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SHORT COMMUNICATION

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

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Abstract

Extracellular matrix plays a pivotal role in biofilm biology. Despite importance of matrix proteins as potential targets for development of antibacterial therapeutics little is known about matrix proteomes. While *P. aeruginosa* is one of the most important pathogens with emerging antibiotic resistance only few studies are devoted to matrix proteomes and there are no studies describing matrix proteome for any clinical isolates. As matrix responsible for some extracellular functions, it is expected that protein composition should be different in comparison with embedded in biofilm cells and this difference reflects possible active processes in matrix. Here we report the first matrix proteome for clinical isolate of *P. aeruginosa* in comparison with embedded cells. We have identified the largest number of proteins in matrix among all published studies. Ten proteins were unique for matrix and not present inside cells, but most of these proteins do not have well described function with respect to extracellular component of biofilm. Functional classification of enriched in matrix proteins resulted in several bioprocess groups of proteins. Top three groups were: oxidation-reduction processes, nucleoside metabolism and fatty acid synthesis. Finally, we discuss obtained data in prism of possible directions for antibiofilm therapeutics development.

Introduction

Biofilms is the most common lifestyle of microorganisms. The key feature of biofilms is a presence of extracellular matrix which cover all member of biofilm and creates microenvironment for communication, protects against different threats, provide opportunity for spatial organization and functional diversification with community. In a case of infections biofilms represented by microbial aggregates with tolerance to host defense mechanisms and antimicrobials. Matrix consists of 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 a term «matrixome» was proposed [1]. Despite accepted idea of a pivotal role of matrix in bacterial biofilms little is known about protein composition of matrix at a level of proteomes. Moreover, for the well-studied and clinically important biofilm forming bacteria *P. aeruginosa* there are only few studies devoted to matrix proteome, while proteomics of the whole biofilm is better described[2, 3]. In comparative study of liquid cultured PAO1 strain and clinical isolates it was shown that cystic fibrosis (CF) isolates expressed a narrower range of transporters and a broader set 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 [4]. Only one study described proteome of matrix in comparison with embedded cells [5] and one else study compared matrix with the total biofilm proteome [6], both studies devoted to reference strain PAO1. The gap in understanding of proteomic difference between matrix and

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embedded cells frustrates development of antibiofilm therapeutics and overall understanding of biofilm biology. Here we performed a proteomic study of matrix composition in comparison with embedded cells for clinical strain of *P. aeruginosa* in order to identify bioprocesses taking place in matrix as a probable target for further research.

Table 1. Studies devoted to biofilm matrix proteome. Biofilm`s compartments and MS methods were named in accordance with 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	[7]
P. aeruginosa PAO1	**Matrix**	LC-MS/MS	Total biofilm 857 **Matrix 60**	[6]
P. aeruginosa PAO1	**Matrix** OMVs Cells	1D-SDS-PAGE combined with nano-LC-ESI-MS/MS	**Matrix 178** OMVs 57 Cells 764	[5]
P. aeruginosa PAO1 at different time points	Cells OMVs	LC-MS/MS	Cells 2443 in total OMVs 1142 in total	[8]
P. aeruginosa CF clinical isolate KB6	Cells Matrix	Orbitrap LC MS/MS	Cells 1642 **Matrix 957**	This study
S. aureus in vivo chronic implant infection	Secretome Surfactome	GeLC-MS/MS	Secretome 33 Surfactome 72	[9]
S. aureus	DNA-binding proteins in biofilm matrix	quadrupole time of flight (Q/TOF) mass spectrometer (Agilent 6520) with a nanospray ionization source	49	[10]
B. multivorans C1575	Matrix OMVs	Gel-based LC-MS/MS	Matrix 161 OMVs 64	[11]
S. acidocaldarius	EPS	nanoRSLC-Orbitrap LC-MS/MS	85	[12]
Shewanella sp. HRCR-1	EPS	LC-MS/MS	58	[13]
Cutibacterium acnes	Matrix	Orbitrap MS	447	[14]
Vibrio cholerae	Matrix	LC electrospray ionization and then entered into an LTQ linear ion-trap mass spectrometer (ThermoFisher)	74	[15]
Haemophilus influenzae (NTHi)	ECM	Gel-based LC/MS/MS	60	[16]

Materials and Methods

Biofilm growth and separation matrix from cells.

P. aeruginosa KB6 (CF clinical isolate) was a gift from Zigangirova N. A. (Gamaleya NRCEM, Moscow, Russia) [17]. For biofilm preparation single colony from TSA plate was picked in liquid LB medium and grown 24 h at 37 C, 210 rpm. Liquid culture was diluted in 50 times with LB medium in a volume of 20 ml

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and placed in petri dishes for 18 h at 37 C under static condition. Then medium was removed, and biofilm was exposed to 10 ml of 1.8 M NaCl. After 5 min of incubation bacterial suspension in dissolved matrix was separated with centrifugation at 5000 g. Liquid phase was filtered through 0.22 nm 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 water bath. Cell debris was removed by centrifugation at 10000 g, 15 min and proteins from 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.

Proteomic sample preparation and peptide identification.

Proteomic sample preparation and peptide identification was made in Advanced Mass Spectrometry Core Facility (Skolkovo Innovation Center, Moscow, Russia). LC MS/MS was carried out on a Q Exactive HF (Thermo Scientific) with a nanoESI interface in conjunction with an Ultimate 3000 RSLC nanoHPLC (Dionex Ultimate 3000).

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 [18][19] 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 [20] [21] was used to estimate identification FDR. The PSMs were filtered at 1% PSM and 1% protein identification FDR. Quantification and MBR was performed with IonQuant [22]. Obtained quantified data were processed for differential expression analyses with limma package R [23], 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 was made with Pseudomonas.com database [24].

For correlation matrix analysis we used GraphPad Prism 9 desktop software (version 9.2.0).

For figures arrangement we used bio-render online software (Biorender.com).

Results

P. aeruginosa KB6 (*exoT+; exoY+; exoU-; exoS+*) is a clinical isolate from bronchoalveolar lavage of cystic fibrosis patient with strong biofilm-forming phenotype [17]. To investigate protein composition of extracellular biofilm matrix we used previously published method of separation of biofilm matrix from embedded cells with NaCl [25] [26]. To confirm absence of cell lysis for KB6 strain during matrix separation we evaluate number of recovered CFU after NaCl treatment for both biofilm and suspension bacterial culture. There was no difference in recovery of CFU with and without NaCl treatment for both suspension and biofilm samples, so matrix isolation with NaCl was used for protein sample preparation. For proteomic study we grown static biofilm for 18 h in LB medium. This time point corresponds biofilm in early stationary phase when the number of embedded bacterial cells reach the plateau and matrix is already formed. Further increase in biofilm biomass occurs mainly due to mass of extracellular components rather

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than number of bacterial cells and significantly impacted with lysed cells. To decrease number of proteins which may represent passive cell leakage or death during 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 liquid medium. Extracellular matrix and embedded cells were separated with NaCl and processed for protein isolation. 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 S1 table.

General overview of proteomes.

In total more than 1600 proteins were identified in all samples. After initial manual inspection of intensity signals as expected we observed matrix-specific proteins, cell-specific proteins, and proteins with presence in both compartments. While inspection of intensity signals is not applicable for comparison of representation of different proteins it is still gave us a consciousness about separation matrix from cells. 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, S7/S12 ribosomal protein has one of the top 20 intensity signals in cell samples but was completely absent in matrix samples. So, in general the absence of this ribosomal and some other proteins in matrix samples is not 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 intensities between cells and matrix was 0.4959 (95% CI 0,4555 to 0,5343) (correlation matrix is presented on figure 1). While correlation was expectedly positive there were several examples of outfitters like lytic murein transglycosylase with well-known localization on the outer surface of bacterial cell wall [27].

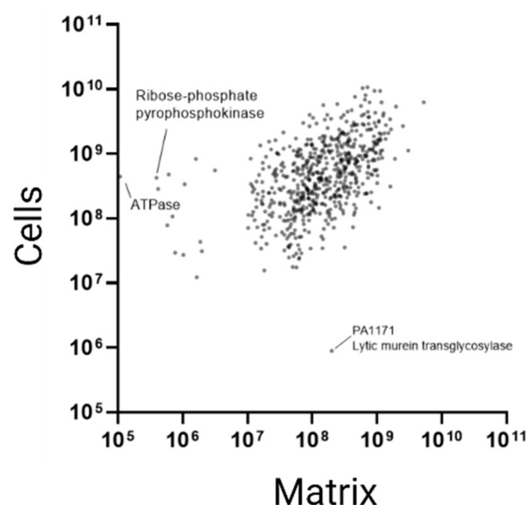

Figure 1. Correlation of individual protein intensities between embedded cells and matrix.

Cells and matrix differ in overall biomolecule content, physicochemical and other properties. That were our premises for rigorous statistical analysis of comparative protein representation. For getting quantitative comparison of representation of proteins we proceeded to logarithm transformation and quantile normalization of protein intensities. Statistical analysis was made with Limma R package [28]. All differentially represented proteins are listed in table S1. For fold difference presentation volcano plot was

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created (figure 2). In total there were more than 1600 proteins, and 974 of them were presented in biofilm matrix.

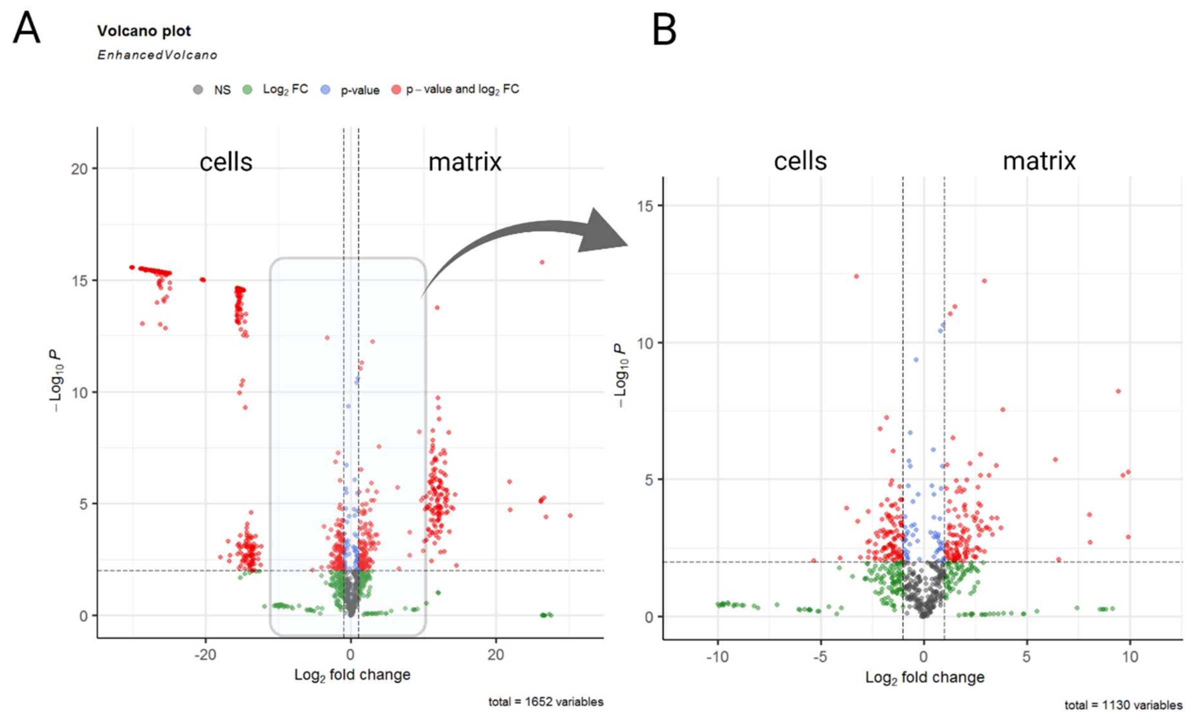

Figure 2. Volcano plot of representation of proteins between cells and matrix. A – overall protein representation; B – enlarged area with proteins with fold change between Log2 from 1 to 10. Red color indicates proteins with fold change more than 2 and significance value p<0.01; green color indicates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

Unique proteins in extracellular matrix.

Matrix unique proteins were defined if they were observed in both experiments in matrix only. Totally ten proteins were present in 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 of 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.

Protein	Protein ID	Role in biofilm	Reference
two-partner secretion system transporter CdrB	WP_010895680.1	Secretion partner of CdrA adhesin	[29]
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	-

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nucleoside hydrolase Nuh (catabolizes adenosine)	WP_003147076.1	QS-controlled private good	[30]
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	[31]

Functional classification of overrepresented proteins in extracellular matrix.

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

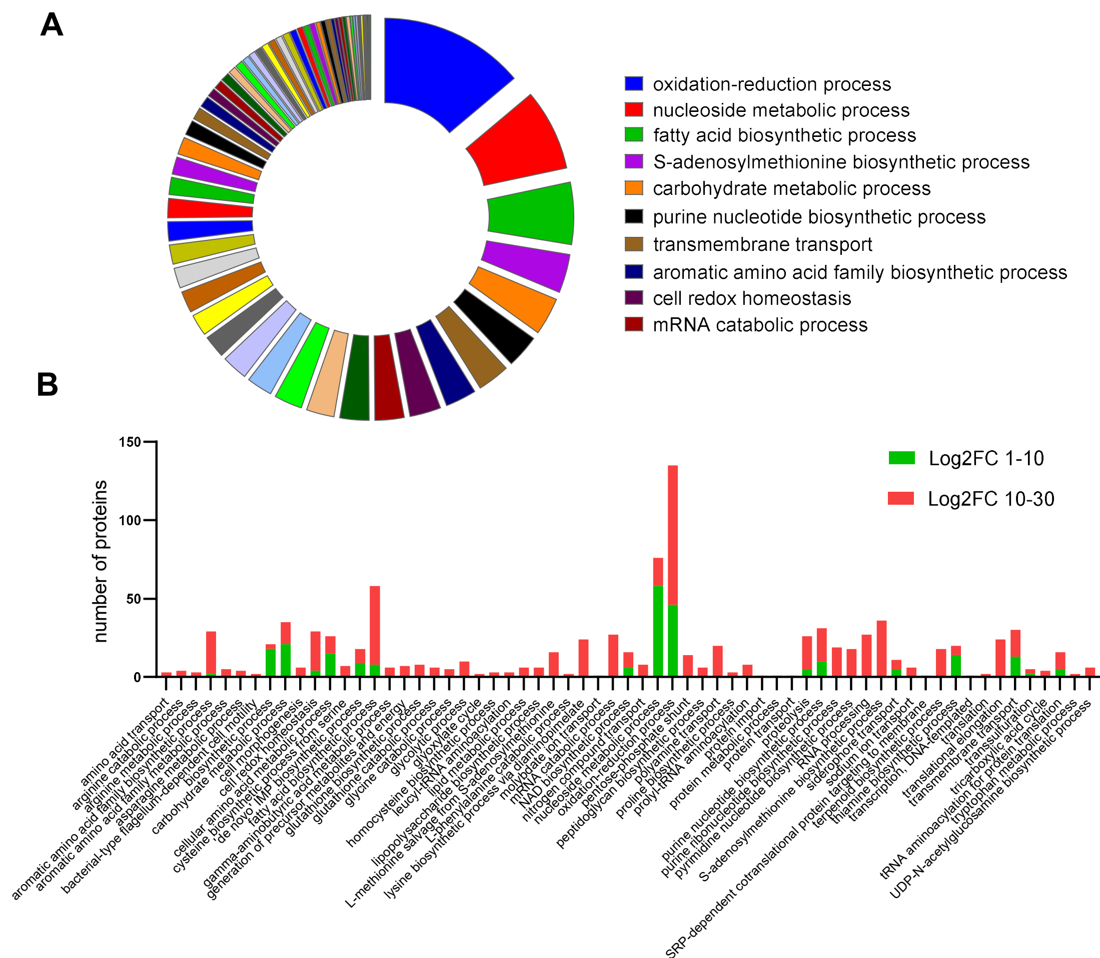

Figure 3. Bioprocess group classification of overrepresented proteins in biofilm matrix. A – proportion of overall proteins in biofilm matrix, each color represents individual bioprocess group, legend indicates top ten of bioprocess groups; B – number of overrepresented proteins in different bioprocess groups with

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respect to log2FC: green color – proteins with log2FC from 1 to 10; red color – proteins with log2FC more than 10, group names are listed in alphabetical order.

Bioprocess groups with the largest number of proteins belongs mainly to oxidation-reduction, nucleoside metabolic processes and fatty acids biosynthesis.

Discussion

Biofilm matrix in most cases contains many proteins. Extracellular proteins in 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 not limited) external digestive system, signaling, protection and maintaining stability of matrix [1, 32–34]. Moreover, many proteins may have moonlight functions.

Understanding of protein composition is critical for resolving both fundamental questions in bacterial lifestyle and 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 identification of matrix protein composition may open new opportunities for 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 matrix [35]. Matrix-disrupting or interrupting agents may reverse tolerant phenotype and increase efficacy of antibiotic therapy [36]. For example, antibody-mediated destabilization of matrix through disruption of IHF-DNA complexes was effective in vitro and in vivo both as single therapy and in combination with antibiotics [37–39]. 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 matrix proteome of reference strain PAO1 of *P. aeruginosa* and some other bacteria (table 1). Here we for the first time describe matrix proteome of CF clinical isolate of *P. aeruginosa* in comparison with embedded cells and discuss obtained data in prism of possible target for therapeutic development. In comparison with other studies we identified the largest number of proteins in 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 more comprehensive picture of bacterial proteomics.

In our study only small number of proteins were unique for matrix only. One of them – CdrB protein is involved in 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 [40]. Surprisingly we did not find CdrA protein neither in cells nor in matrix. In less sensitive gel-based proteomic study authors have observed CdrA protein in matrix [5, 16], so absence of the protein in our data is unlikely due to limits of detection. One of the possible explanations that CdrA/CdrB component are not important for KB6 strain due to limited role of Psl polysaccharides in biofilm matrix, so in the absence of Psl CdrA might be degraded by proteases.

Another matrix unique protein is nucleoside hydrolase (Nuh) - 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 intracellular (periplasmic) private good in a prism of quorum sensing in bacterial communities [30]. In our study, we found Nuh in 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* remains still undiscovered, so if Nuh works outside the cell such factor is not needed. In an environment with sole carbon sources Nuh mutant have impaired growth [41], but therapeutic potential of direct or indirect inhibition of Nuh activity remains elusive due to nutrient rich nature of infected tissues.

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For the rest 8 matrix-unique proteins there is no clear evidence of their possible role in biofilm, so further research is needed. Meanwhile as matrix considered as 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* [42] [43]. Monoclonal antibody Aurograb® entered phase III clinical trial as addition to vancomycin therapy for deep-seated staphylococcal infections (NCT00217841), but trial was stopped due to lack of reaching primary endpoint. Anyway, somewhere positive results in targeting eukaryotic ABC transporters for cancer treatment supports idea of more broad evaluation of the similar capability for prokaryotes.

ATP-dependent Zn proteases are common enzymes in cell envelop of *P. aeruginosa*, they participate in several processes including metabolism, protein transport and removal of misfolded proteins and adaptation to environmental conditions [31]. Also, some proteases may act as a virulence factor - Zn2+-dependent protease *Bacillus anthracis* called Lethal Factor is required for infection [44]. So, 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 [45]. While Zn is important for many bacterial processes (not limited to proteases) host nutritional immunity was shown to be effective against infections caused by *P. aeruginosa* [46]. Deprivation of Zn ions was proposed as a way to combat infections caused by another common pathogen - *S. aureus* [47].

Despite small number of matrix unique proteins, we found a lot of overrepresented proteins in matrix. Bioprocesses classification of these proteins reveals several groups of proteins. The most reach group (more than 130 proteins) was oxidation-reduction processes. Biofilms of *P. aeruginosa* are often reach in molecules involved in virulence and competition with other microorganisms, including redox-active molecules. Due to limited diffusion in matrix factors involved in reactive oxygen species generation, might be harmful for internal bacterial community. *P. aeruginosa* is balancing to maintain oxidation-reduction processes at appropriate level. Also, extracellular electron-transfer (EET) exist inside biofilm matrix [48]. EET manly involves pyocyanin and eDNA, but role of protein component is not yet studied.

The second group of overrepresented proteins was nucleoside metabolism. Importantly this group of proteins includes mainly proteins with significant, but relatively low fold change rate (log2FC 1-10) in comparison with embedded cells. As nucleotide metabolism is one the most active process for any living organism, we believe that some process could occur in matrix, but with respect to relatively low fold change rate of proteins in this group it is highly probable that most members of the group are products of cell lysis or cell leakage. At the same time most biofilms are 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 [49]. Digestion and consumption of polymeric extracellular nucleic acids requires at least extracellular nucleases. Also, nucleosides act as signal molecules and involved in regulation of bacterial community inside biofilm [50], so targeting of proteins involved in these processes is theoretically possible, but at the present time is not clear.

The third group with more than 50 proteins was proteins involved in fatty acid biosynthetic process. Fatty acids are one of the major components of cell envelop. Also, it is well known signal function of cis-2-docenoic acid messenger (DSM) as well as importance of fatty acid component of AHL [51] . In recently published study Altay et al. made comprehensive analysis of essential reactions and affected pathways in *B. cenocepacia* (both planktonic and biofilm) using 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 [52]. That data support further development of fatty acids metabolism inhibitors as promising therapeutics against bacterial infections, including bacterial biofilms.

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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 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 bacterial community extracellularly. As protein degradation rate are vary in broad ranges, some proteins may present in matrix for long time after leakage or secretion from cell, so their occurrence in matrix does not (1) match 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. Limitation of our study is an inability to conclude about if protein is «archeological», bystander or functionally active in matrix and how these proteins distributed in matrix (are they cell attached, part of OMVs or associated with other matrix components). Further research with fucus on function and dynamics of every single protein are needed.

Conclusion

Biofilm matrix of clinical strain *P. aeruginosa* are 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 a case of targeting proteins and processes taking place in biofilm matrix.

Supplementary Materials

Table S1: List of all identified proteins.

Author Contributions

Conceptualization, D.E., A.S., N.P.; methodology, A.S., K.D.; software, A.S., N.P.; validation, A.S.; investigation, D.E., I.K.; resources, Y.R.; data curation, M.D.; writing—original draft preparation, D.E., M.D., I.T.; writing—review and editing, Y.R.; project administration, D.E.; funding acquisition, A.G. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflict of interest.

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