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Pseudomonas aeruginosa develops Ciprofloxacin resistance from low to high level with distinctive proteome changes

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Abstract

Pseudomonas aeruginosa infection is very difficult to treat because of its drug resistance, but how it develops drug resistance remains largely unknown. In this study we investigated Ciprofloxacin resistance development in *P. aeruginosa*. Different Ciprofloxacin concentrations selected different low level resistant mutants, and high level resistant mutants emerged from low level resistant mutants if stressed further by Ciprofloxacin. A deep quantitative proteomic study of the Ciprofloxacin resistant mutants uncovered the cellular pathways that supported such resistances. The two low level resistant mutants had different molecular mechanisms. One was mainly due to the switching to anaerobic respiration, and the other was probably due to DNA repair, polyamine and ion. High level of resistance involved the mexCD-oprJ efflux pump and the downregulation of PQS quorum sensing. Other pathways might also have contributed to high level resistance, like the anaerobic respiration, protein degradation and DNA repair. The intracellular Ciprofloxacin concentration assay indicated that mexCD-oprJ overexpressed mutants had low drug accumulation. This study provided a comprehensive overview of the proteomic landscape in the evolution of Ciprofloxacin resistance in *P. aeruginosa*, and might have implications in diagnosis and treatment of Ciprofloxacin resistant *P. aeruginosa*.

Introduction

The gram negative bacterium *Pseudomonas aeruginosa* is a human pathogen, which accounts for about 10% of hospital acquired infections, and is persistent in airways of cystic fibrosis (CF) patients[1-3]. Ciprofloxacin is very efficacious against wild type *P. aeruginosa* with minimum inhibitory concentration (MIC) of about 0.1 µg/ml, however, resistance emerged during Ciprofloxacin treatment with low level resistance (MIC <2 µg/ml) to clinically significant resistant level[4, 5].

The process of *P. aeruginosa* developed resistance against Ciprofloxacin remains largely unknown. H.C. Su, et al.[6] showed that under laboratory conditions, the resistance developed in multiple steps - after the initial mass killing, the non-inheritable Ciprofloxacin tolerant *P.aeruginosa* cells mutated to be heritably Ciprofloxacin resistant, in which pre-existent cellular networks were involved in drug resistance, though only a few proteins were identified.

Among the proteome of *P. aeruginosa* (5570 open reading frames), thousands of genes are associated to functions related to drug resistance, like cell regulation, import of nutrients, metabolism, efflux systems, protein secretion[7] . We hypothesized that a comprehensive proteomic study might reveal more proteins and cellular networks in Ciprofloxacin resistance. Here we discovered two levels of resistant mutants: the low level resistant mutants had MIC of 1 µg/ml, but these low level mutants could become high level (MIC 16 µg/ml) resistant when further treated with Ciprofloxacin. We performed a deep quantitative proteomics study on different drug resistance mutants, which provided a comprehensive proteomic view of *P. aeruginosa* resistance to Ciprofloxacin.

P. aeruginosa is inherently resistant to drugs because of its less permeable cell wall and a variety of efflux pumps[8]. We measured Ciprofloxacin accumulation in wild type *P. aeruginosa* and in all the mutants to provide direct evidence of permeability and effluxing. The Ciprofloxacin accumulations together with the proteomic changes give insights into the resistant mechanism.

Experiment procedure

Bacterial strain and growth

P. aeruginosa strain PAO1 was purchased from ATCC (BAA-47). All liquid cultures were grown at 37°C with aeration in a 500-ml flask with 50 ml of Tryptic Soy Broth medium. Colony forming unit (CFU/ml) was determined by serial dilution plating on Tryptic Soy Agar after 48-hour incubation at 37°C.

Ciprofloxacin resistance mutant selection

The Ciprofloxacin mutants selection scheme was shown in Figure 1. *P. aeruginosa* PAO1 was cultured overnight followed by 1:100 dilution to 50ml Tryptic Soy Broth medium and cultured for 3 hours at 37°C / 220rpm (cell number reached about 1×10^9 cfu/ml), then Ciprofloxacin was added to a final concentration of 0.125, 0.25, 0.5, 1(μ g/ml), separately in each culture, and continued culturing for 48 hours. Initial mass killing was observed (data not shown), and there was no mutant arose from 1 μ g/ml Ciprofloxacin treatment. Mutant from 0.125 μ g/ml Ciprofloxacin treatment was named mu0125_1, and mutant from 0.5 μ g/ml Ciprofloxacin treatment was named mu05_1. Both mu0125_1 and mu05_1 had MIC of 1 μ g/ml. Then mu0125_1 and mu05_1 were further stressed with higher Ciprofloxacin concentrations – 1, 2, 4, 8 (μ g/ml), mutants could be selected from 2 μ g/ml Ciprofloxacin treatment, and mutant originated from mu0125_1 was named mu0125_h, mutant originated from mu05_1 was named mu05_h, both of which had MIC of 16 μ g/ml.

MIC measurement

MIC was measured by serial broth dilution in 96 multiple well plates. 50 μ l of *P. aeruginosa* culture (2×10^5 cfu/ml) was mixed with 50 μ l Ciprofloxacin solution (serial dilution in Tryptic Soy Broth) and cultured at 37°C for 18 hours. The MIC results were from three independent experiments.

Intro-cellular drug accumulation assay

This was based on a previous publication[9]. Briefly *P. aeruginosa* was cultured to OD_{600nm} 0.5, buffer exchanged into PBS with 0.4% glucose at room temperature, and adjusted OD_{600nm} to 1. Then Ciprofloxacin was dosed at designated concentrations in duplicates. At 5, 15, 30, and 60 (mins), 0.5ml of cells was taken, and centrifuged through a double silicon oil layer at 13,000 xg for 5mins. The cell pellets were extracted by 50 μ l of 50% acetonitrile and 0.1% trifluoroacetic acid for 4 hours. After centrifugation at 10,000 xg for 30mins, the supernatant was used for LC/MS/MS analysis.

Quantification was carried out on an AB Sciex QTRAP4000 triple quadrupole mass spectrometer coupled with Agilent 1200 HPLC. Transition 332.5/314.5 was chosen and all parameters optimized. The HPLC column was Agilent Zorbax SB-18 (15cm x 2.1mm I.D, 5 μ m). Mobile phase A was 0.1% of formic acid, mobile phase B was acetonitrile with 0.1% formic acid, and the flow rate was 0.3 ml/min. The gradient was: 0 mins – 20% B, 4mins -80% B, 5mins -80% B, 6mins 20% B. Ciprofloxacin standards was diluted in 50% acetonitrile 0.1% trifluoroacetic acid extract from *P.ae* to compensate for matrix effect. Ciprofloxacin amount was quantified by external standard. The intracellular concentration was based on the assumption that *P. aeruginosa* cell volume was 1 μ m³.

Proteome sample preparation, LC/MS/MS measurement and data analysis

P. aeruginosa PAO1 wild type and mutants were cultured in 5 independent experiments in Tryptic Soy Broth to OD₆₀₀ 0.5, then 25ml from each batch was

harvested at 3750 xg for 10mins at room temperature followed by three times washing with ice cold PBS. The cell pellets were lysed by 1ml of 1% sodium dodecyl sulfate 50mM Tris (pH 8). Protein concentration was determined by BCA assay.

Protein digestion was carried out using filter assisted digestion[10]. Cell lysate was reduced by 20mM of DTT at 95°C for 5mins followed by alkylated with 50mM of iodoacetamide, then 30 microliter of cell lysate mixed with 300 µl of 8M urea and filtered through 3k cutoff membrane, washed with 50mM ammonium bicarbonate buffer followed by digestion overnight at 37 °C with 1 µg of trypsin. After trypsin digestion, the tryptic peptides were desalted using Waters SepPak C18 cartridge followed by vacuum drying. The tryptic peptides were re-dissolved in 0.1% of formic acid, and its concentration measured by Nanodrop.

Peptides were separated by nano-flow HPLC (Easy-LC 1000, Thermo Fisher Scientific) coupled online to an Orbitrap Fusion (Thermo Fisher Scientific). The column was home packed with 3 µm ReproCil-Pur C18-AQ (Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) with a dimension of 50cm long and 75µm inner diameter. Mobile phase A was 0.1% formic acid, and mobile phase B was acetonitrile with 0.1% of formic acid. The peptides were eluted at 300nl/ml with the following gradient over 2 hours: 3-25%B in 80mins; 25-35%B in 20mins; 35-45%B in 8mins; 45-85%B in 2mins and 85% for 8mins. Data was acquired using the top speed method (3s cycle). In every three second, a full scan MS at resolution of 120,000 at 200 m/z mass was acquired in the Orbitrap with a target value of 4e5 and maximum injection time of 60ms, Peptides with charge states 2-4 were selected from the top abundant peaks by the quadrupole for high energy collisional dissociation (HCD) MS/MS, and the fragment ions were detected in the linear iontrap with target AGC value of 1e4 and maximum injection time of 250ms. The dynamic exclusion time was set at 40s. Precursor ions with no charge states were not fragmented.

Label-free quantitative data analysis was performed using MaxQuant (version 1.5.0.0), integrated with Andromeda, the probabilistic database search engine[11]. A file of 247 common contaminants was used to identify contaminants. The *P. aeruginosa* protein sequences were downloaded from Uniprotkb (5638 sequences)[12]. Enzyme specificity was set as trypsin with 1 missed cleavage site. Carbamidomethylation of cysteine was set as fixed modification while N-terminal acetylation and methionine oxidation were set as variable modifications. The second peptide identification in Andromeda was enabled. False positive rate of 0.01 was required for peptides and proteins. Minimum length of peptide was set to 7 amino acids. At least 2 peptides were required for protein identification. The precursor mass error of 20ppm was set for the first search, and 6 ppm for the second search, while for the fragment ions, the mass error was set as 0.3Da. Label-free quantitation was enabled.

The protein group results from MaxQuant analysis were imported to Perseus 1.4.1.3[13]. After the removal of contaminants, reverse matches and only identified by sites, the LTQ values were logarithm 2 transformed. The biological

replicates were grouped into wild type and mutant groups. Only proteins with at least 3 valid LTQ values in each group was used for quantification. Two-tailed t-tests were performed on mutant versus wild type. Protein-protein interaction network was analyzed by STRING (v10)[14]. Information of protein function and pathway assignment was retrieved from the Pseudomonas genome database (www.pseudomonas.com)[15] and KEGG[16, 17].

Results and discussion

Ciprofloxacin resistance evolved from low resistance to high resistance

We used the same time frame and cell numbers as reported before to generate the Ciprofloxacin resistant mutants[6]. For Ciprofloxacin concentration, since very low Ciprofloxacin might not be clinically relevant, our experiment started from MIC upwards - 0.125, 0.5, 1, 2 ($\mu\text{g/ml}$) of Ciprofloxacin, and found the mutation prevention concentration (MPC) was 1 $\mu\text{g/ml}$. Mu0125_l from 0.125 $\mu\text{g/ml}$ Ciprofloxacin and mu05_l from 0.5 $\mu\text{g/ml}$ Ciprofloxacin all had low level of resistance against Ciprofloxacin (MIC 1 $\mu\text{g/ml}$). The relative high value of MPC against *P. aeruginosa* meant opportunities for *P. aeruginosa* growing in biofilm and in sputum to develop resistance, as the maximum plasma concentration for orally administered 500mg Ciprofloxacin is about 2 $\mu\text{g/ml}$, and it takes time for the drug to diffuse to the *P. aeruginosa* sites[18].

When mu0125_l and mu_05 were further subject to 1, 2, 4, 8 $\mu\text{g/ml}$ of Ciprofloxacin treatment separately, further mutations were selected from 2 $\mu\text{g/ml}$ dosage. Mutant arose from mu0125_l was named mu0125_h and mutant arose from mu05_l was named mu05_h, both of which were highly resistant to Ciprofloxacin and clinically significant with MIC of 16 $\mu\text{g/ml}$. This demonstrated clearly two distinctive steps in the evolution of Ciprofloxacin resistance by *P. aeruginosa*. It is interesting to note that the plasmid coded proteins Qnr(s) could only provide low-level of resistance, but it provided a gateway to high-level of resistance[5]. So it is crucial to understand the mechanism of low level resistance.

Proteomic analysis of *P. aeruginosa*

In this proteomic study, we used label-free quantification, which was also employed by other proteomic studies on *P. aeruginosa*[19-21]. The quantification benefited from the top-speed method in the Orbitrap Fusion which ensured enough MS1 acquisition (every 3s) across the chromatographic peaks, hence more accurate peak shapes and accurate quantification.

All together there were 3251 proteins identified which is 57.6% of the 5638 sequences in the UniprotKB database, 2498 proteins were quantified in mu0125_l and mu_0125_h and 2457 proteins were quantified in mu05_l and mu05_h. These results exceeded previous proteomic studies on *P. aeruginosa* and were the deepest *P. aeruginosa* proteome so far according to our knowledge. There might be more ORFs not been detected, but we settled with this non-fractionation approach for label-free quantification. Five independent biological

replicates were performed and results statistically tested. Only proteins with two fold changes of their abundances were selected for protein-protein interaction analysis by STRING, KEGG pathway analysis and manual interpretation of protein functions.

Two distinctive low level Ciprofloxacin resistant mutants

The low level Ciprofloxacin resistant mutant Mu0125_I

For mu0125_I, 57 proteins were upregulated and 74 down regulated compared to wild type *P. aeruginosa* (>2-fold change, $p < 0.05$) (Table S1, Table S2, Figure 2). Anaerobic respiration was clearly adopted with all the denitrification enzymes up-regulated from nitrate reductase to nitrous oxide reductase. The advantage of anaerobic respiration over aerobic respiration is it might produce less reactive oxygen species as it does not use oxygen as electron acceptor. The mutation to anaerobic respiration was defensive against oxidative stress from Ciprofloxacin[22].

Arginine fermentation and anaerobic respiration are regulated by Anr[23]. Here we found the arginine deiminase pathway, arginine dehydrogenase pathway and urease were all up-regulated. Besides the carbon, nitrogen and energy from arginine fermentation, the main function of arginine deiminase pathway might be the ammonia production, hence raised the cellular pH, which provided a more reductive environment.

KatA, cytochrome C551 peroxidase and Alkyl hydroperoxide reductase AhpD were upregulated. Catalase and peroxidase decompose H_2O_2 thus prevent the highly reactive hydroxyl radical. Among the three known catalases in *P. aeruginosa*, KatA, KatB and KatE, KatA was shown to be crucial for adaptation to H_2O_2 and osmotic stresses[24, 25]. C551 peroxidase takes not only H_2O_2 as substrate but also other peroxides like lipid peroxides, etc., which, together with Alkyl hydroperoxide reductase AhpD added the reductive and repair range against oxidative stress.

NrdJ α and NrdJ β upregulation were class II ribonucleotide reductases, which are essential for cell growth, were upregulated in anaerobic respiration[26, 27]. The upregulation of polyphosphate kinase 2 (PPK2) might protect the cells against antibiotic stress[28]. Peptidylprolyl isomerase (Q9HXD5) was upregulated[26]. Universal stress protein (Q9HW73) was controlled by Anr and upregulated [29].

P. aeruginosa is known to import nutrients from the environment, here branched amino acids, threonine and alanine import were increased. One porin, oprG, was upregulated.

The upregulated beta lactamase (Q9HW53) might provide resistance to lactams.

Drug resistance often comes at the cost of fitness and virulence[30]. This was the case for mu0125_I. The type VI secretion system was downregulated, which

meant mu0125_l might be less virulent. In addition, the exopolysaccharide Psl production was reduced[31], and pyoverdine and pyochelin (siderophores) were downregulated (Table S2).

The low level Ciprofloxacin resistant mutant Mu05_l

For mu05_l, there were 40 proteins upregulated and 24 proteins down regulated compared with wild type *P. aeruginosa* (>2-fold change, $p < 0.05$) (Figure 3, Table S3, Table S4). The nutrients importing proteins were among the most upregulated, like those for ion, polyamine, amino acids, sugar, sulfate and phosphate. Other upregulated pathways were protein translation, protein oxidative folding disulfide isomerases (DsbA and DsbG), and lipid metabolism.

The environmental factors are playing an important role in drug resistance, like the magnesium concentration, polyamine (putrescine, spermine and spermidine) were reported to induce resistance to multiple drugs in *P. aeruginosa*[32]. In addition to binding to LPS and conferring resistance to cationic peptide antibiotic, spermidine accumulation inside the cells was needed to provide Ciprofloxacin resistance[33, 34]. Here potD but not potH was responsible for polyamine uptaking.

P. aeruginosa mainly takes iron in two ways: siderophores and heme, both of which were upregulated. TonB dependent receptor is responsible for siderophores transport, which is the most upregulated (12 fold). Depletion of iron by chelating or replacing iron with Gallium, an iron homologue, has shown to be deleterious to *P. aeruginosa*[32]. Because of the pervasive role of iron in the physiology of *P. aeruginosa*, the signaling pathway affected by iron may vary for different conditions and drugs.

The operon (PA3002-PA3003) was reported to be involved in drug resistance, in which PA3003 was a mutator gene for Ciprofloxacin resistance[35]. PA3003 is a hypothetical protein, but PA3002 displaced the stalled RNA polymerase and facilitated DNA repair. The upregulation of PA3003 might be also involved in DNA repair.

The main downregulated pathway was nitrogen metabolism (Table S4). The downregulation of oxidative phosphorylation reduced the reactive oxidative species.

From low level resistance to high level resistance

High level Ciprofloxacin mutant Mu0125_h

Fifty-eight proteins were upregulated while 91 proteins were downregulated in compared to wild type *P. aeruginosa* (>2-fold change, $p < 0.05$) (Figure 4, Table S5, Table S6). The most upregulated proteins were mexCD-oprJ efflux pump, which has a very low basal expression in mu0125_l and wild type *P. aeruginosa*.

MexCD-oprJ was regulated by nfxB, interestingly the protein product of nfxB was also overexpressed. The mexCD-oprJ has been reported to provide resistance to Ciprofloxacin[36-38], and its efficiency was shown in the section of Ciprofloxacin accumulation assay (Figure 6).

The arginine deiminase pathway was kept from mu0125_l together with some denitrification proteins. Nitrate-inducible formate dehydrogenase is a selenoprotein, hence selenocysteine-specific elongation factor was upregulated. There were more oxidoreductases, like katB, thioredoxin, and ferric reductase to cope with the oxidative stress.

For the downregulated proteins (Table S6), the most affected signaling pathway was the Pseudomonas Quinolone Signal (PQS) quorum sensing[39]. Chitinase[40] was down 11 fold, and PqsB, PqsC and PqsD were all downregulated. Interestingly, quorum sensing was reported lost in clinical isolates from CF patients[41].

High level Ciprofloxacin mutant Mu05_h

Forty-seven proteins were upregulated and 66 proteins were downregulated compared with wild type *P. aeruginosa* (>2-fold change, $p < 0.05$) (Figure 5, Table 7, Table 8). The most pronounced changes were proteins of the efflux pump – mexCD-oprJ. The efflux efficiency was shown in the section of Ciprofloxacin accumulation assay.

Compared to mu05_l, the sugar and dicarboxylate import was increased; only heme import for iron intake while the siderophores were no more upregulated. Metabolisms of branched amino acid, tricarboxylic cycle and oxidative phosphorylation were upregulated.

The increase of ClpA/B protease protects cells from stress by controlling protein aggregation[42]. DNA-binding protein HU- α was annotated with DNA stabilization and DNA repair. Pyocin and bacteriophage proteins were reported to be induced by Ciprofloxacin, and were part of the SOS signaling[43].

Interestingly, among the upregulated protein, there were two proteins (Q9IOX3 and Q9IOF6) involved in fluorobenzoate metabolism were upregulated. Whether or not Ciprofloxacin could be metabolized remains to be seen.

The most downregulated pathway (Table S8) was PQS quorum sensing. Chitinase was downregulated by 26-fold. The two component systems regulator ParR was down regulated.

Ciprofloxacin accumulation in *P. aeruginosa*

The direct way to determine the mutants' cell wall permeability and effluxing is to assay the intracellular Ciprofloxacin concentration. We used the silicon oil method to get rapid and reproducible results[9]. One of the concerns about drug

accumulation assay is the drug toxicity. However, at least for Ciprofloxacin, when the wild type *P. aeruginosa* was treated with 0.125 µg/ml up to 2 µg/ml (2 µg/ml is 16x MIC), the intracellular concentration remained linear (Figure 6). The reason could be the integrity of the cells was not damaged during such short a time.

The low level resistance mutants (mu0125_l and mu05_l) had the same Ciprofloxacin level as the wild type *P. aeruginosa*, which meant there was no permeation barrier and effluxing for Ciprofloxacin in the mutants, and the drug resistance was due to the up- and down-regulated proteins.

The high level resistant mutants (mu0125_h and mu05_h) had far less intracellular Ciprofloxacin from 5mins onwards compared to wild type *P. aeruginosa* and low level resistant mutants. This showed that the mexCD-oprJ efflux pump was very efficient to keep Ciprofloxacin at a low level very early on, and contributed to the high level and clinically significant resistance to Ciprofloxacin.

Conclusion remarks

In this study we discovered that Ciprofloxacin resistance in *P. aeruginosa* PAO1 evolved through two distinctive steps from low level to high level resistance. Low level resistance was the launch pin for high level resistance, like the plasmid transmitted Qnr(s), which only provided low level resistance but clinically significant mutants emerges eventually[5].

A deep quantitative proteomic study of the Ciprofloxacin resistant mutants uncovered the cellular pathways that underpinned such resistance. The two low level resistant mutants showed different molecular mechanisms. One was mainly due to the switching to anaerobic respiration, and the other was probably due to DNA repair, polyamine and ion.

High level of resistance involved the mexCD-oprJ efflux pump and the downregulation of PQS quorum sensing. Other pathways might also have contributed to high level resistance, like the anaerobic respiration, protein degradation and DNA repair. The intracellular Ciprofloxacin concentration assay indicated that mexCD-oprJ had indeed kept low drug accumulation.

This study provided a comprehensive overview of the proteomic landscape in the evolution of Ciprofloxacin in *P. aeruginosa*, and might have implications in diagnosis and treatment of Ciprofloxacin resistant *P. aeruginosa*.

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