety-eight proteins were found to be altered in the ofloxacin-treated group.

The differentially expressed proteins were classified functionally in GO analysis to understand the systematic effect of ofloxacin on *E. coli* at the protein level. First, we classified

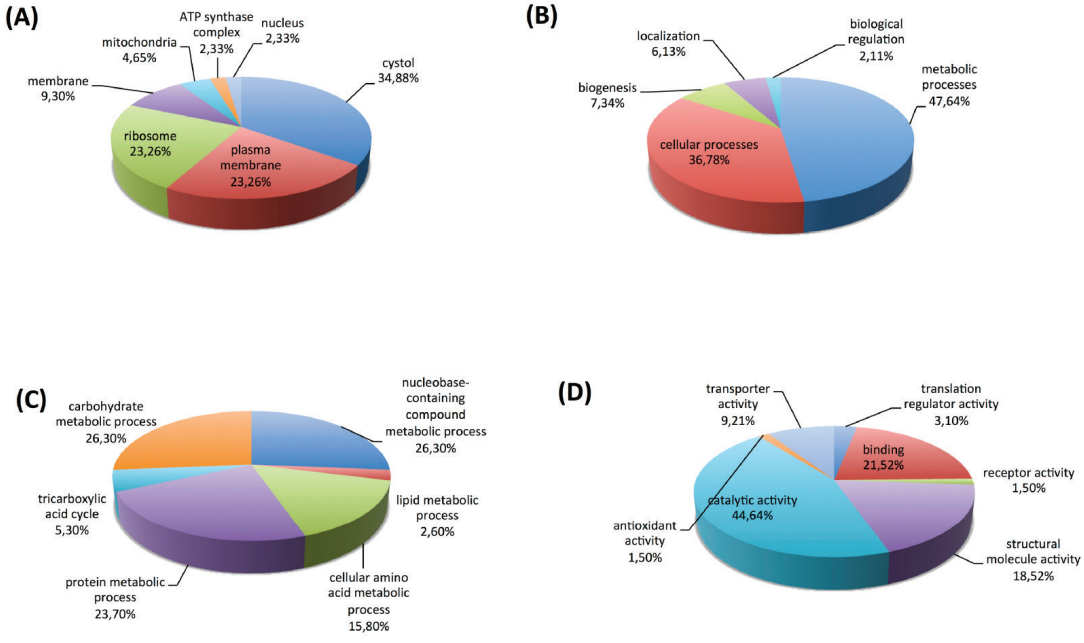

Figure 4. (A) Classification of differentially expressed proteins according to cellular component; (B) functional classification of differentially expressed proteins in cellular component; (C) ofloxacin-induced primary metabolic processes (nucleobase-containing compound metabolic process); (D) functional classification of differentially expressed proteins according to their molecular functions

ATP: Adenosine triphosphate

proteins according to their location (cellular component) in cells. We observed that many differentially expressed proteins in the ribosome were dysregulated, which indicated that ofloxacin has a veritable effect on protein and enzyme synthesis, in addition to its main mechanism of action. In cellular component annotation, another important aspect is the induction of membrane proteins by ofloxacin. Membrane proteins are important regulators involved in the various metabolic processes within cells, additionally facilitating antibiotic resistance. According to our results, five outer membrane proteins, MipA, LamB, OmpW, TorA, and RseB, were found to be downregulated in the ofloxacin-treated group. In recent years, the role of these proteins in antibiotic resistance has been studied extensively, and it has been found that they could act as both positive and negative regulators of antibiotic resistance. Lin et al.[14] showed that increased levels of ompW were related to antibiotic resistance. They also found the outer membrane protein LamB to be downregulated under antibiotic stress.[14] MipA is another extensively studied membrane protein. Li et al.[15] showed that MipA is a novel antibiotic resistance related protein in *E. coli*, conferring resistance to kanamycin. The downregulation of MipA promotes resistance mechanisms. Thus, the downregulation of LamB and MipA could be one of the key factors in the potential resistance mechanism against ofloxacin. A downregulation of outer membrane proteins could cause a decrease in antibiotic intake by bacterial cells. We also classified proteins according to their biological processes. It was observed that ofloxacin has a dramatic effect on metabolic processes. When we analyzed the metabolic processes in detail, we found that ofloxacin affects primary metabolic processes, which are key systems related to cell viability. In carbohydrate metabolic processes, most of the proteins were found to be downregulated.

We analyzed the differentially expressed proteins according to their molecular functions. It was observed that majority of the proteins in the ofloxacin-treated group were involved in catalytic and binding activities.

Nucleic-acid binding proteins modulate transcription processes in bacterial cells. These proteins directly affect cell viability and growth, and some of them are essential for drug-induced DNA repair. In our work, we observed that ofloxacin induces several nucleic-acid binding proteins. Elongation factor G (FusA) is one of the most important proteins involved in translational elongation. It mediates the translocation of mRNA and tRNA through the ribosome and is essential for protein synthesis. Several studies showed that FusA is the main target of some antimicrobial agents.[16] Another nucleic-acid binding protein, RpoB, is the key protein in drug-resistance development. Several studies have showed that mutation in RpoB is one of the major determinants of rifampin resistance.[17] Our results showed that the expression level of RpoB was decreased in the ofloxacin-treated groups. DNA starvation/stationary phase protection protein (Dps), a nucleic-acid binding protein, provides protection to cells under stress conditions. Several studies on the function of Dps in antibiotic resistance indicated that it has multiple properties that function in stress protection: DNA binding, iron sequestration, and ferroxidase activity.[18-20] Therefore, Dps has recently emerged as a potent antimicrobial target. According to our results, the expression level of Dps decreased in the ofloxacin-treated group.

We observed that the NAD-dependent DNA ligase A (LigA), which is an important element in the DNA repair process, was upregulated in the treated group. The DNA repair process is one of the most important steps in the development of antibiotic resistance. The overexpression of LigA may promote DNA repair against ofloxacin-induced stress. In recent years,

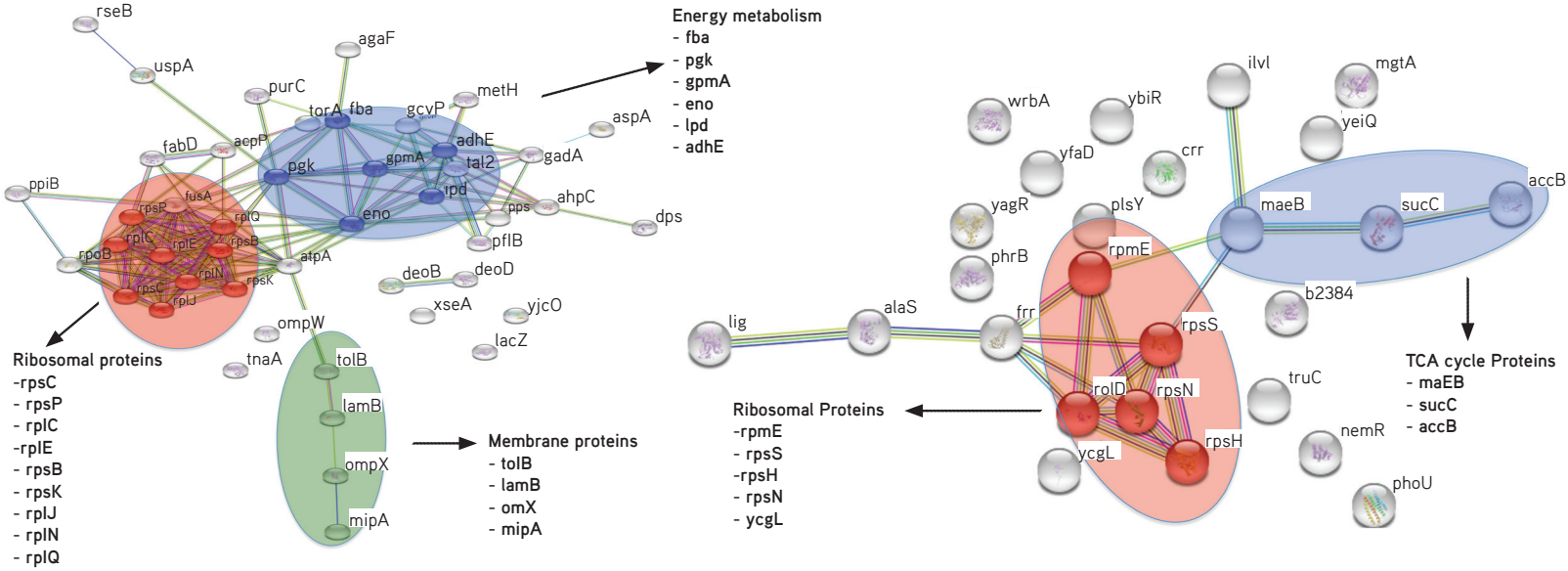

Figure 5. (A) Map of downregulated proteins. Red represents the proteins involved in ribosomal proteins, whereas the blue mark represents glycolytic metabolism. Moreover, membrane proteins are marked in green. (B) Map of upregulated proteins. Ribosomal proteins are marked red, and TCA cycle proteins are marked in the blue zone

TCA: Tricarboxylic acid

LigA has been evaluated as a potential antimicrobial target, and LigA inhibitors have shown promising results.[21,22] We believe that combination therapies including ofloxacin and LigA inhibitors can be more effective for resistant strains of *E. coli*.

We also observed that ofloxacin affects the expression levels of various heterocyclic-compound-binding proteins. Tryptophanase, (TnaA), which is responsible for the production of indole, is an important factor in biofilm formation and antibiotic resistance.[23] Previous studies have shown that the decrease in TnaA levels interrupts inter-species biofilm signaling and subsequently decreases biofilm formation.[24] In our study, TnaA was decreased in the presence of subinhibitory concentrations of ofloxacin. This result suggests that ofloxacin treatment may be considered in the treatment of biofilm-forming infections.

ATP synthase subunit alpha is another heterocyclic-compound-binding protein. Previous studies showed that inhibition of ATP synthase proteins caused dysregulation of energy metabolism and suppressed bacterial growth. ATP synthase is a promising target of many antibiotics.[25] In our study, the presence of the ATP synthase subunit alpha decreased in the ofloxacin-treated groups.

In protein interaction analysis, We investigated interactions of altered proteins in biological pathways. When downregulated proteins were evaluated with String analysis, it was observed that the disruption of ribosomal metabolism was likely to be closely related to energy metabolism, which was also largely affected by the reduction in outer membrane proteins such as LamB and MipA. As discussed above, MipA and LamB are important regulators in membrane-based resistance processes. The exact the interactions between ribosomal proteins, energy metabolism, and membrane proteins have not yet been elucidated. It can be hypothesized that the reduction in MipA and LamB may also lead to reduced intake of the essential factors related to ribosomal and energy metabolism, in addition to reduced antibiotic intake. KEGG pathway analysis also confirmed that ofloxacin treatment mainly affects cells' metabolic functions (especially energy metabolism) and ribosomes.

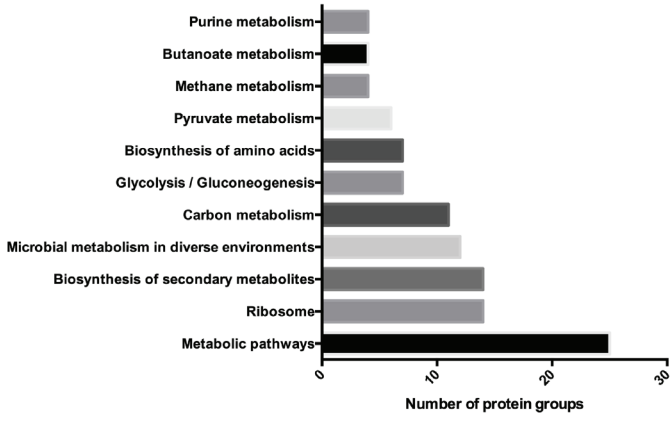

Figure 6. Ofloxacin-induced pathways (FDR <0.05)
FDR: False discovery rate

According to the String analysis of upregulated proteins, the ribosomal proteins RpmE and RpsS were related to pyruvate metabolism and (tricarboxylic acid) TCA cycle proteins (MaeB, SucC, AccB). Rosato et al.[26] showed that the TCA cycle is upregulated under antibiotic stress, and dysregulated production of TCA cycle-mediated reactive oxygen species had an effect on DNA integrity and subsequently triggered activation of the SOS response. Due to the SOS response, RecA is upregulated for DNA repair,[27] which may result in enhanced mutagenesis and the development of resistance to antibiotics as a defense mechanism. We demonstrated upregulation of TCA cycle proteins; however, we did not observe RecA in our study, which might have been due to the concentration and exposure time of ofloxacin.

CONCLUSION

The present study, which was designed to evaluate the effect of ofloxacin on *E. coli* proteomics and to elucidate additional resistance mechanisms, showed that ofloxacin has systematic effects on ribosomal processes, energy pathways, and various antimicrobial targets. Moreover, we found that various mechanisms may play a role in ofloxacin resistance, which requires confirmation by further dose- and time-dependent studies.

Conflicts of interest: No conflict of interest was declared by the authors. The authors alone are responsible for the content and writing of the paper.

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