RESEARCH ARTICLE

An important role of a "probable ATP-binding component of ABC transporter" during the process of *Pseudomonas aeruginosa* resistance to fluoroquinolone

Jinsong Zhou[1, 2], Dongyun Hao[2], Xudong Wang[1], Teimei Liu[1], Chengyan He[1], Feng Xie[1], Yanhong Sun[2] and Jin Zhang[2]

[1] The Third Hospital of Jilin University, Changchun, P. R. China
[2] Key Lab for Molecular Enzymology and Engineering of Ministry of Education, Jilin University, Changchun, P. R. China

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In order to find new drug target to eliminate the fluoroquinolone resistance, the *in vitro* progress of *Pseudomonas aeruginosa* fluoroquinolone resistance was mimicked, and then proteomic analysis was applied to comparing different protein profiles during the resistant process. The results show that the expression of a "probable ATP-binding component of ATP binding cassette (ABC) transporter" existed in ciprofloxacin-intermediate and -resistant strains, but not in sensitive strain. In addition, the ciprofloxacin concentrations in *P. aeruginosa* strains, which were obtained from the progress of *P. aeruginosa* fluoroquinolone resistance, were determined by means of HPLC; the results show that the decrease of the intracellular concentration of drug and the expression of this new protein nearly take place simultaneously. The changes of mRNA levels of the probable ATP-binding component of ABC transporter were detected by virtue of RT-PCR and showed that this protein did not express in the sensitive strains but expressed increasingly in the intermediate and resistant strains. In order to determine the relationships between the development of antibiotic resistance and this protein further, a DNAzyme was designed to aim at the mRNA of the probable ATP-binding component of ABC transporter directly; the ciprofloxacin resistance of *P. aeruginosa* was partially reduced *in vivo* by inhibiting the expression of this protein. This DNAzyme has no effect on sensitive strain. And the comparison of drug intracellular concentrations between DNAzyme-treated strains and its control strains shows that this protein may be included in the course of active drug efflux.

Keywords:
Ciprofloxacin / DNAzyme / Drug resistance / Probable ATP-binding component of ABC transporter / Proteomic analysis

1 Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is an adaptable bacterial saprophyte found in soil, water, sewage, the mammalian gut, and plants. It is also a common opportunistic pathogen for humans; it can cause many illnesses including cystic fibrosis, burn wounds, tissue injury, and immunosuppressive therapy in different patients [1, 2]. The fluoroquinolone is a useful group of antibacterial agents. It can rapidly inhibit DNA synthesis by forming quinolone–gyrase–DNA complexes, promoting the cleavage of bacterial DNA and resulting in rapid bacterial death [3]. However, *P. aeruginosa* can easily develop resistance to the fluoroquinolone during the process of treatment. The mechanism of the resistance is known as: (i) amino acid substitutions in the GyrA (one subunit of gyrase) and ParC (one subunit of topoisomerase IV) caused by gene mutation in the quinolone

Correspondence: Dr. Jinsong Zhou, The Third Hospital of Jilin University, Changchun 130023, P.R. China
E-mail: jinsongzhou@126.com
Fax: +86-431-5646978

Abbreviations: ABC, ATP binding cassette; **MICs**, minimum inhibitory concentrations

resistance determining regions (QRDRs), (ii) active drug efflux, such as the MexA-MexB-OprM, MexE-MexF-OprN, and MexC-MexD-OprJ efflux system, and so on, and (iii) the structural alteration of outer membrane protein, such as loss of oprF and lipopolysaccharide [4–7].

The drug resistance of *P. aeruginosa* has been a major problem in the practice medicine, although sequencing and annotation of *P. aeruginosa* genome is completed [8]. The information on functional genomics and recently developed proteomics provide us with new opportunities to fight against drug resistance.

However, the present studies of *P. aeruginosa* fluoroquinolone resistance focused on the initial and final stages of the drug resistance; some important details about the mechanism could not be clarified. In this study, the process of *P. aeruginosa* fluoroquinolone resistance was mimicked *in vitro*, and then differential proteomic analysis was carried out by comparison of the different protein profiles during the resistant process. In order to find new drug target to eliminate the fluoroquinolone resistance, a DNAzyme inhibition test was performed on the mRNA of protein that was selected by differential proteomic analysis.

2 Materials and methods

2.1 Materials

Six strains of *P. aeruginosa* were used in this study. PA01 (ATCC27853) was obtained from National Center for Clinical Laboratories, PA20, and PA22 were separated from the natural lake in this lab. PA02, PA03, and PA04 were clinical isolates that were collected from the China–Japan Hospital of Jilin University, China. PA03 and PA04 were resistant to fluoroquinolone, with ciprofloxacin minimum inhibitory concentration (MIC) 4 and 8 μg/mL, respectively; PA01, PA02, PA20, and PA22 are sensitive to all antibiotics. All of those six strains were stored at −80°C prior to use. Ciprofloxacin and chemicals used in in-gel digestion were obtained from Sigma-Aldrich. Mueller–Hinton (MH) broth was obtained from BioMérieux. All the materials used in 2-DE were obtained from Pharmacia, except a BlueRanger™ Prestained Protein Molecular Weight Marker which was from Pierce Chemical Company. PVDF membrane was from Millipore. Oligonucleotides (primers and DNAzyme) were synthesized with an Applied Biosystems 394 DNA synthesizer and purified by PAGE. TRIzol® Reagent in RNA isolation was purchased from Molecular Research Center. mRNA Selective PCR Kit was from Takara Shuzo. DNA molecular weight marker was purchased from Promega.

2.2 Mimicking the fluoroquinolone resistance process of *P. aeruginosa in vitro*

Two strains, PA01 and PA02, were used in ciprofloxacin susceptibility test (CST) performed by the macrodilution broth method according to the documents of the National Committee on Clinical Laboratory Standards (NCCLS) [9]. After the first CST, the bacterium representing MICs of ciprofloxacin was collected, a portion of this collection was suspended in sterile 0.9% saline to a density equivalent to the 0.5 McFarland standard for next MICs test, and the rest of the bacterium was stored in glycerol broth (Glycerol: MH-Broth = 1:1 v/v) at −80°C. The next step was to retest the collected bacterium's ciprofloxacin MICs as mentioned above, followed by collecting the bacteria representing ciprofloxacin MICs; the serial ciprofloxacin MIC test was repeated until the ciprofloxacin MICs of *P. aeruginosa* was 8 μg/mL. The code numbers of the two serial strains, which were obtained from the serial ciprofloxacin MIC tests, were represented by the two series of PA01(N) and PA02(N) ("N" representing the number of ciprofloxacin MICs test), these strains together with other *P. aeruginosa* strains were divided into three groups (see Table 1).

2.3 2-DE

All the *P. aeruginosa* strains in groups II, III, and I were used in 2-DE. Samples for the 2-DE analysis were prepared as follows. After being collected by centrifuging (5000 × *g* for 5 min) and washed with MilliQ water five times in order to completely remove proteins in MH-Broth, the deposit was suspended in MilliQ water and the OD was measured spectrophotometrically at 420 nm ($OD4_{420}$). Then centrifuged again, the deposit was lysed by lysis buffer on ice for 5 min according to suspension's OD of approximately 0.7–0.8, the deposit can be lysed in 0.5 mL of lysis buffer, which contained 0.01 M Tris (pH 7.4), 8 M Urea, 1 mM EDTA, 0.05 M DTT, 10% v/v

Table 1. *P. aeruginosa* strains divided into three groups

	P. aeruginosa strains*	Descriptions
Group I	PA01, PA02, PA20, and PA22	Sensitive strains that did not contact with ciprofloxacin
	PA01(1)–PA01(5), PA02(1)–PA02(3)	Sensitive strains obtained from serial MICs test
Group II	PA01(6)–PA01(8), PA02(4), PA02(5)	Intermediate strains obtained from serial MICs test
Group III	PA01(9)–PA01(12), PA02(6)–PA02(9), PA03 and PA04	Ciprofloxacin-resistant strains obtained from serial MICs test except PA03 and PA04

* The number in brackets after the strains name represents the number of ciprofloxacin MICs test.

glycerol, 5% v/v NP40, 6% w/v pharmalytes (pH 3–10), and then the bacterial suspension was sonicated (3 × 30 s). After removing insoluble cell debris by centrifugation on a microfuge at 11 000 × *g* for 10 min, the supernatant that contained the soluble bacterial proteins was stored at −70°C before it was used. IEF was performed on a piece of 18-cm-long Immobiline Drystrip (pH 3–10) according to the instructions provided by Amersham Pharmacia Biotech [10]. Approximately 100 μL of the protein solution (150–200 μg total protein) was applied to the acidic side of each gel. After IEF, the Immobiline Drystrips were submitted to the second-dimension electrophoresis on 12.5% SDS-PAGE slab gels according to published procedure [11]. Five microliters of Blue-Ranger Prestained Protein Molecular Weight Marker (MW from 17.5 to 209 kDa) was used for calibrating the protein molecular weight on each SDS-PAGE gel. After SDS-PAGE, the gels were silver stained according to the method described in Current Protocols in Protein Science (CPPS) [12] for proteomic comparative analysis. Another silver stain method without using glutaraldehyde according to the method described in Short Protocols in Molecular Biology [13] was used for in-gel protein digestion and mass spectroscopy analysis. 2-DE was repeated for each *P. aeruginosa* strain at least three times to confirm the reproducibility of the protein profiles. The images of silver-stained 2-DE gels were acquired *via* a CCD camera (Perkin-Elmer) and comparatively analyzed with the help of Phoretix 2-D software.

2.4 In-gel protein digestion MS

The interested sliver-stained proteins spots were cut out of the gel, destained, reduced, alkylated, and digested *in situ* with tosyl-L-phenylalanine chloromethyl ketone (TPCK-) trypsin as described by Liang and coworkers [14]. After digestion overnight at 37°C, the samples were extracted three times (each 1 h) at 37°C with 50 μL of 5% TFA/50% acetonitrile (CAN). All the extracts were merged, lyophilized completely, and stored at −80°C until LC/MS/MS analysis.

2.5 Protein identification by MS

We used an IT mass spectrometer, LC/MS/MS LCQ™ Deca-XP Plus (ThermoFinnigan), to identify proteins according to MS/MS sequence. The preconcentrated and desalted tryptic peptides (5 μL) were injected into LCQ Deca-XP Microtech C-18 column (Sunnyvale, CA, USA) (180 μm × 10 cm) and automatically eluted directly into the mass spectrometer. All the data (MS/MS spectrum), which were acquired on LCQ Deca-XP Plus, were analyzed by TurboSEQUEST™ search program embedded in Bioworks 3.0.

2.6 N-terminal sequencing

The proteins from the SDS-PAGE gel were electroblotted onto a PVDF membrane with CAPS buffer, and visualized by staining with CBBR250 as CPPS described [15]. The selected spots were excised from the membrane and submitted to N-terminal sequencing by EDMAN degradation, which was performed by Shanghai GeneCore BioTechnologies. An eight-residue peptide at the N-terminal sequence was determined, and tentative identification was made by searching for homology with sequence present in the protein database using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

2.7 Detection of the ciprofloxacin concentration within *P. aeruginosa* strains

Strains, PA01(1)–PA01(12) and PA02(1)–PA02(9) were suspended in 1 mL of chilled 50 mmol/L sodium phosphate buffer (pH 7.0) with a density equivalent to OD_{420} = 1.0. The ciprofloxacin concentrations within strains were represented as milligram *per* liter (OD_{420} = 1.0). The suspended strains were centrifuged in a microcentrifuge at 4°C, 8000 rpm, for 5 min. The bacteria were washed again in the same chilled buffer and recentrifuged for 5 min. The deposit was resuspended in 600 μL of 10 mmol/L Tris-HCl (pH 8.0) buffer; containing 3 μL of 10% SDS and 3 μL of 20 mg/mL Protease K and incubated at 37°C for 1 h. After addition of 2 mL of CAN, the suspension was agitated for 30 s and centrifuged at 10 000 rpm for 5 min. The clear supernatant was prepared for HPLC detection. The method used in determination of ciprofloxacin in solution by HPLC with UV detection (Syltech Model 500) was according to Marika's description [16].

2.8 Detection of mRNA expression by RT-PCR

Total RNA was isolated from *P. aeruginosa* strains according to the instruction of TRIzol Reagent. The amount of strains was OD_{420} = 1.0. The one-step RT-PCR was carried out as described in RNA Selective PCR kit manual. The detailed condition was as follows: 25 μL of 2 × mRNA Selective PCR Buffer I, 10 μL of 25 μM $MgCl_2$, 5 μL of dNTP/analog mixture, 40 U RNase inhibitor, 5 U AMV RTase XL, 5 U AMV-Optimized Tag, and 1 μL of each oligonucleotide primer (20 μM each) containing sense primer (5'-GGGACAAGCGCAACGAAG-3', 380–397 bp in ATP-binding component of ATP transporter mRNA); and antisense primer (5'-GGTAGTGGGTGGTGAGGATGA-3', 586–566 bp in ATP-binding component of ATP transporter mRNA), total volume was 50 μL. The reaction mixture was overlaid with 50 μL of mineral oil and subjected to 50°C for 20 min reverse transcription, followed by 30 cycles at 85°C for 1 min, 45°C for 1 min, and 72°C for 3 min. The PCR products were electrophoresed on 2% agarose gels, and stained with ethidium bromide; 207 bp could be visualized by UV transillumination. The results were quantitatively analyzed by means of Phoretix1-D gel analysis software.

2.9 Design and construction of DNAzyme

A "10–23" DNAzyme [17] was designed against the mRNA of the "probable ATP-binding component of ATP transporter" (see Fig. 1). An AU cleavage site (622–623 bp) of the mRNA

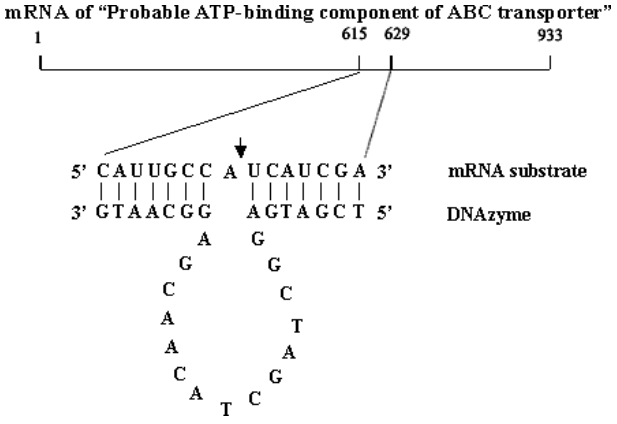

Figure 1. Sequence and location of the DNAzyme target site in the ATP-binding component of ATP transporter mRNA. Design of DNAzyme.

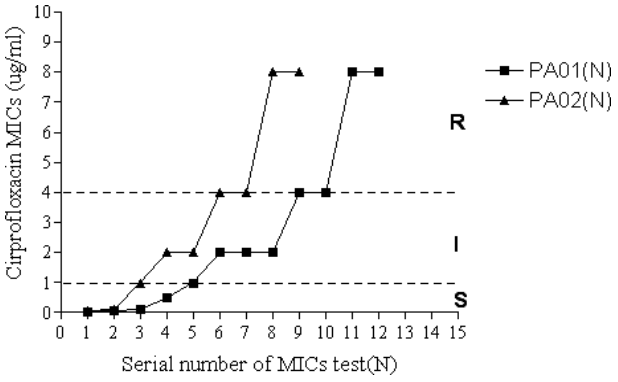

Figure 2. Results of serial ciprofloxacin MICs test. Interpretive standard of ciprofloxacin MIC for *P. aeruginosa*: sensitive (*S*) $\leq$ 1μg/mL; intermediate (*I*) = 2 μg/mL; resistant (*R*) $\geq$ 4 μg/mL.

substrate was chosen such that mRNA could not form second structures by its own stable intramolecular base pairing. The designed DNAzyme consisted of seven nucleotides on both arms with a 15-mer catalytic core sequence. The control DNAzyme was designed with an inverted catalytic core sequence (5'-TCGATGACCGATCGATGTTGCT GGCAATG-3').

2.10 DNAzyme transfection of *P. aeruginosa*

The strains used in this test were divided into three groups (Table 1). After the preparation of electrocompetent *P. aeruginosa* strains, the electroporation apparatus Multiporator (Eppendorf, Germany) was set to 1.6–2.5 kV, 25 μF and the pulse controller to 200 Ω. One to five microliters of DNAzyme (or control DNAzyme) was added to the tubes containing 40 μL of electrocompetent cells (1×10^7 colony forming unit (CFU)) on ice and electroporation was performed. At the end of electroporation, the sample in the cuvette was added to 5 mL of MH-broth, and the cells were transferred to a sterile polypropylene culture tube followed by adding ciprofloxacin whose concentration was equal to the strain's MICs of ciprofloxacin. The cultures were incubated for 24 h at 37°C with moderate shaking to allow *P. aeruginosa* to grow and then the OD was measured at 420 nm. The cultures of group II were collected for the determination of the concentration within *P. aeruginosa* strains with the method described before.

3 Results

3.1 Mimicking the quinolone-resistant process *in vitro*

In serial MICs tests, subcultured *P. aeruginosa* strains in ciprofloxacin containing MH-Broth could increase the MICs of *P. aeruginosa*, which was from 0.03 to 8 μg/mL through no more than 12 tests (see Fig. 2).

3.2 2-DE patterns of *P. aeruginosa*

To identify the changes in protein expression of *P. aeruginosa* with different ciprofloxacin MICs, the silver-stained 2-DE gels were compared with each other. The 2-DE patterns are shown in Fig. 3. Visual examination and comparison by Phoretix 2-D software of these gel patterns showed that some protein spots were absent in the group II and III strains in comparison with the group I strains (data not shown). At the same time, there were 15 protein spots, which were found in the 2-DE pattern of group II and III strains but not in that of group I strains (data not shown). Among these 15 protein spots, one spot (encircled in Fig. 3B and C, MW $\approx$ 35 kDa, p*I* $\approx$ 6.4) appeared when the ciprofloxacin MIC of *P. aeruginosa* strains was $\geq$2 μg/mL. It is expressed less in intermediate pattern and more in resistant pattern (Fig. 3A–C), identifying distinct differences in the spot intensity between the intermediate and resistant patterns.

3.3 LC-MS/MS analysis of protein spot

In order to identify this protein, the selected protein spot was first subjected to in-gel digestion with trypsin. The extracted peptides were separated and analyzed with LS-MS/MS on a Finnigan LCQ IT mass spectrometer (LCQ Deca XP Plus). The uninterpreted MS/MS raw data were used to search for database with TurboSEQUEST search program, which could match each experimental CID spectrum with the theoretical CID spectrum of any isobaric peptide (see Fig. 4). The search results indicate that the protein is a "probable ATP-binding component of ATP binding cassette (ABC) transporter" with high confidence.

3.4 Identification of protein spot by terminal sequencing

To corroborate the LC-MS/MS results, the EDMAN degradation of the protein spot was performed. The result of EDMAN degradation gives the following amino acid

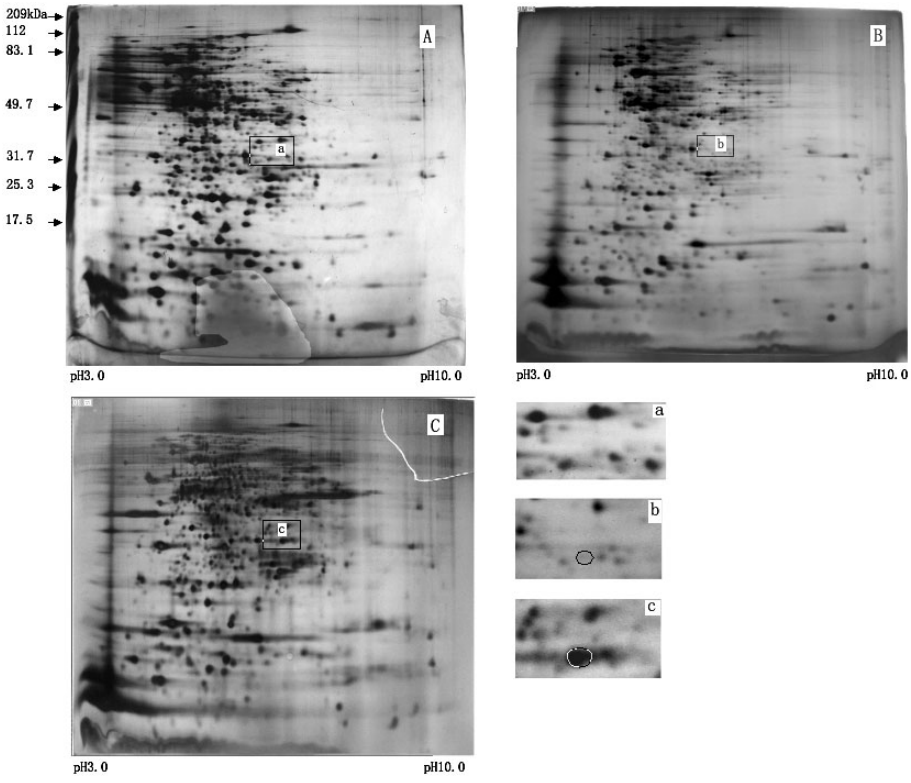

Figure 3. (A–C) representing three different 2-DE patterns during the process of *P. aeruginosa* ciprofloxacin resistance, respectively. (A) 2-DE pattern of Group I strains; (B) 2-DE pattern of Group II strains; (C) 2-DE pattern of Group III strains. Encircled spot in (B) and (C) was interested protein; this spot did not exist in (A). Spot intensity in (C) was much greater than in (B).

sequence: MSSALSIR. A BLAST search result (http://www.ncbi.nlm.nih.gov/BLAST/) of N-terminal sequence exhibited a 100% identity to the amino acid sequence of a probable ATP-binding component of ABC transporter of *P. aeruginosa*.

3.5 Detection of the ciprofloxacin concentration within the *P. aeruginosa* strains

The changes of ciprofloxacin concentrations during the process of fluoroquinolone resistance were determined for serial strains, PA01(N) and PA02(N). Both serial strains showed similar results, *i.e.*, the intracellular concentration of ciprofloxacin decreased but the MICs increased (Fig. 5).

3.6 Detection of the probable ATP-binding component of ABC transporter mRNA levels by RT-PCR

According to the results mentioned above, a set of primers were designed and applied to detecting the gene expression of the probable ATP-binding component of ABC transporter. The results of RT-PCR show that this gene was not expressed although *P. aeruginosa* was sensitive to ciprofloxacin (MICs $\leq$1 μg/mL). But when ciprofloxacin MIC was $\geq$2 μg/mL, mRNA of the protein could be detected. The gray scale of each band showed that the mRNA level increased from intermediate strains to resistant strains (Fig. 6).

3.7 Inhibition of drug resistance by cleavage mRNA of the probable ATP-binding component with DNAzyme

When DNAzymes (or control DNAzyme) were transferred into *P. aeruginosa,* the survival rate of DNAzymes-treated *P. aeruginosa* strains was different from their controls at the same concentration of ciprofloxacin, which was equal to the strains' MICs. (Table 2, Fig. 7). From the results, it seems that DNAzyme had no effect on the strains in group I, and had significant effect on the strains in groups II and III. The extent of the inhibition of ciprofloxacin resistance by DNAzyme was from 19.7 to 67.6%.

The concentrations of ciprofloxacin within the strains of group II were detected after DNAzyme inhibition test and the results are shown in Table 3.

4 Discussion

As an opportunistic pathogen, *P. aeruginosa* increases resistance to a diverse range of antibiotics. This has led to the establishment of the Pseudomonas Genome Project (www.pesudomonas.com). Although genome sequencing and gene annotations of *P. aeruginosa* have been fully published, new drug targets, which could eliminate drug resistance, still remain unclear [18]. This might partially be due to

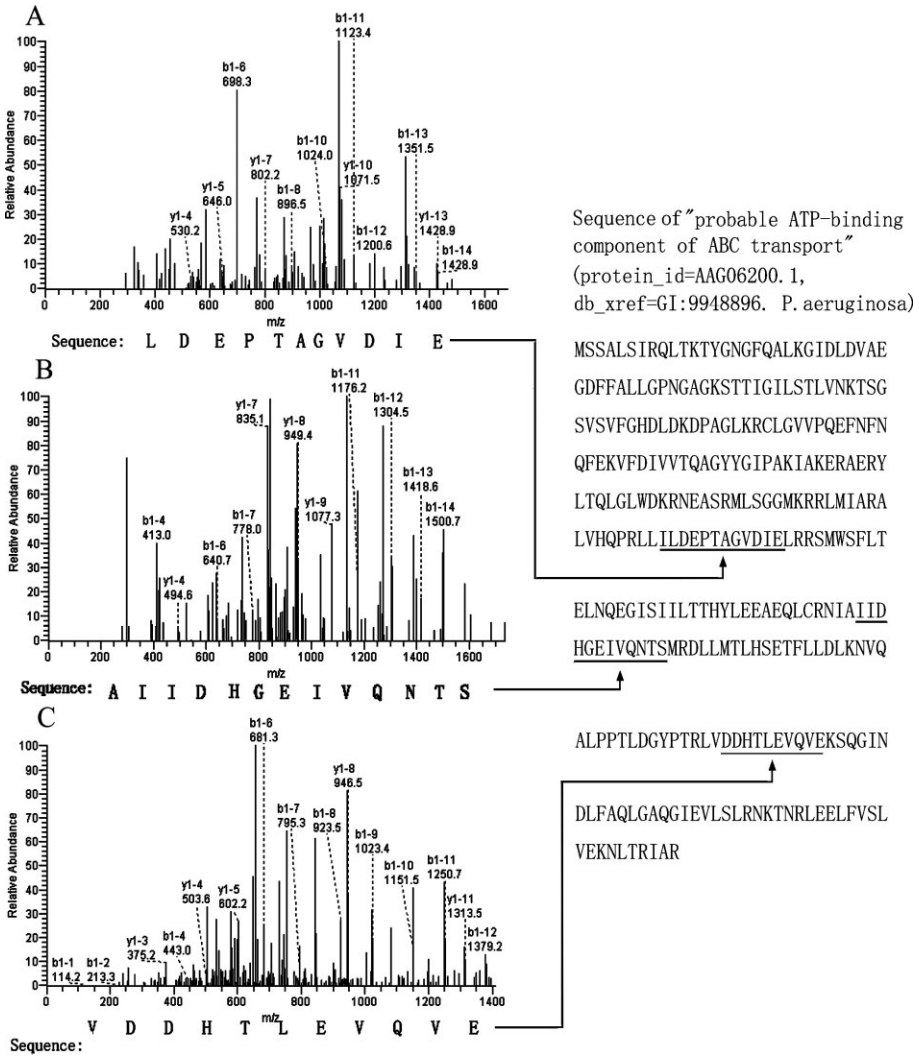

Figure 4. LC-MS/MS analysis of protein spot. (A–C): CID spectra of candidate peptides; the corresponding sequence of the spot could be found in the protein sequence of probable ATP-binding component of ABC transporter.

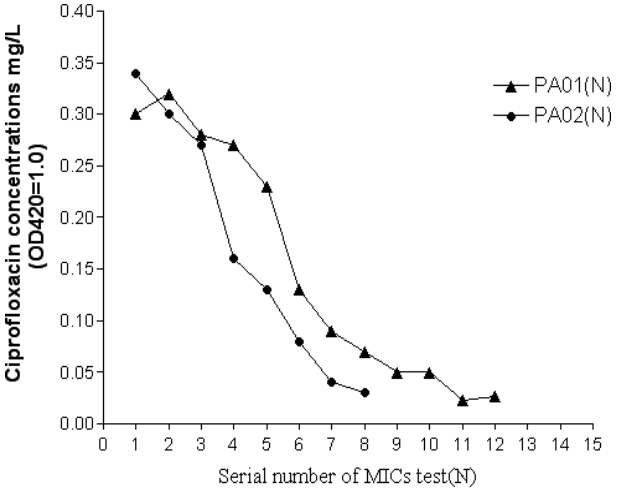

Figure 5. Determination of the strain intracellular concentrations of ciprofloxacin through the whole process of drug resistance.

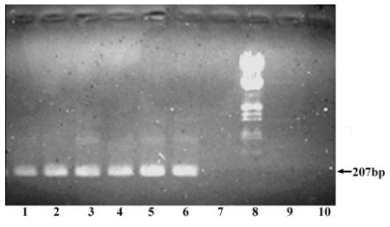

Figure 6. RT-PCR detected mRNA expression in PA02(N). Lane1, PA02(4); lane2, PA02(5); lane3, PA02(6); lane4, PA02(7); lane5, PA02(8); lane6, PA02(9); lane7, PA02(1); lane9, PA02(2); lane10, PA02(3); lane8, marker.

the proteins that are suppressed when bacterial strains become resistant mutants. From the proteomic point of view, drug resistance could be a result of the interactions between drugs and the whole cellular substances including proteins, DNA, RNA, saccharides, and lipids. In the resistant state,

Table 2. The results of DNAzyme inhibition test

Strains		CIP concentration, μg/mL	OD_{420} of control DNAzyme	OD_{420}, DNAzyme treated	Relative inhibition percentage*,%
Group I	PA01	0.03	0.68	0.71	−4.41
	PA02	0.06	0.75	0.74	1.33
	PA20	0.03	0.63	0.57	9.52
	PA22	0.03	0.72	0.77	−6.94
	PA01(1)	0.03	0.57	0.56	1.75
	PA01(2)	0.06	0.61	0.61	0.00
	PA01(3)	0.12	0.79	0.75	5.06
	PA01(4)	0.5	0.79	0.75	5.06
	PA01(5)	1	0.64	0.74	−15.63
	PA02(1)	0.06	0.74	0.73	1.35
	PA02(2)	0.12	0.57	0.64	−12.28
	PA02(3)	1	0.63	0.58	7.94
Group II	PA01(6)	2	0.74	0.51	31.08
	PA01(7)	2	0.62	0.31	50.00
	PA01(8)	2	0.92	0.45	51.09
	PA02(4)	2	0.75	0.54	28.00
	PA02(5)	2	0.76	0.61	19.74
Group III	PA01(9)	4	0.88	0.61	30.68
	PA01(10)	4	0.94	0.55	41.49
	PA01(11)	8	0.76	0.4	47.37
	PA01(12)	8	0.71	0.23	67.61
	PA02(6)	4	0.76	0.4	47.37
	PA02(7)	4	0.69	0.33	52.17
	PA02(8)	8	0.61	0.38	37.70
	PA02(9)	8	0.5	0.37	26.00
	PA03	4	0.79	0.45	43.04
	PA04	8	0.82	0.49	40.24

* Relatively inhibition percentage = (average $OD_{420\text{-Controls}}$ − $OD_{420\text{-DNAzyme}}$)/Average $OD_{420\text{-Controls}} \times 100$.

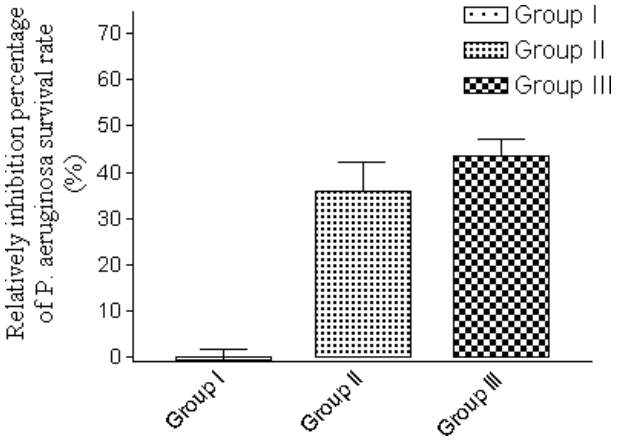

Figure 7. Average relatively inhibition percentage of DNAzyme in Groups I, II, and III. Data are relative inhibition percentage means ± SEM of group I (−0.60 ± 2.25, N = 12) of group II (35.98 ± 6.23, N = 5) and of group III (43.37 ± 3.66, N = 10). t-test showed that means (of group I and II/of group I and III) were significance different ($p < 0.0001$).

Table 3. The concentrations of ciprofloxacin within the strains of group II

Strains	CIP concentration mg/L, OD_{420} = 1.0	
	Control DNAzyme	DNAzyme
PA01(6)	0.12	0.18
PA01(7)	0.05	0.14
PA01(8)	0.02	0.12
PA02(4)	0.13	0.21
PA02(5)	0.12	0.17

some genes may be suppressed and some genes may be activated. These new expressed proteins may play an important role in the resistant mutants because they are essential for bacterial growth in antibiotics-containing environments.

This study describes our attempts to find new drug targets, which could reduce *P. aeruginosa* ciprofloxacin resistance by proteomic method, and we found that a probable ATP-binding component of ABC transporter may be a new one.

We mimicked the *in vitro* progress of *P. aeruginosa* ciprofloxacin resistance and obtained a series of strains that could represent the whole process of ciprofloxacin resistance (from sensitive state to resistant state). The combination of *in vitro* mimicking method, which has been used to study the relationship between antibiotics and bacteria [19–22] and the proteomic analysis on *P. aeruginosa*, which has been focused on phenotypic differences in biofilms [23–25], gene function analysis [26], and protein identification under different culture conditions [27], has not been used to reveal the protein profile changes in the drug resistance process, and this combination could be helpful to find new anti-drug-resistance targets.

As expected, many protein spots are lost as soon as sensitive *P. aeruginosa* were grown in MH-broth containing ciprofloxacin (data not shown) and ciprofloxacin may suppress the expression of some proteins; this is consistent with the other *P. aeruginosa* drug resistance study, such as a loss of 48–49-kDa protein in polymyxin- or imipenem-resistant strains [28, 29]; diminution or a loss of 31–32-kDa outer membrane protein in ciprofloxacin-resistant strains [30]; a loss of 45–48-kDa protein in carbapenem-resistant variants [31]; a loss of acidic-protein spot (pI = 5.2) in beta-lactam-resistant strains [32]. Some studies also reported that there were some proteins overexpression in resistant strains, such as a basic-protein spot (pI = 9.0, beta-lactamase) detected in ceftazidime-resistant strains; OprM, OprJ, and OprN detected in some multiple-drug-resistant mutants [33]. In our study, we found that the protein spot (MW ≈ 35 kDa, pI ≈ 6.4) only existed in ciprofloxacin-intermediate and -resistant strains (MICs ≥ 2 μg/mL).

The methods of protein identification used here was the combination of LC/MS/MS, TurboSEQUEST search, and EDMAN degradation. The raw data of MS/MS and the excellent CID spectrum of selected candidate peptides,

which were filtered under very strict conditions, revealed that the best unique match in *P. aeruginosa* was a probable ATP-binding component of ABC transporter. The BLAST search program (http://www.ncbi.nlm.nih.gov) showed that these candidate peptides had high sequence conservation in ABC transporters belonging to other species (*P. syringae*, *Synechocystis* sp., *P. putida*, *E. coli*, *S. flexneri*, *H. influenzae*, *etc.*). Further identification was N-terminal sequencing by EDMAN degradation that gave the following amino acid sequence: MSSALSIR. BLAST search program showed that the peptide sequence matched only one protein, a probable ATP-binding component of ABC transporter, in *P. aeruginosa*, and had high conservation of ABC transporter in other two *Pseudomonas* species (*P. syringae*, *P. putida*).

The ABC transporter is one of the active transport systems of the cell, which is widespread in archaea, eubacteria, and eukaryotes. It is also known as the periplasmic binding protein-dependent transport system in gram-negative bacteria and the binding-lipoprotein-dependent transport system in gram-positive bacteria [34, 35]. The ATP-binding protein component is the most conserved, the membrane protein component is somewhat less conserved, and the substrate-binding protein component is most divergent [36, 37] in terms of the sequence similarity.

From the determination of intracellular concentrations through the whole process of drug resistance, we could find that when the strains were in the intermediate resistant state, the intracellular concentration of drug suddenly decreased, but at the same time, the probable ATP-binding component of ABC transporter began to be expressed. This hints that the protein may have some relationship with active efflux mechanism and the gene of the protein was activated when the ciprofloxacin concentration inside the cell was over some degree.

The DNAzyme, which was applied to cleave the mRNA of the protein to reduce *P. aeruginosa* ciprofloxacin resistance, has ability to bind a target RNA *via* Watson–Crick bp interactions. The designed DNAzyme avoided all potential secondary structures formed by target RNA own stable intramolecular base pairing, and had total ΔG of both arms achieved the predicted value of free energy <-25 kcal/mol. The efficiency of this designed DNAzyme in reducing ciprofloxacin resistance was marked by the growth of *P. aeruginosa* strains in the broth medium containing ciprofloxacin at different concentrations. The drug resistance was reduced to some extent compared with corresponding control strains. The results showed that when the expression of the probable ATP-binding component of ABC transporter was suppressed, the resistant strains could not live in ciprofloxacin-containing environment perfectly. In addition, we compared the concentrations of ciprofloxacin within the strains of group II after DNAzyme inhibition test; the results show that there was a positive correlation between the inhibited expression of this protein and the accumulation of ciprofloxacin. That is to say this protein must play an important role in the process of ciprofloxacin resistance especially in the course of active drug efflux.

There were a lot of ABC transporters that had been found in eukaryotes, and were associated with eukaryotic multidrug resistance by translocating hydrophobic drugs and lipids from the inner to the outer leaflet of the cell membrane [38–40]; and the family of ATP transporters in prokaryote has been deeply studied since last decade, but the relationship between ATP transporters and antibiotic resistance has not been clear. Banerjee *et al.* (1998) [41] first found an ABC protein, the B-subunit of the phosphate specific transporter, in a fluoroquinolone-resistant *M. smegmatis* colony. Since then on, some prokaryotic drug resistance related to ATP transporter has been described, such as HorA protein in *L. brevis* [42], macrolide-specific ABC-type efflux carrier in *E. coli* [43], glycerol-3-phosphate dehydrogenase in *Streptococcus* mutants [44], and so on. However, no study showed that an ATP-binding ABC transporter could contribute to antibiotic resistance. Our study first revealed that the probable ATP-binding component of ABC transporter may play an important role in fluoroquinolone resistance by proteomic analysis and DNAzyme inhibition test, and that this protein may be a new target to eliminate drug resistance. This study also provided a clue for finding new drug targets against antibiotic resistance by paying close attention to the proteins that might be expressed in intermediate and resistant strains but not in sensitive strains.

5 References

[1] Wang, J., Lory, S., Ramphal, R., Jin, S., *Mol. Microbiol.* 1996, *22*, 1005–1012.

[2] Kharazmi, A., *Immunol. Lett.* 1991, *30*, 201–205.

[3] Davis, R., Markham, A., Balfour, J. A., *Drugs* 1996, *51*, 1074–1119.

[4] Hooper, D. C., *Clin. Infect. Dis.* 2000, *30*, 243–254.

[5] Berger, J. M., Gamblin, S. J., Harrison, S. C., Wang, J. C., *Nature* 1996, *379*, 225–232.

[6] Ng, E. Y., Trucksis, M., Hooper, D. C., *Antimicrob. Agents Chemother.* 1996, *40*, 1881–1888.

[7] Köhler, T., Michea-Hamzehpour, M., Plesiat, P., Kahr, A. L., Pechère, J. C., *Antimicrob. Agents Chemother.* 1997, *41*, 2540–2543.

[8] Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D. *et al.*, *Nature* 2000, *406*, 959–964.

[9] National Committee for Clinical Laboratory Standards, *Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard – Fifth Edition*, M2-A5, NCCLS, Villanova, PA, 1995, pp. 6–10.

[10] *2-D Electrophoresis: Using Immobilized pH Gradients, Principles and Methods*, Amersham Pharmacia Biotech, pp. 14–23.

[11] *2-D Electrophoresis: Using Immobilized pH Gradients, Principles and Methods*, Amersham Pharmacia Biotech, 28–35.

[12] *Electrophoresis, Basic Protocol 2: Silver Staining Current Protocols in Protein Science*, John Wiley & Sons, New York 2002, Chapter 10.

[13] Frederick, M. A., *Short Protocols in Molecular Biology*, 3rd Edn., John Wiley & Sons, New York 1995.

[14] Chen P., Xie J. Y., Liang S. P., *Acta Biochimica et Biophysica SINICA* 2000, *32*, 387–391.

[15] *Electrophoresis, Basic Protocol 2: Silver Staining Current Protocols in Protein Science*, John Wiley and Sons, New York 2002, Chapter 10.

[16] Marika, K., Kimiko, T., Tsutomu, K., Koichi, N., Shigeyuki, N., *Clin. Chem.* 1998, *44*, 1251–1255.

[17] Santoro, W., Joyce, G. F., *Proc. Natl. Acad. Sci. USA* 1997, *94*, 4262–4266.

[18] Low, D. E., Scheld, W. M., *JAMA* 1998, *279*, 365–370.

[19] Gerber, A. U., Craig, W. A., *J. Lab. Clin. Med.* 1982, *100*, 671–681.

[20] Dimitracopoulos, G., Intzes, C., Papavassiliou, J., *J. Clin. Pathol.* 1979, *32*, 723–727.

[21] Loughlin, M. F., Jones, M. V., Lambert, P. A., *J. Antimicrob. Chemother.* 2002, *49*, 631–639.

[22] Wu, Y. L., Scott, E. M., Po, A. L., Tariq, V. N., *J. Antimicrob. Chemother.* 1999, *44*, 389–392.

[23] Steyn, B., Oosthuizen, M. C., MacDonald, R., Theron, J., Brozel, V. S., *Proteomics* 2001, *1*, 871–879.

[24] Sauer, K., Camper, A. K., Ehrlich, G. D., Costerton, J. W., *J. Bacteriol.* 2002, *184*, 1140–1154.

[25] Yoon, S. S., Hennigan, R. F., Hilliard, G. M., Ochsner, U. A. *et al.*, *Dev. Cell* 2002, *3*, 593–603.

[26] Sonnleitner, E., Moll, I., Blasi, U., *Microbiology* 2002, *148*, 883–891.

[27] Madhusudhan, K. T., McLaughlin, R., Komori, N., Matsumoto, H., *J. Basic Microbiol.* 2003, *43*, 36–46.

[28] Gilleland, H. E., Jr., Lyle, R. D., *J. Bacteriol.* 1979, *138*, 839–845.

[29] Lynch, M. J., Drusano, G. L., Mobley, H. L., *Antimicrob. Agents Chemother.* 1987, *31*, 1892–1896.

[30] Daikos, G. L, Lolans, V. T., Jackson, G. G., *Antimicrob. Agents Chemother.* 1988, *32*, 785–787.

[31] Margaret, B. S., Drusano, G. L., Standiford, H. C., *J. Antimicrob. Chemother.* 1989, *24*, 161–167.

[32] Michea-Hamzehpour, M., Sanchez, J. C., Epp, S. F., Paquet, N. *et al.*, *Enzyme Protein* 1993, *47*, 1–8.

[33] Masuda, N., Sakagawa, E., Ohya, S., *Antimicrob. Agents Chemother.* 1995, *39*, 645–649.

[34] Reizer, J., Reizer, A., Saier, M. H., Jr., *Protein Sci.* 1992, *1*, 1326–1332.

[35] Poelarends, G. J., Mazurkiewicz, P., Konings, W. N., *Biochim. Biophys. Acta* 2002, *1555*, 1–7.

[36] Tam, R., Saier, M. H., Jr., *Microbiol. Rev.* 1993, *57*, 320–346.

[37] Saurin, W., Koster, W., Dassa, E., *Mol. Microbiol.* 1994, *12*, 993–1004.

[38] Valverde, M. A., Diaz, M., Sepulveda, F. V., Gill, D. R. *et al.*, *Nature* 1992, *355*, 830–833.

[39] Taylor, J. C., Horvath, A. R., Higgins, C. F., Begley, G. S., *J. Biol. Chem.* 2001, *276*, 36075–36078.

[40] Sauna, Z. E., Smith, M. M., Muller, M., Kerr, K. M., Ambudkar, S. V., *J. Bioenerg. Biomembr.* 2001, *33*, 481–491.

[41] Banerjee, S. K., Misra, P., Bhatt, K., Mande, S. C., Chakraborti, P. K., *FEBS Lett.* 1998, *425*, 151–156.

[42] Sakamoto, K., Margolles, A., Van Veen, H. W., Konings, W. N., *J. Bacteriol.* 2001, *183*, 5371–5375.

[43] Kobayashi, N., Nishino, K., Yamaguchi, A., *J. Bacteriol.* 2001, *183*, 5639–5644.

[44] Tao, L., Tanzer, J. M., *J. Dent. Res.* 2002, *81*, 505–510.

