Time-course proteome analysis of air-liquid interface biofilm of *Pseudomonas aeruginosa* PA14

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ABSTRACT

At an air-liquid interface (ALI), pathogenic bacteria have ability to create robust and three-dimensional biofilms that are resistant to shear force. ALI biofilms not only contribute to heightened antibiotic resistance in response to antimicrobial treatments but also enable pathogens to endure challenging environmental conditions. Despite knowing that *Pseudomonas aeruginosa* is capable of developing ALI biofilms, there is limited knowledge about the underlying mechanisms. This study aimed to investigate the dynamics of *P. aeruginosa* PA14 air-liquid interface (ALI) biofilms over time using comprehensive proteomic analysis. Over the course of 48 to 72 h, the formation of biofilms at the ALI demonstrated a noticeable and discernible increase in thickness. A total of 778 proteins were commonly shared across all time points of ALI biofilm samples, and differential expression analysis identified a varying number of proteins across different time points. Specifically, 1,460, 1,789, 1,959, 1,938, and 1,949 proteins were identified as differentially expressed in ALI biofilms at 48 h (T1), 72 h (T2), 96 h (T3), 120 h (T4), and 144 h (T5), respectively. Among the upregulated proteins, those related to "amino acid transport and metabolism (E)" were the most prevalent, followed by proteins associated with "energy production and conversion (C)" within specific COG categories. KEGG pathway analysis highlighted an abundance of proteins related to "amino acid metabolism" and "carbohydrate metabolism" in both the upregulated and downregulated sets. Several pathways associated with biofilm formation, cAMP/Vfr signaling, QS, Gac/Rsm, c-di-GMP signaling pathway, were substantially altered during ALI biofilm formation. Several proteins involved in biofilm formation, such as AlgE, FliC, FlgE, HcpA, and Icc, were consistently upregulated at all time points. Evidence suggests that the flagella is likely to have a greater significance than pili, particularly in the initial phases of ALI biofilm development. Exopolysaccharides, specifically alginate and Pel, are believed to play a crucial role in the generation of ALI biofilms, whereas Psl was not involved. Proteins associated with virulence, transporters, and iron also showed differential expression throughout the biofilm formation process, indicating their importance in ALI biofilms. These findings provide valuable insights into the dynamics of the proteome and functional pathways associated with *P. aeruginosa* ALI biofilms, contributing to an enhanced understanding of biofilm development and the identification of potential therapeutic targets.

INTRODUCTION

Biofilms are complex communities of bacteria that grow on both living and non-living surfaces. These communities are enveloped by extracellular polymeric substances (EPS), which consist of carbohydrates, extracellular DNA, proteins, exopolysaccharides, and lipids. The EPS plays multiple roles, acting as a structural scaffold, providing protection against environmental stresses like antimicrobial agents and immune defenses, and facilitating genetic material exchange within the bacterial community.

Bacterial biofilms can be developed on various types of interfaces, including both solid-liquid and air-liquid interfaces (ALI). In the early stages, aerobic bacteria can migrate to the air-liquid interface and initiate the formation of a thin biofilm layer. Unlike biofilms formed on solid surfaces, the self-organization of constituent cells is more demanding in ALI biofilms due to the absence of a solid substrate for attachment. Over time, these biofilms can grow, develop into thick structures capable of withstanding shear forces, and exhibit a three-dimensional wrinkled morphology. The ALI provides an ideal habitat for aerobic bacteria as they can access oxygen from the air above and obtain nutrients from the liquid medium below.

***Pseudomonas aeruginosa* is a pathogen responsible for hospital-acquired infections, including cystic fibrosis, pneumonia, and bloodstream infections. It can colonize and form biofilms on medical devices such as urinary catheters and endotracheal tubes. Treating *P. aeruginosa* infections is challenging due to its ability to rapidly mutate and develop resistance to antibiotics. Multidrug-resistant strains of *P. aeruginosa* have been frequently identified in patients with catheter-associated urinary tract infections, bloodstream infections, and ventilator-associated pneumonia. Although *P. aeruginosa* is known to form pellicles, the detailed mechanisms involved are not well understood. This study focuses on a time-course analysis of the quantitative proteome of *P. aeruginosa* PA14 ALI biofilms.**

Fig. ALI biofilm formation on the top of the LB media. 48 h **(A)**, 72 h **(B)**, 96 h **(C)**, 120 h **(D)**, and 144 h **(E)**.

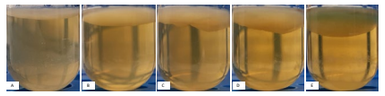


Fig. Heatmap of the proteomic data

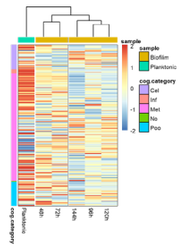


Fig. 3. Venn diagram of the proteomic data

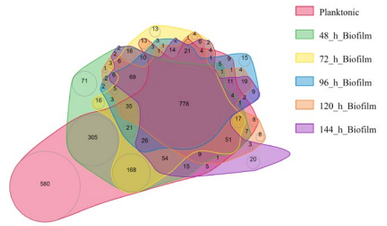


Fig. The number of differentially expressed proteins

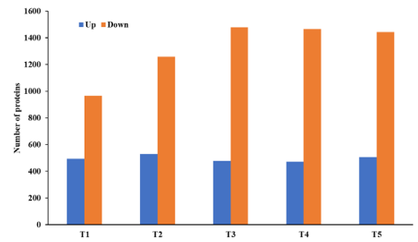
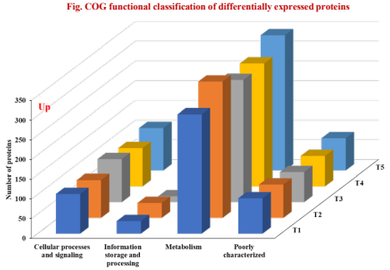


Fig. COG functional classification of differentially expressed proteins



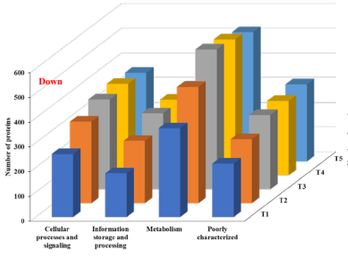
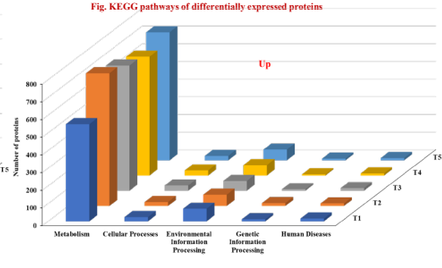
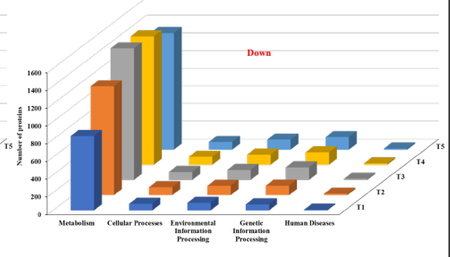
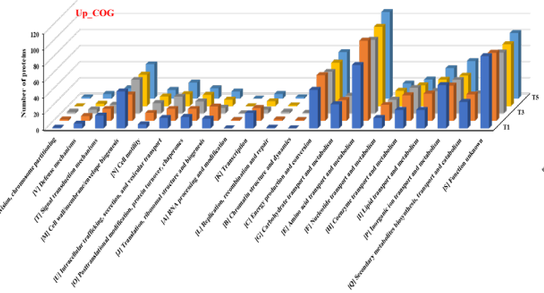
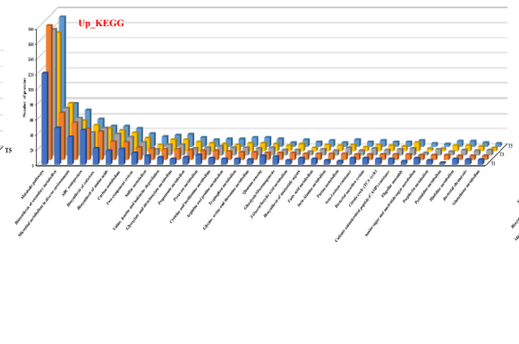


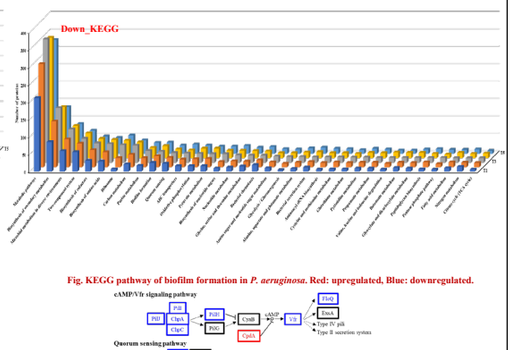
Fig. KEGG pathways of differentially expressed proteins











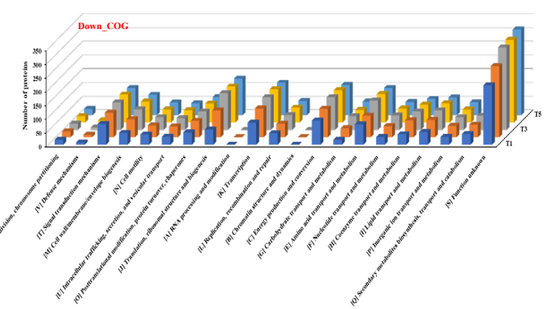


Fig. Protein expression patterns and functional distribution of proteins

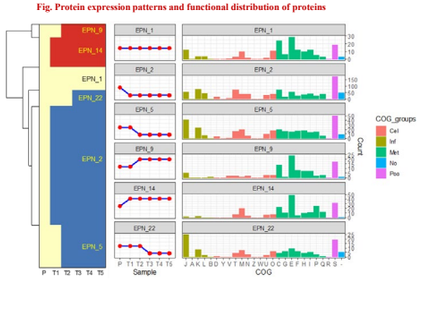
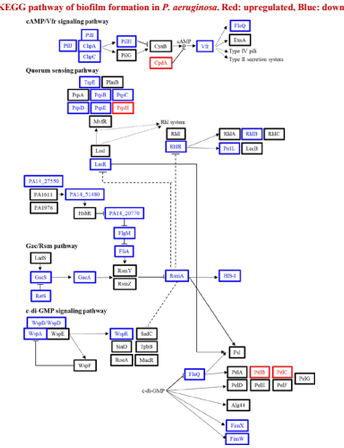


Fig. KEGG pathway of biofilm formation in *P. aeruginosa*. Red: upregulated, Blue: downregulated.



METHODS

Bacterial strain and growth conditions

A single colony was isolated, inoculated in 5 ml of Luria-Bertani (LB) broth, and grown overnight at 37°C with shaking at 200 rpm. Strain PA14 culture was transferred to a microcentrifuge tube and centrifuged. The supernatant was removed, and cells were washed three times with PBS. The bacterial suspension was adjusted to an optical density at 600 nm of 0.1 and ALI biofilms were grown without shaking at 37°C with 4 ml of LB broth for 48, 72, 96, 120, and 144 h. ALI biofilms were harvested from the surface of the culture and pelleted by centrifugation. The pellet was washed with PBS and used for protein extraction. For the planktonic growth, the culture was grown at 37°C overnight under shaking. Cells were harvested by centrifugation and the resulting pellet was washed with PBS and employed for protein extraction.

Protein extraction

Washed planktonic and ALI biofilms were suspended with 500 µl of BugBuster Plus Lysonase kit in Lysing Matrix B tubes containing 0.1 mm silica spheres. Bacterial cells were disrupted by an FP120 reciprocator at speed 6 for 45 sec. Cells then were boiled and vortexed for 5 min and 1 min, respectively. The final protein extract was recovered by centrifuge at 20,817 x g for 20 min at 4°C.

Sample preparation

Ten microgram of each sample was loaded onto a 10% SDS-PAGE gel. The gel was excised into 10 segments per lane and gel slices were processed using a robot, ProGest with the following protocol. The gel was washed first with 25 mM ammonium bicarbonate and then with acetonitrile. Reduction was carried out with 10 mM dithiothreitol at 60°C and alkylation with 50 mM iodoacetamide. Digestion was performed with trypsin at 37°C for 4 h. The reaction was quenched by adding formic acid and the supernatant was analyzed directly without further treatment.

MS analysis

Gel digests were analyzed by nano LC/MS/MS with a Waters NanoAcquity HPLC system interfaced to a ThermoFisher Fusion Lumos mass spectrometer. Peptides were pumped onto a trapping column and eluted over a 75 µm analytical column at 350 nL/min; both columns were packed with Luna C18 resin. The mass spectrometer was run in a data-dependent mode, with MS and MS/MS performed in the Orbitrap at 60,000 FWHM resolution and 17,500 FWHM resolution, respectively. The fifteen most abundant ions were selected for MS/MS. The instrument was operated with a 3s cycle for MS and MS/MS.

Data processing

Data were searched using a local copy of Mascot with the following parameters: Enzyme, trypsin; Database, Uniprot Pseudomonas aeruginosa strain UCBPP-PA14 (concatenated forward and reverse plus common contaminants); Fixed modification, carbamidomethyl (C); Variable modifications, oxidation (M), acetyl (Protein N-term), deamidation (NQ), and pyro-Glu (N-term Q); Mass Values, monoisotopic; Peptide Mass Tolerance, 10 ppm; Fragment Mass Tolerance, 0.020 Da; Max Missed Cleavages, 2. Mascot DAT files were parsed into the Scaffold software for validation, filtering, and creating a nonredundant list per sample. Data were filtered at 1% protein and peptide false discovery rate (FDR) and requiring at least two unique peptides per protein. The cutoff between control and TC-treated groups was ≥ 2.0 (up) and ≤ 0.5 (down). Functional annotation of the proteins was carried out by COG, and the KEGG pathway analysis was employed for systematic analysis of gene function. Heatmap was constructed using the R package 'pheatmap', and the protein expression pattern analysis was conducted in-house python script. The Venn diagram was created using nVenn, a web-based tool, for the analysis of different sets

CONCLUSION

- ***Proteomic analysis identified upregulated proteins associated with amino acid transport and metabolism, while downregulated proteins showed dynamic changes across various cellular processes.***
- ***Proteins involved in biofilm formation, virulence, transporters, and iron exhibited differential expression during biofilm development.***
- ***These findings enhance our understanding of the proteomic dynamics and key pathways involved in P. aeruginosa ALI biofilms, providing valuable insights for biofilm control and management in P. aeruginosa infections.***