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

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11 **Abstract:** Extracellular matrix plays a pivotal role in biofilm biology and proposed as a potential target for
12 therapeutics development. As matrix is responsible for some extracellular functions and influence bacterial cytotoxicity
13 against eukaryotic cells, it must have unique protein composition. *P. aeruginosa* is one of the most important pathogens
14 with emerging antibiotic resistance, but only a few studies were devoted to matrix proteomes and there are no studies
15 describing matrix proteome for any clinical isolates. Here we report the first biofilm matrix proteome of *P. aeruginosa*
16 isolated from bronchoalveolar lavage of patient in intensive care unit. We have identified the largest number of proteins
17 in the matrix among all published studies devoted to *P. aeruginosa* biofilms. Comparison of matrix proteome with
18 proteome from embedded cells let us to identify several enriched bioprocess groups. Bioprocess groups with the largest
19 number of overrepresented in matrix proteins were oxidation-reduction processes, proteolysis, and transmembrane
20 transport. The top three represented in matrix bioprocesses concerning the size of the GO annotated database were cell
21 redox homeostasis, nucleoside metabolism, and fatty acid synthesis. Finally, we discuss the obtained data in a prism
22 of antibiofilm therapeutics development.

23 **Keywords:** biofilm; matrix; proteome; matrixome; *Pseudomonas aeruginosa*; nucleoside metabolism; cell redox
24 homeostasis; fatty acid synthesis, proteolysis; transmembrane transport
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26 ## 1. Introduction

27 Biofilms are the most common lifestyle of microorganisms, including both pathogenic and
28 environmental bacterial species. From a clinical perspective, biofilm cause difficult-to-treat recurrent
29 diseases. Microbial aggregates with tolerance to host defense mechanisms and antimicrobials are found at
30 the site of infection. Currently, there is an urgent need to discover new targets and strategies to overcome
31 the tolerance for the effective treatment of biofilm-associated infections.
32 The key feature of biofilms is an extracellular matrix that covers all members of biofilm and creates a
33 microenvironment for communication, protects against different threats, provides an opportunity for
34 spatial organization and functional diversification within the community. The matrix comprises a broad
35 range of biopolymers, metabolites, and signal molecules. Also, it may include organized compartments
36 like outer membrane vehicles (OMVs). To stress the idea of a rich and complex matrix organization,
37 Karygianni at al. have proposed the term «matrixome» [1]. The sources of proteins in the biofilm matrix
38 might be active secretion, passive leakage from cells, and entrapped into the matrix from the environment
39 molecules [2,3]. Proteins in the matrix have a structural role in maintaining biofilm organization, play a
40 role as a protective barrier, creates microenvironment with limited diffusion. The barrier role is somewhere
41 similar to structural function but also includes hydrophobic features, charge, and so on, rather than just
42 being mechanically stable structure. Moreover, matrix proteins may bind antimicrobial molecules and
43 reduce their diffusion and effect on bacterial cells, i.e. extracellular ribosomal proteins bind antibiotics and
44 prevent their penetration in bacterial cells. Other functions of matrix proteins rely on their enzymatic
45 activity and include degradation of biopolymers, participation in biochemical processes, and signaling
46 function.
47

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48 Biofilms are dynamic communities. Lifecycle can be divided into several major stages: attachment, 49 maturation, and dispersion. During the maturation stage, the matrix may accumulate virulence factors 50 which then come out together with dispersed bacteria. During chronic infections, the dispersion stage is 51 associated with the recurrence of symptomatic infections and colonization of new sites in the body.

52 Importantly, bacteria inside biofilm have diverse phenotypes, so one produces some molecules in 53 extracellular space while non-producers consume these public goods. Matrix as a compartment is also an 54 example of public goods. A part of bacterial population, especially inside multispecies biofilms, may not 55 have tolerance or specific resistance mechanisms, but due to matrix still be irresponsible for the treatment.

56 Targeting of matrix proteins as a strategy to combat infections may act in the following ways: (1) 57 destabilization/disruption of biofilm matrix to improve the action of host defense and/or antibiotic therapy; 58 (2) targeting of extracellular biochemical processes to reduce overall biofilm success in survival and 59 virulence; (3) targeting of extracellular virulence factors associated with biofilm matrix to decrease 60 virulence in a case of mass dispersion.

61 Despite the accepted idea of a pivotal role of matrix in bacterial biofilms, protein composition of the 62 matrix remains poorly discovered. Moreover, for the well-studied and clinically important biofilm-forming 63 bacteria *P. aeruginosa* there are only a few studies devoted to matrix proteome of reference strains (mainly 64 PAO1, Table 1), while proteomics of the whole biofilm is better described [4,5].

65

66

67 **Table 1.** Studies devoted to biofilm matrix proteome. Biofilm`s compartments and MS methods were named by 68 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	[6]
***P. aeruginosa* PAO1**	**Matrix** Total biofilm	LC-MS/MS	Total biofilm 857 **Matrix 60**	[7]
***P. aeruginosa* PAO1**	**Matrix** OMVs Cells	1D-SDS-PAGE combined with nano-LC-ESI-MS/MS	**Matrix 178** OMVs 57 Cells 764	[8]
***P. aeruginosa* PAO1 at different time points**	Cells OMVs	LC-MS/MS	Cells 2443 in total OMVs 1142 in total	[9]
***P. aeruginosa* ATCC27853 at different time points**	**Matrix**	iTRAQ-labeled peptides and LC-MS/MS analysis	**Matrix** 389 in total	[10]
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	[11]
***P. aeruginosa* 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	[12]
***S. aureus* USA300 CA-MRSA strain LAC**	DNA-binding proteins in the biofilm matrix	quadrupole time of flight (Q/TOF) mass spectrometer	49	[13]

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		(Agilent 6520) with a nanospray ionization source		
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	[14]
B. multivorans **C1575**	Matrix OMVs	Gel-based LC-MS/MS	Matrix 161 OMVs 64	[15]
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]

69
70 Comparative study of liquid culture of PAO1 strain and clinical isolates showed that cystic fibrosis
71 isolates expressed a narrower range of transporters and a broader set of enzymes of metabolic pathways
72 for the biosynthesis of amino acids, carbohydrates, nucleotides, and polyamines, but this study did not
73 cover biofilm mode of life as well as extracellular matrix composition [21]. Only one study described the
74 proteome of the matrix in comparison with embedded cells [8] and one else study compared the matrix
75 with the total biofilm proteome [7], both studies were devoted to reference strain PAO1. The gap in
76 understanding the difference in protein composition between matrix and embedded cells frustrates the
77 development of antibiofilm therapeutics and the overall understanding of biofilm biology. Here we
78 performed a proteomic study of matrix composition in comparison with embedded cells for the clinical
79 strain of *P. aeruginosa* to identify bioprocesses taking place in the matrix as probable targets or as factors or
80 as barriers during pharmacological development of antibiofilm therapeutics.

81 ## 2. Results

82 ### *2.1. General overview of proteomes*

83 *P. aeruginosa* KB6 (exoT+; exoY+; exoU-; exoS+; full name - GIMC5015:PAKB6) is a clinical isolate from
84 bronchoalveolar lavage of patient from ICU [22]. This strain has strong biofilm-forming phenotype
85 (Supplementary figure 2).
86 Two independent and separated in time biofilms were grown in a liquid medium. For the proteomic
87 study, we have grown static biofilm for 18 h in LB medium. This time point corresponds to biofilm in the
88 early stationary phase when the number of embedded bacterial cells reaches the plateau, and the matrix is
89 already formed. Further increase in biofilm biomass occurs mainly due to extracellular components rather
90 than an increasing number of bacterial cells. During the further biofilm growth, mass of extracellular
91 substance significantly impacted with lysed cells. To decrease the number of proteins that may represent
92 passive cell leakage or death during the biofilm stationary phase and to avoid interference of these
93 «archeological» proteins with secreted extracellular proteins, we choose this early time point (18 h). To
94 investigate protein composition of extracellular biofilm matrix, we used a previously published method of
95 separation of biofilm matrix from embedded cells with high ionic solution of NaCl [23,24]. Extracellular
96 matrix and embedded cells were separated and processed for protein isolation. Also, one more biological

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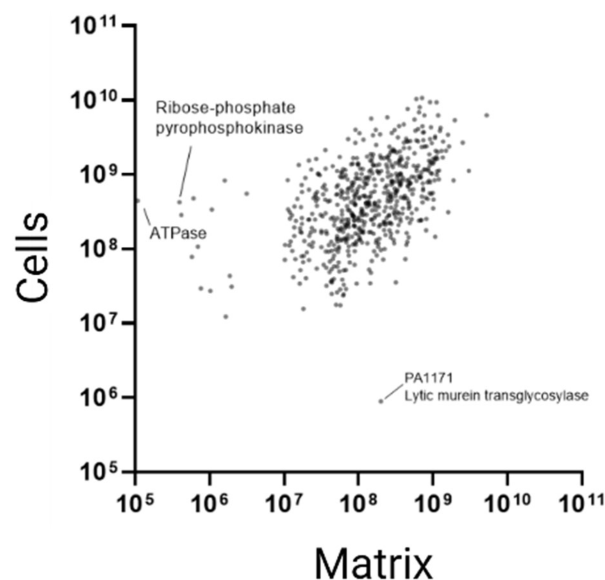
97 replicate (the third) representing embedded cells only was added, while matrix from this biofilm was used
98 to check if protein quantity is enough for proteome analysis (total protein quantity from matrix preparation
99 was more than 100 mkg, while minimum requirements is 50 mkg). We performed proteomic analysis for
100 each sample in three technical replicates. For protein identification, *P. aeruginosa* KB6 strain-specific protein
101 dataset was created based on genome sequence (available in GenBank under accession number
102 NZ_CP034429). For protein identification, we used MSFragger software. The full list of all identified
103 proteins is available in Table S1.

104 In total, we identified more than 1600 proteins in all samples. After initial manual inspection of LFQ
105 intensities, we observed matrix-specific proteins, cell-specific proteins, and proteins with presence in both
106 compartments. Spearman correlation coefficient for proteins LFQ intensities between cells and matrix was
107 0.4959 (95% CI 0,4555 to 0,5343) (Figure 1). While the correlation was expectedly positive, there were several
108 examples of outfitters like lytic murein transglycosylase with well-known localization on the outer surface
109 of the bacterial cell wall [25].

110 While an inspection of intensity signals is not applicable for quantitative comparison due to different
111 nature of cells and matrix samples, it is still giving us an additional consciousness about the proper
112 separation matrix from cells. The absence of several intracellular proteins in matrix samples confirms that
113 our approach to separate matrix from cells does not cause significant cell leakage during sample
114 preparation. For example, proteins involved in ribosome assembly and function (L29, L33, S15, Era),
115 septum formation and division (FtsX and MinC), transcription regulation (Cro/Cl family transcriptional
116 regulator) and some others were absent in the matrix (LFQ intensities and spectral counts were zero in all
117 matrix samples), while for some examples LFQ intensities in cells samples were more than $1*10^8$. So, the
118 absence of these proteins in matrix samples confirms matrix separation without cell lysis.
119



120
121 **Figure 1.** Correlation matrix of individual protein intensities (LFQ) between embedded cells and matrix. Proteins
122 with zero LFQ intensities are out of axis range and not plotted.
123

124 Cells and matrix differ in overall biomolecule content, physicochemical and other properties. That was
125 our premise for rigorous statistical analysis of comparative protein representation. For getting a
126 quantitative comparison of the representation of proteins, we proceeded to logarithm transformation and
127 quantile normalization of LFQ intensities. We made statistical analysis with the Limma R package [26]. All
128 identified proteins are listed in table S1, and proteins differing in their representation in cells and matrix
129 are listed in Table S2. For representation of the fold difference, volcano plot was created (Figure 2).

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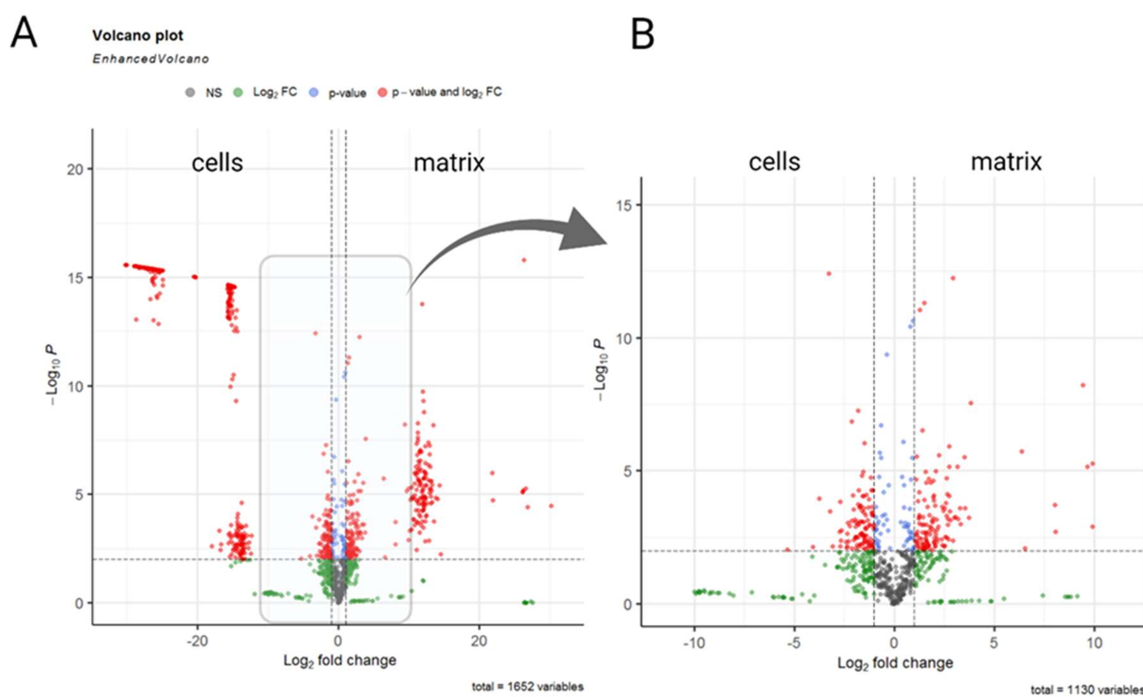

130
131 **Figure 2.** Volcano plot of representation of proteins between cells and matrix. A – overall protein representation; 132 B – an enlarged area with proteins with fold change between Log2 from 1 to 10. Red color indicates proteins with fold 133 change more than 2 and significance value $p<0.01$; green color indicates proteins with fold change more than 2 and 134 significance value $p>0.01$; blue color indicates proteins with fold change less than 2 but significance value $p<0.01$; grey 135 color indicates proteins with fold change less than 2 and significance value $p>0.01$.
136

137 In total, there were more than 1600 proteins, and we found 957 of them in the biofilm matrix (766 138 matrix proteins were present in both biological replicates).

139 *2.2. Unique proteins in the extracellular matrix*

140 Unique proteins were defined if we observed them (MaxLFQ total and unique intensities are non-141 zero) either in all matrix samples or in all cell's samples. Totally ten proteins were present in the 142 extracellular matrix only. We have proceeded with the literature search and manual biofilm-related 143 functional annotation for these matrix unique proteins (Table 2). Nonetheless, for most extracellular matrix-144 only proteins, we did not find straightforward evidence of their importance for biofilm structure or any 145 extracellular biofilm-related function.

146 **Table 2.** Matrix unique proteins. Matrix unique proteins had nonzero intensities in all matrix samples and zero 147 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	[27]
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	[28]
YgdI/YgdR family lipoprotein	WP_003089729.1	Not described	-
dGTPase	WP_003112492.1	Not described	-

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ATP-dependent zinc protease	WP_003113269.1	Not described, but some envelope proteases play role in many biological processes	[29]

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

150 Additionally, to unique matrix proteins, there were proteins with significant and extremely high log2
151 fold change (Log2FC) – more than 20. We considered these proteins as semi-unique matrix proteins (Table
152 3).

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

Protein	Protein ID	Role in biofilm	Reference
malonate decarboxylase subunit alpha	WP_003112666.1	Malonate improve biofilm formation	[30]
OprD family porin	WP_003114177.1	Not described*, carbapenem binding	[31,32]
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	-

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

157

158 *2.3. Functional classification of proteins in the extracellular matrix*

159 All overrepresented in matrix proteins are displayed in Figure 2A. We observed distinguishable
160 clusters of overrepresented proteins depending on their fold change (Log2FC). While a separate cluster of
161 highly overrepresented proteins with log2FC more than 10 is visible on the main volcano plot (Figure
162 2A), for better resolution of the area within log2FC 1-10 frame we also provide an enlarged area of the
163 volcano plot (Figure 2B). Analysis of bioprocess classification of all found in matrix proteins and
164 overrepresented in matrix proteins is displayed in Figure 3. Many bioprocess groups include at least one

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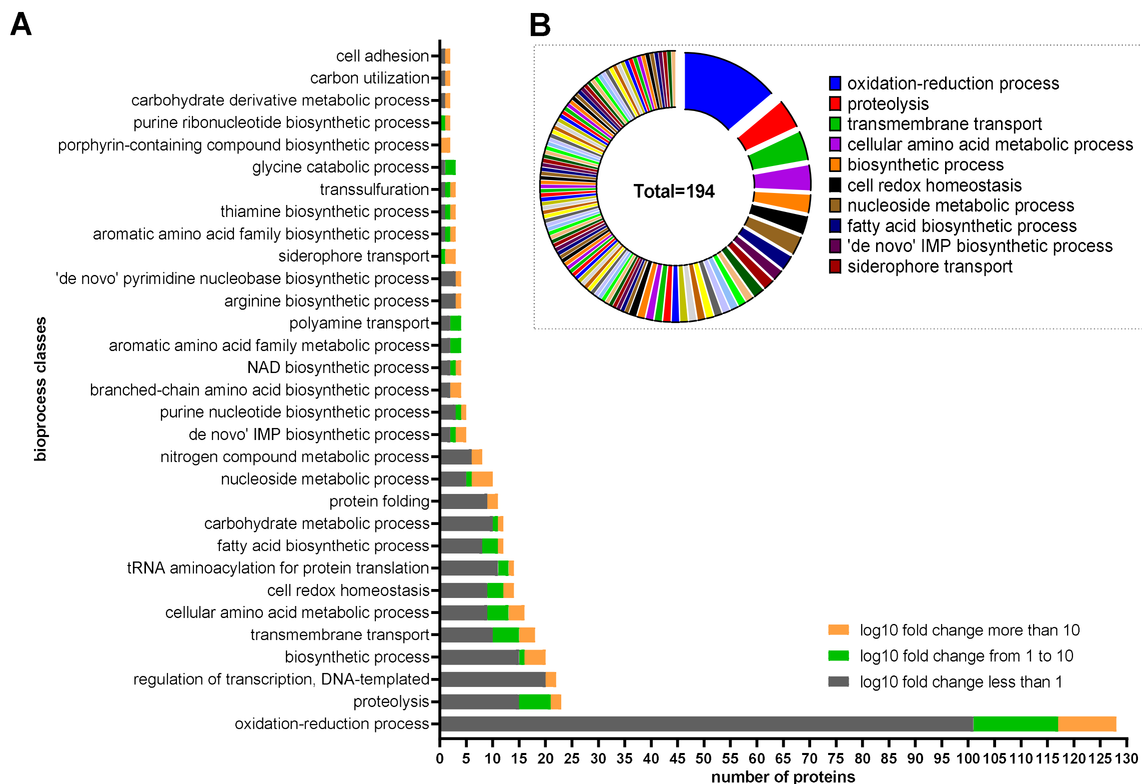
165 overrepresented in biofilm matrix protein.



166

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

174

175 We focused on groups with the largest number of found proteins and the proportion of
176 overrepresented proteins from all found in matrix proteins. For this reason, we applied the following
177 criteria: (1) at least 10 proteins per group found in the matrix; (2) more than 3 found in matrix proteins are
178 overrepresented (4 to 28 overrepresented proteins from 8 bioprocess groups). Such bioprocesses belong to
179 oxidation-reduction and cell redox homeostasis, proteolysis, transmembrane transport, amino acid, and
180 nucleoside metabolic processes, fatty acids biosynthesis. An important consideration is that GO annotated
181 bioprocesses consist of groups with highly different numbers of proteins. Number of proteins involved in
182 bioprocess reflects complex nature and flexibility of protein interactions. As an absolute number of
183 identified proteins may not reflect bioprocess representation, we count for each selected bioprocess group
184 proportion of found proteins from all GO annotated group members as a measure of bioprocess
185 representation (Table 4).

186

187 **Table 4.** Bioprocess representation in biofilm matrix based on the proportion of found proteins from GO-
188 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

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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

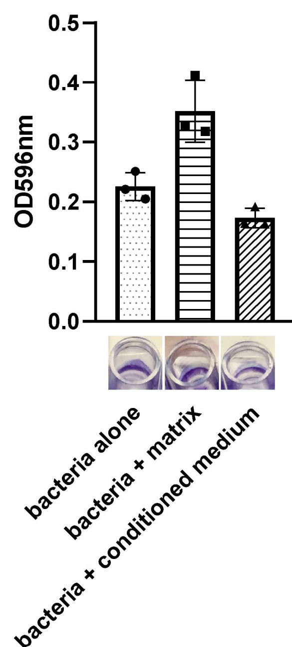
189

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

198

199 *2.4. Matrix decreases cytotoxicity of bacteria against A549 lung epitelial cells*

200 We hypothesized that the biofilm matrix may affect the way how bacteria interact with eucaryotic cells
201 and form biofilm in coculture model. As some compounds may freely diffuse between matrix and
202 surrounding medium, we served biofilm-conditioned medium for comparison. In experimental conditions
203 suitable for eucaryotic cells (DMEM medium supplemented with fetal bovine serum (FBS), atmosphere of
204 5% CO_2) addition of matrix to the isolated from biofilm bacteria improved biofilm formation while biofilm-
205 conditioned LB medium slightly reduce biofilm biomass (Figure 4).
206



207
208 Figure 4. Matrix effect on biofilm formation at conditions suitable for eucaryotic cell culturing. Bacteria
209 isolated from biofilm with addition of 10% of matrix or biofilm-conditioned medium were grown in DMEM
210 medium supplemented with 10% FBS at 5% CO_2 for 18 h, then stained with crystal violet. Diagram
211 represents the quantification of CV staining and well photographs represent biofilm formation at the air-
212 liquid interface.

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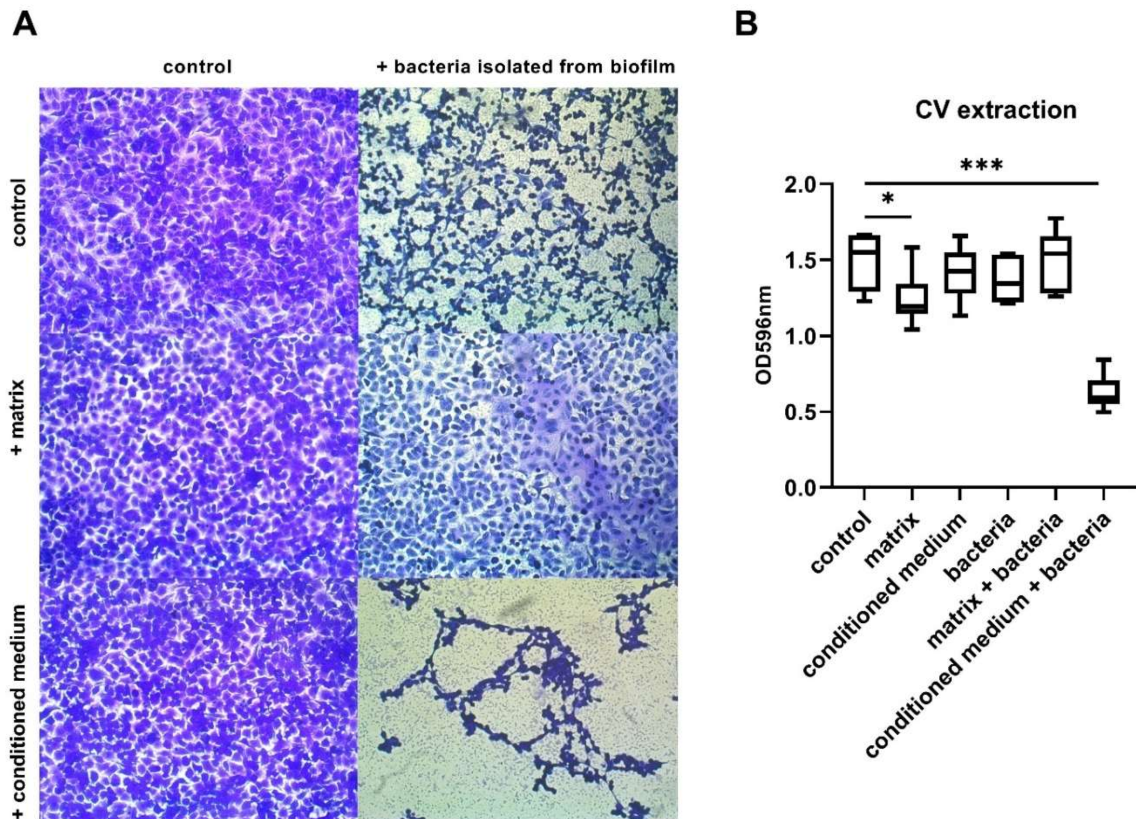
213
214 For coculture experiments, we choose A549 adenocarcinomic human alveolar basal epithelial line as
215 a pulmonary epithelial cell model. Addition of bacterial cells isolated from biofilm at MOI=3 resulted in
216 A549 cell layer disruption and eucaryotic cell death after 18 h of incubation in 5% CO2 atmosphere.
217 Addition of matrix to coculture model caused a significant change of A549 appearance with maintained
218 eucaryotic cell attachment and monolayer integrity, while addition of biofilm-conditioned medium had an
219 opposite action (Figure 5A).
220



221
222 **Figure 5**. Matrix effect on biofilm formation and cytotoxicity in coculture isolated from biofilm bacteria with A549
223 eucaryotic cells. A – light microscopy at 10x magnification of coculture stained with crystal violet. B – cumulative
224 biomass quantification with crystal violet. Asterisks indicate ANOVA p value: * - $p<0.05$; *** - $p<0.001$.
225
226 Importantly at this condition *P. aeruginosa* forms biofilm on both eukaryotic cells and plastic surface
227 as well as pellicle biofilm on the air-liquid interface (Figures 4, 5 and Supplementary figure 2B), so
228 quantification of staining showed cumulative biomass of eukaryotic cells and biofilm (Figure 5B). Bacteria
229 alone disrupted monolayer of A549 cells, but some cells remained attached and biofilm formation on the
230 walls of wells compensate biomass quantification. Matrix alone was slightly toxic to A549 cells but
231 restricted bacterial cytotoxicity and the most A549 cells remained attached, so the overall biomass was as
232 in the control. At the same time, well-tolerated biofilm-conditioned medium promotes bacterial cytotoxic
233 effect, which is clearly visible under microscopy and after dye extraction. So the biofilm matrix was able to
234 restrict bacterial cytotoxicity and prolong the maintenance of cell layer integrity.

235 **3. Discussion**

236 Biofilm Biofilm matrix in most cases contains many proteins. Extracellular proteins in the biofilm
237 matrix provide their functions for the whole bacterial community, so they may be considered as public
238 goods. Extracellular functions of matrix proteins include (but are not limited to) the external digestive
239 system, signaling, protection, and maintaining the stability of the matrix [1,33–35]. Moreover, many
240 bacterial proteins may have moonlight functions [36]. So studies devoted to matrix composition including
241 proteomics are essential for depicting biofilm lifestyle.

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242 We showed that the matrix may suppress bacterial cytotoxicity in coculture with eukaryotic cells.
243 Biofilm-associated infections frequently cause local tissue damage and inflammation without system
244 dissemination. While this complex process includes counteraction of host immune system and virulence of
245 bacterial pathogen, biofilm matrix might play a direct role in limitation of barrier tissue injury and
246 localization of infection process. There are many ways how matrix could restrict bacterial «aggression».
247 Just a few of them include providing favorable nutrients rather than eukaryotic cells, restriction of bacterial
248 sensing of eukaryotic cells, suppression of virulence activation and others. Wang et al. have reported that
249 cytotoxicity is attenuated at high MOI in coculture of *P. aeruginosa* with A549 cells [37]. Authors identified
250 phenylacetic acid (PAA) as compound responsible for T3SS suppression. PAA was identified in the
251 conditioned culture medium from planktonic bacterial culture. In contrast, we did not observe any
252 protective role of biofilm-conditioned medium, but the idea and overall principal of virulence suppression
253 by bacterial population in the stationary phase is similar. Kaya et al. reported 90% survival of PBMC co-
254 cultured with *P. aeruginosa* biofilm [38]. Moreover, PBMC responded to *P. aeruginosa* biofilm and vice versa
255 - biofilm increased bacterial cells number in the presence of PBMC or PBMC-secreted factors. This
256 observation indicates the existence of cross-talk during establishing of chronic infection and our data
257 suggest matrix importance in this communication.
258 Understanding the protein composition of the matrix is critical for resolving both fundamental
259 questions in bacterial lifestyle and the development of tools for manipulating biofilms. Most proteomic
260 studies focus on the whole biofilm and usually compare the whole biofilm proteome with the planktonic
261 cells proteome. At the same time, the identification of matrix protein composition may open new
262 opportunities for the development of antibiofilm drugs. Well known that biofilms of pathogenic
263 microorganisms are tolerant to antibiotics, many other therapeutics, and host immune factors due to the
264 matrix [39]. Matrix-disrupting or interrupting agents may reverse tolerant phenotype and increase the
265 efficacy of antibiotic therapy [40]. For example, antibody-mediated destabilization of the matrix through
266 the disruption of IHF-DNA complexes was effective in vitro and in vivo as a single therapy and in
267 combination with antibiotics [41–43]. Moreover, extracellular targets less probable cause selection of
268 resistant mutants, so targeting of matrix proteins is a perspective way to combat chronic infections,
269 especially infections caused by ESKAPE pathogens. Despite all of these, it is still little known about matrix
270 proteomes. Only numerous studies have described the matrix proteome of reference strain PAO1 of *P.*
271 *aeruginosa* and some other bacteria (Table 1). Here we for the first time describe the matrix proteome of
272 clinical isolate of *P. aeruginosa* in comparison with embedded cells. In comparison with other studies, we
273 identified the largest number of proteins in the matrix. While some proteins might be invisible due to their
274 low concentrations and individual limits of detection, we believe that further improvement of MS
275 equipment and techniques will get a more comprehensive picture of bacterial proteomics.
276 In our study, only a small number of proteins were unique for matrix. One of them – CdrB protein is
277 involved in the transport of CdrA adhesin. This adhesin is important for biofilm formation and its binding
278 to Psl results in increased biofilm structural stability. Antibody-mediated blocking of CdrA inhibit biofilm
279 formation[44]. Also, *cdrAB* is regulated together with Psl. CdrA binding to Psl protects it from endogenous
280 and exogenous protease digestion [45]. Both CdrA and Psl coding operons are present in the genome of
281 KB6. Surprisingly, MaxLFQ unique intensities for CdrA protein were zero in both cells and matrix samples.
282 In a less sensitive gel-based proteomic studies, authors have observed CdrA protein in the matrix [7, 20],
283 but in our data CdrA seems to be under the limit of quantitation. One of the possible explanations is that
284 endogenous proteases had degraded CdrA by the time when we collect biofilm and other mechanisms
285 maintained biofilm structure. In this case found in matrix CdrB might be «archeological» protein. Further
286 dynamic studies in Psl and CdrA presence in the matrix may shed a light on this question.
287 Another matrix unique protein is nucleoside hydrolase (Nuh) – an enzyme that hydrolyzes adenosine
288 and inosine, allowing the cell to grow on these nucleosides as the sole carbon or nitrogen source. Nuh was
289 considered as an intracellular (periplasmic) private good [28]. In our study, we found Nuh in the matrix,
290 but not inside bacterial cells. That means Nuh might be an extracellular public good, at least for some
291 strains like ours. Also, transporters responsible for adenosine transport to periplasmic space in *P. aeruginosa*
292 remain still undiscovered, so if Nuh works outside the cell, the need of transporters is questionable. In an
293 environment with adenosine as the sole carbon source, Nuh mutant has impaired growth [46], but

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294 therapeutic potential of direct or indirect inhibition of Nuh activity remains elusive due to the nutrient-rich
295 nature of infected tissues, and further research is needed.
296 For the rest 8 matrix-unique proteins, there is no clear evidence of their possible role in biofilm.
297 Meanwhile, as matrix is considered a nutrient-rich environment, presence of substrate-binding protein
298 from ABC transporter and its role in nutrient (probably amino acids) acquisition is obvious. Antibody-
299 mediated blocking of some ABC transporters was shown to be effective in vitro and in vivo against *M.*
300 *hominis* and *S. aureus* [47,48]. Monoclonal antibody Aurograb® entered phase III clinical trial as an addition
301 to vancomycin therapy for deep-seated staphylococcal infections (NCT00217841), but the trial was stopped
302 due to lack of reaching the primary endpoint. Anyway, somewhere positive results in targeting eukaryotic
303 ABC transporters for cancer treatment support the idea of a broader evaluation of the similar capability for
304 prokaryotes. Also, transmembrane transport is one of the prevalent bioprocess groups in terms of all found
305 in matrix proteins (n=18) as well as in terms of several overrepresented in matrix protein (n=8).
306 ATP-dependent Zn proteases are common enzymes in the cell envelope of *P. aeruginosa*, they
307 participate in several processes, including metabolism, protein transport and removal of misfolded
308 proteins, and adaptation to environmental conditions [29]. Also, some proteases may act as a virulence
309 factor - Zn2+-dependent protease *Bacillus anthracis* called Lethal Factor is required for infection [49]. The
310 proteolysis bioprocess group was the second represented in the matrix with 8 overrepresented proteins.
311 Proteolysis is a part of the external digestive system and provides peptides and amino acids for bacterial
312 nutrition, so the presence of some proteins involved in amino acids metabolism was expected (this
313 bioprocess group includes 16 found in matrix proteins with 7 overrepresented proteins). Proteolytic activity
314 could be crucial for both bacterial survival and infection process and targeting bacterial proteases could be
315 a perspective way to combat bacterial infections [50]. Moreover, for Zn proteases, host nutritional
316 immunity (including Zn-dependent processes) was effective against infections caused by *P. aeruginosa* [51].
317 Also, deprivation of Zn ions was proposed to combat infections caused by another common pathogen - S.
318 aureus [52].
319 Despite a small number of matrix unique proteins, we found a lot of overrepresented proteins in the
320 matrix. Eight proteins with extremely high log2 fold change (more than 20) were considered as semi-unique
321 for matrix, but we did not find in the literature any role in biofilm lifestyle. So as for unique matrix proteins,
322 there is an unexplored area in biofilm biology.
323 Bioprocesses classification of represented in matrix proteins reveals several groups. Considering log2
324 fold change, we found that groups of oxidation-reduction processes, biosynthetic processes, and nucleoside
325 metabolism had the largest number of highly overrepresented proteins. Obviously, each bioprocess might
326 vary in the number of involved proteins, so we also introduced bioprocess representation as a part of all
327 GO-bioprocess annotated proteins.
328 In the matrix, the most reach group of proteins (128 proteins found in the matrix, 27 overrepresented)
329 belongs to oxidation-reduction processes. Also, a group of proteins involved in cell redox homeostasis was
330 one of the most represented GO-annotated bioprocesses in the matrix (14 proteins found, 8 overrepresented
331 from 26 annotated in GO bioprocess database). Biofilms of *P. aeruginosa* contain molecules involved in
332 virulence and competition with other microorganisms, including redox-active molecules. Self-produced
333 factors involved in the generation of reactive oxygen species might be harmful to the internal bacterial
334 community. So *P. aeruginosa* is balancing to maintain oxidation-reduction processes at the appropriate
335 level, so the balance of oxidation-reduction reactions and redox homeostasis likely play a significant role
336 in the biofilm matrix as an environment with limited diffusion. Several effective therapeutic approaches
337 utilize oxidative stress to combat bacterial biofilms, including photodynamic therapy (PDT) and sanitizers
338 like hydrogen peroxide [53,54]. Also, extracellular electron transfer (EET) exists inside biofilm matrix, but
339 the role of the protein component of EET remains undiscovered [55].
340 The second represented bioprocess included proteins involved in the fatty acid biosynthetic process
341 (4 overrepresented proteins from 12 found in the matrix). Fatty acids are one of the major components of
342 the cell envelope. Also, it is a well-known signal function of cis-2-docenoic acid messenger (DSM) as well
343 as the importance of the fatty acid component of AHL [56]. In a recently published study, Altay et al. made
344 a comprehensive analysis of essential reactions and affected pathways in *B. cenocepacia* (both planktonic
345 and biofilm) using a systems biology approach. From all identified essential reactions, lipid metabolism
346 was responsible for more than half of the single lethal reactions; among this fatty acid biosynthesis was

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347 most frequently found [57]. That data supports the further development of fatty acids metabolism
348 inhibitors as promising therapeutics against bacterial infections, including bacterial biofilms.
349 The most represented bioprocess was nucleoside metabolism – 10 out of 11 GO-annotated proteins
350 were found in the matrix. Biofilms are often enriched with extracellular nucleic acids, which act not only
351 as a structural component or component of EET, but also as a nutrient source [58]. Nucleosides act as
352 substrates and cofactors in many biosynthetic processes, as signal molecules, and are involved in regulating
353 bacterial community inside biofilm [59], so the presence of nucleoside metabolic proteins is required.
354 Therapeutic targeting of proteins involved in these processes is theoretically possible, but at present is not
355 clear. At the same time, nucleoside analogs are common drugs in other nonbacterial diseases, and
356 evaluation of their possible role as antibacterial drugs may open new opportunities [60]. So antimycotic
357 drug 5-fluorocytosine was able to suppress virulence of *P. aeruginosa* in a murine model of lung infection
358 [61].
359 Obviously, sources of extracellular matrix proteins belong to two main categories: (1) active secretion
360 of biomolecules and (2) passive way to increase extracellular content as a consequence of cell leakage or
361 lysis. While some extracellular proteins are «passive» products of bacterial cell lysis, they still might be
362 active outside the cell and provide their function to the bacterial community extracellularly. As protein
363 degradation rates lay in broad ranges, some proteins may present in the matrix for a long time after leakage
364 or secretion from the cell, so their occurrence in the matrix does not (1) match the actual situation inside
365 cells or (if. e. active transcription and translation), or (2) reflect any real extracellular needs for biofilm (i.e.
366 be structurally or/and physiologically involved in extracellular processes). The one limitation of our study
367 is an inability to conclude if the protein is «archeological», bystander, or functionally active in the matrix
368 and how these proteins are distributed in the matrix (are they cell-attached, part of OMVs, or associated
369 with other matrix components). Moreover, in a prism of drug development, some proteins may act as
370 distracting extracellular targets in a way of absorption of active drug and distract from intracellular targets.
371 Also there is a risk of potentiating severe infection in a case of matrix disruption and massive release of
372 bacterial cells. To choose a right strategy for antibiofilm development, there is a need for detailed
373 knowledge about function and dynamic of every single protein. Another important limitation is the fact
374 that biofilm cultured *in vitro* on the plastic surface in bacteriological mediums does not reflect real
375 physiological conditions and extensive study must be done to evaluate the relevance of any *in vitro* results
376 for the understanding of pathogenesis of chronic infections and finding targets for antibiofilm drug
377 development.

378 **4. Materials and Methods**

379 *4.1. Biofilm growth and separation matrix from cells*

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

393 *4.2. Proteomic sample preparation and peptide identification*

394 Proteomic sample preparation and peptide identification were made in Advanced Mass Spectrometry
395 Core Facility (Skolkovo Innovation Center, Moscow, Russia). The protein pellet was subjected to tryptic in-
396 solution digestion. LC-MS/MS was carried out on a Q Exactive HF (Thermo Scientific) with a nanoESI
397 interface in conjunction with an Ultimate 3000 RSLC nano HPLC (Dionex Ultimate 3000). Peptides were
398 loaded onto a trap column and separated on an analytical column (C18) using an H2O/acetonitrile gradient
399 with 0.1% formic acid for 150 min. The Q Exactive HF spectrometer was operated in the data-dependent
400 mode with a nanoESI spray voltage of 1.8 kV, capillary temperature of 210 °C, and S-lens RF value of 55%.

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401 All spectra were acquired in positive mode with full scan MS spectra scanning from m/z 310–1500 in the
402 FT mode at 120,000 resolution. A lock mass of 445.120025 was used. The top 25 most intense precursors
403 were subjected to rapid collision induced dissociation (rCID). Dynamic exclusion with of 70 seconds was
404 applied for repeated precursors.

405 *4.3. Data analysis*

406 To identify and quantify tryptic peptides and the proteins from which the peptides are derived,
407 spectra from the MS/MS experiments were analyzed by GUI FragPipe v. 17.1
408 (https://github.com/Nesvilab/FragPipe). Peptide identification was performed by MSFragger search engine
409 [62,63] using protein sequence database extracted from NCBI (*Pseudomonas aeruginosa* strain
410 GIMC5015:PAKB6 chromosome, complete genome NZ_CP034429) with decoys and contaminants.
411 Oxidation of methionine and acetylation of protein N-termini were set as variable modifications,
412 carbamidomethylation of cysteine was set as a fixed modification. The maximum allowed variable
413 modifications per peptide was set to 3, mass tolerance was set as 20 ppm for precursor and 0.02 Da for
414 fragment ions. Philosopher kit tools [64,65] were used to estimate identification FDR. The PSMs were
415 filtered at 1% PSM and 1% protein identification FDR. Quantification by label-free protein quantitation
416 method and MBR was performed with IonQuant [66]. Obtained quantified data (intensities) were
417 processed for differential expression analyses with limma package R [26], with followed visualization
418 result by EnhancedVolcano package R ("EnhancedVolcano: Publication-ready volcano plots with
419 enhanced coloring and labeling." https://github.com/kevinblighe/EnhancedVolcano).
420 Bioprocess classification and functional annotation were made with the Pseudomonas Genome
421 Database
422 (https://pseudomonas.com/primarySequenceFeature/list?strain_ids=10430&term=Pseudomonas+aerugino
423 sa+GIMC5015%3APAKB6&c1=name&v1=&e1=1&assembly=complete) [67].
424 For correlation analysis, ANOVA comparison and graphs, we used GraphPad Prism 9 desktop
425 software (version 9.2.0). For the graphical abstract and figures arrangement we used bio-render online
426 software (Biorender.com).

427 *4.4. Coculture bacterial cells with eukaryotic cell line A549*

428 Biofilm-conditioned medium, bacterial cells and dissolved matrix were prepared as described in
429 section 4.1. Conditioned medium and matrix were filtered through a 0.22 mkm syringe filter. Bacterial cells
430 pellet was resuspended in DMEM medium (Gibsco), and tenfold serial dilutions was plated on LB agar to
431 count bacterial number. Samples were stored on ice and used within several hours after preparation.
432 Adenocarcinomic human alveolar basal epithelial A549 cell line was maintained in DMEM medium
433 (Gibsco) supplemented with 10% fetal bovine serum (Gibsco). A549 cells were seeded in 96-well tissue
434 culture plate at $2*10^4$ cells per well and cultured to reach 85 % confluence. Then 10% of medium volume in
435 well were replaced with matrix, biofilm conditioned medium and bacterial cells suspension in desired
436 combinations. For the control well we used 1.8 M NaCl diluted as in matrix preparation and DMEM
437 medium as for bacterial dilution. Plates were placed in atmosphere of 5% CO2, 37 ^{o}C for 18 h. After
438 incubation liquid was removed from the plate, wells were washed with 0.9% NaCl, fixed with ice cold
439 methanol and stained with crystal violet (BD) for 30 minutes. After washing with tap water plates
440 proceeded to light microscopy. For quantification crystal violet dye was extracted with 30% acetic acid and
441 OD495nm was measured on plate spectrophotometer (ThermoScientific).

442 5. Conclusions

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

449 **Supplementary Materials:** The following supporting information can be downloaded:

450 Table S1: All identified proteins.

451 Table S2: Differentially represented proteins.

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452 Supplementary Figure S1: Number of recovered CFU after matrix separation

453 Supplementary Figure S2: Biofilms of *P. aeruginosa* KB6 stained with crystal violet

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

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466

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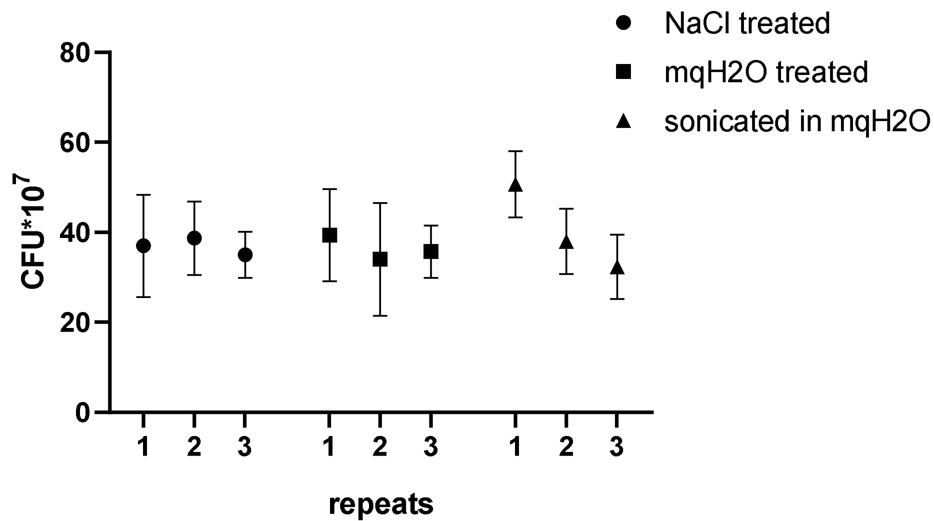

Figure S1. Number of recovered CFU after matrix separation. Biofilms in 96-well plate were separated with NaCl, intensive resuspension in mqH2O or sonication in US bath for 5 min. Graphs represent three replicated wells. Bacteria from each well were seeded in triplicates. Number of recovered CFU was calculated from number of CFU in last dilution with more than 20 CFU per plate.

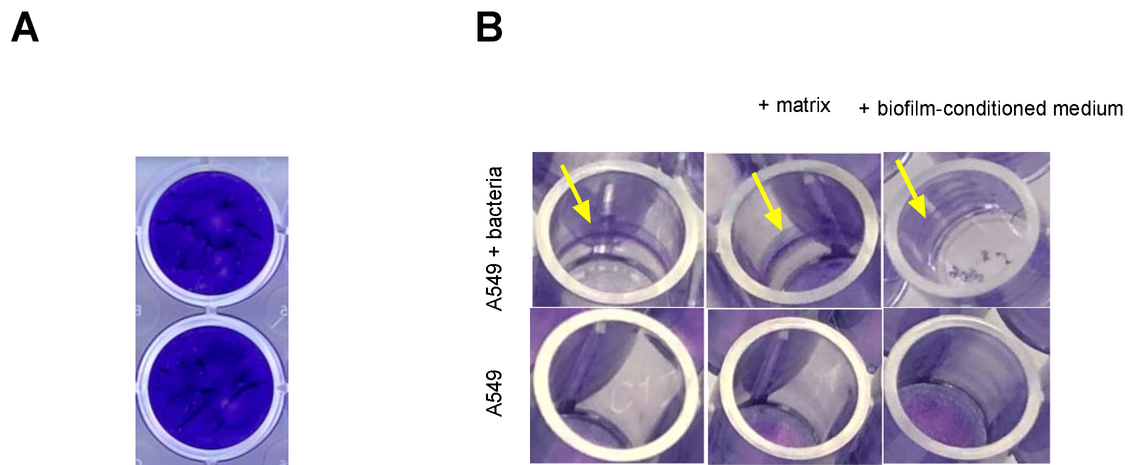

Figure S2. Biofilms of *P. aeruginosa* KB6 stained with crystal violet. A – KB6 demonstrates strong biofilm-forming phenotype. Crystal violet (CV) staining of biofilm formed in LB medium, 37 °C, 18 h. **B** – bacterial cells isolated from biofilm form biofilm in coculture model with A549 eukaryotic cells, MOI=3, 5% CO2, 37 °C, 18 h. Photographs demonstrate formation of biofilm at air-liquid interface (also biofilms are present at the bottom of wells in close proximity and in direct contact with A549 cells), so biofilm biomass has a significant impact in quantification of CV staining in coculture experiments.