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Comparative analysis of proteins, drug resistance, and biofilm formation in free-swimming bacteria and their surface-attached counterparts

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In this study, Pseudomonas *aeruginosa* PAO1 cells were cultured to confluence, and the bactericidal activity of ceftazidime, imipenem, levofloxacin, ciprofloxacin, amikacin, gentamicin, and tobramycin against planktonic bacteria and 1-day-old, 3-day- old, and 5-day-old biofilm cells were measured. The results showed that the bactericidal activity of the above-mentioned antibiotics against 5-day-old biofilm bacteria were more than 256- , more than 256-, 32-, 16-, 64-, 128- and 128-fold lower than that against planktonic cells, respectively. The biofilm tagged with green fluorescent protein were observed with confocal laser scanning microscopy and the protein expression in biofilm bacteria of all three different ages and their planktonic counterparts were compared. The differentially expressed proteins were further identified by peptide mass fingerprinting. The results showed that the thickness of the biofilms increased from 4 µm on the first day to 20 µm on the fifth day. The increasing biofilm thickness and some of the identified proteins such as GDP-mannose dehydratase might partially explain the difference of the bactericidal activity of the above antibiotics against biofilm cells. Although the role of the differentially expressed proteins in biofilm drug resistance needs to be explored further, the results showed that fluoroquinolone antibiotics are the better choice for treatment of *P. aeruginosa* biofilm-associated infections.

Key words: *P. aeruginosa*, biofilm, planktonic, proteome, drug resistance;

INTRODUCTION

When single bacterial come together to form a community that is adherent to a solid surface and embedded in an exopolysaccharide matrix, then the biofilm forms (Costerton et al., 1994). Once the bacterial exist in the form of biofilm , their susceptibility to antimicrobial agents will decrease 10 - 1000 times (Hoyle et al. 1991). Bacterial can form biofilm in any kind of materials, from glass, metal to animal tissues, even on the surface of teflone (Mah et al., 2001). With the increase of invasive procedures, Biofilm related infectious disease has become more and more important to medical science. It is estimated that 65% nosocomial infectious are associated with biofilm, and it will cost more than 1 billion dollar to treat these biofilm-related diseases (O'Toole, 2002). *Pseudomonas aeruginosa* is a ubiquitous Gram-negative bacterium common in soil and water environments that has become one of the most common and deadly opportunistic pathogens (Stover et al., 2000). Owing to its ability to easily form biofilms, *P. aeruginosa* has become one of the most common and deadly opportunistic pulmonary pathogens that is capable of causing refractory chronic lung infections (Ishida et al., 1998). The infections are often life threatening, and is attributed to the production of various degradation enzymes, virulence factors, and the enhanced ability to form biofilms (Branda et al., 2005). However, the drug-resistance mechanism of *P. aeruginosa* biofilms is still unclear.

In this study, the bacteria were cultured in a flow medium, and the bactericidal activity of various antibiotics against 1-day-old, 3-day-old, and 5-day-old biofilm bacte-

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Abbreviations: CLSM; Confocal laser scanning microscopy, **GFP;** Green fluorescent protein.

ria were measured. CLSM were used to observe the biofilm architecture on the first, third, and fifth day after inoculation. The whole-protein extracts obtained from the biofilm cells and their planktonic cells and their biofilm counterparts on all the 3 days were analyzed using 2-D electrophoresis. The differentially expressed proteins were selected for further MS analysis, and after identification, they were used to elucidate the mechanism of biofilm resistance and helpful to find the new targets for the treatment of biofilm-related infectious diseases caused by *P. aeruginosa*.

MATERIALS AND METHODS

Bacteria and antibiotics

The planktonic bacteria *P. aeruginosa* PAO1 were cultured in Luria broth (LB) medium for 12 h at 37°C. SOC medium (2.0% tryptone, 0.5% yeast extract,10 mM magnesium chloride, 10 mM NaCl, 2.5 mM KCl, 10 mM magnesium sulfate, 20 mM glucose) and SMH buffer (300 mM fructose, 1 mM magnesium chloride, 1 mM HEPES, pH 7.0) were used in electrotransformation. Ceftazidime, Imipenem, Amikacin, Gentamicin, Tobramycin, Levofloxacin, Ciprofloxacin were used for bactericidal activity, which were purchased from the Chinese CRM/RM information center.

Bactericidal activity of various antibiotics against planktonic bacteria and biofilm bacteria at different stages

Susceptibility test for antibiotics against planktonic bacteria was performed using the broth dilution method according to CLSI guidelines, then the contents of nonturbid liquid at 24 h were sub-cultured onto LB agar, and viable cells were counted after incubation for 24 h at 37°C. Bactericidal activity of antibiotics against biofilm-forming cells were performed according to previously described protocols (Abdi-Ali et al., 2006). A continuous-flow reactor was designed according to Karin Sauer with small changes (Sauer et al., 2002). Briefly, medium was pumped through the silicone tubes via a BT100L-DG-4 pump to a closed effluent medium reservoir. The tubes within which the biofilms were cultivated had a regular geometry throughout (2.4 × 0.8 mm, TygronR-3603). *P. aeruginosa* Overnight culture were adjusted to 0.5 McFarland with LB broth, twenty milliliters of diluted cultures were injected through the tubing wall and into the lumen (length 2 m) and allowed to attach for 2 h before the flow of LB(0.5 ml/min) was initiated. After various times (1day , three days and 5 days), the tubes were cut into fragments of 1 cm length, followed by wash gently with PBS , then were transferred to Mueller–Hinton broth (MHB) containing a given antibiotic with various concentrations for 12 h at 37°C. The silicone tube pieces were transferred into 1 ml PBS, vortexed violently for 10 min. The suspensions were diluted and plated on LB agar plates and viable cells were counted after incubation for 24 h at 37°C.

Biofilm mode of growth and microscopy

An enhanced GFP expression plasmid (pGFPuv20) was constructed by cloning a 736 bp *EcoR* I-*Hind* III fragment from plasmid pGFPuv into *EcoR* I *Hind* III digested pUCP20, according to David G (Davies et al., 1998). pGFPuv20 was transformed into *P. aeruginosa* by electroporation according to Stapper (Stapper et al., 2004). The 488 nm laser lines of an ArKr laser were used to excite GFP. Each silicone tube of continuous-flow reactor was ligated to a μ-slide IV flow kit (ibidi), which have a square glass in the bottom that can be used for noninvasive microscopic evaluation of biofilm morphology by CLSM using an OLYMPUS FV500 system.

Preparation of crude protein extract

After 1day, 3 days and 5 days biofilm cells were harvested from the interior surface by pinching the tube along its entire length, resulting in extrusion of the cell material from the lumen. The resulting cell paste was collected on ice. Prior to sampling, the bulk liquid was purged from the tubing to prevent interference from detached cells. Biofilm- grown *P. aeruginosa* cells were centrifuged at 12,000 g for 10 min at 4°C, then washed immediately with PBS three times followed by 5.48% sorbitol three times, and resuspended in U9 lysate buffer (urea 7mol/l, thiourea 2 mol/l, CHAPS 4%(m/v), DTT 65 Mmol/L, IPG Buffer 0.5%(V/V), PMSF 2 μg/l(m/V) supplemented with 50 μg/ml RNase and 200 μg/ml DNase. All cell samples were lysed by sonication on ice using six 10 s bursts at 4 W and centrifuged at 14000 g, 30 min, 4°C. Total protein concentration was determined by the Bradford method. Bovine serum albumin was used as the standard. 30 μg total proteins as calculated from the initial concentration were loaded to perform SDS-PAGE and the final protein concentrations were calibrated with Gray-scale analysis software. Experiments for each time point were repeated at least three times.

Two-dimensional gel electrophoresis and MALDI-TOF MS

The protocols were described in detail elsewhere (Yang et al., 2007; Zhang et al., 2008). Total cell protein (1000 μg) was loaded onto GE Healthcare 18 cm IPG strips (PH 4 - 7 NL) (GE Healthcare; Amersham Biosciences) by active overnight rehydration. After the IEF and twice equilibration, the second-dimension SDS–PAGE was performed in 12.5% acrylamide gels using Ettan Dalt twelve (GE Healthcare; Amersham Biosciences). Proteins were detected by coomassie brilliant blue stain and scanned at 300 dpi (Power Look 2100XL, UMAX company). Image elaboration and analysis were carried out with ImageMaster 2-D Platinum version 5 software (GE Healthcare; Amersham Biosciences). Two-dimensional gels were repeated for each growth condition independently for three times to confirm the reproducibility of the protein pattern under attached growth conditions. Only the differences in protein spots that were reproduced three times are described here. After In- gel digestion, MS analysis was performed using an AUTOFLEX II TOF-TOF (Bruker Daltonics, Germany).

RESULT AND DISCUSSION

Bactericidal activities of antimicrobials against biofilm-forming sessile cells and floating (planktonic) cell

In this study, we tested the bactericidal activity of various antibiotics on bacterial biofilms cultured for varying time in a continuous -flow device. We observed that the bactericidal activity of all the antimicrobial agents decreased during the incubation period, and the order of reduction in activity was ceftazidime > imipenem > tobramycin > amikacin > gentamicin > levofloxacin > ciprofloxacin. The bactericidal activity for each of the above-mentioned antibiotics against 5-day-old biofilm cells were more than

Table 1. Bactericidal activities of antimicrobials against biofilm-forming sessile cells and floating (planktonic) cell (μg/ml).

		Biofilm		
	Planktonic	**BF-1**	**BF-3**	**BF-5**
ceftazidime	8	128	1024	2048
imipenem	8	128	1024	2048
levofloxacin	2	8	32	64
ciprofloxacin	1	4	8	16
amikacin	8	32	256	512
gentamicin	4	16	256	512
tobramycin	4	16	256	512

BF-1, BF-3, BF -5 represent the biofilm cells of 1 -day- aged, three –day aged and 5-day-aged respectively.

256-, more than 256-, 32-, 16-,64-,128- and 128-fold (Table 1).

Biofilm formation

P. aeruginosa displayed significantly different substratum coverage and thickness of biofilms (Figure 1). On the first day of biofilm formation, the substratum was covered by single-layer of bacterium, only a few formed relative larger microcolonies, the thickness of biofilm was 2 - 3 μm; When the biofilms was incubated for three days, the substratum was all covered by multiple-layers of bacteria, with uniform biofilm thickness of 10 - 14 μm. The biofilm thickness increased further up to 20 μm when the biofilms was incubated for five days

Protomics differentially expressed between biofilm-forming sessile cells and planktonic cell

Two-dimensional gel electrophoresis images stained by Coomassie brilliant blue R350 are shown in Figure 2. Image analysis revealed that 29 proteins were differentially expressed in the biofilm bacteria; among these, 5 proteins (spot 1, 2, 3, 4 and 7) were down regulated, and the rest were upregulated (Table 2). The up- regulated proteins, such as Arginine deiminase, ornithine carbamoyl transferase, serine hydroxymethyl transferase, iron - sulfur protein, ribosome recycling factor and 50S ribosomal protein have been shown to be upregulated in *P.aeruginosa* biofilm bacteria in other studies (Sauer et al., 2002; Vilain et al., 2004; Vilain et al., 2004) Svensäter reported that the expressions of DnaK, ribosome recycling factor and the elongation factor Tu were upregulated in *Streptococcus mutans* biofilms; these results were similar to those obtained in our experiments (Svensater et al., 2001). However, due to differences in experimental strains, the production of serine hydroxymethyl- transferase enzymes, ornithine carbamoyl transferase enzyme, arginine deiminase displayed contradictory results (Sauer et al., 2001; Sauer et al., 2002). The expression of pyruvate dehydrogenase was up-regulated in our experiment , which is contradictory to Svensäter's reports (Svensater et al. 2001). The proteins down- regulated were identified as malate synthase G, agonistic hydratase 2, sec-independent translocase protein tatA/E homolog undefined protein yydD and deoxyguanosine triphosphate hydrolase-like protein. To date, similar reports of these down- regulated protein expressions have not appeared in other literatures. There were 4 proteins identified in our experiment failing to find the corresponding locus in *P. aeruginosa* PAO1 genome. Considering that the score of the MS analysis was not high, the credibility of the proteins identified was not high either.

The effect of the biofilm thickness on drug resistance

The sensitivity of the 1-day-old, 3- day-old, and 5-day-old biofilm bacteria to antibiotics was 4 - 16 times, 8 - 128 times, and 16 - 512 times lower, respectively, than the sensitivities of the planktonic cells. The significant increase in drug resistance in biofilm bacteria with the increase in incubation time may be related to the gradual increase in the thickness of the biofilm. In a 10-cell-thick biofilm, the diffusion time shows a 100-fold increase (Stewart, 2003) . In our experiment, the thickness of the 3-day- old biofilm was equivalent to that of a 20-cell-thick and that of the 5-day-old biofilm was equivalent to that of a 40-cell-thick. The consequent delay in diffusion of oxygen and nutrients may contribute to increased drug resistance. Philp reported that hydrogen peroxide can only penetrate into 13-μm -thick biofilms (Stewart, 2003); however, the thickness of the 3-day-old biofilm was 10 - 14 μm and that of the 5-day-old biofilm was up to 20 μm. Therefore, the obstacles in antibiotic diffusion may markedly decrease the sensitivity of biofilm cells to antibiotics. Philp (Stewart, 2003) also found that biofilms with thickness of up to 25 μm are sufficient to create anaerobic conditions, and under anaerobic conditions, the bacteria are less susceptible to antibiotics, especially the aminoglycoside antibiotics (del Pozo et al., 2007). Coincidentally, in our experiments, the 5-day-old biofilm cells were found to be the most resistant to antibiotics. In addition, biofilm bacteria with a diameter of only 4 μm can initiate the Quorom-Sensing system, thereby inducing gene expression with protection(Stewart, 2003). Further, high densities of bacteria can produce widespread pressure stress and increase the expression of stress factors such as algT and rpoS along with catalase. The results of our experiments were consistent with these findings, because the expression of the proteins involved in adaptation and protection, transcription, and translation were upregulated, and maximum production of catalase was observed on the thickest biofilm.

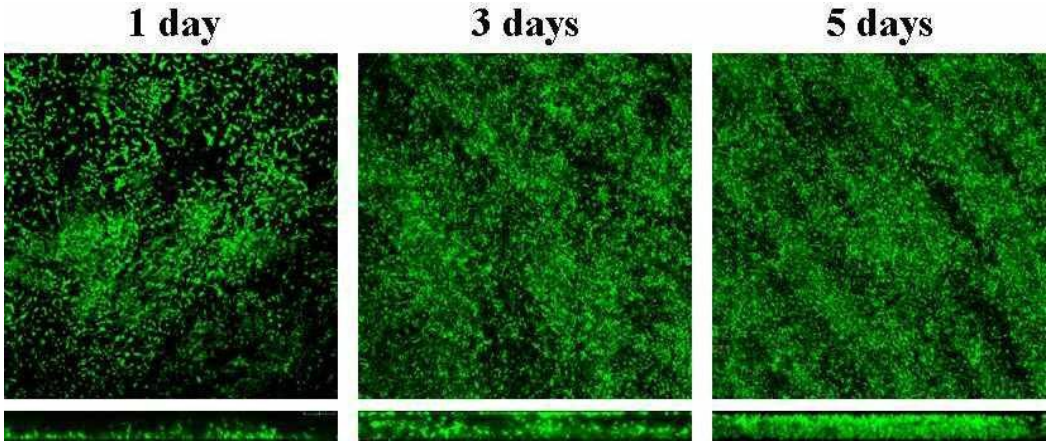

Figure 1. Confocal photomicrographs of P. aeruginosa biofilms.
Right panels are day 1, middle is day 3, left is day 5.The strains were tagged with GFP and grown in LB medium in a continuous flow cell as described in Methods. Panels on the bottom are verticla sections through the biofilms collected on the middle of the images, which represented the thickness of biofilms.

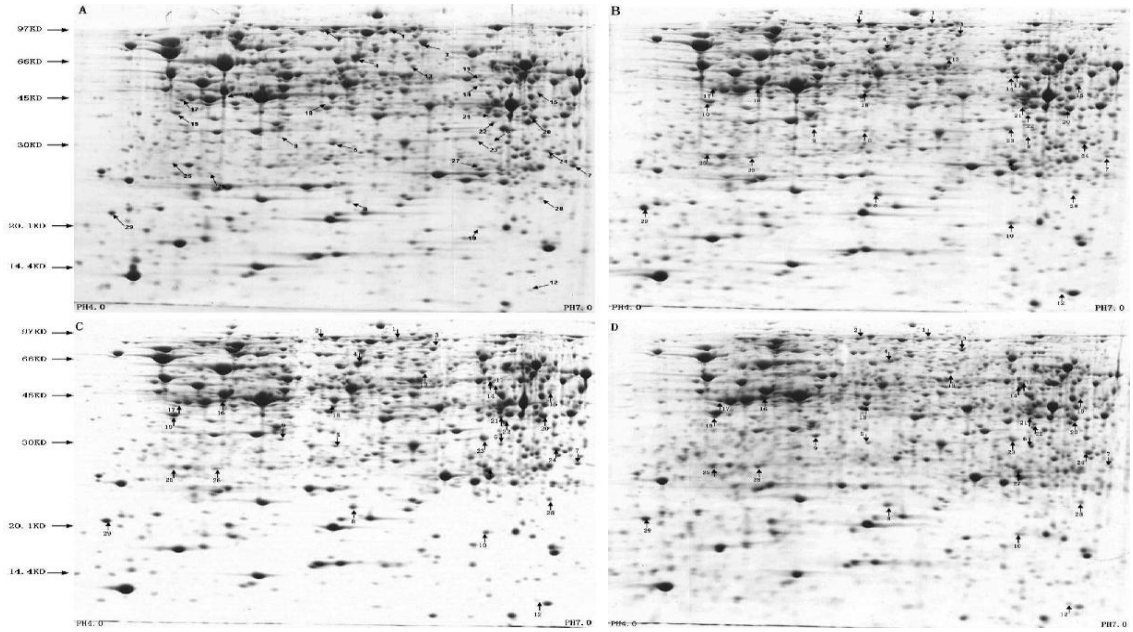

Figure 2. Two-dimensional images of crude protein extracts of *P. aeruginosa* PAO1 planktonic bacteria (A), biofilm cells attached for 1 day (B), as well as attached for 3 days (C) and 5 days(D); The crude protein extracts (1000 µg) were separated on pH 4 - 7 nonlinear Immobiline Dry strips (GE Healthcare; Amersham Biosciences), followed by SDS polyacrylamide gel electrophoresis; Gels were stained with Coomassie brilliant blue R350; arrows pointing indicate proteins that were differentially expressed and were picked up for matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDITOF-MS) (Table 2).

Fluoroquinolone antibiotics might be the better choice for the treatment of *P. aeruginosa* biofilm-associated infections

In comparison with the planktonic cells, the biofilm bacteria showed the most enhanced resistance to cefyaazidime and imipenem (more than 256 times higher). Further, resistance to amikacin, gentamicin, and tobramycin increased to a lesser extent (up to 64-, 128- , and 128 times, respectively), and the lowest increase in resistance was observed for ciprofloxacin and levofloxacin (up to 16 times higher); this finding can be attributed to the fact that

Table 2. Protomics differentially expressed between planktonic cell and biofilm-forming sessile cells during course of biofilm development (one day, three days and five days).

function	spot	Locus[a]	Protein[b]	MW (KDa)	PI	MS Score	Fold Change[c] 1D	3D	5D
Carbon catabolism and amino acid metabolism cofactor	1	PA0482	Malate synthase G	78.898	6.05	64	0.64	0.29	0.54
	2	PA1787	aconitate hydratase 2	94.196	5.22	202	0.66	0.54	0.64
	5	PA5171	Arginine deiminase	46.806	5.52	178	2.48	2.56	4.13
	12	PA0871	Putative pterin-4-alpha-carbinolamine dehydratase	12.865	5.91	36	2.65	8.29	1.96
	20	PA4602	Serine hydroxymethyltransferase 3	45.414	5.70	151	1.62	1.81	2.03
	21	PA3537	Ornithine carbamoyltransferase	38.255	6.13	102	1.98	2.52	2.06
Energy metabolism, fatty acid, metabolism phospholipid	9	PA3416 PA3417	Pyruvate dehydrogenase E1 component	99.843	5.56	98	3.32	2.54	5.36
	16	PA4848	Biotin carboxylase	49.256	5.92	114	1.69	1.60	2.81
	18	PA0447	glutaryl-CoA dehydrogenase	43.626	5.84	90	2.09	1.71	3.84
	24		Indole-3-glycerolphosphate synthase	33.787	8.46	67	1.50	1.77	2.21
adaptation, protection	8	PA3529	probable peroxidase	21.922	5.37	48	3.44	2.38	3.15
	13	PA1596	HSP90	72.577	4.89	55	1.53	1.93	1.70
	14	PA4236	Catalase	55.612	6.21	180	1.53	1.78	2.72
	19	PA4761	Chaperone protein dnaK	68.405	4.81	42	1.75	1.89	0.60
Nucleotide synthesis Transcription translation Elongation	6	PA4258	50S ribosomal protein L22	12.401	10.55	34	2.07	2.54	5.15
	7	PA1124	Deoxyguanosinetriphosphate triphosphohydrolase-like protein	45.664	5.94	59	0.36	0.44	0.42
	10	PA3653	ribosome recycling factor	20.473	5.85	76	2.90	2.09	2.78
	11	PA3134	Glutamyl-tRNA synthetase	54.170	5.79	41	2.81	1.53	2.79
	17	PA4265 PA4277	Elongation factor Tu	43.684	5.23	135	1.98	2.34	2.29
	23	PA4742	tRNA pseudouridine synthase B	33.617	6.41	76	1.56	2.17	2.27
	25	PA0019	Peptide deformylase	20.432	4.87	66	2.45	2.40	4.92
	28	PA0770	Ribonuclease 3	24.895	6.32	62	2.38	1.55	2.11
	29	PA3620	DNA mismatch repair protein mutS	92.676	5.40	66	2.57	1.64	2.16
Protein transport Translocation	3	PA5068	Sec-independent translocase protein tatA/E homolog	85.03	7.93	43	0.37	0.53	0.37
	15	PA4938	Adenylosuccinate synthetase	48.373	8.32	66	2.14	1.54	2.30
	22	PA5453	GDP-mannose4,6-dehydratase	36.491	6.13	110	1.75	2.23	2.08
	27		Thiazole biosynthesis protein thiG	26.767	5.76	62	2.07	2.15	1.74
others	4		Uncharacterized protein yydD	69.406	6.55	64	0.43	0.6	0.31
	26		Probable GTP-binding protein eng B	22.289	6.75	53	2.65	2.01	5.50

[a] the locus designates the gene number according to the *Pseudomonas Genome Project* (http://www.pseudomonas.com)
[b] Proteins identified by peptide mass fingerprinting.
[c] Fold change calculated as the IOD % value of the protein in the biofilm vs. the planktonic cells. 1D, 3D, 5D represent the biofilm cells of 1 -day- aged, three –day aged and 5-day-aged, respectively.

eftazidime and imipenem can only effectively kill bacteria in the breeding season. Oxygen depletion and nutrient-diffusion limitations ensure that the underlying bacteria are in a non- progressive state, thereby causing a significant decline in antibiotic susceptibility. Fluoroquinolone antibiotics can kill quiescent bacteria and can be transferred more quickly to the lower layers (Vrany et al., 1997); therefore, these antibiotics showed the lowest reduction in efficacy. The reason why the resistance of biofilm cells to aminoglycoside antibiotics with the same ability to kill quiescent bacteria is more than that to the fluoroquinolones might be related with the hyper-expression of alginates. Because the negatively-charged alginates can impede the movement of the positively charged amino glycoside antibiotics, thereby reducing the efficacy of the antibiotics (Shigeta et al., 1997). Our study showed that the expression of GDP-mannose dehydratase, a key enzyme involved in the synthesis of alginates, on the third and fifth day of biofilm formation was 2 times higher than the expression in age-matched planktonic cells, which are consistent with the findings in Waite's reports (Waite et al., 2005). Ramsey reviewed some different opinion with regard to the alginate synthesis in biofilms (Ramsey et al., 2005). Some reports using gene chips did not show upregulation of the alginate synthesis gene in biofilms, some other reports using alginate-specific antibodies and β-galactosidase method found alginate expression in the biofilms formed by non-mucoid *P. aeruginosa* immediately after adhesion (Davies et al., 1995).

Conclusion

The differences in the experimental techniques and methods employed by each laboratory may cause discrepancies in the results. However, many proteins showed similar results under different experimental conditions. This study revealed the differential expression of a number of proteins in biofilm bacteria and their planktonic counterparts, which has not been reported previously. Although the role of the differentially expressed proteins in biofilm drug resistance needs to be explored further, the results showed that fluoroquinolone antibiotics are the better choice for treatment of *P. aeruginosa* biofilm-associated infections.

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