Original Article
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COMPARATIVE PROTEOMIC ANALYSIS OF *ESCHERICHIA COLI* UNDER OFLOXACIN STRESS

OFLOKSASIN STRESI ALTINDA *ESCHERICHIA COLI* 'NIN PROTEOMIK ANALIZI

Short tittle: Comparative proteomic analysis of *Escherichia coli*
*Escherichia coli'*nin proteomik analizi

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ABSTRACT
INTRODUCTION: In our present study, proteomics has been utilized to evaluate changes in Escherichia coli (E. coli) proteins in response to ofloxacin to understand mode of action of ofloxacin and resistance mechanism against ofloxacin in E.coli.
METHODS: Proteomics analysis of E. coli was performed by using Liquid chromatography quadrupole time-of-flight mass spectrometry (LC/QTOF-MS) followed by a data processing step by MaxQuant. Functional classification and pathway analysis showed systematic effect of ofloxacin over E.coli proteome structure.
RESULTS: In total, 649 common proteins were identified in untreated and ofloxacin-treated groups, while 98 proteins were significantly different in ofloxacin treated group. Functional classification and pathway analysis showed that ofloxacin has systematic effect over ribosomal process, energy pathways (TCA cycle and glycosis), membrane proteins, microbial targets and biofilm formation.
DISCUSSION AND CONCLUSION: Result showed ofloxacin effects many cellular processes and pathways. In addition proteomic analysis revealed that E.coli develops resistance mechanism with different biological processes.
Keywords: Proteomics, LC/MS, Antibiotic resistance, ofloxacin, E.coli

ÖZET
GİRİŞ ve AMAÇ: Bu çalışmanın amacı ofloksasinin oluşturduğu stres ortamında E.coli proteom yapısında meydana gelen değişimlerin proteomik analiz ile incelenmesidir.
YÖNTEM ve GEREÇLER: Bu çalışmada kontrol ve ofloksasin ile muamele edilmiş gruplar Sıvı kromatografisi/kuadrupol uçuş zamanlı kütle spektroskopisi ile analiz edilmiştir. Yolak ve fonksiyonel sınıflandırma çalışmaları yapılmıştır
BULGULAR: Çalışmada 649 tane proteinin yapısı tayin edilmiştir. Bu proteinlerden 98 tanesi gruplar arasında anlamlı olarak değişmiştir. Sonuçlarda ofloksasinin ribozomal süreçlerde; enerji yolaklarında, membran ve antimikrobial hedefler üzerinde etkin olduğu gösterilmiştir.
TARTIŞMA ve SONUÇ: Sonuçlarda ofloksasinin etki mekanizması ve E.coli içerisinde gelişen direnç mekanizmaları protein seviyesinde gösterilmiştir.
Anahtar Kelimeler: Proteomik, LC/MS, Antibiyotik direnci, ofloksasin, E.coli
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INTRODUCTION

Antibiotic resistance is one of the most important health issues worldwide. Improper use of antibiotics leads to the emergence of resistant microorganisms [1, 2]. Further understanding of antibiotic–bacteria interactions is of importance for more efficient use of antibiotics as well as developing novel combination treatment strategies. In recent years, omics-based technologies have been emerged in bacterial antibiotic resistance [3, 4]. Mass spectrometry-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 in understanding the effect of antibiotics on bacteria and resistance mechanisms against antibiotics [5-7].

Ofloxacin is one of the second-generation fluorinated quinolones with a broad-spectrum antibacterial activity against most Gram-negative and, -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 needs to be explained in order to deeply understand the bacteria-fluoroquinolone interactions.

The present study aimed to evaluate the effects of ofloxacin on *Escherichia coli* (*E. coli*) at 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 our present study is also important in order to enlighten the potential resistance mechanisms against ofloxacin.

MATERIALS AND METHODS

Bacterial Culture and Minimum Inhibitory Concentration Experiments

E. coli ATCC 25922 was cultured on Tryptic Soy Agar (TSA). In order to confirm the minimum inhibitory concentration (MIC) value of ofloxacin against *E. coli*, standard broth micro dilution was performed according to the method reported by the Clinical Laboratory Standards Institute (CLSI) [12]. The MIC value of ofloxacin was determined and compared with the latest breakpoint tables of EUCAST [13]. For proteomics experiments, 0.5XMIC value for ofloxacin was used (0.01 mg/L). *E. coli* ATCC 25922 was cultured on Mueller Hinton Broth (MHB) and incubated under 37ºC 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 control group, the same concentration of the bacterial culture was also prepared without adding any antibiotic. Flasks were incubated at 37°C for 20 h for proteomics experiments. 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 (PBS) in triplicate. The bacterial cells were disrupted using lysis buffer containing lysozyme. Protein concentrations of ofloxacin treated and control groups were calculated by using Bio-Rad DC assay. Chloroform (Sigma)/Methanol (Sigma)/Water co-solvent system was used for protein extraction.

Extracted proteins were suspended in 100 mM Ammonium bicarbonate solution (Sigma). 200 mM Dithiothreitol (DTT) (Sigma) was used as reducing agent. Proteins were incubated with DTT at 56ºC for 20 min. After reduction, proteins were incubated with 100 mM Iodoacetamide (sigma) at room temperature. Proteins were digested by Trypsin (1:100 (w/w) at 37 ºC for 16 hours. After desalting, tryptic peptides were dissolved in acetonitrile that contained 0.1 % formic acid.

LC/QTOF-MS analysis

Peptides were analyzed in a LC/QTOF-MS platform. They were separated in C18 columns (Zorbax C18 column 150 × 0.05 mm, 5 column 1Å (Agilent)) at 55°C by performed Agilent 1290 HPLC. 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. It finally reached its initial condition at 90 min. Post run was adjusted at 25 minutes.

Peptides were analyzed in an Agilent 6530 QTOF-MS system. They were ionized in the positive mode of the ESI 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 most intense six ions were selected for MS–MS analysis. The ion charge states were +2, +3, and +4. The fragmentation energy was adjusted to 45 V. 20 µg protein was loaded to the LC/MS system for each run. Four technical replicates were analyzed for both 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). *E. coli* database was downloaded from UniprotKB. Peptide 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. Two missed cleavages were allowed. In the identification process, the false discovery rate (FDR) value was selected as 0.01 for reliable identification.

Label-free quantification algorithm in Maxquant (MLFQ) was used for the semi-quantification between ofloxacin treated and control groups.

Perseus software (https://www.biochem.mpg.de/5111810/perseus) was conducted as statistical analysis. Principal component analysis showed the overall effects of ofloxacin on *E. coli*. Two-sample t test ($p<0.05$) was performed with the number of randomizations being 250 and FDR being 1%, in order to find the differentially expressed proteins between 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 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, 0.5XMIC value of 0.01 mg/L was used for proteomics

experiments. The growth curve for ***E. coli*** ATCC 25922 after treatment with 0.01 mg/L of ofloxacin was constructed during 48 hours. The colony counts for treated bacterial cells were significantly different from control at 6, 20 and 48 hours (Figure 1).

Proteins were extracted and digested from control and ofloxacin treated groups and analyzed with four technical replicates by using the LC-QTOF-MS system. Chromatograms have been given in Figure 2. Proteins were identified with a conservative threshold (FDR ≤ 0.01 and matched peptide numbers ≥2). Totally, 649 common proteins were identified in the control and ofloxacin treated groups. In our 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 relatively less proteins compared to nano LC based proteomics.

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 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. Principal component analysis (PCA) was used to observe the global effect of ofloxacin on *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 the *E. coli* proteome structure (PC1 50.8 %, PC2 19.2%).

The MLFQ intensities of proteins were evaluated using the statistical software called Perseus. The two-sample t test showed alterations in the expression levels of 98 proteins. Total of 30 proteins were increased and 68 proteins were decreased (Altered proteins and fold changes were given in 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 cystol (34.88%), plasma membrane (23.26%), and ribosome (23.26 %). Also some of them were categorized as integral to membrane proteins (9.30%), mitochondrial (4.65%), 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 involved in the 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 activity (44.6%), binding activity (21.5%), structural molecular activity (18.5%), translation regulator activity (3.1%), transporter activity (9.2%), antioxidant activity (1.5%), and receptor activity (1.5%).

Protein interaction analysis

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

KEGG pathway analysis

Next, we evaluated increasing and decreasing proteins together in the KEGG pathway (FDR < 0.05). The ofloxacin-induced metabolic pathways were presented in Figure 6. KEGG pathway analysis showedd 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, the 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*. It was found that 98 proteins altered in ofloxacin treated group.

The differentially expressed proteins were classified functionally in GO analysis to understand systematic effect of ofloxacin on *E. coli* at protein level. First, we classified proteins according to their location (cellular component) in cells. We observed that many differentially expressed proteins in ribosome were dsyregulated, 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 present 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 on antibiotic resistance have been studied extensively, and it has been found that they could act as both positive and negative regulators in antibiotic resistance. Lin et al. showed that increased levels of ompW was related with antibiotic resistance. They also found that outer membrane protein LamB is downregulated under antibiotic stress. [14]. MipA is another membrane protein that has been studied extensively. Zhang et al. showed that MipA is a novel antibiotic-resistance related protein in *E. coli*, against canamycin. The downregulation of MipA promotes resistance mechanisms [15]. Thus, the downregulation of LamB and MipA could be one of the key factors in the potential resistance mechanism against ofloxacin. A downregulation in 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 process, most of the proteins were found to be down regulated.

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 activity and binding activity.

Nucleic-acid binding proteins, modulate the transcription processes in bacterial cells. These proteins directly affects 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. The 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 researches showed that FusA is the main target for some antimicrobial agents [16]. Another nucleic-acid binding protein, RpoB, is the key protein for the development of the drug-resistance process. Several studies have showed that RpoB mutation is one of the major determinant 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 during stress conditions. Several studies on the function of Dps in antibiotic resistance indicated that it has multifunctional properties for

stress protection: DNA binding, and iron sequestration and its ferroxidase activity [18-20]. Therefore, Dps has been recently emerged as a potent antimicrobial target. According to our present results, the expression level of Dps decreased in ofloxacin-treated group.

We observed that the NAD-dependent DNA ligase LigA (ligA), which is an important element in the DNA repair process, was upregulated in the treated group. 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, LigA has been evaluated as a potential antimicrobial target and LigA inhibitors have shown promising results [21, 22]. We believe that the combination therapies of ofloxacin and LigA inhibitors can be more effective for resistant strains of *E. coli*.

We also observed that ofloxacin affects the expression level 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 the biofilm formation [24]. In our study, TnaA was decreased under subinhibitory concentration of ofloxacin. This result suggests that ofloxacin treatment may be considered in biofilm infections.

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

In protein interaction analysis, we aimed to evaluate interactions of altered proteins. When downregulated proteins were evaluated with String analysis, it was observed that the disruption of ribosome metabolism likely to be closely related with 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 been enlighten yet. It can be hypothesized that the reduction in MipA and LamB may also leads to the reduced intake of the essential factors related to ribosome and energy metabolism, in addition to the reduced antibiotic intake. KEGG pathway analysis also confirmed that the ofloxacin treatment mainly affects cells' metabolic functions (especially energy metabolism) and ribosomes.

According to the String analysis of upregulated proteins, ribosomal proteins RpmE and RpsS was related with pyruvate metabolism and TCA cycle proteins (MaeB, SucC, AccB). Rosato et. al. [26] showed that TCA cycle is upregulated under antibiotic stress and dysregulated production of TCA cycle-mediated reactive oxygen species (ROS) had an effect on DNA integrity and subsequently triggered the activation of the SOS response. Due to 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 showed the upregulation of TCA cycle proteins. However, we couldn't observe RecA in our study, which might be due to concentration and exposure time of ofloxacin.

CONCLUSION

In conclusion, our present study which was designed to evaluate effect of ofloxacin on *E .coli* proteomics and to enlight additional resistance mechanisms, showed that ofloxacin has systematic effect on ribosomal processes, energy pathways and various antimicrobial targets. Moreover, we found that various mechanisms may play a role in ofloxocin resistance which needs to be confirmed by further dose- and time- dependent studies.

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On behalf of all authors, the corresponding author states that there is no conflict of interest

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FIGURE LEGENDS

Figure 1. Growth curve of *E. coli* ATCC 25922 with or without 0.02 mg/L ofloxacin treatment (0.5XMIC). The sampling for proteomic experiments was made at 20th hour. Two-way ANOVA was performed for multiple comparisons. *p<0.05; ****p<0.0001.

Figure 2. Chromatograms of control and ofloxacin treated groups.

Figure 3. PCA analysis of treated and untreated groups. Blue zone shows the technical replicates of untreated groups and the red zone shows the techncal replicates of the treated group.

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

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

Figure 6. Ofloxacin induced pathways (FDR<0.05)