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ORIGINAL PAPER

Decoding *Acinetobacter baumannii* biofilm dynamics and associated protein markers: proteomic and bioinformatics approach

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Abstract

Biofilm formation by *Acinetobacter baumannii* is one of the major cause of its persistence in hospital environment. Biofilm phenotypes are more resistant to physical as well as chemical stresses than their planktonic counterparts. The present study was carried in quest of biofilm-associated protein markers and their association with various biological pathways of *A. baumannii.* The study was designed with an aim to highlight the crucial common factor present in the majority of the *A. baumannii* strains irrespective of its resistance nature. A label-free proteome comparison of biofilm and planktonic phenotypes of *A. baumannii* was done using QExactive tandem mass spectrometry. Our investigation suggests key elevation of adhesion factors, acetate metabolism, nutrient transporters, and secretion system proteins are required for biofilm formation in *A. baumannii*. Elevation of biofilm-associated proteins revealed that biofilm is the unique phenotype with the potential to form robust matrix-embedded colonies and defeat stress condition. Further, core protein markers of biofilm phenotypes could be used as targets for new clinical interventions to combat biofilm-associated infections.

Keywords *Acinetobacter baumannii* · Biofilms · Planktonic · Antimicrobial resistance · Comparative proteomics

Introduction

Acinetobacter baumannii is a member of ESKAPE group of pathogens, which is endemic in many regions and responsible for nosocomial infections worldwide, particularly in the intensive care units (ICU) (Rice 2008; Karampatakis et al 2017). *A. baumannii* has an ability to persist on diverse surfaces; including hospital associated medical devices and biological tissues in the form of 'biofilms' (Rodríguez-Baño et al. 2008; Fiester and Actis 2013).

Bacterial biofilms are universal survival strategy that consists of embedded bacterial cells within self-produced extracellular polymeric substances (EPS). The EPS are primarily composed of exo-polysaccharides, lipids, proteins and nucleic acids (Monds and O'Toole 2009). These EPS embedded biofilm formation is hostile environment for bacterial survival (Gupta et al. 2016).

A. baumannii forms robust biofilms, which abide challenging environmental conditions. Biofilm growth modes are known to be a predominant state for persistent survival of *A. baumannii* in hospital settings that may increase the probability to cause infections and outbreaks (Rao et al. 2008; He et al. 2015). The emergence of multidrug resistant strains of *A. baumannii* with high biofilm forming ability not only increase the time required for its treatment, but also intensify the chance of recurrence of the pathogen and raise the difficulty to treat associated infections (Eze et al. 2018).

Since the last two decades, majority of the drug discovery efforts have been focused on the eradication of planktonic phenotypes of *A. baumannii* (Fishbain and Peleg 2010; Poulikakos et al. 2014). However, it's an established fact that *A. baumannii* biofilm forming cells are more resistant against antimicrobials and extermination of such cell communities is more troublesome than their planktonic counterparts (Espinal et al 2012; Qi et al. 2016). The biofilm forming ability is not restricted to antimicrobial resistant strains of

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A. baumannii but has also been observed in susceptible strains (Tomaras et al. 2003; McQueary and Actis 2011). Therefore, understanding the molecular mechanisms which trigger biofilm formation is of utmost importance to cure biofilm-associated infections and development of specific countermeasures.

Several proteomic and transcriptomic studies have been carried for the identification of the important factors contributing towards biofilm formation by *A. baumannii* (Rumbo-Feal et al. 2013; Shin et al. 2009; Cabral et al. 2011). These studies, led to the identification of several important factors associated with biofilm formation, including *csu*A/BABCDE operon, quorum sensing, two-component regulatory systems as key contributory factors. The dynamics of molecular mechanisms during air–liquid pellicle formation in *A. baumannii* has also been explored using gel free comparative proteomics (Kentache et al. 2017).

Although the previous reports revealed several important factors associated with *A. baumannii* biofilm, however a single strain was usually considered (Rumbo-Feal et al. 2013; Shin et al. 2009; Cabral et al. 2011). Majorly, the physiology of 24 h mature biofilms of *A. baumannii* was only investigated. The present study was designed with an aim to highlight the crucial common factor present in the majority of the *A. baumannii* strains irrespective of its resistance nature. Elucidation of common factors responsible for biofilm formation may help in the development of new therapeutics to combat with *A. baumannii* biofilm associated infection. To our knowledge, no comparative study has been done, which include susceptible as well as MDR isolates to explore protein expression dynamics of 48 h mature biofilms.

In the present study, a gel-free quantitative global proteome comparison between biofilm and planktonic growth states of five biological replicates (four antimicrobial resistant clinical isolates and a susceptible type strain ATCC 19606) of *A. baumannii* was carried out. Differentially expressed statistically significant proteins associated with biofilm formation were further utilized for bioinformatics analysis to elucidate important biological pathways contributing to *A. baumannii* biofilms formation.

Methods

All culture media and antibiotics were purchased from HiMedia Pvt. Ltd., India. Other analytical grade chemicals used for protein extraction and quantification, were purchased from Merck, and Bio-Rad Laboratories.

Bacterial cultures and antimicrobial susceptibility

The bacterial isolates of *A. baumannii* were collected from the Department of Microbiology, AIIMS, New Delhi. Clinical isolates were designated as Ab1, Ab2, Ab3, and Ab4 whereas the type strain ATCC 19606 was labelled as ATCC. All strains were stored as glycerol stock (10%v/v glycerol in Luria broth) at -80 °C. Routine growth was achieved in 10 ml Luria broth (LB) at 37 °C with constant shaking. Antimicrobial susceptibility and minimum inhibitory concentration for nine antibiotics (as recommended by ICMR, India) were determined as per CLSI guidelines using Kirby Bauer and E-test strips diffusion methods, respectively.

Quantitative biofilm assay

Biofilm forming ability of *A. baumannii* isolates was determined in triplicates using standard quantitative crystal violet assay (O'Toole 2011; Qi et al. 2016). Briefly, overnight grown cultures were adjusted to 0.5 McFarland standard. Each culture was further diluted 1:20 in 200 µl LB into 96-well plates. The diluted cultures were incubated at 37 °C for 48 h. Planktonic fractions were discarded, and adhered cells were washed thrice with phosphate-buffered saline (PBS) and stained using 0.1% w/v of crystal violet for 20 min. Crystal violet was discarded and excess stain was removed by washing plates twice with PBS. The stain retained by adhered cells was further solubilized in 200 µl of 95% ethanol by incubating for 20 min, and optical density was determined at 570 nm. *A. baumannii* ATCC 19606 and un-inoculated LB was used as positive and negative control, respectively for the determination of relative extent of biofilm formation by clinical isolates. Two criteria were considered for identification of high biofilm former. These criteria were adopted from study of Qi et al. 2016 and Bardbari et al. 2017. Isolates with mean $OD_{570} \geq OD_{570}$ of ATCC 19606 were characterized as strong biofilm former (Qi et al. 2016). Similarly as per another criterion, strains with mean $OD_{570} \geq 4 \times OD_{570}$ of negative control were designated as strong biofilm former and isolates with $OD_{570} \geq 2 \times OD_{570}$ of negative control as medium biofilm formers (Bardbari et al. 2017). The purpose of using two different criteria for classification of biofilm formers was to validate results obtained using first criterion, ensuring correctness of the high biofilm formers.

Growth of planktonic and biofilm phenotypes

An overnight grown culture of *A. baumannii* was used to inoculate 100 ml of LB to achieve a final concentration of 10^7 CFU per ml. An overview of experimental approach used to compare protein profiles of biofilm forming and planktonic phenotypes of *A. baumannii* is given in Fig. 1. For planktonic growth, the cultures were incubated at 37 °C with continuous shaking at 180 rpm for 48 h. Simultaneously, for biofilm formation 100 ml culture of each strain was dispensed into polystyrene plates and incubated at 37 °C

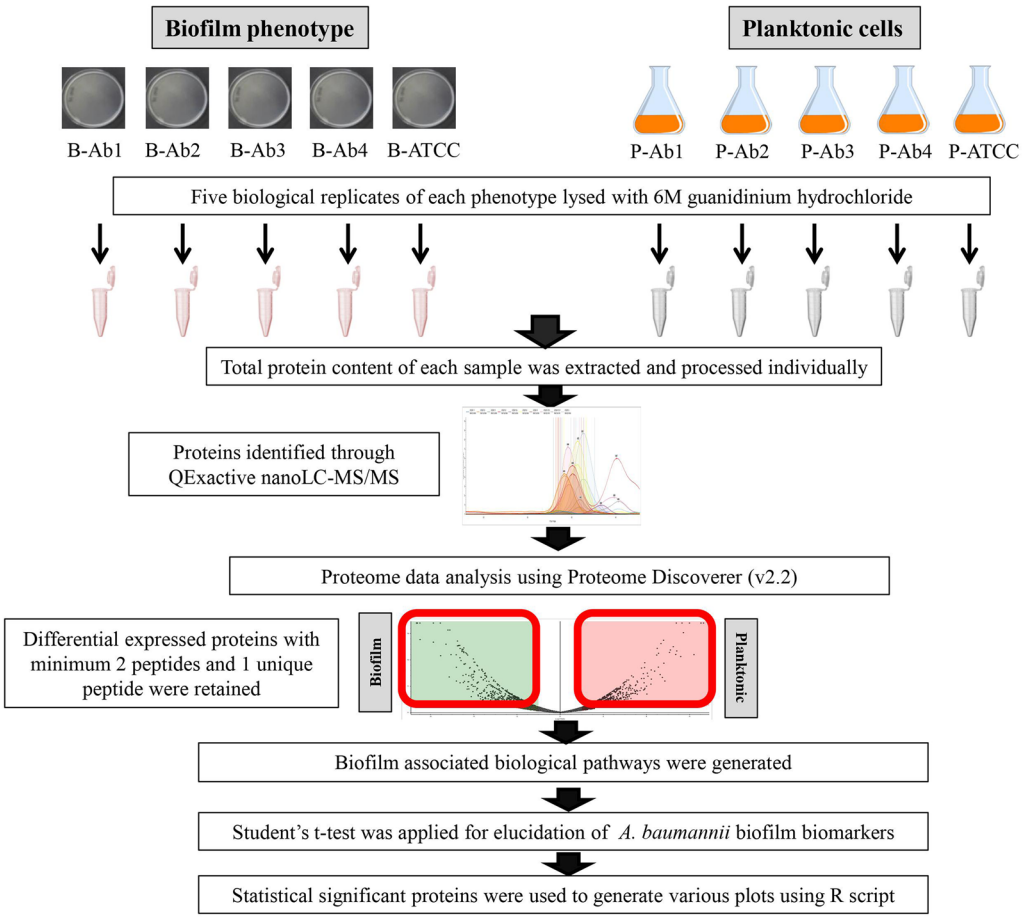

Fig. 1 Schematic design of the workflow used for protein extraction and quantitative proteome comparison of biofilm and planktonic phenotypes of *A baumannii* The sample Ab1, Ab2, Ab3, and Ab4 represents multidrug resistant clinical isolates of *A baumannii* whereas sample ATCC is type strain ATCC 19606 Biofilm and planktonic phenotypes are designated as B and P respectively

under static conditions for 48 h. The supernatant culture was discarded and plates washed twice with PBS to remove any un-adhered cells. Biofilm encased cells of each culture were scraped pooled and processed individually. Cells of each strain from two phenotypes were harvested by centrifugation at 3,500 g for 10 min at 4 °C and washed twice with deionized water prior to protein extraction.

Protein extraction from biofilms and planktonic cells

Total protein content of each strain from its two phenotypes was extracted employing mass spectrometry compatible protocol. Briefly, ten culture pellets from two growth phenotypes were treated individually with 600 µl preheated lysis buffer (6 M Gn-HCl, 0.1 M Tris, pH 8). The cells were vortexed thoroughly and heated at 95 °C for 10 min, followed by three cycles of ultra sonication on ice for 30 s with an interval of 5 s. Unbroken cells from the lysate were removed by centrifugation at 10,000 g for 10 min at 4 °C. The protein concentration in each sample was quantified by Bio-Rad protein assay.

Quantitative gel free proteome analysis

Gel free proteome analysis of all ten samples was carried at VProteomics, New Delhi. All experiments were performed using EASY-nLC 1000 system (Thermo Fisher Scientific) coupled to a QExactive mass spectrometer equipped with nano-electrospray ion source.

Sample preparation

25 µg of each protein sample was reduced and alkylated with a final concentration of 5 mM tris (2-carboxyethyl) phosphine (TCEP) and 50 mM iodoacetamide respectively. The samples were further diluted to achieve 0.6 M final concentration of Gn-HCl with 25 mM ammonium bicarbonate buffer and digested with trypsin (1:50, trypsin: lysate ratio) for 16 h at 37 °C. Digests were cleaned up using C18 silica

cartridge (The Nest Group, Southborough, MA) according to the manufacturer's protocol and dried using speed vac. The dried pellets were resuspended in Buffer A (5% acetonitrile, 0.1% formic acid).

Mass spectrometric analysis of peptide mixtures

1 µg of the peptide mixture was loaded on precolumn and resolved using 15 cm Pico Frit filled with 1.8 µm C18-resin. The peptides were loaded with Buffer A (5% acetonitrile, 0.1% formic acid) and eluted with a 0–40% gradient of Buffer-B (95% acetonitrile, 0.1% formic acid) at a flow rate of 300 nl/min for 90 min. The QExactive was operated using the Top 10 HCD data-dependent acquisition mode with a full scan resolution of 70,000. MS/MS scans were acquired at a resolution of 17,500. Lock mass option was enabled for polydimethylcyclosiloxane (PDMS) ions (m/z = 445.120025) for internal recalibration during the run.

Data processing

Five biological replicates of each phenotype were processed and generated 10 raw files. The proteome data was analyzed with Proteome Discoverer (v2.2) against the Uniprot *Acinetobacter baumannii* (ACIB) customized dataset. For Sequest search, the precursor and fragment mass tolerances were set at 10 ppm and 0.5 Da, respectively. The protease used to generate peptides, *i.e.* enzyme specificity was set for trypsin/P (cleavage at the C terminus of "K/R: unless followed by "P") along with maximum missed cleavages value of two. Carbamidomethyl on cysteine, oxidation of methionine and N-terminal acetylation and phosphorylation on site (S, T, Y) were considered as variable modifications in a database search. Protein false discovery rate (FDR) for peptide spectrum match (PSM) was set to 0.01 FDR. Identified proteins were considered to have at least 2 peptides and 1 unique peptide. Abundance values were normalized based on "total peptide amount method". Missing values were imputed using random values by low abundance resampling method. The 10 samples were divided into two groups *i.e.,* biofilm "B" and planktonic "P" and the proteins varied by at least 1.2 fold log2 abundance with threshold p value of less than 0.05 between two growth conditions were retained. The mass spectrometry raw data files were deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier "PXD023113".

Statistics rationale

The differential expressions of proteins of biofilm and planktonic phenotypes were further calculated to search for specific protein markers, which were at least present in 6 out of 10 samples. Missing values were imputed based on the normal distribution. Abundance values were log2 normalized followed by Z-score standardization. Z score values were further processed using Student's t test to calculate the significance of proteins. Significance was calculated using Benjamini Hochberg and 0.05 FDR was used as significance cutoff. All the significant proteins were then used for data visualization and representation using in-house R scripts.

Relationship between each sample was visualized as correlation plot (Pearson's correlation coefficient). Principal component analysis (PCA) was performed to evaluate variations among biological replicates and within biological replicates. The proteins with similar expression pattern along with cellular components and biological functions were visualized using heat map.

Gene ontology (GO) term enrichment using ClueGO

The GO enrichment analysis of proteins of biofilm and planktonic phenotypes was performed using the ClueGO, a plug-in of Cytoscape 3.7.2. ClueGO enables the comparison of clusters or groups and highlights their functional specificity (Bindea et al. 2009). Two protein sets were uploaded in ClueGO (Cluster 1 biofilm proteins; and Cluster 2 planktonic proteins) and compared with each other. The enrichment/depletion of biological terms of two clusters was performed using two-sided hyper-geometric test. The Kappa-statistics score threshold was set to 0.4 and GO term fusion was enabled. GO tree intervals were selected between 2 to 8 levels, with minimum 1 gene that should account at least 2% of genes for each pathway. Cytoscape preferred layout was selected to visualize significant enriched biological processes of two phenotypes.

Results and discussion

Antimicrobial susceptibility and biofilm formation of *A. baumannii*

The type strain *A. baumannii* ATCC 19606 showed susceptibly to all tested antibiotics. Whereas, four clinical isolates were resistant to all tested antibiotics except amikacin and cefoperazon for which isolate Ab3 and Ab4 were susceptible and intermediate resistant, respectively (supplementary table 1). Thus, the clinical isolates were characterized as MDR as per CLSI guidelines because they were resistant to three or more antibiotics from different classes of antibiotics.

The extent of biofilm formation is summarized in supplementary Fig. 1. The mean OD_{570} for reference strain and negative control was $0.44 \pm 0.034$and 0.14 ± 0.012, respectively. The OD_{570} for clinical Ab1, Ab2, Ab3 and Ab4 were 2.11 ± 0.17, 2.44 ± 0.07, 1.99 ± 0.05, and 2.22 ± 0.02, respectively. As per the criteria used for classification of

biofilm formers, the four clinical isolates used in the study were categorized as high biofilm formers. The high biofilm forming MDR clinical isolates along with susceptible type (reference strain) *A. baumannii* ATCC 19606 were further used for quantitative proteome comparison under biofilm and planktonic growth modes.

Abundance of proteome profiles in biofilm and planktonic phenotypes

Consideration of susceptible and resistant isolates in one group for whole proteome comparison might reveal core regulatory pathways for biofilm formation by *A. baumannii.* The detection of common factors that contribute to biofilm formation might be helpful for development of novel treatment strategies to combat biofilm associated infections.

The proteomics data identified a total of 2154 proteins in whole cell lysate with minimum 2 peptides and 1 unique peptide (supplementary table 2). The quantitative analysis using Proteome Discoverer revealed a total 499 (23%) proteins, which showed statistical significant difference ($p \leq 0.05$ and log2 fold change ≥ 1.2) among two growth conditions. Biofilm cells showed up-regulation of 232 proteins, while 267 proteins were get down regulated in comparison to planktonic phenotype. The cellular locations of 499 proteins of biofilm and planktonic growth modes are shown in supplementary Fig. 2.

The up-regulated biofilm associated proteins were further categorized in biological processes according to their molecular functions (Fig. 2 and supplementary table 3). We have noticed that the proteins were majorly associated with functions of cell adhesion, secretions across membrane, nutrient uptake, responses to external stimuli, amino acid biosynthesis, and acetate metabolism. On other hand, the proteins of planktonic phenotypes were involved in maintaining cell physiology, energy production (carbohydrate catabolism) transcription, and translation. It was observed from the data that the biofilm mode of growth evolved with mechanisms for prolong survival, whereas planktonic conditions are ideal for increase in biomass. Similar phenotypic behavior of two phenotypes has also been observed in other research findings (Sauer et al 2002; Kostakioti et al 2013; Kentache et al 2017).

Proteins contribute to formation and persistence of biofilms

Membrane transporters and secretion systems

Biofilm cells showed higher expression of several proteins, which contribute to the transport of biomolecules. One such secretion system is Sec-dependent transporter of proteins across cytoplasmic membranes. In present study, abundance of multifunctional protein secA (3.7 fold) was observed, which involved in export of proteins across inner

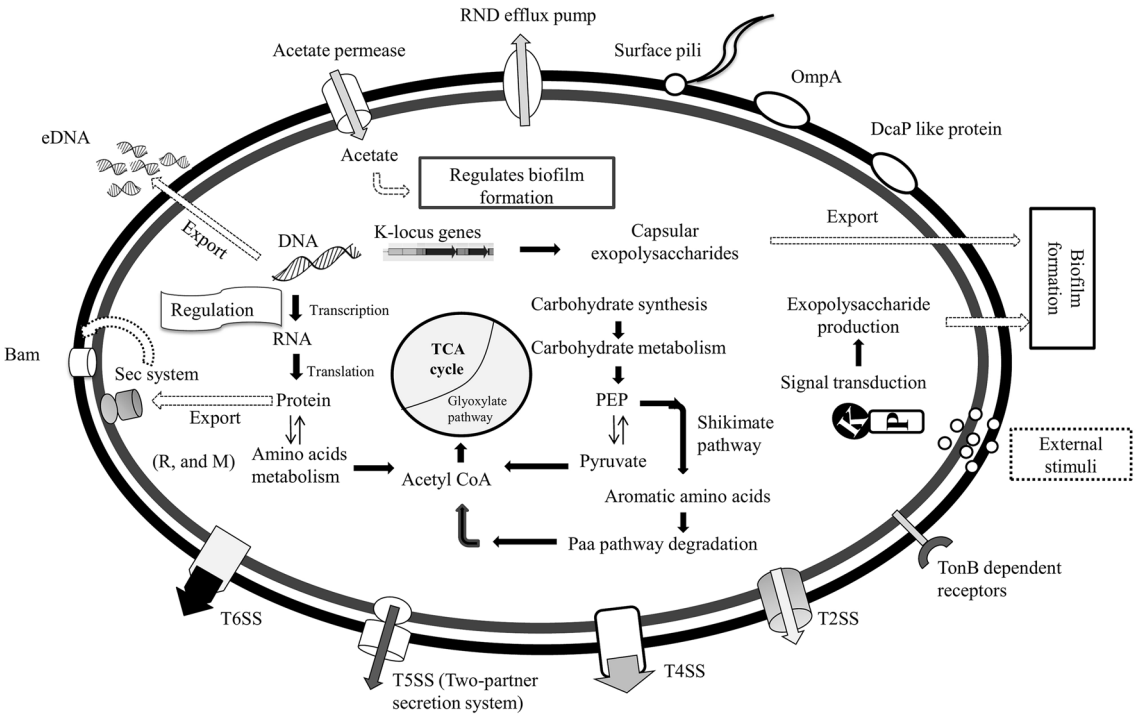

Fig. 2 Representation of contributory biological processes of the *A baumannii* biofilm phenotype

membrane channel SecYEG (Green and Mecsas 2016). Concurrently, the upregulation of periplasmic chaperone SurA, supports previous findings related to its role in the delivery of unfolded proteins through Sec-dependent mechanism. The upregulation of secA and SurA may be linked with proteins transport to the external milieu of the bacteria, and reinforce the fact that sec-dependent mechanisms are associated with biofilm formation (Pérez et al. 2017).

Biofilm cells also showed up-regulation of a central component of autotransporter β-barrel assembly machinery (BAM); BamA protein, helps in translocation of molecules to the external milieu. Recently, BamA protein have been reported to have immunoprotective potential and may serve as a vaccine candidate to confront *A. baumannii* associated infections (Singh et al. 2017). Thus, our study also confirms that outer membrane BamA protein may serve as an important biomarker along with therapeutic potential against biofilm forming *A. baumannii*.

Other crucial secretion mechanisms needed for biofilm formation were also noted, which export substances across the membrane. The majority of these secretion system proteins have also been reported as contributors of antimicrobial resistance, virulence, and survival of bacterial pathogen like *Escherichia coli* (Wallden et al. 2010). *A. baumannii* biofilm cells showed 2.7 fold higher expression of general secretion system protein GspG of type II secretion system (T2SS). This T2SS is reported to export lipases for utilization of exogenous lipids and associated with virulence of *A. baumannii* (Elhosseiny and Attia 2018). Biofilm cells over expressed four proteins of type IV secretion pili: TraB, TraD, TraH and TraV (5 to sixfold). Plasmid encoded type IV secretion pili forms a conjugation machinery that transfers proteins as well as single stranded DNA–protein complexes in a cell contact-dependent or independent mechanism. The importance of type IV secretion system (T4SS) in biofilm formation and antimicrobial resistance has also been previously explained in bacterial pathogens (Zechner et al. 2012). The biofilm phenotypes over expressed filamentous hemagglutinin protein (FHA), a part of two-partner secretion (TPS) pathway of type V secretion system (T5SS). Current findings corroborate the previously reported roles of T5SS in *A. baumannii* adhesion and biofilm formation (Pérez et al. 2017). Interestingly, the study highlighted up-regulation of type VI secretion system (T6SS) proteins: Hcp family protein, EvpB family protein, and contractile sheath small subunits TssB. T6SS has been engaged in different processes like biofilm formation and pathogenesis (Silverman et al. 2012). Similar results were also reported for the development of pellicle cells in *A. baumannii* (Kentache et al. 2017). Considering the current findings and simultaneous agreement with earlier reports suggests the presence of T2SS, T5SS, and T6SS helps in the biofilm formation of *A. baumannii.*

Cell adhesion proteins and export of exopolysaccharide

Cell adhesion with a surface is initial step of biofilm formation that is accomplished by surface adhesion proteins. Two fimbrial proteins and csuA pilus protein were significantly up regulated in biofilm cells. Fimbrial proteins and Csu operon encoded proteins are reported to account for biofilm formation via adhesion with surfaces, motility, and DNA transfer (Tomaras et al. 2008; Gaddy and Actis 2009). We also noted abundance of ompA protein, which facilitates cell adhesion with eukaryotic cells and important for biofilm formation (Gaddy and Actis 2009).

The importance of exopolysaccharide (EPS) biosynthesis and export during biofilm formation can be confirmed with significant elevation of polysaccharide biosynthesis tyrosine autokinase, which is involved in extracellular polysaccharide biosynthesis. Apart from EPS biosynthesis, the event of phosphorylation and dephosphorylation is important for signal transduction mechanisms. We noted a similar higher expression of tyrosine kinase and exopolyphosphatase in *A. baumannii* biofilm phenotypes. Although a complete understanding of molecular mechanism of these proteins needs to be investigated in *A. baumannii*. However, the up-regulation of *ppx* encoded exopolyphosphatase is similar to previous reported importance for survival and biofilm formation of *P. aeruginosa* (Gallarato et al. 2014). Recently, Tiwari et al. had reported the role of tyrosine kinase and phosphatase in exopolysaccharide production of *A. baumannii,* which ultimately contributes to biofilm phenotypes (Tiwari et al. 2020).

Interestingly, we noted higher expressions of different proteins of capsular polysaccharide biosynthesis K locus genes along with outer core glycosyltransferase (gtrOC) (supplementary table 3). Growing chains of polysaccharides get exported to periplasmic space only after glycosylation by gtrOC (Kenyon and Hall 2013). Capsular exopolysaccharides have diverse functions in *A. baumannii* such as protection from antimicrobial agents and host-immune responses (Singh et al. 2019); glycosylated exopolysaccharides are also reported to be important for biofilm phenotype in *A. baumannii* (LeesMiller et al. 2013). Current findings supported the surface capsular polysaccharide of *A. baumannii* to be a critical contributor to biofilm development and virulence.

Antimicrobial resistance in biofilm cells

A significant up-regulation of various antimicrobial resistance factors like efflux pumps, membrane transporter adeB, and beta-lactamase were observed in biofilm cells. The resistance related efflux operons *AdeABC* and *AdeIJK* are reported to perform essential membrane associated functions during biofilm formation and also contributes to the fitness of pathogenic *A. baumannii* (Yoon et al. 2016). The

up regulation of antimicrobial resistant factors in biofilm cells is a common observation reported in previous studies (Cabral et al. 2011; Kentache et al. 2017). We noted concurrent presence of antimicrobial resistance related proteins in biofilm and planktonic phenotypes. Concurrent presence of antimicrobial resistance related proteins in both phenotypes is due to the utilization of MDR *A. baumannii* clinical isolates for differential proteomics. Efflux systems are common in all cells because the play physiological role in cell metabolism and removal of byproducts (Vila et al. 2007).

Nutrients uptake and biosynthesis of cofactors

In the complex biofilm community, bacterial cells produce extracellular matrix that protects embedded cells against a number of environmental stresses with limited access to fresh nutrients. Similar feature was noted in our study, where biofilm cells showed significant elevation of proteins like DcaP-like protein, NLPA lipoprotein, and acetate permease etc. Biofilm encased cells transport hydrophilic nutrients via means of diffusion. Thus, increased expressions of transmembrane channels seem to be a necessary prerequisite for the uptake of important nutrients. Moreover, these proteins are not only required for transportation of nutrients in biofilm but also contribute towards establishment of the biofilm community by enhancing cell adhesion and EPS production in *A. baumannii* (Cabral et al. 2011).

The DcaP-like protein is required for the uptake of dicarboxylic acid and found to be important for *A. baumannii* biofilms (Bhamidimarri et al. 2019). However, its role in biofilm biogenesis is still unclear and need to be investigated. The NLPA lipoprotein in *A. baumannii* is required for methionine import and may evoke immunogenic responses (Hashemzehi et al. 2018). The NLPA lipoprotein may act as a vaccine candidate to fight against biofilm forming *A. baumannii*. The significant higher expression of acetate permease is required for acetate uptake in biofilm growth modes. Acetate has also been reported as an important response regulator for increasing cell division and biofilm formation in *E. coli* (Lynnes et al. 2013). Thus, elevated expressions of above mentioned membrane proteins may be important requisite in biofilm development.

Interestingly, we also observed twofold elevation of 4-aminobutyrate aminotransferase, which is involved in biosynthesis of gamma-aminobutyric acid (GABA). Many bacterial cells are reported to utilize GABA to communicate with other bacteria or host cells. It has already reported that treatment of GABA with *P. fluorescens* has increased its adhesion with glass surface and also induced apparent increase in biofilm formation (Dagorn et al. 2013). Hence, the above observations may suggest the need of GABA for *A. baumannii* biofilm formation. Cobalamin is a cofactor for a number of enzymes, which are involved in carbohydrate and fatty acid metabolism. We noted up regulation of vitamin B12 receptor in biofilm phenotypes. Biofilm phenotypes also showed exclusive abundance of biotin synthase, required for biotin biosynthesis. Cobalamin and biotin are well known essential cofactor for a number of enzymes of key metabolic pathways like fatty acid biosynthesis, TCA cycle, and amino acid metabolism. Thus elevation in cobalamin uptake and biotin synthesis can be linked to survival and pathogenesis of *A. baumannii* as reported earlier for other bacterial pathogens (Rowley and Kendall 2019; Salaemae et al. 2016).

The biofilm phenotypes showed higher expressions of iron and copper uptake proteins. Ferrous iron transporter and tonB-dependent siderophore receptors are involved in iron uptake, which might have vital function in quorum sensing, biofilm formation, survival and regulation of gene expression. The iron has been reported as a vital nutrient for bacterial survival, virulence quorum sensing and biofilm formation (Eze et al. 2018; Ratledge and Dover 2000). Likewise, the importance of iron regulated proteins has been critically evaluated in previous reports (Kentache et al. 2017; Lin et al. 2012). Furthermore, biofilm phenotypes expressed two fold higher expressions of TonB-dependent copper receptor in comparison of planktonic growth modes. TonB-dependent copper receptor of *A. baumannii* mediates its attachment with the host cells. The deletion of copper receptor results in abolished biofilms and reduced virulence of *A. baumannii* (Abdollahi et al. 2018).

Acetyl-CoA metabolism and bioenergetics of biofilm cells

Carbohydrate catabolism is a fundamental biological process for generating energy in terms of ATP for virtually all cells. To generate energy, biological molecules are metabolized towards a common central intermediate 'acetyl-CoA'. The proteins of carbohydrate metabolism up-regulated in biofilm cells are mentioned in supplementary table 3. We observed significant elevation (1.5 to 6.6 fold) of several proteins belonging to acetate uptake and metabolism to acetyl-CoA. It is the case of acetate permease, acetate kinase, phosphate acetyltransferase pyruvate decarboxylase, and aldehyde dehydrogenase that are, to our knowledge, described for the first time. The acetate conversion into acetyl-CoA may be predicted for providing energy through central TCA cycle. The presence of isocitrate lyase in glyoxylate pathway has been reported as a crucial factor for antimicrobial tolerance, persistence, and biofilm formation in *P. aeruginosa* and *Mycobacterium tuberculosis* (Muñoz-Elías and McKinney 2005; Ahn et al. 2016). The acetate metabolism and glyoxylate pathway has not yet described in *A. baumannii* biofilms and our study suggests their potential role in the development of *A. baumannii* biofilms.

Amino acids metabolism

The proteome comparison of planktonic versus biofilm growth modes of *A. baumannii* highlighted several proteins of amino acid metabolism in biofilm phenotypes. We noted an elevation of enzymes of methionine biosynthesis in biofilm growth modes (supplementary table 3). In bacteria, methionine is crucial for protein translation as well as a precursor of methyl group donor S-adenosyl-l-methionine (SAM), which is requisite for DNA and protein methylation, and biosynthesis of polyamine. The importance of methionine for biofilm development in other bacteria like *S. aureus* and *P. aeruginosa* was reported by Jochim et al., as methionine auxotrophs were inefficient to form and maintain biofilms (Jochim et al. 2009). Moreover, the methionine biosynthesis pathway has been suggested as a putative target for development of novel anti-microbial agents (Jochim et al. 2009). Biofilm phenotypes showed higher expressions of three proteins of arginine biosynthetic process: ArgC, ArgD, and ArgG. It is already proved that arginine helps in intercellular attachment of *Staphylococcus aureus*, which is required for biofilm formations (Zhu et al. 2007). In agreement with Zhu et al., we can suggest that arginine biosynthesis is crucial for *A. baumannii* biofilms formation.

We noted elevation of several enzymes involved in aromatic amino acid metabolism. Biofilm cells expressed 6.6 fold higher expression of 3-phosphoshikimate 1-carboxyvinyltransferase (EPSP synthase), which is involved in the shikimate pathway for aromatic amino acid biosynthesis. Concurrently, two proteins: tryptophan synthase (trpA) and indole-3-glycerol phosphate synthase (trpC) of tryptophan biosynthetic pathway were up-regulated in biofilm cells. Harrison et al., have recently depicted the important role of tryptophan biosynthesis in development of mature biofilm architecture and prolong survival of *Haemophilus influenza* (Harrison et al., 2019). The tryptophan production can be linked with the production of indole that induce biofilm formation, stress tolerance and virulence of *A. baumannii* has reported earlier (Lin et al. 2018). Concurrent findings validated the need of tryptophan biosynthesis in *A. baumannii* biofilm formation.

Furthermore, enzymes of phenylacetic acid (paa) degradation pathway were over expressed in biofilm phenotypes. Six proteins designated as *paaA*, *paaC*, *paaE*, *paaI*, *paaK* and *pcaF* were highly expressed in comparison to planktonic counterparts. Degradation *paa* pathway finally produce "Acetyl-CoA" and "succinyl-CoA", which integrate TCA cycle to generate energy required for biofilm formation. These results corroborate previous finding of Kentache et al. highlighted a connection between aromatic compound degradation and biofilm formation in *A. baumannii* (Kentache et al. 2017).

Stress responses and regulation of biofilm cells

Presence of several stress adaptation proteins observed in *A. baumannii* biofilms (supplementary table 3). Among these, cold shock protein showed an elevation (6.6 fold) as compared to their planktonic counterparts, which may be possibly, engaged in virulence, stress tolerance, and cellular aggregation of *A. baumannii* biofilms as evident in other bacteria (Eshwar et al. 2017). The presence of chaperone and stress adaptation proteins was noted in *A. baumannii* biofilms. The chaperone proteins (ClpX and DnaK) and stress adaptation proteins (catalase, alkyl hydroperoxide reductase, and glutathione transferase) usually increase their expression for survival, invasion and biofilm formation (Frees et al. 2004; Capestany et al. 2008).

Furthermore, two regulatory proteins Fis and LysR were noted with elevated levels, which have the function of expression regulation of biofilm adhesion and quorum sensing in bacteria like *Pseudomonas putida* (Moor et al. 2014; Gao et al. 2014). Current findings are in agreement with previous reports, which suggested the need of stress responses and regulatory proteins for *A. baumannii* biofilms.

GO enrichment analysis of up and down-regulated proteins of biofilm phenotypes

The bioinformatics analysis using Cytoscape plug-in "ClueGO" revealed the integration of functionally enriched biological processes of biofilm and planktonic growth state of *A. baumannii* (Fig. 3). The enrichment analysis showed that *A. baumannii* cells under two growth conditions trigger various biological processes related to phenotypes. The processes highlighted in red showed a significant role in biofilm biogenesis. The enrichment analysis emphasizes the importance of glyoxylate cycle, acetyl-CoA biosynthesis from acetate, amino acid biosynthesis, diacylglycerol biosynthesis, Entner–Doudoroff pathway, and propionate catabolism are major contributor for *A. baumannii* biofilm formation. Therefore, biological processes associated with biofilm formation can further be considered as antibiofilm targets. Contrastingly, the biological processes of planktonic phenotype are highlighted in blue. The GO enrichment reveal the

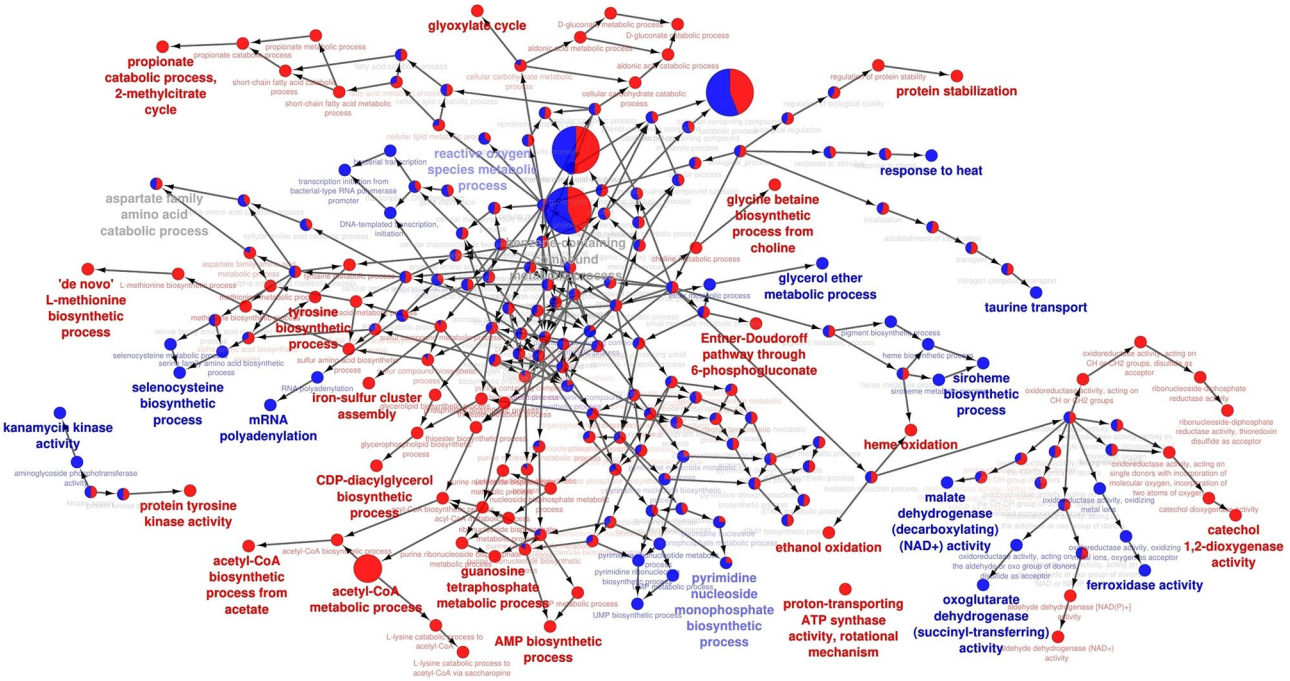

Fig. 3 Schematic representation of gene ontology (GO) enrichment *A baumannii* biofilm and planktonic phenotypes visualized with ClueGO (kappa score = 04). Each node represents GO function and edges represent connections between the nodes GO terms with up/downregulated expression in biofilms are shown in red/blue, respectively The mixed color edges show the gene proportion of each phenotype associated with the term

growth of *A. baumannii* as planktonic phenotype is related to the process of carbohydrate metabolism and translation.

Protein markers of biofilm and planktonic phenotype of *A. baumannii*

Student t test was further applied upon all differentially expressed 499 proteins with an aim to identify specific protein markers associated with two growth conditions. Statistical analysis identified only 56 proteins, which differentiate biofilm and planktonic growth conditions of *A. baumannii*. The heatmap clearly discriminate the proteins of both phenotypes. The proteins were classified according to their correlation in abundance variation within two phenotypes along with cellular location and biological processes (Fig. 4). The PCA and correlation plot revealed good homogeneity within biological replicates, since two phenotypes were clearly differentiated (Fig. 5A, B).

A total 26 proteins, which involved in acetate metabolism, stress adaptation, secretion system, along with several structural proteins are exclusive contributor of *A. baumannii* biofilm. These biofilm associated protein are potential protein markers (supplementary table 4).

Contrastingly, planktonic cells have exclusive abundance of several proteins related to survival and structure maintenance under stationary phase. For example, enzymes of carbohydrate metabolism, survival, cell division, and stress adaptation proteins (supplementary table 4). Oxidative stress conditions are favorable for destruction of intracellular proteins and cell envelope that could lead to cell death. The over expression of universal stress protein, stress induced protein, glutathione transferase, and superoxide dismutase in planktonic cells were in accordance in Cabral et al. 2011, which suggest that *A. baumannii* late stationary cells are more tolerant to oxidative stress. Under nutrient deprived conditions bacteria constantly sense metabolic changes and adapt accordingly for its survival. For this, they need to consume amino acids as an alternative energy source (Zampieri et al. 2019). These findings suggest that stationary phase *A. baumannii* also have mechanisms to evade stress conditions as reported earlier (Fiester and Actis 2013).

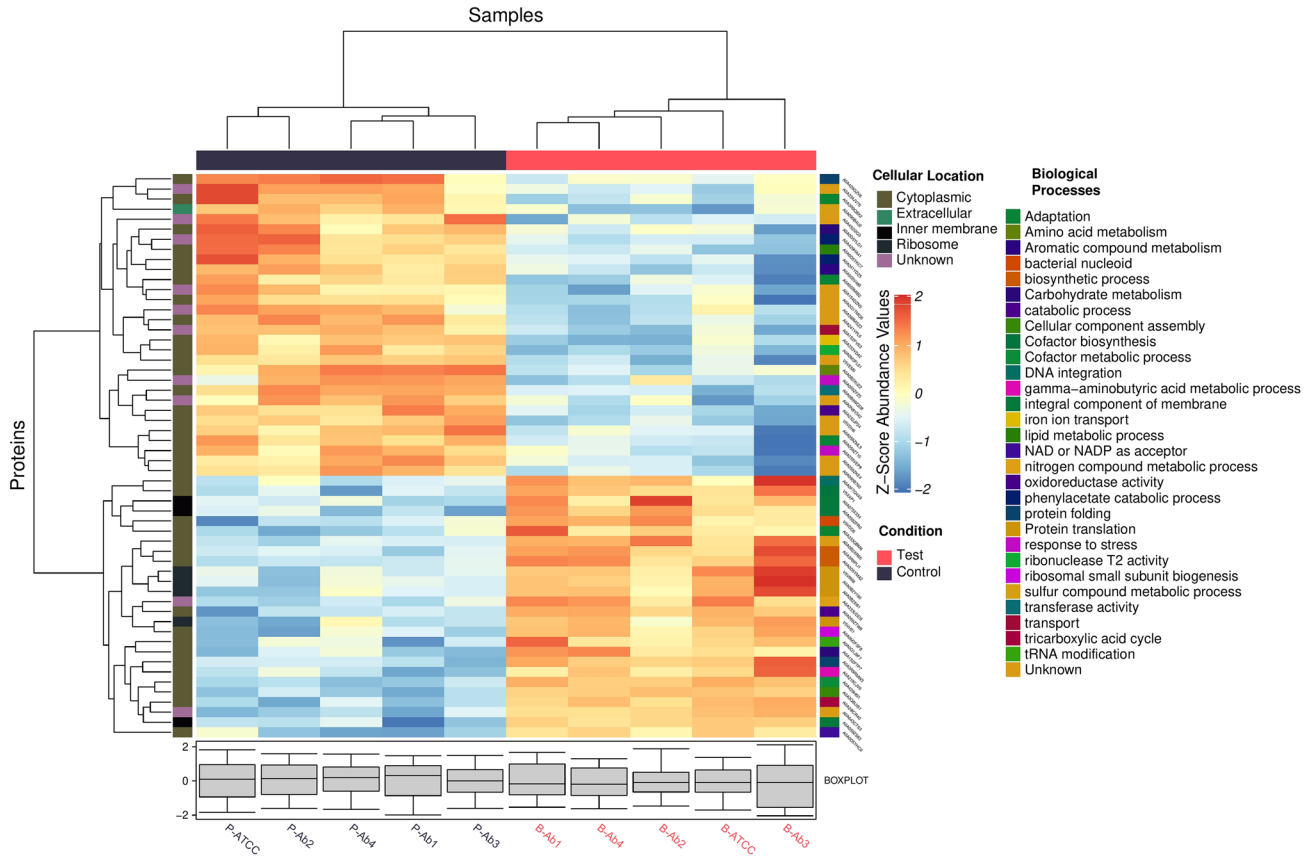

Fig. 4 A heatmap hierarchical clusters showing correlation among 56 statistical significant proteins of two phenotypes Z-score abundance, experimental conditions, cellular location and biological functions are indicated on left and right side respectively A box plot of the protein abundance intensity (Log 2) for each sample also presented on the bottom

Conclusion

Ubiquitous biofilms on abiotic surface are an important pathogenicity factor. We have compared global proteome of biofilm and planktonic phenotypes of *A. baumannii* to elucidate its biofilm protein markers. Present study observed that the variations in the expression profiles of membrane transporter BamA protein, csuA pilus proteins, and capsular exopolysaccharide biosynthesis proteins, lead to promote the biofilm formation. The peculiar biofilm phenotype is achieved with the virtue of bacterial adhesion, nutrient uptake, EPS production and their secretion to external milieu. Apart from structural contributors, the metabolism of acetate, glyoxylate, methionine and arginine are requisite for biofilm phenotype, which are triggered with elevated expression of isocitrate lyase, argC, argD, argG and clpX chaperone proteins. Unlike biofilm phenotype, planktonic growth state is committed to survive and maintain cell physiology under stationary phase, achieved with the elevated expressions of enzymes of carbohydrate metabolism, survival, cell division, and stress adaptation proteins. Biofilm associated proteins observed in the present study, may offer wide scope to screen multiple compounds against biofilm forming *A. baumannii* with the use of *in-silico* drug screening approach.

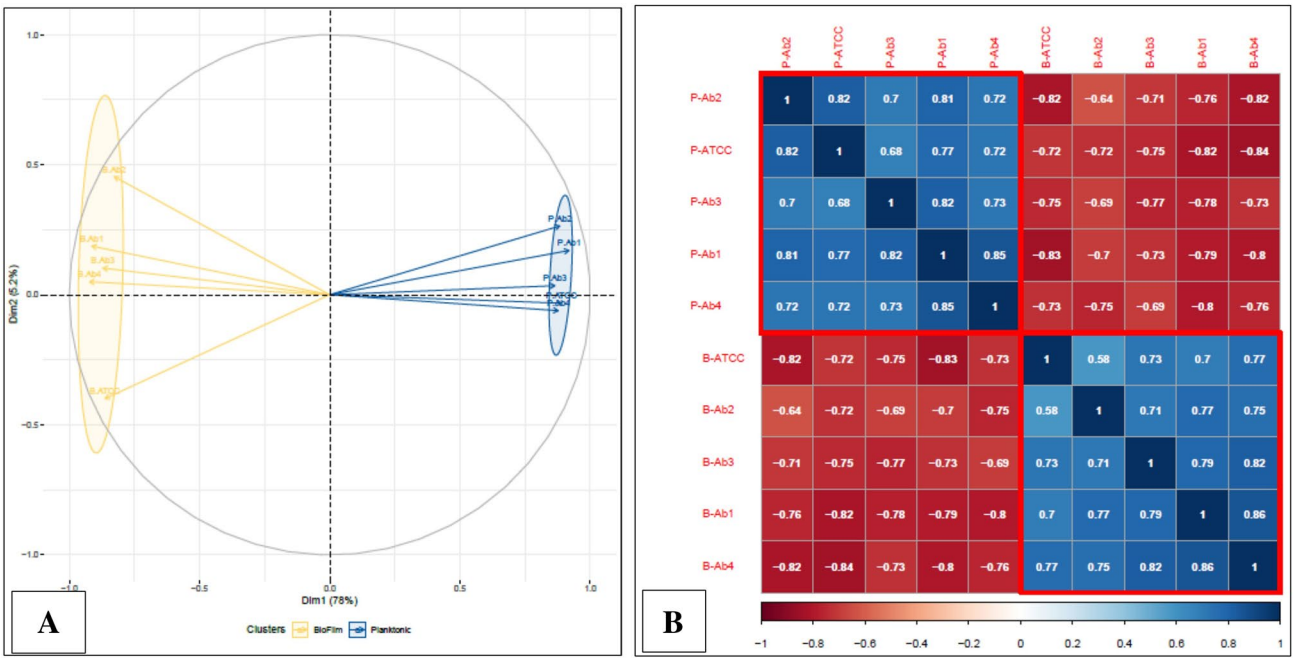

Fig. 5 **A** Plot of principal component analysis (PCA) performed on *A baumannii* isolates measured in quadruplicate The Thermo Proteome Discoverer was utilized for PCA The arrows indicate loading of the isolates Biofilm and planktonic phenotypes of MDR clinical isolates are homogenous and they are significantly variable to each other, while both phenotypes of susceptible type ATCC 19606 are homogenous to each other. **B** Correlation plot representing similarity among biological replicates and clearly discriminating two growth conditions

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Author contributions JV: Conceptualization and methodology design. MC: Methodology and writing- original draft preparation. SK: Data analysis and visualization. Dr. AK: Collection of bacterial isolates. RS: Reviewing and editing.

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Declarations

Conflict of interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethical approval This retrospective study was approved by Institutional Ethics Committee (Number IEC/project no-21-2015).

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