Nontargeted Metabolomics Analysis of Oxacillin Resistance in Staphylococcus Aureus

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Research Article

Keywords: Staphylococcus aureus, antibiotic resistance, oxacillin, nontargeted metabolomics

Posted Date: July 13th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-540791/v2

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Abstract

Background

*Staphylococcus aureus* has acquired resistance to antibiotics in the long-term struggle against antibiotics. Treatment of *Staphylococcus aureus* infection has become more difficult. In this study, based on nontargeted metabolic figure printing technique, the metabolome of a pair of isogenic methicillin-susceptible and resistant *Staphylococcus aureus* (MSSA and MRSA) strains treated with the sublethal dose of oxacillin was characterize to investigate the mechanism of antibiotic resistance.

Results

Massive alternations of metabolite expression were observed in both MSSA and MRSA treated with oxacillin. The results of accurate mass and mass fragmentation analysis showed that 7 and 29 metabolites of MRSA and MSSA have changed significantly after oxacillin treatment. The dysregulated metabolites suggested that CoA and fatty acids could help *Staphylococcus aureus* survive under antibiotic stress. Metabolic pathways engaged in antibiotic resistance were discovered through pathway enrichment analysis. The enriched pathways suggested that DNA repairing and flavin biosynthesis are universal pathways to help MSSA and MRSA survive under antibiotic stress. Compared with MSSA, MRSA systematically and effectively fight against oxacillin through precisely controlling energy producing, PBP2a substrate biosynthesis and antioxidant function.

Conclusions

Coenzyme A and fatty acids help both MSSA and MRSA survive under the antibiotic stress. MSSA was susceptible to oxacillin and was forced to response. On the contrary, MRSA systematically and effectively fight against oxacillin. The different metabolome responses of MSSA and MRSA provide us with new insights into how *Staphylococcus aureus* develops antibiotics resistance.

Background

*Staphylococcus aureus* (*S. aureus*) produces virulence factors and causes infection diseases ranging from minor skin infections to life-threatening deep infections [1]. Methicillin is a β-lactam antibiotic and was used to treat infections caused by penicillin-resistant *Staphylococcus aureus* since 1959. However, *S. aureus* had acquired resistance to methicillin through the infection treatment [2]. This pattern of resistance then spread to the community and has become an inherent pattern of antimicrobial resistance [3]. According to statistics, methicillin-resistant *Staphylococcus aureus* (MRSA) infections have been prevalent in hospitals around the world in the past 40 years [4,5]. Infections caused by MRSA have increased morbidity and mortality, and greatly increased the burden of medical care resources [6,7]. Treatment of these infections has become more difficult. Understanding the MRSA antibiotic resistance is of great importance in new antibiotics development and infection treatment.
Studies have shown that the methicillin resistance gene (mecA) is present in all MRSA strains and is part of the mobile genetic element. mecA directs the synthesis of penicillin-binding protein 2a (PBP2a; also called PBP2') \[8,9,10\]. PBPs catalyze the transpeptidation reaction, which is necessary for cell wall formation and peptidoglycan chains cross-linking \[11\]. MRSA synthesizes PBP2a instead of PBPs, which could block all β-lactam binding without affecting the transpeptidation reaction \[12,13\]. The low affinity of PBP2a to all β-lactam antibiotics allows MRSA to survive even when exposed to extremely high concentrations of β-lactam antibiotics.

The researchers proved that the acquisition of microbial antibiotic resistance was a global change from a transcriptome and proteome view \[14,15,16\]. Antibiotics can affect many signal transduction or metabolic pathways by triggering specific transcriptional responses. Alterations within the metabolic pool reflect the adaptive cascade at transcriptome and proteome levels, and represent the definite physiological status of bacteria. In addition, metabolites have signal transduction and regulatory functions, they are important links between gene expression, protein biosynthesis and metabolic pathway regulating. Thus, a comprehensive metabolome study on metabolic response of the S. aureus to a given antibiotic stress should help to better understand the physiology of S. aureus antibiotic resistance. Profiling antibiotic-induced metabolic changes of S. aureus by metabolome methods will provide new insights into the key players in the antibiotic response of the bacteria and contribute to infection treatment.

There were a few studies about the antibiotic resistance of S. aureus. The metabolic changes of S. aureus under different kinds of antibiotics were monitored. Targeted metabolic profiling of MRSA in response to methicillin using HPLC-MS/MS was conducted by Zhu et al. \[17\]. In Zhu’s study, massive metabolic shifts were induced by antibiotics in S. aureus. Somerville et al. investigated metabolic adaptation in the vancomycin-intermediate S. aureus (VISA). VISA gained adaptability to vancomycin by promoting acetogenesis and purine biosynthesis \[18\]. Overall, metabolites that engaged in purine and pyrimidine synthesis, central carbon and amino acid metabolism were dysregulated in antibiotics treated S. aureus \[19\].

Metabolomics is an efficient way to study phenotype variations caused by gene mutation, environmental influence, or disease. Currently, metabolic profiling and metabolic fingerprinting are the two complementary approaches for metabolomic investigation. Metabolic profiling is also called targeted metabolomics, it focuses on the analysis of a group of compounds either a class of chemicals or metabolites related to a specific metabolic pathway \[20\]. While metabolic fingerprinting is nontargeted metabolomics which globally compares metabolic patterns or “fingerprints” that changed in response to toxin exposure, disease, genetic or environmental alteration \[21\]. In this study, nontargeted metabolomics was employed to study oxacillin resistance in S. aureus for the first time. We profiled the global metabolic changes of MRSA and MSSA under sublethal dose of oxacillin to understand the resistance mechanism from a systematic view. Significantly changed metabolites in MRSA and MSSA were identified and characterized by combined accurate mass and mass fragmentation analysis. Metabolic pathways that
involved in oxacillin resistance were uncovered by pathway enrichment analysis. The commonalities and differences of MSSA and MRSA against oxacillin were unraveled.

**Methods**

**Cell culture and antibiotic treatment**

The *S. aureus* strains ATCC 25923 (MSSA) and ATCC 43300 (MRSA) were obtained from Huashan Hospital (Shanghai, China). Minimum inhibitory concentration (MIC) is determined by broth dilution method to establish the level of resistance and to determine an appropriate dosage. Both strains were cultured in Muller Hinton broth (Sigma-Aldrich, St. Louis, MO) with a series of concentrations of oxacillin at 35 ºC in an ambient air incubator for 24 h. MIC was determined as the lowest concentration of oxacillin in the medium without visible bacterial growth.

For antibiotic treatment, ∼1 × 10^6/mL bacterial cells were seeded in 50 mL of fresh culture medium and 1/8 of the MIC oxacillin were given to both MRSA and MSSA. Accordingly, MRSA and MSSA were treated with oxacillin at the concentrations of 2.0 and 0.0625 μg/mL, respectively. For both strains, control samples were seeded with the same density of bacterial cells and cultured under the same conditions but without any oxacillin treatment. Each group (oxacillin treated and untreated) has four biological replicates. When OD_{600} value (optical density at 600 nm) reached 0.7, the *S. aureus* cells were harvested.

**Sample preparation**

The *S. aureus* cells were harvested by centrifugation at 5000 × g for 10 min at 4 ºC. Cell pellets were collected and washed with 0.6% NaCl aqueous solution. 0.1 g wet cell pellets were suspended in 1 mL chilled extraction buffer (methanol/acetonitrile/water, 2:2:1, v/v/v). The cell disruption was done by fast cooling using liquid nitrogen, sonication at 4 ºC for 5 minutes and storage at -20 ºC for 1 h. The extraction mixture was centrifuged, the supernatant was condensed by SpeedVac and stored at -80 ºC before LC-MS/MS analysis.

**LC-MS/MS**

The ultrahigh performance liquid chromatography system (Waters, USA) coupled to a TripleTOF 4600 mass spectrometer (Sciex, Singapore) was used to analyze the metabolome extract samples. Both HILIC column (ACQUITY UPLC BEH HILIC, 1.7μm, 2.1× 150mm, Waters) and C18 column (ACQUITY UPLC BEH C18, 1.7μm, 2.1× 100mm, Waters) were used and the flow rate was set at 0.3 mL/minute, and the injection volume was 10.0 μL. When it is HILIC column, the mobile phase consisted of two components: (A) water/acetonitrile (95/5, v/v) with 5 mM ammonium acetate, (B) acetonitrile/water (95/5, v/v) with 5 mM ammonium acetate. The gradient began at 0 % solvent A, increased to 2 % in 3 min, 2 % to 7 % in 7 min, 7 % to 15 % in 4 min, 15 % to 20 % in 21 min, 20 % to 33 % in 3 min, 33 % to 63 % in 12 min, retained at 63 % B for 1 min, followed by 4 min 100 % solvent B. For C18 column analysis, mobile phase A (0.06% acetic acid in water) and B (pure acetonitrile) were used. The LC gradient began at 5% solvent B and
maintained for 3 min, increased to 25% in 8 min, 25% to 50% in 5 min, 50% to 100% for 20 min, retained at 100% B for 3 min and then back to 5%. The parameters of MS in positive ionization mode were applied for both chromatographic modes: IonSpray Voltage to 5000 V; Ion Source Gas flow to 35 L/h; Curtain Gas to 30 L/h, Source Temperature to 450 °C; Collision Energy to 35 V, Collision Energy Spread to 15 V.

Before the data acquisition, the mass spectrometer would be cleaned and adjusted to its best performance. The mass spectrometer is calibrated using commercial PPG solution. PPG solution was directly infused into the mass spectrometer at every six injection intervals to minimize the mass deviation. MS data were obtained by data-dependent acquisition (DDA) and the same setting for both HILIC and C18 analysis. The mass range for both TOF MS scan and Product Ion scan was set at 68–1300 mass/charge (m/z), the accumulation time was set at 0.1 second for TOF MS scan and 0.05 second for Product Ion scan. For the Switch Criteria, exclude isotopes with no exclusion were chosen for both scan types. The dynamic background subtract was selected for the whole data acquisition.

Quality Control

All the extracted metabolic samples were dissolved in 50 µL acetonitrile aqueous solution (1:1, v/v). Pooled samples for quality control purpose were prepared by mixing 10µL of each redissolved metabolic sample. QC samples were injected to monitor and overcome analytical drifts at regular intervals in UPLC–MS/MS data acquiring. Features with coefficient of variation (CV) < 30% were considered reproducible, as suggested elsewhere [22].

Data analysis

All the UPLC-MS/MS files were firstly converted to mzXML format via ProteoWizard software [23], and subsequently the generated mzXML data files were submitted to the XCMS online at https://xcmsonline.scripps.edu/. Parameters for feature detection were set according to the MS data overview [24]. The mass tolerance was set at 15 ppm, peak width from 0.1 to 3 min, prefilter intensity at 10 and noise filter at 3. Bandwidth was set at 5 for alignment, and analysis of variance (ANOVA) test was used for statistical analysis. The other parameters were set as the default. XCMS analysis was carried out in two steps. First, mzXML data files of three groups (control, oxacillin treated and QC) were submitted to XCMS for variations assessment. Then, mzXML data files of control and oxacillin treated groups were submitted to XCMS for statistical analysis (ANOVA). The overlapped features with unique m/z and retention time, as well as CV < 30%, were defined as metabolic features. Volcano plot of the metabolic features was generated by MetaboAnalyst. Metabolic features with p < 0.01, fold change > 2 or < -2 were considered as significantly changed metabolites due to the oxacillin treatment. Systematic pathway analysis was done by XCMS using the tentative identified significantly changed metabolites. The MS2 spectrums of significantly changed metabolites were extracted for further structure confirmation using MetFrag based on the METLIN metabolite database, Human Metabolome Database (HMDB) and LIPID MAPS structure database (LMSD) [27].

Data Availability Statement
The converted LC-MS data in mzXML format has been deposited to MassIVE [39]. The data files could be downloaded via ftp://MSV000087501@massive.ucsd.edu (User name: MSV000087501_reviewer, password: 3371) while the dataset is private. After publication, the data should be accessible at ftp://massive.ucsd.edu/MSV000087501/.

**Results And Discussion**

**Overview of the metabolome MS data**

Metabolic profiling of two *S. aureus* strains treated with or without oxacillin were analyzed by XCMS. Retention time correction was applied and the time deviations were illustrated in Figure S1. No peak drift was observed in total ion chromatograms after the retention time correction (Figure 1). The stability of data profiles demonstrated the robustness of our analytical method.

**Principal component analysis**

A number of detected ions aligned by their accurate masses, retention times, and peak areas were yielded after data processing. In order to verify whether *S. aureus* responded differently to oxacillin comparing to normal medium, a principal component analysis (PCA) model was established to evaluate the global changes in the metabolome. The plots (Figure 2) showed that for both MRSA and MSSA, the oxacillin treated samples were clustered together and separated from the oxacillin untreated samples, indicating that metabolic responses of *S. aureus* in the control and oxacillin treated group were different.

**Identified metabolites**

With the use of all two chromatographic modes (HILIC and C18) for the enhancement of metabolome coverage, around 8000 features were found, with the majority of the features obtained from HILIC chromatographic mode. The features were further processed and statistically evaluated with fold change analysis. Control and treated groups were compared, for both HILIC and C18 identification, features with a fold change ≥ 2 and p-value ≤ 0.01 were selected for metabolite identification. Features with low quality and intensity (chromatographic peak height < 10) were removed. All the remained features were searched against databases (HMDB, LMSD, and METLIN) for preliminary metabolite identifications. Metabolic profiles between oxacillin treated group and untreated group of MSSA and MRSA were compared in parallel, and metabolic changes were studied. In total, combining the preliminary identifications of HILIC and C18, 133 metabolites from MRSA and 523 metabolites from MSSA changed significantly after oxacillin treatment (Figure 3). In general, MSSA was susceptible and had more greatly changed metabolites in response to oxacillin comparing with MRSA, suggesting MRSA may has a systematic and effective way to fight against oxacillin while MSSA is forced to response.

The preliminary identified metabolites that only exist in plants and animals were removed. To further confirm the significantly changed metabolites, mass fragmentation analysis was performed. Mass fragments of all the remained differentially expressed metabolites were extracted and searched against
databases (HMDB, LMSD, KEGG) together with their parent ions to predict compound IDs using MetFrag [27]. Compound predictions with matching score > 0.9 were deemed as reliable identifications. In our study, through combined accurate mass and mass fragmentation analysis, eight differentially expressed metabolites were characterized in MRSA (table S1). Most of the differentiated metabolites were fatty acids and sterols which were located on cell membrane. It is reported that lipids biosynthesis will facilitate functional membrane microdomains (FMMs) and promote PBP2α oligomerization to resist β-lactam antibiotics [28]. Pantothenate and Glutamyl-Proline were the mostly changed metabolites in MRSA due to oxacillin exposure. The two metabolites and their derivatives were also observed in Zhu’s study using targeted metabolomics. Pantothenate is the precursor for CoA biosynthesis and was the mostly up-regulated metabolite in MRSA due to oxacillin treatment. This finding is consistent with our previous proteomic research result that pantothenate and CoA biosynthesis is critical for MRSA antibiotic resistance [29]. Glutamyl-Proline was mostly down regulated in MRSA after oxacillin treatment. In MSSA, through accurate mass and mass fragmentation analysis, twenty nine dysregulated metabolites were further characterized (table S2). Among the twenty nine metabolites, more than half were also observed in Zhu’s study. The consistency between our results and Zhu’s results using targeted metabolome approach provided independent validation that our nontargeted metabolomics method and data analysis workflow were reliable [17]. Figure 4 showed two representative metabolites of MSSA, adenine and (E)-4-stilbenol, which were up-regulated or down-regulated by more than 50 times after oxacillin treatment. It is speculated that oxacillin caused DNA damage to MSSA, thus MSSA synthesized large amounts of adenine in order to repair the DNA damage. A few metabolites related to fatty acids synthesis were dysregulated in MSSA treated with oxacillin. For example, stearoylglycine and pristanoylglycine were involved in fatty acids synthesis. In parallel, N-((R)-Pantothenoyl)-L-cysteine was up regulated as it was the essential component in CoA biosynthesis. By taking the union of the intersecting dysregulated metabolites from MRSA and MSSA due to the oxacillin treatment, the biosynthesis of CoA and fatty acids is believed to contribute to the survival of S. aureus under antibiotic stress.

**Metabolic pathway and function analysis**

A metabolic pathway analysis facilitating further biological interpretation was performed using XCMS online to reveal the most relevant pathways in oxacillin resistance. Bacterial metabolic pathways with p-value ≤ 0.01 and putative metabolites overlapping percentage larger than 50% were considered to be relevant pathways of oxacillin resistance (table 1). Common oxacillin-resistant pathways in MSSA and MRSA were found. The results showed that the metabolic pathways belonging to nucleoside and nucleotide biosynthesis or nucleoside and nucleotide degradation were enriched in MSSA and MRSA after oxacillin treatment, suggesting a widespread DNA damage in both MSSA and MRSA [30,31]. Besides, the DNA damage was caused by oxacillin induced oxidative stress. It seems that DNA repair and other metabolic pathways could counteract DNA damage in MRSA instead of MSSA. The speculations were demonstrated by only detecting extremely high intensity (ten times of untreated group, table S2) of 8-Hydroxy-deoxyguanosine (8-oHdG) in MSSA. 8-oHdG is a biomarker of oxidative DNA damage [32]. Flavin biosynthesis was influenced by oxacillin in both MRSA and MSSA. Flavin was proved to play an important role in the antibiotic tolerance of Escherichia coli [33], deficiency in flavin biosynthesis caused
a 10 fold mortality rate in antibiotic treated *Escherichia coli*. In our study, flavin biosynthesis has been shown to be related to the antibiotic resistance of *S. aureus* for the first time.

Compared with MSSA, MRSA has more pathways involved in oxacillin resistance (table 1). The enriched metabolic pathways in oxacillin treated MRSA proved that antibiotic treatment significantly perturbed the metabolism of bacterial, which is consisted with several other published studies [34,35]. Coenzyme A biosynthesis pathway was enriched in oxacillin treated MRSA. This finding further confirmed the result of our previous proteome study on MRSA's antibiotic resistance [29] that Coenzyme A contributed to MRSA antibiotic resistance. The greatly altered central carbon metabolism (succinate to cytochrome bd oxidase electron transfer) in antibiotics treated MRSA was also found by other researchers [18]. Nucleotide metabolism, TCA cycle and amino acids biosynthesis are enriched in MRSA to provide proteins/enzymes and energy for oxacillin resistance, which means antibiotic resistance is an energy-consuming and enzyme-rich biological process. According to some previous studies by other researchers, PBP2a expression is critical in MRSA survival under antibiotic stress because PBP2a has low affinity to antibiotics [8-10]. In our study, the biosynthetic pathways of PBP2a substrate (UDP-N-acetylmuramoyl-pentapeptide biosynthesis II (lysine-containing), UDP-N-acetylmuramoyl-pentapeptide biosynthesis I (meso-DAP-containing)) were firstly proved to be associated with MRSA antibiotic resistance from a direct metabolome approach. A number of enriched metabolic pathways indicated that oxidation and reduction were important ways of detoxication in MRSA. Flavoprotein cofactors (flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD)) participated in cellular redox metabolism [36], and in our study flavin biosynthesis I was observed to be enriched in oxacillin treated MRSA. 4-hydroxy-2-nonenal detoxification pathway could alleviate cytotoxic stress and improve the ability of MRSA to resist oxidative stress [37]. In addition, mevalonate biosynthetic pathway leads to the production of isoprenoid quinones which are the most important compounds participated in biological detoxication of living organisms. Isoprenoid quinones contribute to MRSA's anti-oxidative stress activity by being the electron and proton carriers that involved in redox balancing [38,39]. All statistically significant enriched pathways revealed the systematic survival mechanisms of MRSA under antibiotic stress, of which anti-oxidative stress is a crucial part.

**Conclusion**

In our study, MRSA and MSSA were exposed to a subinhibitory dose of oxacillin. Nontargeted metabolome analysis was utilized to reveal the *S. aureus* cellular responses to oxacillin. The metabolic profile shifts of both strains were robust and significant when treated with oxacillin. By relative quantification, 133 metabolites from MRSA and 523 metabolites from MSSA were found to be differentially expressed due to oxacillin treatment. Among the metabolites, eight and twenty-nine metabolites were further characterized by combined accurate mass and mass fragmentation analysis. Metabolic pathway analysis showed that DNA damage was caused by antibiotic in both MSSA and MRSA. DNA repairing and flavin biosynthesis are metabolic pathways that contribute to antibiotic resistance in both MRSA and MSSA. Compared with MSSA, MRSA systematically and effectively
responded to oxacillin stress by enriching a number of metabolic pathways with anti-oxidative stress function.

**Abbreviations**

MSSA: methicillin-susceptible *Staphylococcus aureus*

MRSA: methicillin-resistant *Staphylococcus aureus*

*S. aureus*: *Staphylococcus aureus*

mecA: methicillin resistance gene

PBP2a: penicillin-binding protein 2a

VISA: vancomycin-intermediate *S. aureus*

PCA: Principal component analysis

ANOVA: Analysis of variance

FMMs: functional membrane microdomains

FMN: flavin mononucleotide

FAD: flavin adenine dinucleotide

HMDB: Human Metabolome Database

LMSD: LIPID MAPS structure database (LMSD)

KEGG: Kyoto Encyclopedia of Genes and Genomes

8-oHdG: 8-Hydroxy-deoxyguanosine

**Declarations**

**Acknowledgments**

We thank Mr. Kam Tim TANG from the Department of Chemical and Biological Engineering of HKUST for his assistance in the LC-MS instrument management.

**Funding**

This study was supported financially by the Research Grants Council of the Hong Kong Special Administrative Region Government (Grant No. 16100415 and C5031-14E), National Natural Science
Supplementary information

Supplementary

Consent for publication

Not applicable.

Competing interests

The authors declare that there are no conflicts of interest.

Author Contributions Statement

J JL designed the experiments. J JL, ZCY and TYW performed the experiments. J JL and CCQ performed the data analysis. J JL and TYW prepared the manuscript. XFS and HL provided technical and editorial assistance.

References


Tables
Table 1 A systemic view of metabolic pathways that associate with oxacillin resistance in this study.
<table>
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<tr>
<th>Strains</th>
<th>Pathway</th>
<th>Overlapping putative metabolites&lt;sup&gt;1&lt;/sup&gt;</th>
<th>All metabolites&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Overlapping percent</th>
<th>p-values</th>
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<td>5</td>
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1 Number of significantly dysregulated metabolites found in pathway.

2 Total number of putative metabolites found in the pathway using the entire LC-MS analysis results.

*Nucleoside and Nucleotide Biosynthesis

Δ Nucleoside and Nucleotide Degradation

**Figures**

**Figure 1**

Typical total ion chromatographs after correction. (A) Chromatographs of MRSA global metabolites using HILIC column, (B) Chromatographs of MRSA global metabolites using C18 column. Biological samples and replicates are represented by using different color, (C) Chromatographs of MSSA global metabolites using HILIC column, (D) Chromatographs of MSSA global metabolites using C18 column. Biological samples and replicates are represented by using different color.

**Figure 2**

PCA results of oxacillin treated and untreated S. aureus strains (MRSA and MSSA) using HILIC column (A, C) and C18 column (B, D). Biological replicates are used and marked in the same color, the blue dots
represent the QC pool samples.

**Figure 3**

Volcano plot of differentially expressed features in oxacillin treated and untreated S. aureus strains (MRSA and MSSA) using HILIC column (A, C) and C18 column (B, D). Differentially expressed features are deemed significant if \( p < 0.01 \) and absolute fold change > 2. Pink dots represent significantly up-regulated and down-regulated features, and black dots represent other features.
Figure 4

Representative metabolites that have mostly changed in MRSA and MSSA due to oxacillin treatment.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementaryexcel.xlsx
- supplementaryv2.doc