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Original Article

Short-term adaptive metabolic response of *Escherichia coli* to ciprofloxacin exposure

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ABSTRACT

Background and Aims: Antibiotic resistance is one of the most critical global health problems. Understanding the pathogen-antibiotic relationship at molecular level could lead to the discovery of new routes to overcome antibiotic resistance. In our present work, we evaluated early responses of *E. coli* against ciprofloxacin within 30 min by analyzing metabolome structure. Our main goal was to understand the initial steps of the adaptation and resistance process of pathogens under antibiotic stress.
Methods: Metabolomics analysis was performed by processing GC/MS and followed with the MS-DIAL metabolomics platform. In addition, Metaboanalyst 4.0 and the KEGG database were used for statistical and pathway analysis.
Results: In total, 207 metabolites were identified while 47 metabolites were significantly different under ciprofloxacin stress condition. Pathway analysis showed that amino acid, fatty acid, and aminoacyl-tRNA metabolisms were altered as an effect of ciprofloxacin at 30 min.
Conclusion: Our results suggest that the understanding of bacterial metabolism in early phase bacterial responses to antibiotics could be key to reducing the adaptation and resistance process.

Keywords: Metabolomics, GC/MS, ciprofloxacin, *E. coli*

INTRODUCTION

Antibiotic resistance is a global problem causing high morbidity and mortality rates. Increasing use and the inappropriate use of antibiotics are the major causes of antibiotic resistance (Ventola, 2015). There is an increasing effort to overcome the antibiotic resistance problem worldwide. However, classical antibiotic discovery approaches are not sufficient for this growing problem (Mills, 2006; Moloney, 2016). In recent years, researchers have focused on the antibiotic-bacteria relationship by using the omics approach to understand the mode of action of antimicrobial agents and the adaptation and resistance mechanisms of pathogens. This new approach has offered great opportunities in finding new routes to overcome antibiotic resistance. However, there is a long way ahead of us. Genomics, transciptomics, proteomics, and metabolomics have drawn attention toward analyzing the cellular process at a molecular level. Metabolites are side or byproducts of biological reactions. A metabolome structure contains thousands of metabolites and is relatively dynamic when compared to a proteome structure. Metabolomics is the analysis of metabolome structures and has been used intensively for many biological systems. In microbiology, metabolomics techniques have gained popularity in analyzing cellular processes in experimental and clinical studies. It has been used to explain phenotypic resistances and adaptation processes (Pulido, Garcia-Quintanilla, Gil-Marques, & McConnell, 2016; Vranakis et al., 2014).

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Ciprofloxacin, which is a member of the floroquinolone group, has been used to treat urinary tract infections, including acute uncomplicated pyelonephiritis (Iravani et al., 1995; Talan et al., 2004; Talan, Naber, Palou, & Elkharrat, 2004). In recent years, fluoroquiolone resistance has become a clinical problem as also encountered in many other antibiotic groups (Hooper & Jacoby, 2015; Piddock, 1998). Omics-based techniques have been used to understand floroquinolone resistance with various bacterial strains, mainly focusing on *E. coli* as a model microorganism. Li et al. analyzed the effect of ciprofloxacin on *E. coli* with proteomics and metabolomics analysis. They explored the middle-phase effect on metabolome within 3 hours (Li et al., 2018). Machuca et. al. (2017) used transcriptomics analysis to observe the cellular response of *E. coli* to ciprofloxacin in a very early phase. In addition, other members of the floroquinolone group have been investigated by omics techniques focusing on bacterial response in different growth stages (Erickson, Otoupal, & Chatterjee, 2017; Lata et al., 2015).

Early-phase antibiotic response (within 0-1 hour) has recently become a promising approach in microbial-omics studies. This analysis could allow the observation of initial regulatory pathways of the adaptation process. Zampieri, Zimmermann, Claassen, & Sauer (2017) have recently used this approach for *E. coli* by using various antibiotics.

In our present work, we focused on the initial metabolic response (30 min) of *E. coli* against ciprofloxacin stress. We used a gas chromatography/mass spectroscopy (GC/MS) based metabolomics approach and evaluated altered pathways in early metabolic perturbation. We believe that our findings will contribute to the current literature in order to further understand the antibiotic-pathogen relationship in early phases.

MATERIAL AND METHODS

Bacterial culture and sample preparation

The standard bacterial strain used in this study was *E. coli* ATCC 25922. Overnight fresh cultures on Nutrient Agar were used for the experiments. The exact minimum inhibitory concentration of ciprofloxacin was determined by using the broth micro dilution method according to the Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI, 2015). For metabolomics experiments, a sub-inhibitory concentration (MIC/2) of ciprofloxacin was used.

In order to determine growth rate of *E. coli* in our experimental conditions, a growth curve was constructed by plotting OD600 nm values to time. Thus, we confirmed our sampling point.

A single colony was picked from fresh cultures and inoculated into 20 mL of nutrient broth (NB), incubated overnight. Late-stationary phase cells (OD600=2.6) were then sub-cultured with or without a MIC/2 concentration of ciprofloxacin in 20 mL NB and incubated at 37°C for 30 min, which corresponds to late lag phase, in order to evaluate the initial adaptation of bacterial cells to antibiotic stress. The cultures were immediately centrifuged after the incubation period at 5000 g, 4°C for 10 min, followed by a washing step with phosphate buffered saline (PBS) 3 times. The pellet was re-suspended in PBS, and cells were disrupted using a lysis buffer containing lysozyme in order to extract intracellular metabolites from cells.

Extraction of metabolites

In the extraction method, a methanol:water (9:1 v:v) co-solvent system was used to isolate metabolites from other biomolecules. A total of 1 mL of co-solvent was added to cell lysates, and they were incubated for 2 hours at 4°C. The mixtures were centrifuged 15000 g for 10 min at 4°C. Proteins were gathered at the bottom of the tube. Metabolites were taken, and the co-solvent was evaporated in a vacuum centrifuge. In the first step of derivation, 20 µL of a methoxyamine solution in pyridine (20 mg/mL) (Sigma, USA) was added to the dried extract and incubated at 70°C for 90 min. Then, 100 µL of MSTFA (sigma, USA) was added and incubated at 37°C for 30 min.

Analysis of metabolites

Metabolites were separated and analyzed with a GC/MS system. Our GC/MS system was performed as described previously (Gonulalan et al., 2019). The Shimadzu GCMS-QP2010 Ultra GC/MS system was performed with a DB-5MS stationary phase column (30 m + 10 m DuraGuard × 0.25 mm i.d. and 0.25 µm film thickness). Samples were injected in a non-split mode. The injection volume was adjusted as 2 µL. A gradient system was used for metabolite separation. In the gradient system, the temperature was set at 70°C for one min and then increased up to 325°C. The total gradient time was 37.5 min. The MS detector was used in EI mode, and data acquisition was performed in full scan mode with a mass range between 50-650 m/z.

Data processing

The raw GC/MS data was analyzed in MS-DIAL (Lai et al., 2018) (version 3.96, http://prime.psc.riken.jp/Metabolomics_Software/MS-DIAL/). The minimum peak height was adjusted as 1000 amplitude, and the mass range was set for 0-650 m/z. The retention time tolerance was 2 min. In the identification process, the Kovats retention index database was used with 15302 records (http://prime.psc.riken.jp/Metabolomics_Software/MS-DIAL/). The peak area of metabolites were calculated and normalized according to total ion intensity. A metaboanalyst 4.0 platform (Chong et al., 2018) was used for statistical analysis. A volcano test was used with p.vaule <0.05 and fold change >1.5. Altered metabolites were evaluated with a pathway analysis (Pathway analysis module in metaboanalyst 4.0 and KEGG mapper system).

RESULTS AND DISCUSSION

The confirmed MIC of ciprofloxacin against *E. coli* ATCC 25922 was 0.015 µg/mL. The growth curve for the MIC/2 concentration and unexposed controls are presented in Figure 1. The doubling of cells started around 30 min, of which we collected samples for metabolomics analysis. There was no significant difference between the control and ciprofloxacin-exposed groups in terms of growth rate at our sampling time of 30 min (Mean OD600 values were 0.071±0.0005 and 0.072±0.0005 for control and treated, respectively). We continued measuring until the 3 hour mark. The only significant decrease in the ciprofloxacin-exposed group (12.99% decrease when compared

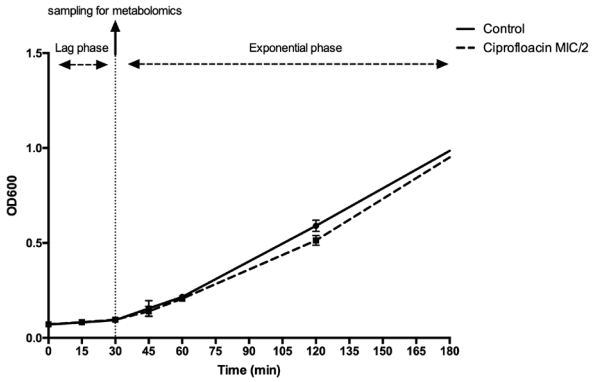

Figure 1. Growth curve for ciprofloxacin-exposed and -unexposed *E. coli* ATCC 25922.

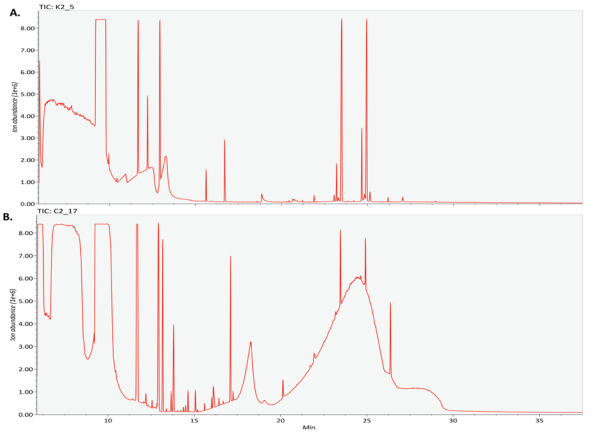

Figure 2. Total ion chromatograms of control (A) and ciprofloxacin treated (B) groups.

to controls) was observed at 120 min (Mean OD600 values were 0.59±0.03 and 0.51±0.02 for control and treated, respectively; p<0.0001).

Metabolites were separated and analyzed with a GC/MS system. The base peak chromatograms of the control and ciprofloxacin-treated groups are given in Figure 2. In the MS-DIAL platform, metabolites were identified with spectral similarity and retention index. In total, 1106 features were detected, and 207 of them were identified. The peak intensity of each metabolite was calculated and then normalized according to total ion intensity. Normalized MS-DIAL data was uploaded to a metaboanalyst 4.0 platform. A principal component analysis (PCA) was applied for all detected features in order to observe the changes of the general metabolome structure of *E. coli* under ciprofloxacin stress. The PCA results showed that there was a dramatic shift in the metabolome structure of *E. coli* in an early phase (Figure 3A). This result indicated that metabolic perturbation includes not only a few single targets but also various cellular processes simultaneously.

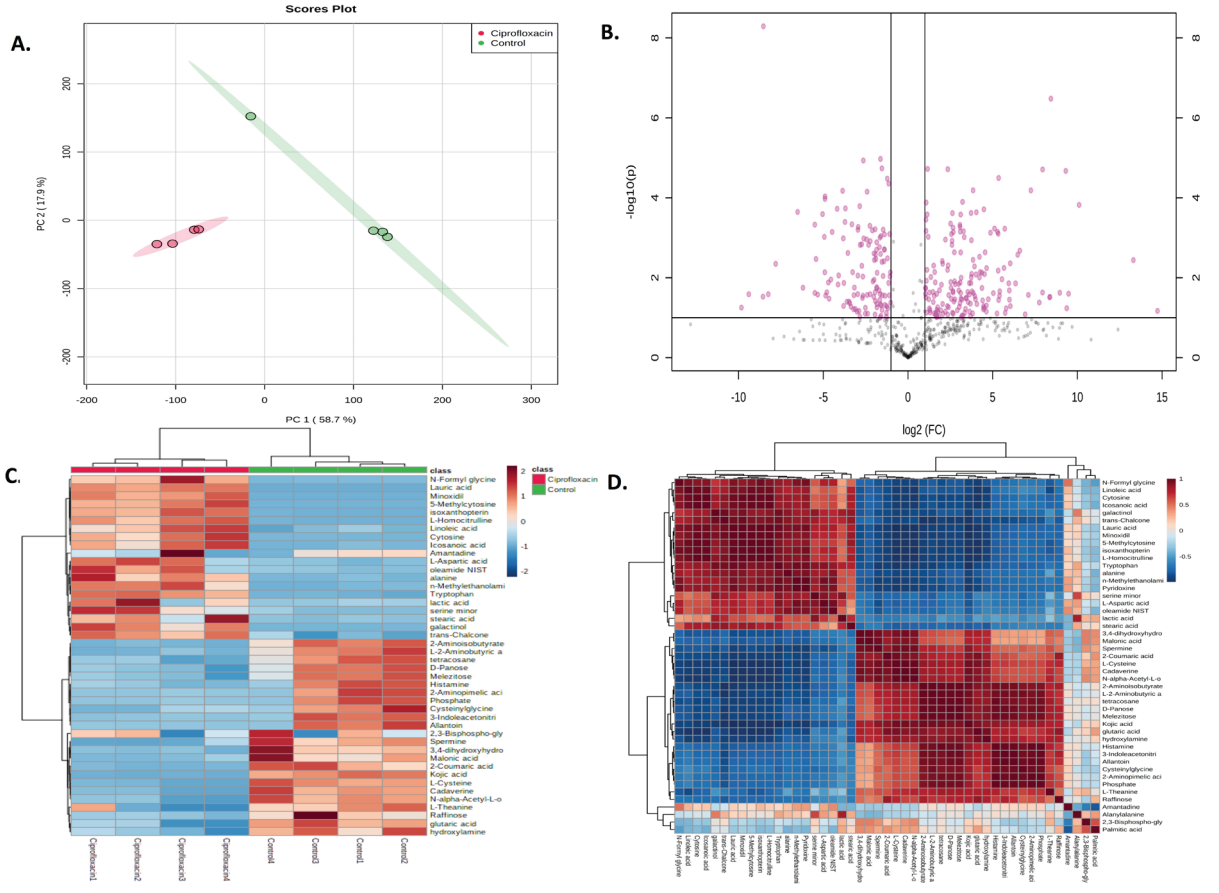

Figure 3. A) Principal component analysis of control and ciprofloxacin treated groups. B) Volcano plot analysis of detected metabolites with p value < 0.05 and fold change >1.5 cut off value. C) Heat map of altered metabolites between two groups. D) Correlation matrix analysis.

Altered metabolites were determined by a volcano plot test with strict rules (p value <0.05 and fold change > 1.5) (Figure 3B). The results showed that 47 metabolites were altered under ciprofloxacin stress. Altered metabolites with fold change and p-value were given in supplementary information. A heat map and a correlation matrix are given in Figure 3C and 3D to visualize the relative abundance of metabolites with color intensity.

A pathway enrichment analysis is the key tool to understand the systematic effect of antibiotics over pathogens. In the present work, we used a metaboanalyst pathway analysis module (Figure 4A) and a KEGG mapper system (Figure 4B). The results showed that various metabolic pathways were altered significantly under a ciprofloxacin stress condition. The amino acid biosynthesis was the most affected metabolic process (Figure 5A). Amino acids play important roles in many cellular process, pathogenicity, and biofilm formation. In previous studies, it was shown that amino acid biosynthesis was changed under antibiotic stress conditions. Moreover, amino acid biosynthesis could be one of the initial steps of the resistance mechanism (Dorries, Schlueter, & Lalk, 2014; Zampieri et al., 2017).

Alanine, aspartate, serine, tryptophan, and cysteine are involved in aminoacyl-tRNA biosynthesis and glycine, serine, and threonine metabolism. Aminoacyl-tRNAs play a central role in protein synthesis. Also, they are very important for the cell envelope that affects how the cell interacts with antibiotics (Kim, Lee, Choi, & Choi, 2003). Also, glycine, serine, and threonine metabolism is another important pathway for adaptation and resistance (Cheng et al., 2019; Ye et al., 2018). Our result on altered amino acid biosynthesis pathways may suggest an immediate transcriptional adaptation, which may be clarified with further transcriptomics studies.

Another critical point was the alteration of fatty acid metabolism (Figure 5B). Since fatty acid biosynthesis is essential for

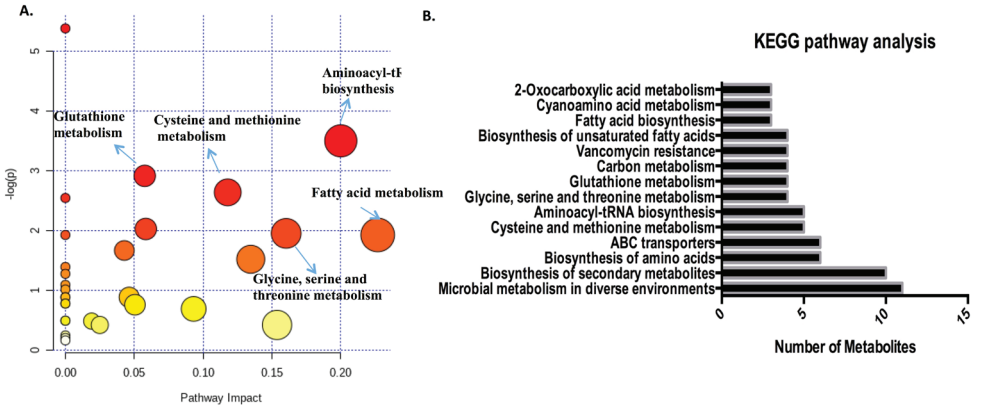

Figure 4. Pathway enrichment analysis; A) Metaboanayst B) KEGG mapper platform.

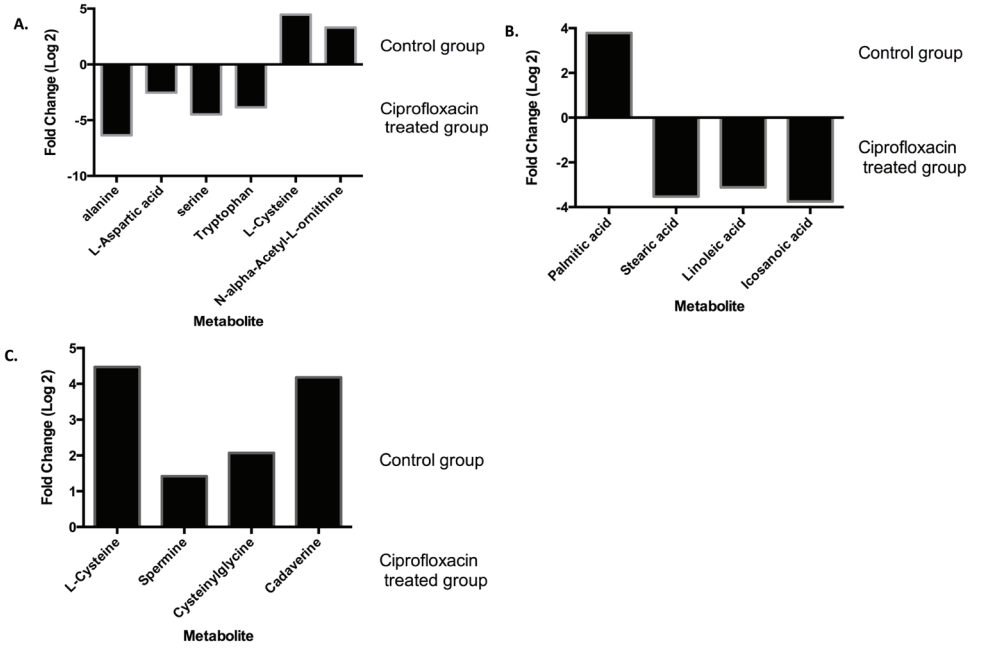

Figure 5. Differentially expressed metabolites in A) Amino acid biosynthesis B) Fatty acid metabolism C) Glutathione metabolism.

many bacterial pathogens, it has recently become a promising antimicrobial target (Yao & Rock, 2017). Some fatty acids are part of the phospholipid layer of the cell membrane, and their composition mostly depends on environmental conditions, which confirms their essential role in environmental adaptation (Yano, Nakayama, Ishihara, & Saito, 1998). Previous studies showed that fatty acid metabolism was different between antibiotic resistant and susceptible strains of various bacteria (Dunnick & O'Leary, 1970; Maifiah et al., 2017). Maifiah et. al. (2017) showed that fatty acid composition was significantly changed in *Acinetobacter baumannii* under colistin stress within 15 min. In our work, we observed similar results in 30 min. Together, these results show that fatty acid metabolism is one of the most immediately affected cellular processes under stress.

Glutathione is mainly involved in bacterial redox regulation and adaptation to stress conditions (Smirnova & Oktyabrsky, 2005). In our present study, we found that various metabolites which are involved in glutathione metabolism decreased in ciprofloxacin-treated group (Figure 5C). Previous studies showed that glutathione metabolism up-regulated to protect cells against bactericidal antibiotics (Cameron & Pakrasi, 2010, 2011). However, the exposure timing was different from ours. They used 11 hours exposure time. Thus, it could be argued that the effect of ciprofloxacin was not sufficient to trigger glutathione metabolism in 30 min.

We acknowledge the following limitation of our study. We mainly focused on a fluoroquinolone antibiotic, ciprofloxacin in our present study, due to its widespread use in clinical settings. However, we cannot exclude the possibility that these early-phase metabolic differences may be a common stress response to various antibiotics or other chemical inducers. Indeed, Zampieri et al. (2017) showed that the patterns for selective metabolites were similar for antibiotic and hydrogen peroxide-exposed bacterial cells. Further studies with various antibiotic groups and chemicals are needed to clarify this issue.

CONCLUSION

Herein, we focused on the early phase response of *E. coli* against ciprofloxacin in order to determine the short-term adaptive metabolic response. We conclude that amino acid biosynthesis-related pathways and fatty acid metabolism are early immediate responses of *E. coli* cells to ciprofloxacin. In further experiments, we will focus on these pathways with targeted approaches. We believe that our findings will contribute to the current literature in understanding early pathogen-antibiotic interactions at the molecular level.

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Conflict of Interest: The authors have no conflict of interest to declare.

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