Biofilm matrix proteome of clinical strain of *P. aeruginosa*

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Abstract: Extracellular matrix plays a pivotal role in biofilm biology. Despite the importance of matrix proteins as potential targets for the development of antibacterial therapeutics little is known about matrix proteomes. As matrix responsible for some extracellular functions, it is expected that protein composition should be different compared to embedded in biofilm cells. This difference reflects possible active processes in the matrix. While *P. aeruginosa* is one of the most important pathogens with emerging antibiotic resistance only a few studies are devoted to matrix proteomes and there are no studies describing matrix proteome for any clinical isolates. Here we report the first matrix proteome in comparison with embedded cells for the clinical isolate of *P. aeruginosa*. We have identified the largest number of proteins in the matrix among all published studies devoted to *P. aeruginosa* biofilms. Functional classification of enriched in matrix proteins resulted in several bioprocess groups. Bioprocess groups with the largest number of overrepresented in matrix proteins were oxidation-reduction processes, proteolysis, and transmembrane transport. The top three represented in matrix bioprocesses concerning the size of the GO annotated database were: cell redox homeostasis, nucleoside metabolism, and fatty acid synthesis. Finally, we discuss obtained data in a prism of possible directions for antibiofilm therapeutics development.

Keywords: biofilm; matrix; proteome; matrixome; *Pseudomonas aeruginosa*; nucleoside metabolism; cell redox homeostasis; fatty acid synthesis, proteolysis; transmembrane transport

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1. Introduction

Biofilms are the most common lifestyle of microorganisms including both pathogenic and environmental bacterial species. From a clinical perspective, biofilms are often represented by microbial aggregates with tolerance to host defense mechanisms and antimicrobials, so they cause difficult-to-treat recurrent diseases. Currently, there is an urgent need to discover new targets and strategies to overcome the tolerance for the effective treatment of biofilm-associated infections.

The key feature of biofilms is the presence of an extracellular matrix that covers all members of biofilm and creates a microenvironment for communication, protects against different threats, provides an opportunity for spatial organization and functional diversification within the community. The matrix consists of a broad range of biopolymers, metabolites, and signal molecules. Also, it usually includes organized compartments like outer membrane vehicles (OMVs). To stress the idea of rich and complex matrix organization the term «matrixome» was proposed [1]. Proteins in the biofilm matrix might be actively secreted, passively leaked from cells, and entrapped into the matrix from the environment [2, 3]. Proteins in the matrix have a structural role in maintaining biofilm organization, play a role as a protective barrier, creates microenvironment with limited diffusion. The barrier role is somewhere similar to structural function but also includes hydrophobic features, charge, and so on rather than just being mechanically stable structure. Moreover, matrix proteins may bind antimicrobial molecules and reduce their diffusion and effect on bacterial cells i. e. extracellular ribosomal proteins bind antibiotics and prevent their penetration in bacterial cells. Other functions of matrix proteins rely on their enzymatic activity and include degradation of biopolymers, participation in biochemical processes, and signaling function.

Biofilms are dynamic communities with lifecycle consisting of attachment, maturation, and dispersion stages. During the maturation stage matrix may serve as depo for virulence factors which then come out together with dispersed bacteria. In the case of chronic infections dispersion stage is associated with the recurrence of symptomatic infections and colonization of new sites in the body.

Importantly bacteria inside biofilm have diverse phenotypes and some extracellular molecules (public goods) are consumed by non-producers. Matrix in general and some components in matrix are examples of public goods. So, a part of bacterial population, especially inside multispecies biofilms, may not have resistance mechanisms, but due to matrix still be irresponsible for the treatment.

Targeting of matrix proteins as a strategy to combat infections may act in the following ways: (1) destabilization/disruption of biofilm matrix to improve the action of host defense and/or antibiotic therapy; (2) targeting of extracellular biochemical processes to reduce overall biofilm succeed in survival and virulence; (3) targeting of extracellular virulence factors associated with biofilm matrix to decrease virulence in a case of mass dispersion.

Despite the accepted idea of a pivotal role of matrix in bacterial biofilms little is known about the protein composition of the matrix at a level of proteomes. Moreover, for the well-studied and clinically important biofilm-forming bacteria *P. aeruginosa* there are only a few studies devoted to matrix proteome of reference strains (mainly PAO1, Table 1), while proteomics of the whole biofilm is better described [4, 5].

Table 1. Studies devoted to biofilm matrix proteome. Biofilm`s compartments and MS methods were named by original publications.

Bacteria	Biofilm compartments	Method	Number of proteins	Reference
***P. aeruginosa* PAO1**	**Matrix** OMVs	Gel-based vs Gel-free 2D LC-MS/MS	**Matrix 327** OMVs 207	[9]
***P. aeruginosa* PAO1**	**Matrix** Total biofilm	LC-MS/MS	Total biofilm 857 **Matrix 60**	[8]
***P. aeruginosa* PAO1**	**Matrix** OMVs Cells	1D-SDS-PAGE combined with nano-LC-ESI-MS/MS	**Matrix 178** OMVs 57 Cells 764	[7]
***P. aeruginosa* PAO1 at different time points**	Cells OMVs	LC-MS/MS	Cells 2443 in total OMVs 1142 in total	[10]
***P. aeruginosa* ATCC27853 at different time points**	**Matrix**	iTRAQ-labeled peptides and LC-MS/MS analysis	**Matrix** 389 in total	[11]
Dynamics in dual-species biofilm – *P. aeruginosa* PAO1 and *S. aureus* ATCC 25923	Surfaceome Exoproteome	Orbitrap QExactive Plus LC-MS/MS	Surfaceome by PAO1 495 Exoproteome by PAO1 762	[12]
***P. aeruginosa* CF clinical isolate KB6**	Cells **Matrix**	Orbitrap LC-MS/MS	Cells 1652 **Matrix 957**	This study
***S. aureus* UAMS-1 *in vivo* chronic implant infection**	Secretome Surfactome	GeLC-MS/MS	Secretome 33 Surfactome 72	[13]
***S. aureus* USA300 CA-MRSA strain LAC**	DNA-binding proteins in the biofilm matrix	quadrupole time of flight (Q/TOF) mass spectrometer (Agilent 6520) with a nanospray ionization source	49	[14]
***S. aureus* HG001**	intracellular extracellular (ECM and flow-through)	LC-MS/MS on LTQ-Orbitrap-Velos mass spectrometer coupled to an EASY-nLC 1000	ECM 1407 flow-through 1400 intracellular 1621	[63]
***B. multivorans* C1575**	Matrix	Gel-based LC-MS/MS	Matrix 161	[15]

	OMVs		OMVs 64	
S. acidocaldarius	EPS	nanoRSLC-Orbitrap LC-MS/MS	85	[16]
***Shewanella sp.* HRCR-1**	EPS	LC-MS/MS	58	[17]
Cutibacterium acnes	Matrix	Orbitrap MS	447	[18]
Vibrio cholerae	Matrix	LC electrospray ionization and then entered into an LTQ linear ion-trap mass spectrometer (ThermoFisher)	74	[19]
Haemophilus influenzae (NTHi)	ECM	Gel-based LC/MS/MS	60	[20]

In a comparative study of liquid culture of PAO1 strain and clinical isolates, it was shown that cystic fibrosis (CF) isolates expressed a narrower range of transporters and a broader set of enzymes of metabolic pathways for the biosynthesis of amino acids, carbohydrates, nucleotides, and polyamines, but this study did not cover biofilm mode of life as well as extracellular matrix composition [6]. Only one study described the proteome of the matrix in comparison with embedded cells [7] and one else study compared the matrix with the total biofilm proteome [8], both studies were devoted to reference strain PAO1. The gap in understanding the difference in protein composition between matrix and embedded cells frustrates the development of antibiofilm therapeutics and the overall understanding of biofilm biology. Here we performed a proteomic study of matrix composition in comparison with embedded cells for the clinical strain of *P. aeruginosa* to identify bioprocesses taking place in the matrix as probable targets or as factors or as barriers during pharmacological development of antibiofilm therapeutics.

2. Results

3.1. General overview of proteomes

P. aeruginosa KB6 (exoT+; exoY+; exoU-; exoS+) is a clinical isolate from bronchoalveolar lavage of cystic fibrosis patient with strong biofilm-forming phenotype [21]. To investigate protein composition of extracellular biofilm matrix we used previously published method of separation of biofilm matrix from embedded cells with high ionic solution of NaCl [29, 30]. To confirm the absence of cell lysis for KB6 strain during matrix separation we evaluated CFU number after NaCl treatment for both biofilm and suspension bacterial culture. There was no difference in the recovery of CFU with and without NaCl treatment for suspension bacteria, while CFU number for biofilm samples was comparable after NaCl treatment and after common biofilm-disruption approach – mild sonication for 5 minutes (data not shown). So, matrix isolation with NaCl was used for protein sample preparation. For the proteomic study, we have grown static biofilm for 18 h in LB medium. This time point corresponds to biofilm in the early stationary phase when the number of embedded bacterial cells reaches the plateau, and the matrix is already formed. Further increase in biofilm biomass occurs mainly due to mass of extracellular components rather than increasing number of bacterial cells. During the further biofilm growth mass of extracellular substance significantly impacted with lysed cells. To decrease the number of proteins that may represent passive cell leakage or death during the biofilm stationary phase and to avoid interference of these «archeological» proteins with secreted extracellular proteins we choose this early time point (18 h).

Two independent and separated in time biofilms were grown in a liquid medium. Extracellular matrix and embedded cells were separated with NaCl and processed for protein isolation. Also, one more biological replicate (the third) representing embedded cells only was added to the analysis from another set of experiments (data not shown). Proteomic analysis was performed for each sample in three technical replicates. For protein identification, *P. aeruginosa* KB6 strain-specific protein dataset was created based on genome sequence (available in GenBank under accession number NZ_CP034429). Protein identification was made with MSFragger. The full list of all identified proteins is available in Table S1.

In total, more than 1600 proteins were identified in all samples. After initial manual inspection of LFQ intensities we observed matrix-specific proteins, cell-specific proteins, and proteins with presence in both compartments. While an inspection of intensity signals is not applicable for comparison of the representation of different proteins it is still gave us an additional consciousness about proper separation

matrix from cells. The absence of several intracellular proteins in matrix samples confirms that our approach to separate matrix from cells does not cause significant cell leakage during sample preparation. For example, S18, L29, L33, S15, S20 proteins involved in ribosome assembly and function were absent in the matrix (LFQ intensities and spectral counts were zero in all matrix samples), but these proteins were observed in all cell's samples with LFQ intensities more than $1*10^8$. So, in general, the absence of this ribosomal and some other proteins in matrix samples is associated neither with cell disruption during matrix separation nor with low detection efficacy as a specific feature for some proteins.

Spearman correlation coefficient for proteins LFQ intensities between cells and matrix was 0.4959 (95% CI 0,4555 to 0,5343) (correlation matrix is presented in Figure 1).

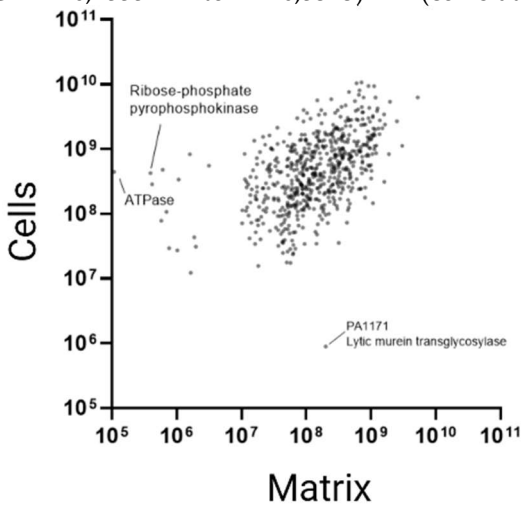

Figure 1. Correlation of individual protein intensities (LFQ) between embedded cells and matrix.

While the correlation was expectedly positive there were several examples of outfitters like lytic murein transglycosylase with well-known localization on the outer surface of the bacterial cell wall [31].

Cells and matrix differ in overall biomolecule content, physicochemical and other properties. That was our premise for rigorous statistical analysis of comparative protein representation. For getting a quantitative comparison of the representation of proteins we proceeded to logarithm transformation and quantile normalization of LFQ intensities. Statistical analysis was made with the Limma R package [32]. All identified proteins are listed in table S1, and proteins differing in their representation in cells and matrix are listed in Table S2. For fold difference presentation volcano plot was created (Figure 2).

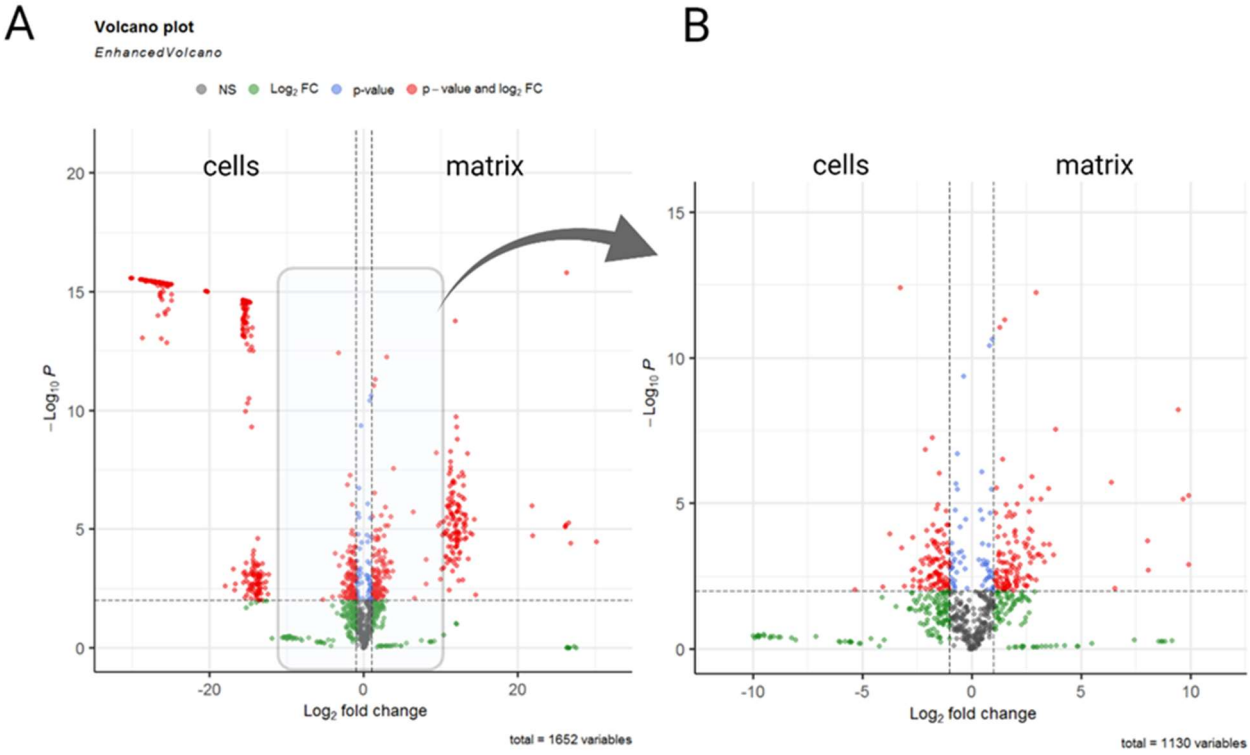

Figure 2. Volcano plot of representation of proteins between cells and matrix. A – overall protein representation; B – an enlarged area with proteins with fold change between Log2 from 1 to 10. Red color indicates proteins with fold change more than 2 and significance value $p<0.01$; green color indicates proteins with fold change more than 2 and significance value $p>0.01$; blue color indicates proteins with fold change less than 2 but significance value $p<0.01$; grey color indicates proteins with fold change less than 2 and significance value $p>0.01$.

In total there were more than 1600 proteins, and 957 of them were found in the biofilm matrix, 766 matrix proteins were present in both biological replicates.

3.2. Unique proteins in the extracellular matrix

Matrix unique proteins were defined if they were observed (LFQ intensity was nonzero) in both experiments in the matrix, while LFQ intensities in cells samples were zero. Totally ten proteins were present in the extracellular matrix only. We have proceeded with literature search and manual biofilm-related functional annotation for these matrix unique proteins (Table 2). Nonetheless, for most extracellular matrix-only proteins we did not find clear evidence of their importance for biofilm structure or any extracellular function.

Table 2. Matrix unique proteins. Matrix unique proteins had nonzero intensities in all matrix samples and zero intensities in all cell samples.

Protein	Protein ID	Role in biofilm	Reference
two-partner secretion system transporter CdrB	WP_010895680.1	Secretion partner of CdrA adhesin	[33]
DUF3298 and DUF4163 domain-containing protein	WP_003102069.1	Not described*	-
hypothetical protein	WP_003113151.1	Not described	-
hypothetical protein	WP_003102379.1	Not described	-
ABC transporter substrate-binding protein (Probable amino acid-binding protein)	WP_003113778.1	Not described, proposed transport function	-
NAD(P)/FAD-dependent oxidoreductase	WP_003114698.1	Not described	-
nucleoside hydrolase Nuh (catabolizes adenosine)	WP_003147076.1	QS-controlled private good	[34]
YgdI/YgdR family lipoprotein	WP_003089729.1	Not described	-
dGTPase	WP_003112492.1	Not described	-
ATP-dependent zinc protease	WP_003113269.1	Not described, but some envelope proteases play role in many biological processes	[35]

*Not described means absence of evidence of significant involvement specifically in biofilm formation and/or pathogenesis. That does not reflect a described role in any other non-related to biofilm functions.

Additionally to unique matrix proteins, there were proteins with significant and extremely high log2 fold change (Log2FC) – more than 20. We consider these proteins as semi-unique matrix proteins (Table 3).

Table 3. Semi-unique matrix proteins. Semi-unique proteins had nonzero intensitiesLFQ in both cells and matrix samples and log2FC more than 20.

Protein	Protein ID	Role in biofilm	Reference
malonate decarboxylase subunit alpha	WP_003112666.1	Malonate improve biofilm formation	[36]
OprD family porin	WP_003114177.1	Not described*, proposed transport function	-
ABC transporter substrate-binding protein	WP_003098136.1	Not described, proposed transport function	-
carbon-nitrogen hydrolase family protein	WP_003112739.1	Not described	-
bifunctional riboflavin kinase/FAD synthetase	WP_003102619.1	Not described; flavin nucleotide biosynthesis	-
M48 family metallopeptidase	WP_003112546.1	Not described	-

transporter substrate-binding domain-containing protein	WP_003115039.1	Not described, proposed transport function	-
division/cell wall cluster transcriptional repressor MraZ	WP_003103101.1	Not described	-

*Not described means absence of evidence of significant involvement specifically in biofilm formation and/or pathogenesis. That does not reflect a described role in any other non-related to biofilm functions.

3.3. Functional classification of proteins in the extracellular matrix

All overrepresented in matrix proteins are displayed in Figure 2A. We observed distinguishable clusters of overrepresented proteins depending on their fold change (Log2FC). While a separate cluster of highly overrepresented proteins with log2FC more than 10 is visible on the main volcano plot (Figure 2A), for better resolution of the area within log2FC 1-10 frame we also provide an enlarged area of the volcano plot (Figure 2B). Analysis of bioprocess classification of all found in matrix proteins and overrepresented in matrix proteins is displayed in Figure 3. Many bioprocess groups include at least one overrepresented in biofilm matrix protein.

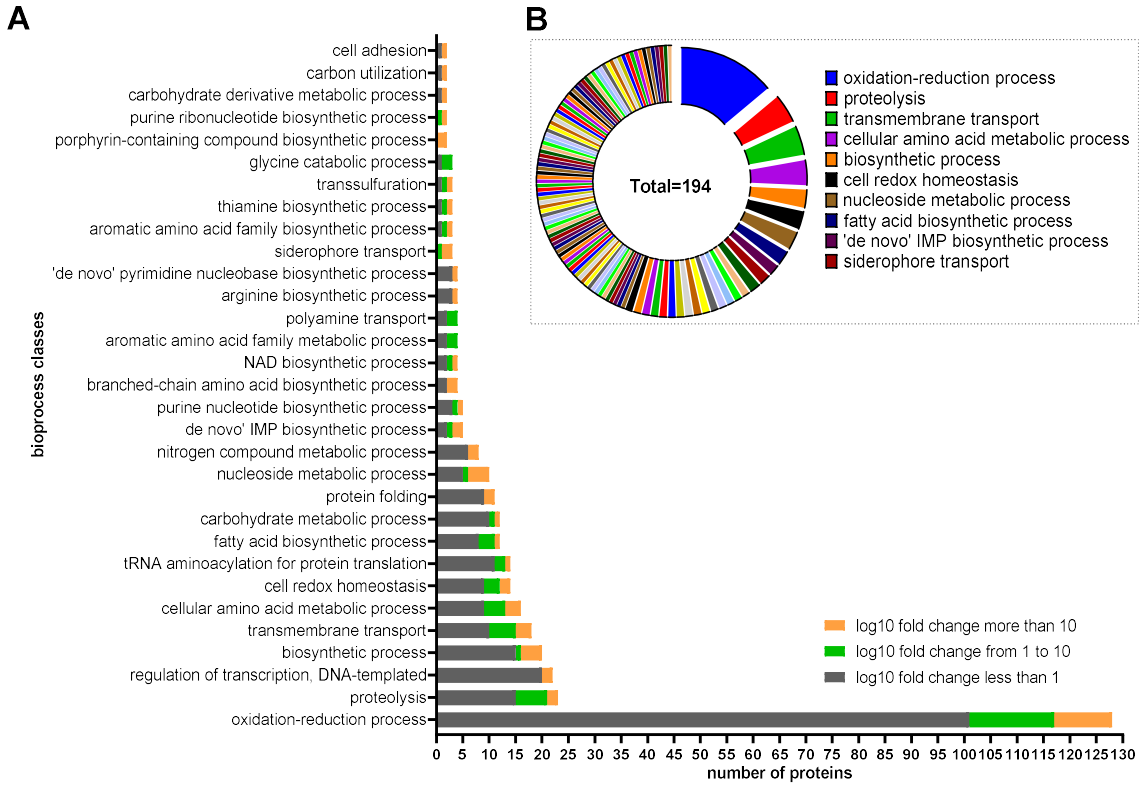

Figure 3. Bioprocess group classification of proteins found in the biofilm matrix. A – number of represented proteins in different bioprocess groups concerning log2 fold change in comparison with embedded cells: grey color – proteins found in the matrix but not overrepresented in comparison with embedded cells; green color – overrepresented proteins with log2 fold change from 1 to 10; orange color – overrepresented proteins with log2 fold change more than 10, only groups with at least 2 found in matrix proteins and at least one overrepresented protein are displayed; B – distribution of overrepresented proteins in biofilm matrix, each color represents individual bioprocess group, the legend indicates top ten of bioprocess groups.

We focus on groups with the largest number of found proteins and the proportion of overrepresented proteins from all found in matrix proteins. For this reason, we applied the following criteria: (1) at least 10 proteins per group found in the matrix; (2) more than 3 found in matrix proteins are overrepresented (4 to 28 overrepresented proteins from 8 bioprocess groups). Such bioprocesses belong to oxidation-reduction and cell redox homeostasis, proteolysis, transmembrane transport, amino acid, and nucleoside metabolic processes, fatty acids biosynthesis. An important consideration is that GO annotated bioprocesses consist

of groups with highly different numbers of proteins. Number of proteins involved in bioprocess reflects complex nature and flexibility of protein interactions. As an absolute number of identified proteins may not reflect bioprocess representation, we count for each selected bioprocess group proportion of found proteins from all GO annotated group members as a measure of bioprocess representation (Table 4).

Table 4. Bioprocess representation in biofilm matrix based on the proportion of found proteins from GO-annotated for the bioprocess proteins.

	number of found in matrix proteins	**number of overrepresented in matrix proteins**	**GO-annotated number of proteins in the database**	**%% of found in matrix proteins from GO-annotated in database**
oxidation-reduction process	128	27	448	29
proteolysis	23	8	87	26
biosynthetic process	20	5	985	2
transmembrane transport	18	8	905	2
cellular amino acid metabolic process	16	7	141	11
cell redox homeostasis	14	5	26	54
fatty acid biosynthetic process	12	4	26	46
nucleoside metabolic process	10	5	11	91

In such analysis, the top three represented bioprocesses were cell redox homeostasis, nucleoside metabolism, and fatty acid synthesis. Considering annotated number of proteins for each group nucleoside metabolic process was represented with ten proteins in the matrix out of eleven GO annotated proteins. In contrast to the nucleoside metabolic process, DNA-templated regulation of transcription (this group includes only 2 overrepresented in matrix proteins) and biosynthetic process groups were represented in matrix with a greater number of proteins (22 and 20, respectively), but it was less than 5 % from GO annotated in database proteins for these groups (22 proteins from 472 and 20 from 985, respectively).

3. Discussion

Biofilm matrix in most cases contain many proteins. Extracellular proteins in the biofilm matrix provide their functions for the whole bacterial community, so they may be considered as public goods. Extracellular functions of matrix proteins include (but are not limited to) the external digestive system, signaling, protection, and maintaining the stability of the matrix [1, 37–39]. Moreover, many bacterial proteins may have moonlight functions.

Understanding protein composition is critical for resolving both fundamental questions in bacterial lifestyle and the development of tools for manipulating biofilms. Most proteomic studies focus on the whole biofilm and usually compare the whole biofilm proteome with planktonic cells proteome. At the same time, the identification of matrix protein composition may open new opportunities for the development of antibiofilm drugs. It is well known that biofilms of pathogenic microorganisms are tolerant to antibiotics, many other therapeutics, and host immune factors due to the matrix [40]. Matrix-disrupting or interrupting agents may reverse tolerant phenotype and increase the efficacy of antibiotic therapy [41]. For example, antibody-mediated destabilization of the matrix through the disruption of IHF-DNA complexes was effective *in vitro* and *in vivo* as a single therapy and combined with antibiotics [42–44]. Moreover, extracellular targets less probable cause selection of resistant mutants, so targeting of matrix proteins is a perspective way to combat chronic infections especially infections caused by ESKAPE pathogens. Despite all of these it is still little known about matrix proteomes. Only numerous studies described the matrix proteome of reference strain PAO1 of *P. aeruginosa* and some other bacteria (Table 1). Here we for the first time describe the matrix proteome of CF clinical isolate of *P. aeruginosa* in comparison with embedded cells and discuss obtained data in a prism of possible targets for therapeutic development. In comparison with other studies, we identified the largest number of proteins in the matrix. While some proteins might be invisible due to their low concentrations and individual limits of detection, we believe that further improvement of MS equipment and technics will get a more comprehensive picture of bacterial proteomics.

In our study, only a small number of proteins were unique for matrix only. One of them – CdrB protein is involved in the transport of CdrA adhesin. This adhesin is important for biofilm formation and its binding to Psl results in increased biofilm structural stability. Also, it is known that *cdrAB* is regulated together with Psl. CdrA binding to Psl protects it from endogenous and exogenous protease digestion [45]. Surprisingly we did not find any nonzero LFQ for CdrA protein neither in cells nor in the matrix. In a less sensitive gel-based proteomic study authors have observed CdrA protein in the matrix [7, 20], so the absence of the protein in our data is unlikely due to limits of detection but seems to be under the limit of quantitation (LFQ calculation). One of the possible explanations is that CdrA/CdrB components are not important for KB6 strain due to the limited role of Psl polysaccharides in the biofilm matrix, so in the absence of Psl CdrA might be degraded by proteases to quantities under the detection limit.

Another matrix unique protein is nucleoside hydrolase (Nuh) – an enzyme that hydrolyzes adenosine and inosine, allowing the cell to grow on these nucleosides as the sole carbon or nitrogen source. This protein was studied as an intracellular (periplasmic) private good in a prism of quorum sensing in bacterial communities [34]. In our study, we found Nuh in the matrix, but not inside bacterial cells. That means Nuh might be an extracellular public good at least for some strains like ours. Also, transporters responsible for adenosine transport to periplasmic space in *P. aeruginosa* remain still undiscovered, so if Nuh works outside the cell such a factor is not needed. In an environment with adenosine as the sole carbon source Nuh mutant has impaired growth [46], but therapeutic potential of direct or indirect inhibition of Nuh activity remains elusive due to the nutrient-rich nature of infected tissues, and further research is needed.

For the rest 8 matrix-unique proteins there is no clear evidence of their possible role in biofilm. Meanwhile, as matrix is considered a nutrient-rich environment presence of substrate-binding protein from ABC transporter and its role in nutrient (probably amino acids) acquisition is obvious. Antibody-mediated blocking of some ABC transporters was shown to be effective in vitro and in vivo against *M. hominis* and *S. aureus* [47] [48]. Monoclonal antibody Aurograb® entered phase III clinical trial as an addition to vancomycin therapy for deep-seated staphylococcal infections (NCT00217841), but the trial was stopped due to lack of reaching the primary endpoint. Anyway, somewhere positive results in targeting eukaryotic ABC transporters for cancer treatment support the idea of a broader evaluation of the similar capability for prokaryotes. Also, transmembrane transport is one of the prevalent bioprocess groups in terms of all found in matrix proteins (n=18) as well as in terms of several overrepresented in matrix protein (n=8).

ATP-dependent Zn proteases are common enzymes in the cell envelope of *P. aeruginosa*, they participate in several processes including metabolism, protein transport and removal of misfolded proteins, and adaptation to environmental conditions [35]. Also, some proteases may act as a virulence factor - Zn2+-dependent protease *Bacillus anthracis* called Lethal Factor is required for infection [49]. The proteolysis bioprocess group was the second represented in the matrix with 8 overrepresented proteins. Proteolysis is a part of the external digestive system and provides peptides and amino acids for bacterial nutrition, so the presence of some proteins involved in amino acids metabolism was expected (this bioprocess group includes 16 found in matrix proteins with 7 overrepresented proteins). Protease`s functions could be crucial for both bacterial survival and infection process and targeting bacterial proteases could be a perspective way to combat bacterial infections [50]. Moreover, for Zn proteases, host nutritional immunity (including Zn-dependent processes) was shown to be effective against infections caused by *P. aeruginosa* [51]. Also, deprivation of Zn ions was proposed to combat infections caused by another common pathogen - *S. aureus* [52].

Despite a small number of matrix unique proteins, we found a lot of overrepresented proteins in the matrix. Eight proteins with extremely high log2 fold change (more than 20) were considered as semi-unique for matrix, but we did not find any related to biofilm role for these proteins in literature. So as for unique matrix proteins, there is an unexplored area in biofilm biology.

Bioprocesses classification of represented in matrix proteins reveals several groups. Considering log2 fold change we found that groups of oxidation-reduction processes, biosynthetic processes, and nucleoside metabolism had the largest number of highly overrepresented proteins. Obviously, each bioprocess might vary in the number of involved proteins, so we also introduced bioprocess representation as a part of all GO-bioprocess annotated proteins.

In the matrix, the most reach group of proteins (128 proteins found in matrix and 27 of them are overrepresented in comparison with embedded cells) belongs to oxidation-reduction processes. Also, a group of proteins involved in cell redox homeostasis was one of the most represented GO-annotated bioprocesses in the matrix (14 proteins found in matrix, 8 were overrepresented from 26 annotated in GO bioprocess database). Biofilms of *P. aeruginosa* are often enriched in molecules involved in virulence and

competition with other microorganisms, including redox-active molecules. Self-produced factors involved in the generation of reactive oxygen species might be harmful to the internal bacterial community. *P. aeruginosa* is balancing to maintain oxidation-reduction processes at the appropriate level, so maintaining the balance of oxidation-reduction reactions and redox homeostasis likely play important role in the biofilm matrix as an environment with limited diffusion. Several effective therapeutic approaches utilize oxidative stress to combat bacterial biofilms including photodynamic therapy (PDT) and sanitizers like hydrogen peroxide [53, 54]. Also, recently was shown that extracellular electron transfer (EET) exists inside biofilm matrix [55]. In matrix EET mainly involves pyocyanin and eDNA, but the role of the protein component is not yet studied.

The second represented bioprocess included proteins involved in the fatty acid biosynthetic process (4 overrepresented proteins from 12 found in the matrix). Fatty acids are one of the major components of the cell envelope. Also, it is a well-known signal function of cis-2-docenoic acid messenger (DSM) as well as the importance of the fatty acid component of AHL [56]. In a recently published study, Altay et al. made a comprehensive analysis of essential reactions and affected pathways in *B. cenocepacia* (both planktonic and biofilm) using a systems biology approach. From all identified essential reactions lipid metabolism was accounted for more than half of the single lethal reactions; among this fatty acid biosynthesis was most frequently found [57]. That data support the further development of fatty acids metabolism inhibitors as promising therapeutics against bacterial infections, including bacterial biofilms.

The most represented bioprocess was nucleoside metabolism – 10 out of 11 GO-annotated proteins were found in the matrix. Biofilms are often enriched with extracellular nucleic acids which act not only as a structural component or component of EET, but also were considered as a nutrient depo [58]. Nucleosides act as substrates and cofactors in many biosynthetic processes, as signal molecules, and are involved in regulating bacterial community inside biofilm [59], so the presence of nucleoside metabolic proteins is required. Therapeutic targeting of proteins involved in these processes is theoretically possible, but at present is not clear. At the same time, nucleoside analogs are common drugs in other nonbacterial diseases, and evaluation of their possible role as antibacterial drugs may open new opportunities [60]. So antimycotic drug 5-fluorocytosine was able to suppress virulence of *P. aeruginosa* in a murine model of lung infection [61].

Obviously, sources of extracellular matrix proteins belong to two main categories: (1) active secretion of biomolecules and (2) passive way to increase extracellular content as a consequence of cell leakage or lysis. While some extracellular proteins are «passive» products of bacterial cell lysis, they still might be active outside the cell and provide their function to the bacterial community extracellularly. As protein degradation rates are varied in broad ranges, some proteins may present in the matrix for a long time after leakage or secretion from the cell, so their occurrence in the matrix does not (1) match the actual situation inside cells or (i. e. active transcription and translation), or (2) reflect any real extracellular needs for biofilm (i. e. be structurally or/and physiologically involved in extracellular processes). The limitation of our study is an inability to conclude if the protein is «archeological», bystander, or functionally active in the matrix and how these proteins are distributed in the matrix (are they cell-attached, part of OMVs, or associated with other matrix components). Moreover, some proteins may act as distracting extracellular targets in a way of absorption of inhibitor and distract from intracellular targets, so further research with a focus on the function and dynamics of every single protein is needed.

4. Materials and Methods

2.1. Biofilm growth and separation matrix from cells

P. aeruginosa KB6 (CF clinical isolate) was a gift from Zigangirova N. A. (Gamaleya NRCEM, Moscow, Russia) [21]. For biofilm preparation single colony from TSA plate was picked in liquid LB medium and grown 24 h at 37 C, 210 rpm. The liquid culture was diluted 50 times with LB medium in a volume of 20 ml and placed in Petri dishes for 18 h at 37 C under static conditions. Then the medium was removed, and biofilm was exposed to 10 ml of 1.8 M NaCl. After 5 min of incubation bacterial suspension and dissolved matrix were separated with centrifugation at 5000 g. Liquid phase (dissolved matrix) was filtered through a 0.22 mkm syringe filter and protein was precipitated with cold acetone (up to 80 %) 18 h at -20 C. Cells pellet was resuspended in lysis buffer (2% SDS; 50 mM Tris-HCl; 180 mM NaCl; 0,1 mM EDTA; 1 mM MgCl2) and boiled for 15 min in a water bath. Cell debris was removed by centrifugation at 10000 g, 15 min, and proteins from the liquid phase were precipitated with 80% cold acetone as for matrix samples. Precipitated proteins were pelleted with centrifugation at 10000 g, 20 min, 4 C. Pellet was washed two times with 80 % cold acetone and proceeded for proteomic sample preparation.

2.2. Proteomic sample preparation and peptide identification

Proteomic sample preparation and peptide identification were made in Advanced Mass Spectrometry Core Facility (Skolkovo Innovation Center, Moscow, Russia). The protein pellet was subjected to tryptic in-solution digestion. LC-MS/MS was carried out on a Q Exactive HF (Thermo Scientific) with a nanoESI interface in conjunction with an Ultimate 3000 RSLC nano HPLC (Dionex Ultimate 3000). Peptides were loaded onto a trap column and separated on an analytical column (C18) using an H2O/acetonitrile gradient with 0.1% formic acid for 150 min. The Q Exactive HF spectrometer was operated in the data-dependent mode with a nanoESI spray voltage of 1.8 kV, capillary temperature of 210 °C, and S-lens RF value of 55%. All spectra were acquired in positive mode with full scan MS spectra scanning from m/z 310–1500 in the FT mode at 120,000 resolution. A lock mass of 445.120025 was used. The top 25 most intense precursors were subjected to rapid collision induced dissociation (rCID). Dynamic exclusion with of 70 seconds was applied for repeated precursors.

2.3. Data analysis

To identify and quantify tryptic peptides and the proteins from which the peptides are derived, spectra from the MS/MS experiments were analyzed by GUI FragPipe v. 17.1 (https://github.com/Nesvilab/FragPipe). Peptide identification was performed by MSFragger search engine [22, 23] using protein sequence database extracted from NCBI (*Pseudomonas aeruginosa* strain GIMC5015:PAKB6 chromosome, complete genome NZ_CP034429) with decoys and contaminants. Oxidation of methionine and acetylation of protein N-termini were set as variable modifications, carbamidomethylation of cysteine was set as a fixed modification. The maximum allowed variable modifications per peptide was set to 3, mass tolerance was set as 20 ppm for precursor and 0.02 Da for fragment ions. Philosopher kit tools [24, 25] were used to estimate identification FDR. The PSMs were filtered at 1% PSM and 1% protein identification FDR. Quantification by label-free protein quantitation method and MBR was performed with IonQuant [26]. Obtained quantified data (intensities) were processed for differential expression analyses with limma package R [27], with followed visualization result by EnhancedVolcano package R ("EnhancedVolcano: Publication-ready volcano plots with enhanced coloring and labeling." https://github.com/kevinblighe/EnhancedVolcano).

Bioprocess classification and functional annotation were made with the Pseudomonas Genome Database (https://pseudomonas.com/primarySequenceFeature/list?strain_ids=10430&term=Pseudomonas+aeruginosa+GIMC5015%3APAKB6&c1=name&v1=&e1=1&assembly=complete) [28].

For correlation analysis, we used GraphPad Prism 9 desktop software (version 9.2.0). For the Figures arrangement, we used bio-render online software (Biorender.com).

5. Conclusions

Biofilm matrix of clinical strain *P. aeruginosa* is enriched with proteins. There are several unique for matrix and many overrepresented in matrix proteins, which reflects several bioprocesses. Development of antibiofilm therapeutics may benefit in the case of targeting proteins and processes taking place in biofilm matrix as the sole mechanism of action or in combination with antibiotics. Altogether matrix protein composition is important for choosing successful strategy in antibacterial drug development and reaching unmet needs of curing biofilm infections.

Supplementary Materials: The following supporting information can be downloaded:

Table S1: All identified proteins.

Table S2: Differentially represented proteins.

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Data Availability Statement: The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [62] partner repository with the dataset identifier PXD031480.

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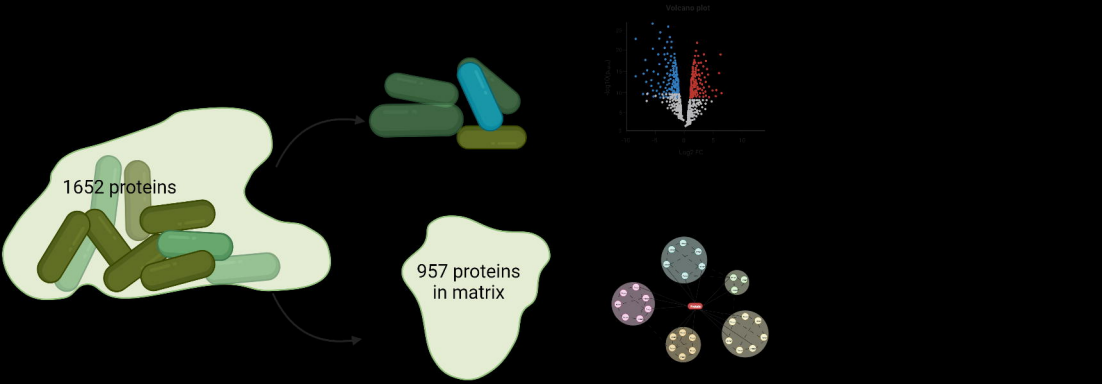
1652 proteins
957 proteins
in matrix
Volcano plot
Log2 FC