At an air-liquid interface (ALI), pathogenic bacteria have the ability to divide and form three-dimensional biofilms that are resistant to environmental stress. ALI biofilm not only contributes to heightened antibiotic resistance in response to antifungal treatments but also enables bacteria to endure challenging environmental conditions. Despite knowing that Pseudomonas aeruginosa is capable of developing an ALI biofilm, there is limited knowledge about the underlying mechanisms. This study aimed to investigate the dynamics of P. aeruginosa ALI air-liquid interface (ALI) biofilm over time using comprehensive proteomic analysis. Over the course of 48 to 72 h, the formation of an ALI biofilm at the ALI demonstrated a novel and discernible increase in thickness. A total of 776 proteins were identified across all time points of ALI biofilm samples, and differential expression analysis identified a varying number of proteins across different time points. Specifically, 600, 679, 800, 616, and 631 proteins were identified as differentially expressed in ALI biofilms at T0 (48 h), T1 (72 h), T2 (96 h), T3 (120 h), and T4 (144 h), respectively. The upregulated proteins, those classified as “up” and “up +”, were associated with “energy production and conversion (C)” within specific COG categories. The COG pathway analysis highlights an abundance of proteins related to “amino acid metabolism” and “nucleotide metabolism” in both the upregulated and downregulated sets. Several pathways associated with biofilm formation, cAMP/PAS signaling, and Cys pathways were significantly enriched during ALI biofilm formation. Several genes involved in biofilm formation, such as FlgE, HcpA, and Icc, were consistently upregulated at all time points. These findings suggest that the flagella is likely to have a greater significance than pili particularly in the initial phases of ALI biofilm development and the identification of pivotal therapeutic targets.

**INTRODUCTION**

Biofilms are complex communities of bacteria that grow on both inanimate and living surfaces. These communities are capable of producing metabolites that help protect themselves against environmental stressors. Biofilm formation is a physiological process by which bacteria can spontaneously adhere to solid surfaces, leading to the development of biofilms. This study focuses on a time-course analysis of the quantitative proteome of P. aeruginosa PA14 ALI biofilms.

**METHODS**

**Sample preparation**

Samples were collected from a 10% LB agar plate and grown for 24 h at 37°C under shaking. For the planktonic growth, the culture was grown at 37°C with shaking. Strain PA14 culture was incubated on LB media with shaking at 200 rpm. Strain PA14 culture was centrifuged to recover the biomass. The supernatant was discarded by a 20 mL pipette tip at speed of 2,000 rpm. The pellet was washed with PBS and used for protein extraction.

**Protein extraction**

Washed planktonic and ALI biofilms were suspended with 500 μL of BugBlot Plus (Lysis kit in lysis Matrix B tubes containing 0.1 mm silica spheres). Bacterial pellets were disrupted by an EPPendorf microspatula at speed of 800 rpm for 30 s (up and vertical for 5 s and 1 min, respectively). The final protein extract was stored at −80°C for future use.

**Data processing**

Proteins were 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 100 mM dithiothreitol at 60°C and alkylation with 50 mM iodoacetamide. Digestion was performed with trypsin at 37°C for 2 h. The reaction was quenched with formic acid and the supernatant was analyzed by nano LC/MS/MS with a Waters NanoAcquity HPLC system interfaced to a ThermoFinnigan LTQ linear ion trap mass spectrometer. Peptides were pumped onto a trapping column and desalted at 37°C with analytical column at 0.3 μL/min for 30 min and then eluted at 0.5 μL/min for 25 min. The fifteen most abundant ions were selected for MS/MS. The instrument was operated with a 3s cycle for MS and MS/MS.

**CONCLUSION**

Proteomic analysis identified upregulated protein associated with amino acid transport and metabolism, while downregulated proteins showed dynamic changes across various cellular processes. P. aeruginosa was known as a pathogenic bacteria as they can access oxygen from the air above and create biofilm. Bacterial biofilms can be developed on various types of interfaces, including both solid-liquid and air-liquid interfaces (ALI). In the early development of biofilm formation, the role of bacterial biofilms is to provide protection against environmental stresses like antimicrobial agents and immune defenses, and facilitating genetic material exchange within the bacterial community. These findings enhance our understanding of the proteomic dynamics and key pathways involved in P. aeruginosa; providing valuable insights for biofilm control and management in P. aeruginosa infections.

**References**

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