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Investigation on response of the metabolites in tricarboxylic acid cycle of *Escherichi coli* and *Pseudomonas aeruginosa* to antibiotic perturbation by capillary electrophoresis

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Abstract

Metabolomics is a new branch of systems biology exerting its influence in many aspects. In order to appraise the effects of antibiotics on central carbon metabolism, a CE based method was set up. With this platform, we estimated the organic acid metabolite pools' fluctuation of *Escherichia coli* and *Pseudomonas aeruginosa* cultured under 11 different antibiotics' stimuli. Multivariate data analysis showed that different antibiotics had clustered distributions for each strain and could be easily distinguished. Genetic, metabolic and antibiotic mechanism differences could also be deduced by the aid of further correlation analysis. For *P. aeruginosa*, even synergy action amid antibiotics could be ascertained.

Keywords: Capillary electrophoresis; Tricarboxylic acid cycle; Carboxylic acids; Antibiotic

1. Introduction

The recently introduced functional genomics concept is revolutionizing research in the biological sciences. Accordingly, three major high throughput analysis technologies—transcriptomics, proteomics and metabolomics, are recognized as the keystones to solve the problem of aligning genotypes to phenotypes. Metabolomics is a new addition to the functional genomics toolbox. It involves the non-targeted, holistic analysis of changes in the complete set of metabolites in the cell in response to environmental or cellular changes [1]. Bundy et al. reported the metabolic profiling strategy to discriminate six different *Bacillus cereus* strains from two different ecotypes: (1) three commonly used laboratory strains that are considered avirulent, and (2) three clinical isolates from meningitis patients. They indicated that screening of genomic DNA for the presence of genes encoding known toxins gave no candidate ones that were unambiguously able to distinguish between the two groups. However, the application of multivariate pattern-recognition methods to metabolite profiles derived from the different strains using ^{1}H nuclear magnetic resonance (NMR) spectroscopy (metabolomics) was able to classify the different groups [2]. Ideally, the metabolome of the cell is determined selectively in every compartment by non-invasive in vivo methods. However, in vivo methods such as NMR and IR are, at present, not very sensitive and moreover do not separate individual metabolites, their utilities are a little limited to some extents. Although many hyphenated techniques, such as GC–MS, LC–MS, GC × GC–MS and so on [3–5], are developing rapidly; the unbiased, holistic analysis of metabolome is still far to approach. Up to now, the study of metabolites was limited to a handful of compounds at a time expected by the researchers to be of particular importance in a given situation—so-called target analysis.

Studying metabolites from the tricarboxylic acid (TCA) cycle in the real sample (e.g., bacterial cell extract) can be considered as a part of metabolomic analysis [6]. The main metabolites (intermediates) in the TCA cycle are di- and tricarboxylic acids (Fig. 1). This cycle plays a key role in energy supplying via aerobic oxidation in most of the prokaryotic and eukaryotic cells; some intermediates are the main nodes to connect the metabolism of carbohydrates, amino acids and nucleotides. Recently, capillary electrophoresis (CE) has been utilized to analyze cell extracts in view of metabolome [7,8],

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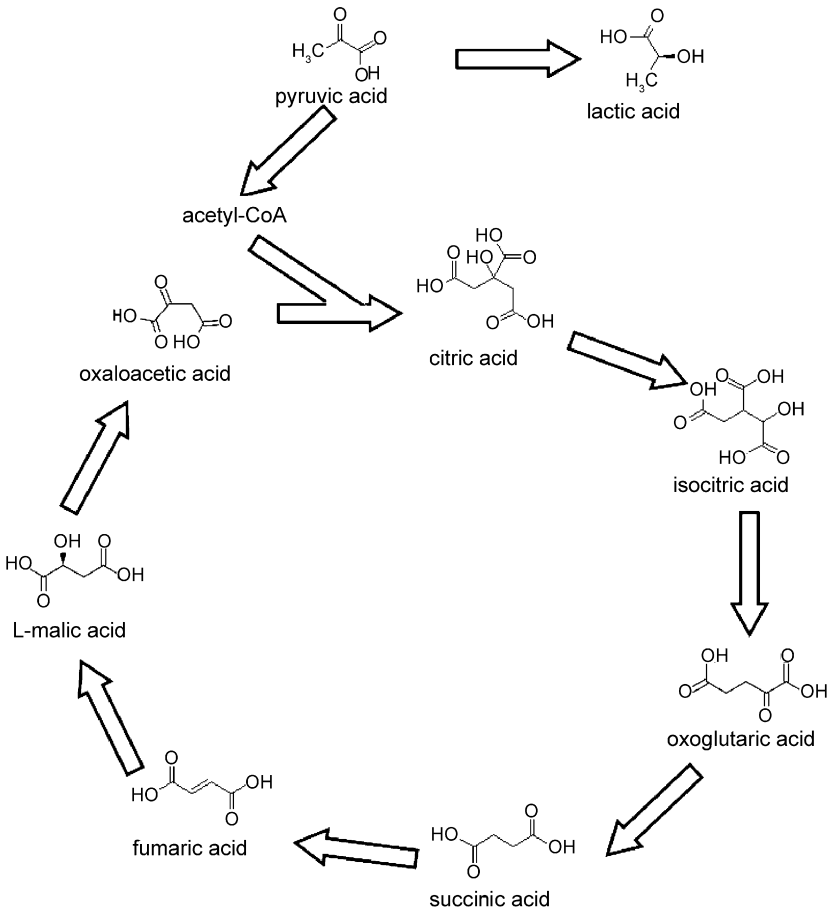

Fig. 1. Schematic representation of metabolites in TCA cycle and partial glycolysis metabolic pathway considered in this study.

and CE approaches are regarded as the first step towards a future use of laboratory-on-a-chip or micro total analytical systems for the analysis of metabolomes due to its lower sample volume request, higher separation efficiency and relative simple operation [9,10], which are the basic prerequisites to satisfy clinical demand. In industrial microorganism territory, Markuszewski et al. [11] reported the first attempt to analyze organic acids in the TCA cycle from real cell extracts, but using an indirect photometric detection method by the aid of 2,6-pyridinedicarboxylic acid as a highly ultra violet (UV) absorbing carrier electrolyte; a relative important intermediate metabolite—oxaloacetic acid, was also excluded in their consideration.

To our knowledge, utilizing CE tactic to evaluate antibiotics effects on pathogenic microbes was seldom found. In this study, firstly, we developed a direct UV-based detection method to analyze the main TCA cycle carboxylic acids and some organic acids (from glycolysis pathway) relating to TCA cycle tightly. Then two typical strains—*Escherichia coli* (*E. coli*) and *Pseudomonas aeruginosa* (*P. aeruginosa*), were tested under the same culture conditions with varied antibiotics. The concentration fluctuation of carbolic acids from TCA cycle was determined to evaluate the response of the cells to the environmental perturbation, and according to this CE-based method, we also tried to verify metabolomics could be used as an alternative for profiling and possible classifying of clinical microbial strains into groups of taxology interests in view of microbial genetics and antibiotic mechanisms. By using multivariate statistical techniques, we intended to explore the information implied, which might provide some hints to antibacterial mechanism differences, and deduce the reasonable explanations for the effects on central carbon metabolic intermediate pools from the selected antibiotics.

2. Experimental

2.1. Apparatus

All experiments were performed with a Beckman P/ACE System MDQ capillary electrophoresis system (Palo Alto, CA, USA) equipped with a DAD detector. Analytes separation was carried out in the 75 cm long fused-silica capillaries (70 cm effective length) with the inner diameter of 50 μm provided by Yongnian Optical Fiber Company (Hebei, China). Detection wavelength was set at 200 nm and the capillary columns temperature was maintained at 20 °C. In order to minimize sample evaporation, the sample compartment was thermostated at 10 °C. Samples were injected by a −10 kV in 10 s and separated by a constant voltage of −20 kV. Prior to first use, new capillary columns were rinsed with 1 and 0.1 M NaOH for 10 min,

respectively, and then treated with deionized water for 20 min. To ensure repeatability, the capillaries were only rinsed with background buffer solution for 5 min between every runs and the electrode buffer was replaced after every third run. Multivariate data analysis was performed using SIMCA-P 10 demo version (Umetrics AB, Umeå, Sweden).

2.2. Reagents

Unless otherwise stated, all chemicals were of analytic purity or higher. Citric acid, succinic acid, malic acid, fumaric acid, lactic acid, pyruvic acid, isocitric acid, α-ketoglutaric acid and oxalacetic acid were purchased from Sigma–Aldrich (Shanghai, China). NaH_2PO_4 and Na_2HPO_4 were the products of Lianbang (Shenyang, China). Water used in the experiments was purified with Mili-Q system (Millipore, Bedford, MA, USA). Methanol was the HPLC purity product from Tedia (Fairfield, OH, USA). Aztreonam (ATM) and Cefepime Hydrochloride (FEP) (Bristol-Myers Squibb, Shanghai, China), Ceftazidime Pentahydrate (CAZ) and Ticarcillin/Clavulanic Acid (TIC) injectable powder (GlaxoSmithKline, Hong Kong), Ceftriaxone Sodium (CRO) (Roche Co., Ltd., Shanghai, China), Ciprofloxacin Lactate (CIP) (Lunan Pharmaceutical Co., Ltd., Shandong, China), Levofloxacin Hydrochloride (LVF) (Kelun Pharmaceutical Co., Ltd., Sichuan, China), Piperacillin Sodium/Tazobactam Sodium (TZP) (Qilu Pharmaceutical Co., Ltd., Shandong, China), Piperacillin Sodium (PIP) (Qilu Pharmaceutical Co., Ltd., Shandong, China), Meropenem (MEM) (Merk & Co., Inc., New Jersey, USA), and Imipenem/Cilastatin Sodium (IPM) (Merk & Co., Inc., New Jersey, USA) were all donated by Dalian Municipal Central Hospital. Basic information about the 11 antibiotics was given in Table 1.

2.3. Bacteria and medium

E. coli (25922) and *P. aeruginosa* (27853) were the strains of American Type Culture Collection (Manassas, VA). Nutrient broth powder was provided by Chinese Institute of Import & Export Commodity Inspection Technology (Beijing, China). Its basic gradients consist of peptone (10 g/l), beef extract (3 g/l), sodium chloride (5 g/l), glucose (1 g/l), and trace additives. This powder was boiled to dissolve in appropriate deionized water according to the instructions and then dispatched to 50 ml bottles with the same volume of 10 ml for each. After autoclaving steam sterilization under pressure for 15 min at 121 °C, the pH of this medium was about 7.4 ± 0.1.

2.4. Bacterial growing, quenching and extraction

The bacteria used in this study were grown by conventional methods. Typically, *E. coli* (25922) and *P. aeruginosa* (27853) were cultured in autoclaved broth with aeration for 24 h at 35 °C separately. These fresh cultures were rinsed twice and diluted with 0.85% (w/v) sterile physiological saline to reach the final concentration of 1.5×10^8 using a bioMerieux (France) DENSIMAT transmissometer. 10 ml autoclaved broth samples, which had been mingled individually with different antibiotics reaching to the final concentrations listed in Table 1, were prepared in aseptic bottles (volume 50 ml). A 10 μl bacterial solution was added to each and then cultured for 10 h under constant agitation (200 rpm). Every antibiotic was prepared freshly to prevent casual degradation taking place.

To halt cellular metabolism, the whole cultured samples were immediately quenched in two-fold volume of methanol at −45 °C for 20 min before their corresponding bacterial turbidities had been measured. The organic acids were obtained by chloroform extraction modified according to that described by Ruijter and Visser [12]. In brief, quenched samples were centrifuged at $3300 \times g$ for 5 min at 4 °C and the supernatant was discarded. Cell sediments were collected to 5 ml centrifugation tubes with 1 ml ice-cooled methanol. To the tubes, 1 ml of chloroform and ca. 500 μl of deionized water was added and then vortexed for 1 min. The mixture was dialyzed in 50 ml deionized water overnight through 5 kDA filter (Mufengyuan; Dalian, China). The dialyzed liquid for each was collected and lyophilized at −50 °C by freeze dry system (LABCONCO, Missouri, USA). The residues were dissolved in 1.5 ml methanol and stored at −80 °C until further processing. To ensure comparability, all artificial processing was performed at 4 °C as possibly as we could.

Table 1
Basic information about the 11 antibiotics applied in the study

Class	Antimicrobial mechanism	Name of applied antibiotics	Concentrations (μg/ml)
β-Lactam	Interfering synthesis of cell wall	Ceftazidime	0.003
		Ceftriaxone	0.0015
		Cefepime	0.0015
		Piperacillin	0.05
		Piperacillin/tazobactam	0.011
		Ticarcillin/clavulanic acid	0.0425
Quinolone	Targeting DNA gyrase and topoisomerase IV	Levofloxacin	0.0005
		Ciprofloxacin	0.00025
Monobactam	Blocking the synthesis of cell wall	Aztreonam	0.003
Carbapenem	Disturbing the synthesis of cell wall	Imipenem/cilastatin	0.001
		Meropenem	0.001

2.5. Recovery determination by HPLC

Metabolite recovery was determined by HPLC. In brief, *E. coli* was cultured under the conditions mentioned above without any addition of antibiotics. Bacterial suspension was dispatched to 10 tubes with each containing 10 ml. When quenched with methanol, nine standard acids were added separately with the same volumes. Appropriate water was pipetted into the left one as control. All 10 samples were extracted as stated in Section 2.4. For HPLC analysis, a Waters 2695 Alliance HPLC System (Waters, USA) was employed with C18 column (Elite, China). Mobile phase consisted of 2.5 mM $NH_4H_2PO_4$ with 5% methanol (v/v). Column temperature was set at 30 °C. Detection wavelength was at 210 nm.

3. Results and discussion

3.1. Optimization of the separation

The optimal separation conditions were determined by varying pH of the background solution (BGS), the total concentration of electrolytes and the addition of organic modifiers. Finally, in BGS of pH 5.65 consisting of 0.4 M NaH_2PO_4, 0.1 M Na_2HPO_4 and 6% (v/v) methanol, baseline resolution of the carboxylic acids was achieved. Because in the TCA cycle, *cis*-aconitic acid is a transitory existence compound and is easily catalyzed to isocitric acid and scarcely involved in other metabolic pathways, it was not treated as a target compound to be separated. Pyruvic acid plays a significant role in central carbolic metabolism both in the prokaryocytes and the eukaryocytes; it was taken into consideration [13]. Accordingly, lactic acid, a reduction product coming from pyruvic acid, which can indicate the alternative pathway's activating instead of TCA cycle, is also regarded as one of great importance metabolite to be separated [1,13]. The typical electrophorogram of these nine carboxylic acids separated under the optimized conditions is shown in Fig. 2.

3.2. Analytical characteristics of the method

Usually, sample introduction in CE is accomplished by electrokinetic or hydrodynamic methods. Initially, those two modes were all investigated. At the expense of sacrificing reproducibility to a limited extent, electrokinetic mode could improve sensitivity to reach relatively lower limits of detection (LODs). Referred to the criteria of signal/noise ratio of 3 [14], extrapolated LODs were in the range of 10^{-7} to 10^{-8} M (Table 2). The extraction recovery of the acids were 64.3% (oxalacetic acid), 79.3% (lactic acid), 83.5% (citric acid), 86.9% (pyruvic acid), 88.2% (fumaric acid), 91.5% (malic acid), 98.1% (succinic acid), 104.6% (α-ketoglutaric acid) and 112.5% (isocitric acid).

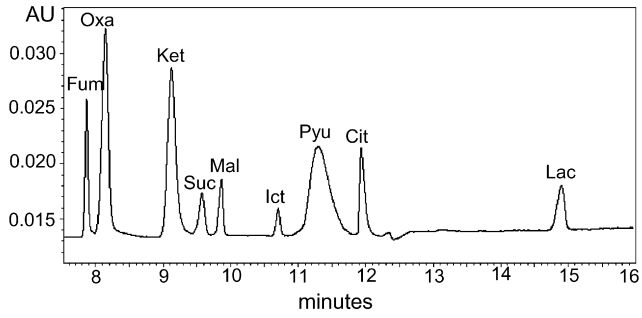

Fig. 2. Electropherogram of nine standard carboxylic acids. Capillary, 75 cm × 75 μm; separation, −20 kV; injection, −10 kV for 10 s; temperature, 20 °C; BGS, 0.4 M NaH_2PO_4, 0.1 M Na_2HPO_4 and 6% (w/v) methanol, pH 5.65. Fum, fumaric acid; Oxa, oxalacetic acid; Ket, α-ketoglutaric acid; Suc, succinic acid; Mal, malic acid; Ict, isocitric acid; Pyu, pyruvic acid; Cit, citric acid; Lac, lactic acid.

3.3. Appraisal of bacterial response to antibiotics by fluctuation of organic acid metabolite pools

All the cell extracts were subjected to analysis by CE. A representative electrophoregram is shown as Fig. 3. For both of the strains, every antibiotic was utilized to culture once at a day and total to 10 days. Owing to the facts that it is not easy to determine the water content of individual bacterial cell, the organic acid concentrations were converted to the unit of M/cell/ml broth (mentioned as derivate concentration hereafter). The average concentrations of the nine organic acids under different antibiotic stimuli for each strain were depicted in Fig. 4. From Fig. 4, it could be discerned that the concentrations of the carboxylic acids

Table 2
Linear equations, LODs and R.S.D. obtained under optimized conditions ($n = 3$)

Name	Regression equation		Detection limits (s/n = 3), LOD (10^8 M)	Migration time (min)	R.S.D. (%, $n = 3$)		
	$y = ax + b$	R			Migration time	Corrected peak area[a]	Peak area
Fumaric acid	$y = 0.7700x + 9.089$	0.993	1.05	7.88	2.08	2.55	0.54
Oxalacetic acid	$y = 0.9113x + 9.256$	0.992	1.70	8.17	3.27	8.74	4.92
α-Ketoglutaric acid	$y = 0.3525x + 7.270$	0.994	1.56	9.14	3.57	5.46	2.23
Succinic acid	$y = 0.8187x + 8.420$	0.991	3.03	9.59	3.06	2.59	8.52
Malic acid	$y = 0.8804x + 8.701$	0.991	3.88	9.84	2.82	0.43	2.35
Isocitric acid	$y = 0.9465x + 8.410$	0.995	25.9	10.72	3.64	5.26	5.94
Pyruvic acid	$y = 1.4649x + 10.447$	0.991	62.6	11.29	4.51	9.84	9.88
Citric acid	$y = 1.3052x + 11.060$	0.997	15.6	11.96	3.98	5.94	5.79
Lactic acid	$y = 0.6420x + 6.809$	0.994	6.08	14.88	4.79	9.05	9.08

[a] Corrected peak area = peak area/migration time.

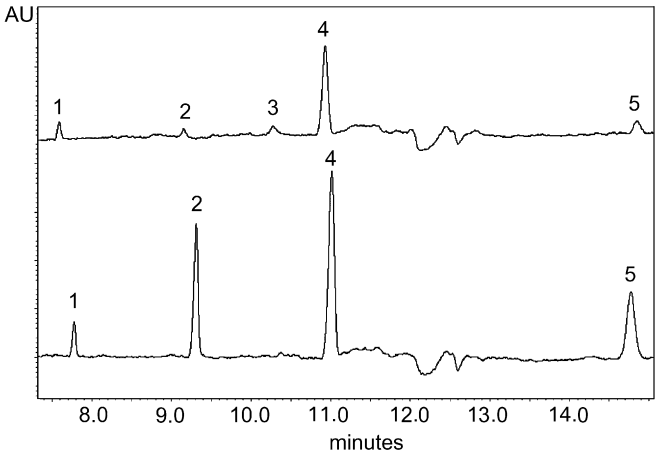

Fig. 3. Capillary electrophoregram of cell extracts coming from *E. coli* (25922) (the lower one) and *P. aeruginosa* (27853) (the upper one) cultured at the ciprofloxacin concentration of 0.25 ng/ml. The main peaks are: fumaric acid (1); α-ketoglutaric acid (2); isocitric acid (3); pyruvic acid (4); and lactic acid (5).

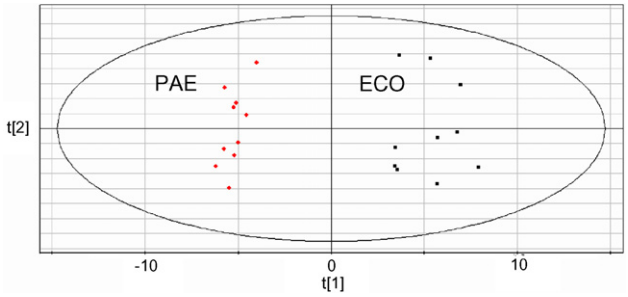

Fig. 5. Two strains differentiated through principal components analysis (PCA) based on the derivate concentrations (M/cell/ml broth) of the organic acids under stimuli from 11 antibiotics. ECO, *E. coil* (right); PAE, *P. aeruginosa* (left).

recycling in TCA cycle were at different orders of magnitude. By the aid of principal components analysis (PCA), both of the strains could be clearly separated (Fig. 5). For each strain, taking the varied antibiotics into consideration, based on the derivate concentrations of the carboxylic acids, the different antibiotics exhibited distinct clustering localization trends (Fig. 6) when projected to three-dimensioned statistic diagrams. The whole data were then transformed into a 99×10 matrix and their Pearson's correlation coefficients were computed orthogonally. Variables with $|C_{ij}| \geq 0.8$ were screened out to construct a network (Fig. 7) using the Graph Package provided by Tom Sawyer Software (http://www.tomsawyer.com/home/products.php.) to facilitate bacterial responsive variance inference.

Metabolomics involves the non-targeted and target analysis of the changes in the complete set of metabolites (small organic compounds, MW < 1000) in the cell (the metabolome), body fluids, or tissues [15]. As the biochemical level of the metabolome is the closest to that of the function of a cell (the phenotype), metabolome studies are becoming more and more predominant as a key to understanding biological function [16]. By analyzing differences between metabolomes using biostatistics (multivariate data analysis, pattern recognition), metabolites relevant to a specific phenotypic characteristics can be identified. Microbiology is one of the most successful arenas benefiting from metabolomics [17]. Unlike the hybrid approaches, although their undoubted resolutions and sensitivities, prevailing in nowadays analytical chemistry world, CE method is still having its advantages in simplicity, rapidness and lower costs, which defines its destine that it cannot be excluded from metabolomic study totally [13]. In this study, after optimization, the carboxylic acids in TCA cycle could be separated properly (Figs. 2 and 3). Their LODs and linear response scales were also satisfied (Table 2). Inspected through this strategy, we found that the metabolic pools of the interested acids were not stabile under different antibiotic stimuli no matter whether inter- or intra-species (Fig. 4). The antibiotics introduced into this research were all of clinical popularity and recommended by Clinical and Laboratory Standards Institute (Wayne, Penn-

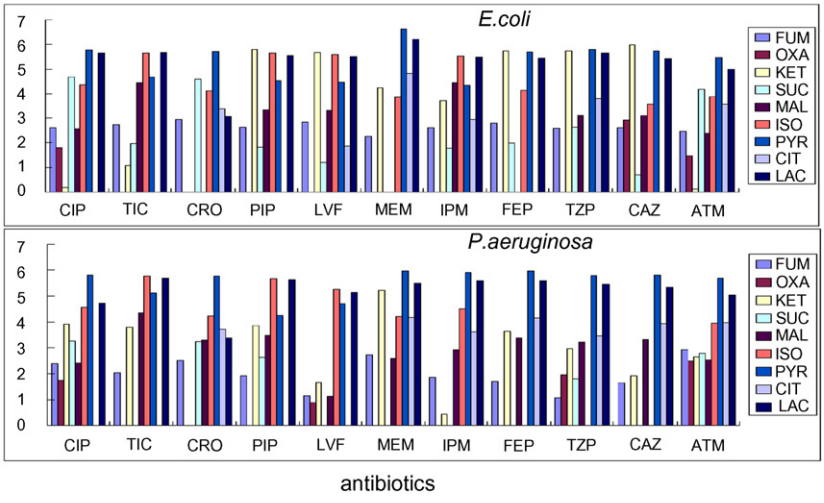

Fig. 4. The average derivate concentrations of nine carboxylic acids under different antibiotics' stimuli. FUM, fumaric acid; OXA, oxalacetic acid; KET, α-ketoglutaric acid; SUC, succinic acid; MAL, malic acid; ICT, isocitric acid; PYR, pyruvic acid; CIT, citric acid; LAC, lactic acid. *Y*-axis: (19+lg C) M/cell/ml broth. For antibiotic abbreviations, see Section 2.

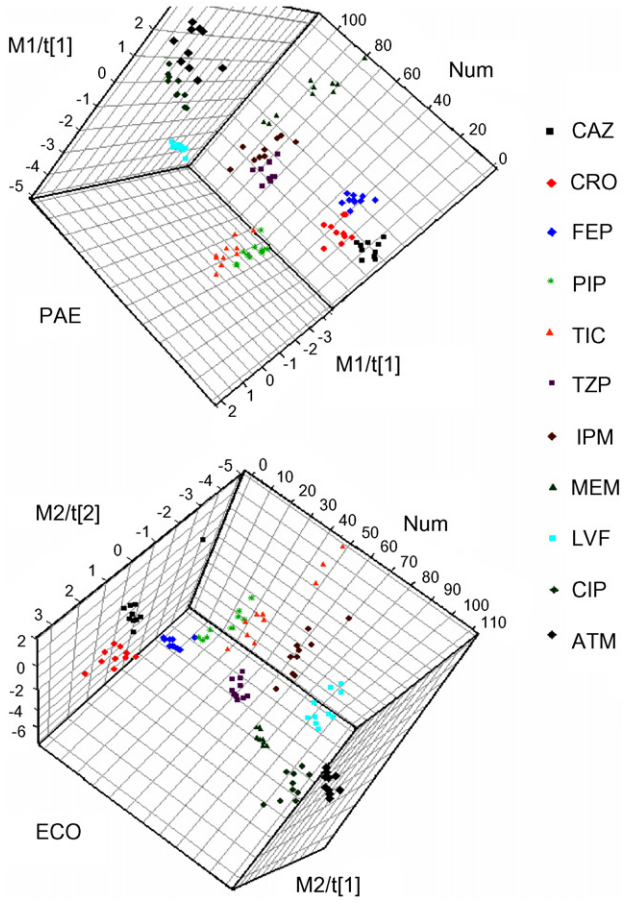

Fig. 6. Antibiotic differentiation from *E. coil* (ECO) and *P. aeruginosa* (PAE) strains according to their derivate acid concentrations from cell extract. Different shapes represent varied antibiotics. For meanings of the abbreviations, see Section 2.

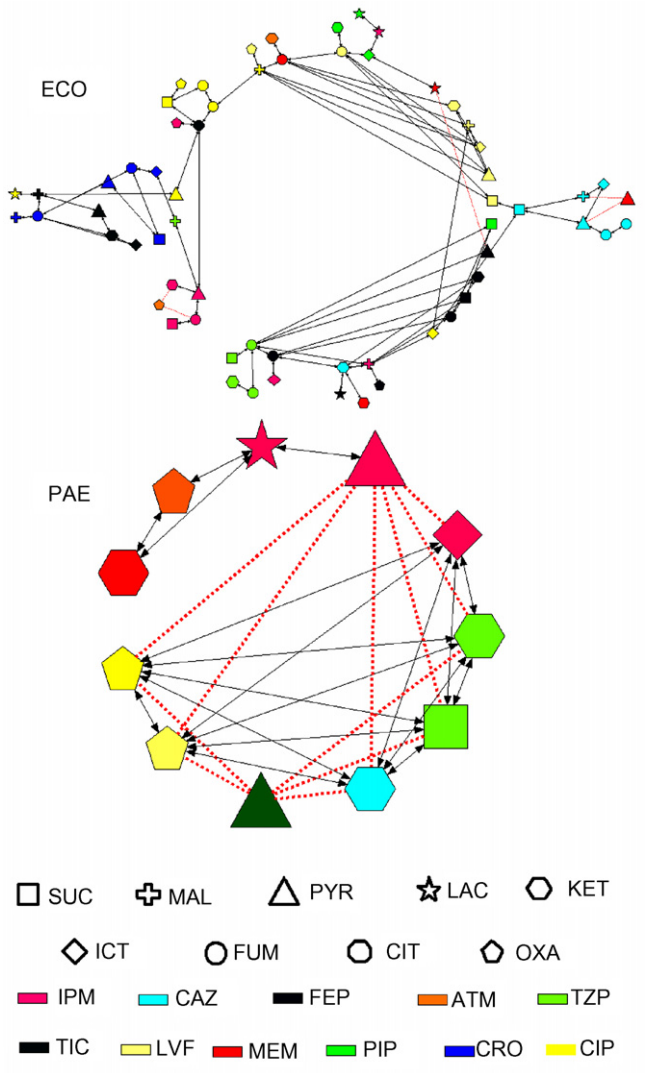

Fig. 7. Major interaction networks of nine organic acids under the presence of different antibiotics from *E. coil* (ECO) and *P. aeruginosa* (PAE). Dotted line indicates negative correlation and solid line represents positive correlation. For meanings of abbreviations, see Section 2. Different colors represent different antibiotics. Different shapes show different acids.

sylvania, USA) for quality control tests to *E. coli* (25922) and *P. aeruginosa* (27853) [18]. Except that the applied concentrations of the antimicrobial agents were significantly modified to lower than the documented minimal inhibitory concentrations (MICs) [18] to allow the tested strains' growth, other procedures strictly abided by Ref. [18] as possibly as we could. Basically, these agents belong to quinolone, β-lactam, monobactam, and carbapenem classes according to their molecular structures. Their antimicrobial mechanisms are either frustrating bacterial DNA replication (the first one) or blocking the cell wall synthesis (the last three classes). Combing the derivate concentrations of the individual acids, each of the antibiotics showed a localized distribution after projected to the 3D plots through PCA for both strains (Fig. 6).

The culture time for each strain was limited to 10 h because longer time will cause some antibiotic degradation. By correlation analysis, we could discern that most of the metabolites having tight relationships were confined to the agents of similar mechanisms (Fig. 7). What should be paid more attention to was the phenomenon that, for *P. aeruginosa*, the same metabolite—oxaloacetic acid, from the two quinolone agents had the similar relationships to other acids from antibiotics working on cell wall synthesis. For example, oxalacetic acid of CIP showed negative correlation with pyruvic acid of TIC and IPM, so did oxalacetic acid of LVF. α-Ketoglutaric acid and succinic acid from TZP had positive correlation with oxalacetic acid both from CIP and LVF, and so on. We attributed this trait to the fact that *E. coil* is a facultative aerobe and *P. aeruginosa* is usually described favoring aerobic growth conditions [19]. In TCA cycle, oxalacetic acid reacting with acetyl-coenzyme A (Acetyl-CoA) is the first step, and pyruvic acid is the center to connect TCA cycle and glycolysis. When pyruvic acid was largely used to participate in anaerobic fermentation, TCA cycle would be suppressed. So pyruvic acid showed negative relationship with most of the TCA metabolites (Fig. 7 PAE). In *E. coil*, pyruvic acid anaerobic fermentation and partaking in TCA cycle can exist simultaneously depending on the oxygen pressure which will modify individual pathway's efficiency. But only in extreme conditions does *P. aeruginosa* survive relying on pyruvic acid

anaerobic fermentation [20]. The exact relations among TCA cycle intermediates and glycolysis metabolites may be confused in *E. coil* just because of its facultative properties.

P. aeruginosa is well known for its intrinsic resistance to various structurally unrelated antimicrobial agents. This broad-spectrum resistance is largely due to the possession of an outer membrane with relatively low permeability [21,22] coupled with secondary resistance mechanisms, such as efflux [23]. The efflux operon mexA/mexB-oprM has been identified in *P. aeruginosa*, and its products have been demonstrated to contribute to the high intrinsic antibiotic resistance of this organism as well as lead to multiple antibiotic resistance [24]. It has been suggested that the relatively hydrophilic and often negatively charged β-lactams, which have targets in the periplasm, can also be extruded directly from the periplasm or from the surface of cytoplasmic membrane through this system. But this efflux resistance mechanism to β-lactams is energy-dependent [24]. This pump like system's operation needs more ATP and thus may accelerate the TCA efficiency under the stimuli of antibiotics. On the contrary, the glycolysis pathway will be frustrated because of its lower energy conversion capability. Reflected in Fig. 7, we got the positive correlations amid most of the acids from TCA cycle but negative correlations between pyruvic acid (from quinolones) and the other compounds of TCA cycle from other agents. As of quinolone antibiotics, they are mechanism-based inhibitors of DNA gyrase and act by forming a complex with the enzyme and the doubly cleaved DNA which is covalently tethered to the gyrase A subunits. The irreversible binding causes the gyrase not to relegate the cleaved DNA and, as a consequence, ultimately set off the SOS repair system that even lead to bacterial cell death [25]. This will also help us to understand the meaning dwelling in Fig. 7 PAE. Based on the components of cell wall [26], we postulated that in the presence of interfering cell wall synthesis antibiotics, cell transformed more pyruvic acid to alanine which is the crucial amino acid containing in peptidoglycan that confers cell wall strength to resist lysis on changes in osmotic pressure. Unlike the mechanism of quinolones, cell wall synthesis interfered antibiotics were very likely to result in competing pyruvic acid consuming in TCA cycle and alanine synthesis.

In *E. coli*, except the relatively tight connection of metabolites stemming from the same antibiotics, quinolone antibiotics have something to do with certain agents whose targets are penicillin-binding proteins (PBPs) (Fig. 7 ECO). This could be explained that PBPs-targeted agents can trigger SOS responses through DpiBA two-component signal transduction system in *E. coli* [27], and SOS responses mostly take place under the circumstance of DNA damage. Quinolones also cause DNA damage, so acids from quinolones and others antibiotics' tight connection maybe the results of similar stimuli origins. From Fig. 7, another distinguished difference was that quinolones had more negative correlation with cell wall-targeted agents in *P. aeruginosa* than in *E. coli*. This might give some hints, at least for *P. aeruginosa*, that quinolones and cell wall-targeted agents showed synergy when used in combination [28]. The answer may be found from differential control of Rpo S regulatory systems in these two bacteria [29].

4. Conclusions

The established CE method proved its utility for metabolomic study. This metabolomic profiling strategy targeted at TCA cycle and partial glycolysis pathways. By investigation of the acid concentration fluctuation from two typical strains with varied oxygen dependence properties, we could differentiate both species under different antibiotics' stimuli. After correlation analysis, mechanism difference could be deduced. Subsequently, synergy action among antibiotics also could be discerned. In our point of views, if the metabolites entered into the detection scope augment, much more informational results will be obtained, and maybe cause the antimicrobial agents study more easily and simplify the clinical research in antibiotic therapy and antibiotic resistance which are becoming a more and more alarming problems worldwide.

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