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Exogenous Protein as an Environmental Stimuli of Biofilm Formation in Select Bacterial Strains

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ABSTRACT

A screening of environmental conditions that would elicit robust biofilm in a collection of *Serratia marcescens* isolated from soil revealed that exogenous milk protein increased biofilm productivity up to ten-fold. A select screening of fish pathogens, freshwater and human isolates identified several other species that responded similarly to exogenous protein. The optimal protein concentration was species specific; *S. marcescens* at 5% milk protein, *Aeromonas* sp. at 2-3%, *Flavobacterium columnare* at 1% and *Pseudomonas aeruginosa* at 0.1-0.4%. Media supplemented with milk protein also increased the cell counts in biofilm as well as the protein incorporated into the biofilm matrix. These data suggest that relatively high concentrations of exogenous protein may serve as an environmental trigger for biofilm formation, particularly for pathogenic bacteria exposed to relatively high concentrations of protein in bodily fluids and mucosal surfaces.

INTRODUCTION

Since the concept of biofilm was first viewed through the eyes of molecular microbiology three decades ago, our appreciation of its importance in ecology has grown exponentially. We now recognize biofilm as an alternative life strategy for many, if not all species of microbes across three domains. The importance of biofilm in industry(1), public health (2), medicine(3-5) and the environment(6, 7) have been well documented, leading us to frequently include the analysis of a bacterial strain's ability to form biofilm as part of its species' description.

Growth of a microbe in a biofilm removes it from a pelagic lifestyle that is characterized by mass action events (or close to it). As a pelagic entity, change in location and concomitant change

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38 in access to nutrients could happen quickly. Once attached to a surface and embedded in a
39 macromolecular matrix, a very different lifestyle ensues – one in which the tempo and mode of
40 life is slowed, the ambient conditions change more gradually (in general) and cell physiology is
41 altered (8).

42 Biofilm, as part of a life history, is reversible (3, 9, 10). A pelagic microbial species can attach
43 to a substratum, multiply and then return to the surrounding solution as the biofilm structure
44 becomes more susceptible to turbulence or the bacterium senses suboptimal conditions for sessile
45 life. Environmental signals play an essential role in informing the microbe so that optimal survival
46 strategies are selected for, including attachment and release. Consistent with the substantial
47 phylogenetic and physiological diversity of the microbial world, biofilm, as an eco/evo strategy,
48 has been employed in many different ecosystems in response to a broad range of environmental
49 conditions. Of interest to us are specific environmental signals that elicit behavioral changes
50 leading to the formation of biofilm. There are logical candidates for signals including carbon and
51 energy sources, essential micronutrients and even inhibitors. For example, in *Janthinobacterium,*
52 both violacein and biofilm production were stimulated by glycerol and inhibited by glucose (11).
53 The presence of calcium ion has also been found to influence biofilm productivity in *Pseudomonas*
54 (12), *Pseudoalteromonas* (13), *Xyella* (14) and *Citrobacter* (15). In addition, $CaCl_2$, $MgCl_2$, $CuSO_4$,
55 sucrose and sodium dodecyl sulfate produced greater biofilm in *Yersinia pestis* (16). In some
56 circumstances it is difficult to distinguish between a primary trigger and a secondary adjuvant of
57 biofilm formation.

58 We report herein on two converging lines of investigation in our laboratory, the identification
59 of environmental signals for biofilm formation in a collection of *Serratia* isolated from soil, and
60 in several fish bacterial pathogens. Our observations with *Serratia* clearly indicated that exogenous
61 skim milk protein at relatively high concentrations was sufficient to stimulate biofilm formation
62 ten-fold. We extended this observation to *Flavobacterium columnare*, *Aeromonas salmonicida*,
63 two fish pathogens, as well as a collection of freshwater isolates. We also observed that exogenous
64 protein had little or no effect on some isolates from mammalian hosts, but stimulated others. These
65 data indicate that exogenous protein promote biofilm production in select strains of bacteria.

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69

70 **MATERIAL AND METHODS**

71 **Strains and cultivation.** *Pseudomonas aeruginosa* PA01 was a gift of Dr. M. Bagdasarian
72 (MSU). *Hydrogenophaga F14*, *Brevundimonas F16*, *Acidovorax* F19 and *Pseudomonas* strain
73 C22 were isolated from lake sturgeon eggs (17) and *Serratia marcescens* strains RL-1-RL16 were
74 selected on Pseudomonas Isolation Agar (Difco) from soil under an arborvitae on the Michigan
75 State University campus in East Lansing, MI (GenBank Accession MF581042-MF581057). The
76 strains described in Figures 2 & 3 were isolated from the Red Cedar River on the Michigan State
77 University (MSU) campus by selection on Pseudomonas Isolation agar (GenBank Accession
78 MG386765-MG386811). *Flavobacterium columnare* 090702-1 and *Aeromonas* strain 060628-1
79 were provided by Dr. Thomas Loch at MSU and were from fish necropsies. *Aeromonas* strain SM,
80 unpigmented *Serratia* and the low biofilm forming *Escherichia coli* were isolated from human
81 feces (18) and generously provided by Dr. Shannon Manning at MSU, as was the high biofilm
82 forming bovine *E. coli* isolate. Isolates were stored at -80°C and resuscitated on Trypticase Soy
83 Agar (Difco) or R2A (Difco). Broth cultures when needed were grown on either TSB or R2B
84 (Difco R2A recipe without agar). Media supplemented with milk protein (Hardy Diagnostics) was
85 made by first sterilizing 2x media stocks (TSB or R2B) and 2x milk protein in separate bottles and
86 then mixing the two shortly after removing the liquids from the autoclave.

87 **Phylogenetic analysis.** The rRNA of *S. marcescens* strains (RL-1-RL-16) specific to this study
88 were sequenced at the MSU genomics facility using the 27F 16S rRNA primer (Sanger chemistry).
89 The *Serratia* sequences (RL-1 through RL-16) were initially screened with the Ribosomal
90 Database Project *Classifier* and *Sequence Match* (19). Phylogenetically related Proteobacteria
91 sequences were downloaded from the Ribosomal Database project and analyzed along with the
92 *Serratia* sequences (RL-1 – RL-16) in SeaView V4 (20) using BioNJ with HKY distance
93 correction and Maximum Likelihood. The maximum likelihood tree and results from *Classifier*
94 and *Sequence Match* are presented in the supplementary materials. All strains isolated from the
95 Red Cedar River were identified by 16S rRNA sequencing using the 27F primer and identified
96 using *Sequence Match* and *Classifier* in the Ribosomal Database Project (19).

97 **Standard Biofilm Assay.** The protocol for biofilm measurement as described by Merritt et al.
98 (21) was used with the following modifications. We used either 96 well or 24 well microtiter plates
99 (Corning Costar) depending on the specific experimental requirements. The 24 well plates were
100 used in experiments when growth, cell and protein concentrations of biofilms were to be

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101 determined. Overnight cultures of strains in either Typticase Soy Broth (Difco) or R2B (3-5 mls)
102 were grown at 25°C in a rotating rack (Cole-Parmer). Sterile broth (75-100µl) was added to all
103 wells and then 50-75µl of broth culture was inoculated into the wells. In all experiments, the
104 amount of culture and broth totaled 150 µl for 96 well plates. When using 24 well plates for cell
105 growth measurements, 750µl of sterile broth and 50 µl of overnight culture were added to all wells,
106 excluding the uninoculated controls. When using 24 well plates for cell and protein concentrations,
107 700 µl of sterile broth and 100 µl of overnight broth culture were added to all wells, excluding the
108 uninoculated controls. After inoculation, plates were sealed with sterile foil (VWR) and incubated
109 at 25°C on an orbital shaker (100 rpm) for 24 or 48 hours depending on the experiment. After
110 incubation, the seal was removed and when processed for biofilm determination, the plates were
111 washed gently (x3) in reverse osmosis (RO) water as described by Merritt et al. (21), stained with
112 150 µl (800 µl for 24 well plates) of 0.5% filtered (0.2 µ filter) crystal violet for 15 minutes, washed
113 x3 in RO water, blotted and allowed to dry overnight in the dark. The following day 150 µl of 30%
114 acetic acid was added to each well (800 µl for 24 well plates) and the plate was incubated for 25
115 minutes at 25°C shaking at 100 rpm. Absorbance at 595 nm was measured in a Biotek EPOCH
116 plate reader with 2 measurements for each well. Each sample had at least four replicates within the
117 plate and each media formulation had at least four uninoculated wells that served as negative
118 controls. The average absorbance of uninoculated wells was subtracted from sample biofilm wells.
119 **Measuring cell growth in milk protein supplemented media.** To test for the effects of milk
120 protein on growth of *P. aeruginosa, S. marcescens, Aeromonas sp.* and *F. columnare*, we measured
121 growth in R2B supplemented with 0.1%, 0.2% and 0.4% milk protein in 24 well microtiter plates.
122 We intentionally selected low concentrations at which milk protein is completely soluble. At
123 higher concentrations (>1%) milk protein solutions are colloidal, making it difficult to measure
124 optical density. Growths were performed in 24 well microtiter plates with 4x replication and
125 shaking at 100 rpm on an orbital shaker at 25°C. Optical density measurements were made at 0,
126 110 min, 210 min, 300 min 390 min 450 min and 24 hours on a Biotek EPOCH plate reader at
127 600nm. At 24 hours the wells were tested for biofilm formation as described above. Uninoculated
128 controls were subtracted from growths at each time point and in biofilm quantitation. Uninoculated
129 controls for each protein concentration were also replicated x4.
130 **Determining Viable Cells Within Biofilm.** To determine the viable cell count within biofilm
131 formed in supplemented and unsupplemented media, cultures of *P. aeruginosa* PA01, *Aeromonas*

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132 060628-1 and *S. marcescens* RL-5 were established in 24 well plates by inoculating 700 µl of R2B
133 ± milk protein with 100 µl of overnight culture. Control wells contained 800 µl of uninoculated
134 media. Plates were sealed with sterile foil and incubated for 24 hours shaking at 100 rpm and 25 °C.
135 These experiments were set up in duplicate so that biofilm determination with crystal violet
136 (Sigma) and viable cell counts could be performed in parallel. Each plate contained four replicates
137 of each strain and media combination. After 48 hours of growth, one plate was stained with crystal
138 violet as described above for quantitation of biofilm and the duplicate plate was used to determine
139 the viable cells count within biofilms, as follows. The plate was gently rinsed three times in sterile
140 water and the washed biofilm was scrapped off using 600 µl of sterile water and a sterile applicator.
141 The cell slurry was transferred to 1.5 ml eppendorf tubes, vortexed to break up cell aggregates and
142 10-fold serially diluted for plating onto R2A. Plating was in triplicate and CFUs are reported as
143 the total CFUs per well.

144 **Determination of protein content in biofilm.** To determine the protein concentration of
145 biofilms, 24 well plates were used as described. In this experiment we tested *P. aeruginosa*,
146 *Aeromonas* 060628-1 and *F. columnare*. Biofilm of *P. aeruginosa* and *Aeromonas* were prepared
147 in R2B and R2B-5%MP while *F. columnare* was tested in R2B-1%MP. Duplicate plates were
148 inoculated so that both protein concentration and crystal violet staining could be tested in parallel.
149 In each plate, all unique media conditions were replicated four times. After 24 hours of incubation
150 at 25°C and shaking at 100 rpm, one plate was stained with crystal violet (as described above) and
151 the duplicate plate was used to determine protein content within biofilm as follows. After 24 hours
152 of incubation the media was removed and the wells were washed twice by adding 800 µl of sterile
153 water and shaking at 100 rpm for 2 minutes. The final wash was removed and 200 µl of sterile
154 water was added to each well. The biofilm was removed by manually scrapping with a sterile glass
155 rod and then transferred to a 1.5 ml Eppendorf tube. The solution was vortexed and centrifuged at
156 4°C and12,000 rpm for 20 minutes in a microfuge to remove the cells. The supernatant was
157 transferred to a new tube and 400 µl of 100% Ethanol was added to precipitate protein. After
158 overnight storage at -20°C, the tubes were centrifuged at 4°C and 12,000 rpm for 20 minutes to
159 pellet all protein. The supernatant was decanted and the pellets were air dried for 15 minutes and
160 then resuspended in 150 µl of 1x PBS. To determine the protein concentration in these samples
161 the Coomassie protein assay (Thermo-Scientific) was employed, using the vendors recommended
162 protocol. Briefly, 150 µl of Coomassie reagent plus 150 µl of sample was added to a microtiter

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163 plate well, mixed and incubated for 10 minutes in the dark. The plate was read at 595 nm using a
164 Biotek EPOCH plate reader. The standard curve was as recommended by the vendor.
165 **Confocal microscopy.** A two-well chamber (Lab-TekII, Nalge Nunc International,USA) was
166 inoculated with 700 µL of media and 100 µl of *P. aeruginosa* or *F. columnare*. 5% Skim Milk
167 Protein at 1/2x Tryptic Soy Broth (Becton, Dickinson and company, France) medium and 100 µL
168 of *F. columnare* overnight broth. The chamber was then wrapped in Parafilm (Bemis, USA) to
169 seal it. Next, the sample was incubated for 72 hours at 100 RPM. At 48-hour incubation the media
170 was gently removed and new 700 µL of fresh medium was added into the wells. At 72 hours, the
171 medium was removed and discarded, and the chamber was gently washed 3 times with 1 mL of
172 sterile water. 1 mL of fluorescent solution containing 0.5mL of 20x Nano Orange dye (Molecular
173 Probes Protein Quantitation Kit N10271) and 0.5mL of FM4-64 dye (Molecular Probes FM4-64)
174 was added into one well and incubated in the dark for five minutes. The well was washed with 1
175 mL of sterile water two times. The sample was kept hydrate during microscopy. A confocal
176 microscope (Olympus FluoView FV1000) was used for imaging at 20x and 90x.
177
178 **RESULTS**
179 During the screening of a variety of environmental conditions designed to stimulate the
180 formation of biofilm by soil isolates of *Serratia*, we detected a significant increase in biofilm when
181 our standard growth media, R2B, was supplemented with milk protein (MP). Using standard
182 media-grade skim milk protein (Hardy Diagnostics) at 5%, biofilm formation of 16 independently
183 isolated soil *Serratia* strains increased significantly. In **Figure 1** we show the response of five
184 *Serratia* strains to media supplemented with different concentrations of milk protein (0.05, 0.5,
185 2.5 and 5.0%). All five isolates responded to 5% MP supplementation with a ten-fold increase in
186 biofilm formation. All remaining isolates responded with similar increases (data not shown). In
187 our biofilm assays we routinely use *Pseudomonas aeruginosa* PAO1 as a positive control. Under
188 all conditions we have tested, PA01 produced a robust biofilm when grown on R2B or TSB but
189 had little response to the presence of exogenous protein when at 0.5% or greater and, in many of
190 our assays, high concentrations of protein in the media slightly inhibited biofilm formation by *P.*
191 *aeruginosa*.
192 To extend these observations to other species we tested the ability of 5% MP to stimulate
193 biofilm production in 200 freshwater bacterial isolates. In **Figure 2** we report on 48 randomly
194 picked isolates that were selectively isolated on *Pseudomonas* isolation agar and were therefore

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195 resistant to irgasan, a broad spectrum antimicrobial that targets fatty acid synthesis in bacteria.
196 Among these isolates, 14 showed substantial increase in biofilm production in milk protein
197 supplemented media at least 2-fold greater than the unsupplemented control (eg. one *Yersinia*,
198 *Shewanella* and *Rahnella*). Of 13 *Pseudomonas* isolates, only two showed more than a 2-fold
199 increase in biofilm with protein-supplemented media. Of the eight genera in this test, *Aeromonas*
200 consistently showed a robust response to exogenous protein in the media. Six of the twenty
201 *Aeromonas* isolates had 10-fold increases in biofilm formation and 8/20 had at least a 2-fold
202 increase.

203 We have tested several hundred freshwater isolates in this manner and when assaying that
204 many strains, we routinely make a single-pass evaluation with the crystal violet assay, accounting
205 for the lack of error bars in **Figure 2**. To statistically confirm our results, we examined six isolates
206 from this freshwater collection in greater detail, three *Aeromonas*, two *Rahnella* and one
207 *Pseudomonas* at four different concentrations of milk protein in TSB. These data, presented in
208 **Figure 3**, show that the *Aeromonas* isolates responded to MP concentrations of 1-5% while a
209 freshwater *Pseudomonas* isolate revealed little response until 5% and *Rahnella* was unresponsive.

210 To extend this analysis to isolates associated with eukaryotic hosts, we also investigated
211 the relationship between biofilm formation and exogenous protein in five isolates from fish, three
212 from humans and an *E. coli* strain from bovine. These data are presented in **Figure 4**. Among the
213 isolates from fish, *F. columnare, Hydrogenophaga, Brevundimonas* responded strongly and
214 positively to exogenous protein by producing abundant biofilm, but at different optimal protein
215 concentrations (**Fig 4. Panel A**). *F. columnare* and *Hydrogenophaga* had greatest biofilm
216 productivity at 1% while *Brevundimonas* was more productive at 5%. *Pseudomonas* C22 does not
217 form abundant biofilm in unsupplemented media and productivity increased only modestly at 1%
218 and 5% MP. *Acidovorax* was unresponsive to exogenous protein. **Panel B** reports on the human
219 and bovine isolates. *Aeromonas* sp, an unpigmented *Serratia* and a low biofilm forming *E. coli*
220 were isolated from human feces and had varied response to exogenous protein. Both *Aeromonas*
221 and the *Serratia* isolates responded with greater biofilm productivity at 1% and 5% but *E. coli* was
222 unresponsive. Included in this experiment was one of our soil *Serratia* isolates (RL-4) for
223 comparison. Interestingly, biofilm formation by the *E. coli* isolate from bovine, identified as a high
224 biofilm forming strain, was inhibited in media supplemented with 1% and 5% MP. Note that this
225 experiment was conducted in LB broth without salt to mimic the conditions used in the initial

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226 characterizations of the *E. coli* strains. Both *P. aeruginosa* PA01 and our soil *Serratia* RL-4 had
227 biofilm profiles in supplemented and unsupplemented TSB similar to what we have seen in R2B.
228 As can be seen from these biofilm assays, in some cases the amount of crystal violet
229 staining material was quite large. In many of the *Aeromonas* strains tested an opaque disk formed
230 at the bottom of the wells, particularly if the incubation period was extended to 48 hours and the
231 96 well format was used. An obvious concern was the possibility that crystal violet was staining
232 protein and biofilm matrix atypically and providing a false positive for biofilm formation. To test
233 for this, we ran several analyses in 24-well microtiter plates that prevented the formation of any
234 opaque disk by virtue of the large well diameter. In these experiments, we measured biofilm
235 formation using crystal violet and performed viable plate counts on biofilm from replica plates.
236 These data are presented in **Figure 5** and show that, as observed above, both *Aeromonas* and
237 *Serratia* responded strongly to exogenous protein, producing at least a 10-fold increase in crystal
238 violet signal while *Pseudomonas* had little response. The cell viability from a replica plate revealed
239 a 1.2-3 order of magnitude increase for *Serratia* and *Aeromonas* when grown with 5% exogenous
240 protein, while *P. aeruginosa* had a robust viable count in the absence of protein and only a modest
241 increase with protein when compared with *Aeromonas*.
242 An obvious question regarding the effect of exogenous protein on the formation of biofilm
243 is whether the biofilm becomes enriched in protein. To test for this, we established biofilm in 24
244 well plates (4 replicates of each strain on a plate) and replicated the whole plate so that both biofilm
245 formation and the amount of protein within the biofilm matrix could be measured. Because of our
246 interest in fish pathogens we tested *Aeromonas* and *F. columnare* with *P. aeruginosa* as our
247 positive control. The results are presented in **Figure 6**. As shown previously, both *Aeromonas* and
248 *F. columnare* responded strongly to exogenous protein by producing more biofilm while *P.*
249 *aeruginosa* PA01 was unresponsive. In this test, we used the optimal protein concentrations of 5%
250 for *Aeromonas* (and *Pseudomonas*) and 1% for *F. columnare*. The biofilm from the replica plate
251 was washed and manually scrapped from the wells and the protein concentration was determined
252 using the Bradford assay, after removing the cells by centrifugation. The amount of protein
253 detected in the biofilm for *P. aeruginosa* was 8.2 and 5.4 µg/ml for growth without and with
254 protein, respectively. For *Aeromonas*, the increase in biofilm in response to exogenous protein was
255 accompanied by an increase in matrix protein concentration from 0.55 to 47.3 µg/ml. For *F.*
256 *columnare*, the 20-fold increase in biofilm was accompanied by a nearly 20-fold increase in matrix

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257 protein (0.18 > 3.11 μg/ml). The optical densities of the cultures are revealing as well. As expected,
258 the initial OD of cultures in unsupplemented media was relatively low, representing a 1:8 dilution
259 from overnight cultures, but clear evidence of growth was detected after 24-hour incubation. The
260 initial OD of the protein-supplemented wells was dominated by the opacity contributed by the milk
261 protein, ~1.7 for a 5% solution and ~0.5 for a 1% solution. After incubation for 24 hours the OD
262 of the *P. aeruginosa* wells dropped to 0.45, suggesting the presence of protease activity.
263 Presumptive protease activity was also detected in the *F. columnare* wells, evidenced by a drop in
264 OD from 0.5 to 0.16. Interestingly the wells containing *Aeromonas* showed no reduction in OD.
265 To determine the effect of exogenous protein on cell growth we incubated *P. aeruginosa, S.*
266 *marcescens, Aeromonas* strain 060628-1 and *F. columnare* strain 090702-1 at three concentrations
267 (0.1%, 0.2% and 0.4%) of milk protein in R2B and monitored growth by optical density at 600nm.
268 Low concentrations were selected to avoid colloidal solution conditions present at higher
269 concentrations. *P. aeruginosa* grew well under these experimental conditions but optical density
270 was diminished in a concentration dependent manner when the media was supplemented with
271 protein (**Panel A, Fig. 7**). In contrast, *S. marcescens* (**Panel B, Fig 7**) grew robustly, regardless of
272 the exogenous protein through 450 min. Statistical differences were detected only after 24 hours
273 of growth when exogenous protein appeared to modestly boost growth. *Aeromonas* grew slowly
274 (**Panel C, Fig 7)** through 450 minutes with no appreciable difference with protein addition. The
275 greatest growth was between 450 min and 24 hours. At 24 hours growth was inhibited at 0.4%
276 exogenous protein. *F. columnare* grew well in the absence of exogenous protein and poorly, if at
277 all, in its presence (**Panel D, Fig 7**). The tendency of this strain to form aggregates in solution
278 accounted for the substantial inter-replicate variability. After 24 hours the plates were processed
279 for biofilm formation (**Figure 8**). *P. aeruginosa* PA01, as mentioned above, is a robust biofilm
280 forming strain. Under conditions of growth in this experiment, enhanced biofilm productivity was
281 detected at all concentrations of exogenous protein. While *S. marcescens* grew vigorously, biofilm
282 productivity was quite low at the tested protein concentrations. *Aeromonas* also lacked biofilm
283 productivity at the lower concentrations of protein but did increase substantially at 0.4% milk
284 protein, in spite of the apparent growth inhibition at this concentration. Biofilm production by *F.*
285 *columnare* increased in a concentration dependent manner when the media was supplemented with
286 protein. This robust biofilm production was in contrast to pelagic growth which appeared inhibited
287 by exogenous protein.

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288 In addition to measuring the protein content with a standard Bradford assay we used nano-
289 orange to visualize the biofilm–associated protein. Using the standard microtiter plate protocol,
290 we established biofilm on sterile coverslips with and without exogenous milk protein (1%) using
291 *P. aeruginosa* and *F. columnare* as the test strains. After growth, the biofilm was washed with
292 sterile water (x3) and stained with Nano-orange and FM4-64 using the vendors protocol. The
293 biofilm was viewed on an Olympus FluoView FV1000 Confocal Microscope at 20X and 90X
294 magnification (**Figure 9**). Numerous *P. aeruginosa* cells were detected at 20X magnification but
295 there was little evidence of a robust contiguous biofilm. Intensely orange spots could be detected
296 suggesting concentrations of protein spotted the surface. At 90X magnification well isolated cells
297 were seen with little evidence of a protein matrix. In contrast, the biofilm formed by *F. columnare*
298 showed a thick branched proteinaceous complex at 20X magnification. Cells were clearly outlined
299 with the lipophilic FM4-64 stain at 90X magnification and showed morphological variation as
300 describe previously (22). In addition, irregularly shaped orange forms as well as cells decorated
301 with Nano-orange were detected.
302
303 **DISCUSSION**
304 These investigations began with repeated unsuccessful attempts to form a robust biofilm of *S.*
305 *marcescens* isolated from soil. Different temperatures, carbon sources, nutrient availability,
306 osmolarity and substrata were tested without effect on biofilm formation. However, one
307 environment in which *S. marcescens* can colonize is the human respiratory system and this
308 provided clues to a possible environmental signal initiating biofilm formation in *Serratia*. Alveolar
309 fluid from human lungs is generally at 5-13% protein (23). This environmental feature of the lung
310 led us to test biofilm formation at several concentrations of protein and identify robust biofilm of
311 *S. marcescens* at 5% milk protein. Moreover, the increase in biofilm productivity was frequently
312 an order of magnitude or greater above that observed in unsupplemented media. Our positive
313 control strain, *P. aeruginosa* PA01, appeared unresponsive to high concentrations of protein in the
314 medium.
315 These observations were extended to 48 freshwater isolates, four strains from sturgeon eggs
316 (17), two known fish pathogens, three strains isolated from human gut and one from bovine and
317 the results showed biofilm production that was dependent on two variables, species and protein
318 concentration (a total of 74 strains including the *Serratia* isolates). Based on these data *Serratia*
319 isolates from both soil and human gut were highly responsive to 5% exogenous protein, producing

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320 5 to 10 times the amount of biofilm that they made in unsupplemented media. In all cases tested,
321 *Serratia* required concentrations around 5% and failed to respond to lower concentrations (0.1%,
322 0.2%, 0.4%, 0.5%, 1% & 2%). *Aeromonas* strains were also sensitive to exogenous protein in the
323 same manner, increasing biofilm production, although lower concentrations of protein (0.4% - 2%)
324 would suffice for some strains. The one strain of *F. columnare* reported on herein was particularly
325 responsive to exogenous protein with an optimum at 1% protein and evidence of increased biofilm
326 productivity at as low as 0.1% protein. Additional studies within the *Flavobacterium* and
327 *Chryseobacterium* lineages indicated that all isolates of *F. columnare* tested thus far are responsive
328 to 1% milk protein (Loch & Marsh, unpublished). Biofilm production by *P. aeruginosa* PA01 was
329 unresponsive to high concentrations of protein (1%-5%) and showed growth inhibition but
330 enhanced biofilm production at low concentrations (0.1%, 0.2% & 0.4%). Those strains that
331 appeared unresponsive at high concentrations included freshwater isolates *Kluyvera*, *Erwinia*,
332 nearly all *Pseudomonas* (11 of 12), all *Rahnella aquatilis* isolates, 3 of 4 *Yersinia* isolates and
333 human and bovine *E. coli* isolates.
334 **Protein as a surface conditioning agent.** A number of investigators have reported that soluble
335 protein can serve as a "conditioner" to surfaces that enhance or inhibit the development of biofilm.
336 Frequently serum is used as a "natural" protein-containing solution to condition surfaces (total
337 protein in serum is typically 60-80g/L). For example, Patel et al. (24) showed that initial binding
338 of *S. epidermidis* cells to hydrophobic polyurethanes was suppressed by serum at 2 hours but
339 enhanced when incubated for 24 hrs. The opposite trend was observed for hydrophilic surfaces
340 where serum inhibited biofilm formation. Similarly, Frade et al. (25) found that serum enhanced
341 biofilm productivity of *Candida albicans* on metallic and non-metallic surfaces. Finally, using
342 methodologies most similar to our approach, Kipanga et al. (26) demonstrated that polystyrene
343 microtiter plates (Costar) conditioned with foetal calf serum showed reduced biofilm formation by
344 *C. albicans*. These assays are in general difficult to compare given the diversity of surfaces, strains
345 and complexity of serum. The Patel et al. work used human serum diluted to 20% as the incubation
346 medium whereas Frade et al. and Kipanga et al. used undiluted foetal calf serum only to condition
347 surfaces, but not as the media of incubation. In contrast our experiments used microbiological
348 media grade skim milk protein, autoclaved separately from other media components to eliminate
349 any temperature induced media-protein interactions. Our fully constructed media containing
350 protein up to 5% was used as the incubation media in which biofilm was formed. The observations

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351 that different phylogenetic taxa have different optimal protein concentrations for growth, biofilm
352 formation and protein assembled into the biofilm matrix suggest that caution must be used in
353 drawing generalizations regarding the influence of serum (or alveolar fluid) on biofilm formation
354 by any single isolate. The various effective ranges of biofilm enhancement exhibited by different
355 strains in our study suggests that conditioning of the surface was not a relevant factor (our
356 concentrations were beyond saturation levels for polystyrene) but that species dependent
357 sensitivity to protein in the media was driving enhanced biofilm production at various protein
358 concentrations. Direct tests of milk protein as a surface conditioning agent for *Serratia* were
359 negative (data not shown).

360 **Exogenous protein – a trigger or adjuvant to biofilm formation?** As mentioned above, we
361 were particularly interested in identifying environmental triggers of biofilm formation. While our
362 results with exogenous protein are provocative in this regard, we cannot identify milk protein
363 supplement as a trigger as opposed to an adjuvant in biofilm formation. The experiments described
364 in Figures 5 & 6 clearly indicate that the addition of exogenous protein increased cell concentration
365 within the biofilm matrix (and biofilm biomass as measured with crystal violet) as well as the
366 concentration of matrix protein in *A. salmonicida* and *F. columnare*. *A. salmonicida* was
367 particularly efficient at incorporating protein into the matrix, increasing 80-fold over controls
368 lacking milk protein. Interestingly, the primary strains of this study, *P. aeruginosa*, *A. salmonicida*,
369 *F. columnare* and *S. marcescens*, produce extracellular proteases when grown on R2A or TSA
370 plates with 5% milk protein (data not shown). Other isolates of these strains have a well-
371 documented history of producing extracellular proteases (27-33). Consistent with this was our
372 observations in Fig. 6 that when cultivated in microtiter plates for biofilm production, both *P.*
373 *aeruginosa* and *F. columnare* reduced the opacity of exogenous protein in the media, indicating
374 that extracellular proteases were actively degrading milk protein under the conditions of our
375 biofilm test. However, *A. salmonicida* showed no such activity in broth but did add an abundance
376 of protein to the biofilm matrix, suggesting that exogenous protein was at least a biofilm adjuvant
377 for *A. salmonicida*. Concluding that exogenous protein is the environmental trigger for *F.*
378 *columnare* biofilm formation is consistent with the complete absence of detectable pelagic growth
379 in broth supplemented with the milk protein but with concurrent construction of abundant biofilm
380 and incorporation of substantial protein into the matrix. Nonetheless, we do not have direct
381 evidence that exogenous protein is an environmental trigger. Finally, we note that skim milk

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382 protein is a common microbiological media additive that is not well defined because of proprietary
383 information claims. The protein concentration range that we employed is not attainable with pure
384 casein.

385 **Protein, Proteases and Virulence**. Some proteases are identified as virulence factors in
386 pathogens including *Serratia* (29) and *Pseudomonas aeruginosa* (30, 33). The simplistic view of
387 these extracellular proteases is that they are foraging for nutrients and clear habitats to occupy as
388 well as impeding host immune responses that are protein based. While extracellular proteases have
389 been linked to biofilm formation in *Enterococcus* (34-36), from our observations it is unclear if
390 extracellular proteases influence the formation of biofilm in *P. aeruginosa, F. columnare*, *S.*
391 *marcescens* and *A. salmonicida,* under our experimental conditions. With a simple plate assay, we
392 can detect extracellular proteases in these strains but the response to exogenous protein in the
393 production of biofilm is strain specific and *Aeromonas* does not appear to degrade MP in broth
394 when testing for biofilm. Whether or not the proteases generate small peptides that are triggers or
395 adjuvants of biofilm production remains to be determined.

396 **The host-pathogen evolutionary dance.** The analogy of an arms race has been used
397 repeatedly for host-pathogen interactions as they evolve over time (37-40). Within this construct,
398 each actor endeavors to detect the strengths and weaknesses of the other and evolve a strategy that
399 increases the odds of survival, usually at the other's expense. Biofilm is recognized as a strategic
400 response of bacteria to host defenses in that it protects the inhabitants from antibiotics, host
401 defensins, macrophages and eosinophil networks (5, 10, 41, 42). The studies herein began with *S.*
402 *marcescens* isolated from soil, a habitat with its own unique set of challenges but one that does not
403 usually include pockets with high concentrations of protein. However, *S. marcescens* is adaptable
404 and can infect both nematodes and humans. In nematodes, infection can initiate in the gut after
405 ingestion (43). Based on the results from our *S. marcescens* strains we would predict that biofilm
406 would be stimulated upon contact with the high protein content of the intestine and the epithelial
407 lining of the nematode (the initial targets for infections caused by *A. salmonicida* and *F. columnare*
408 include the fins, gills and intestinal tract are all sites with elevated protein concentrations).
409 Similarly, in the respiratory system of humans we would predict that *S. marcescens* would form
410 biofilm upon contact with the high protein concentrations of alveolar fluid. With respect to alveolar
411 fluid and infections of the respiratory system, the lung, in contrast to our friendly media with
412 benign milk protein, is designed to be a hostile environment for microbes. The protein content of

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413 alveolar fluid is complex and contains many different proteins of which four proteins are abundant,
414 SP-A, SP-B, SP-C, SP-D, and were originally described as hydrophobic (B & C) and hydrophilic
415 (A & D) surfactants that facilitate gas exchange on the mucosal surface (44). These proteins can
416 represent up to 10% of the dry weight of bronchial lavage fluid (45). Of particular interest are SP-
417 A and SP-D, now recognized as collectins, that participate in host defense along with their role as
418 surfactants. Both bind bacterial LPS and in addition, SP-D binds peptidoglycan. These proteins
419 have also been implicated in clearance of pathogens, activation of macrophages, modulation of
420 inflammatory response and regulation of innate immunity functions in the lung (44-46). We posit
421 that the second virulence strategy of *Serratia* (and *Aeromonas*, and *F. columnare*) is the
422 sequestration of proteins from the environment of their host, into the biofilm matrix. This is
423 consistent with the biofilm matrix as a multifunctional extracellular 'organ' of a bacterial
424 consortium (47). Incorporation of substantial amounts of SP-A into the biofilm as a structural
425 component would locally reduce its concentration in alveolar fluid and mute the host's immune
426 response. Targeting SP-A has been previously documented for *P. aeruginosa* (48).
427 In demonstrating the substantial influence of exogenous protein on biofilm productivity we
428 hope that this stimulates further work on this aspect of biofilm formation. Responses to exogenous
429 protein appeared to be strain specific, suggesting that the different environments in which these
430 strains are colonizing may have a range of exogenous protein concentrations to which cognate
431 strains have adapted. Protein and/or peptides in the concentrations ranges where we have detected
432 enhanced biofilm formation would saturate protein binding sites on the cell surface. Some of these
433 sites, as in the case of *Enteroco*ccus (34, 49, 50), are linked to two-component regulatory systems,
434 hence exogenous protein may be an environmental trigger for biofilm formation.
435
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439 *Serratia marcescens* strains were performed in an undergraduate laboratory (MMG-302) at MSU.
440 DY, JK, & LB were undergraduates at MSU and responsible for Figures 1, 2, 3, 5 & 6. MMC was
441 a visiting undergraduate scholar from Brazil, supported by Science Without Borders funded by
442 Coordination for the Improvement of Higher Education Personnel and performed experiments for
443 Figures 4 & 9); MS was a visiting undergraduate scholar from Kalamazoo College and performed
444 the initial characterization of the *S. marcescens* isolates.
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FIGURE LEGENDS

Figure 1. Biofilm formation by *S. marcescens* soil isolates in response to elevated concentrations of milk protein in broth (0.05%, 0.5%, 2.5% & 5.0%). Incubation in microtiter plates was for 24 hours at 25°C on an orbital shaker at 100 RPM.

Figure 2. Biofilm formation by freshwater isolates in response to milk protein at 5% in broth. All isolates were from the Red Cedar River, East Lansing, MI. All strains were isolated from a direct plating of river water on Pseudomonas Isolation Agar.

Figure 3. Biofilm formation by six freshwater isolates in response to elevated protein concentrations in broth. Six isolates from the biofilm screening described in Fig.2 were tested for biofilm production at four different concentrations of milk protein (0.5%, 1.0%. 2.0% and 5.0%) in TSB.

Figure 4. Biofilm formation by bacterial isolates from lake sturgeon eggs, *Homo sapiens* and Bovine. *P. aeruginosa* PA01 was used as a positive control for biofilm formation. Panel A; isolates from fish (*F. columnare* 090702-1, *Hydrogenophaga* F14, *Brevundimonas* F16, *Acidovorax* F19, and *Pseudomonas* C22) tested on R2Broth with 1.0% and 5.0% milk protein supplemented media. Panel B. Biofilm assay performed in LB broth without NaCl at 1% and 5% milk protein on *Aeromonas*, unpigmented *Serratia* and *E. coli* from *H. sapiens* and *E. coli* from Bovine.

Figure 5. The effect of exogenous protein on the concentration of cells within biofilm. These assays were performed in 24 well plates in R2Broth (gray) and R2Broth supplemented with 5% milk protein (black).

Figure 6. The effect of exogenous protein on the protein concentration within biofilms. These experiments were performed in 24 well plates (4 replicates for each treatment) and each plate was replicated for measuring biofilm (crystal violet) and protein (Bradford assay). *P. aeruginosa* PA01 and *Aeromonas* strain 060628-1 were tested at 5% milk protein and *F. columnare* 090702-1 was tested at 1% milk protein.

Figure 7. The effect of exogenous protein on growth of *P. aeruginosa* PA01, *S. marcescens* RL-5, *Aeromonas* strain 060628-1and *F. columnare* 090702-1. Growth was measured in R2Broth unsupplemented and supplemented with 0.1%, 0.2% and 0.4% milk protein.

Figure 8. Quantitation of biofilm from growth experiment described in Fig. 7.

Figure 9. Confocal images of *P. aeruginosa* (A & B) and *F. columnare* (C & D) biofilm at 20X and 90X grown with 2.5% milk protein.

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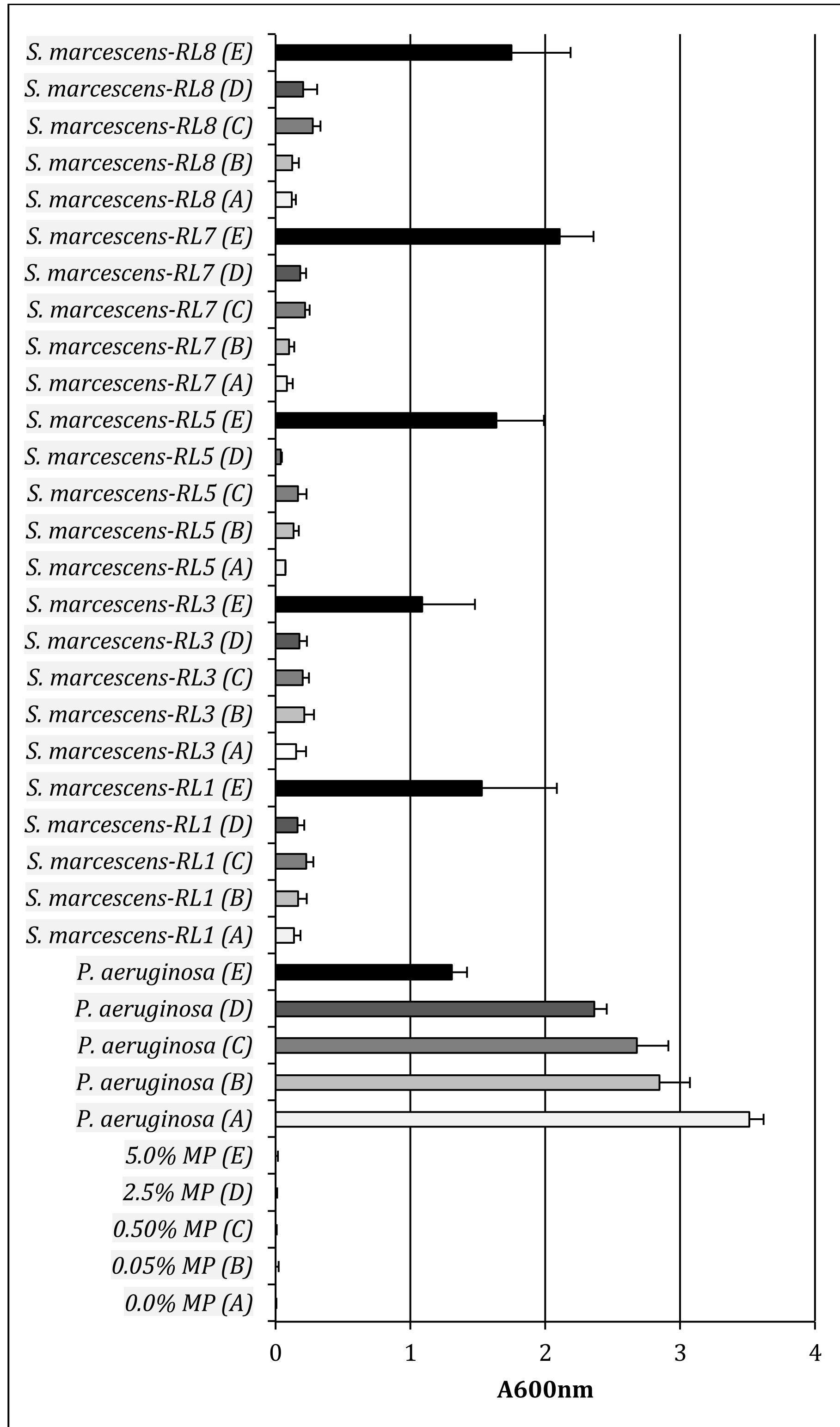

Figure 1

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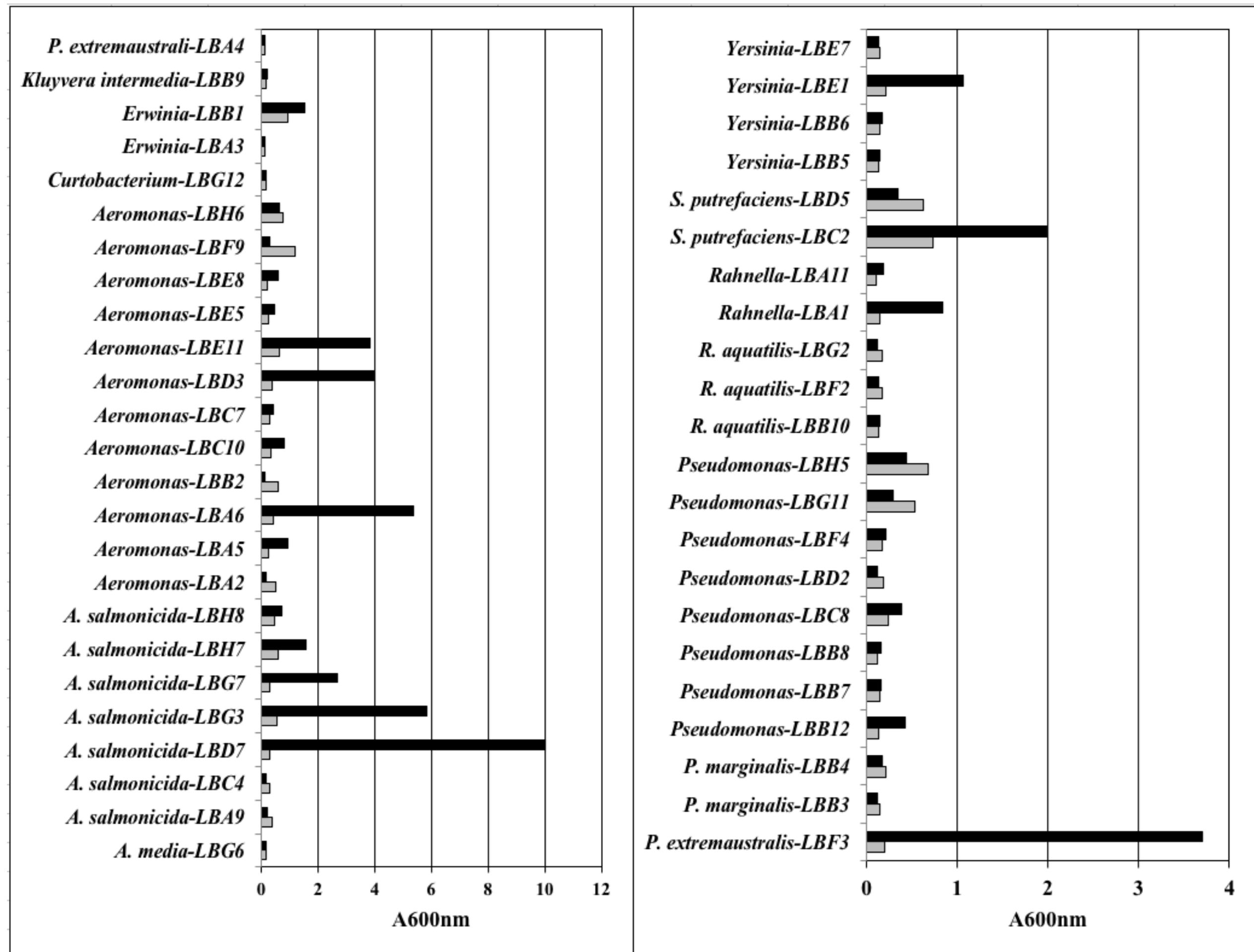

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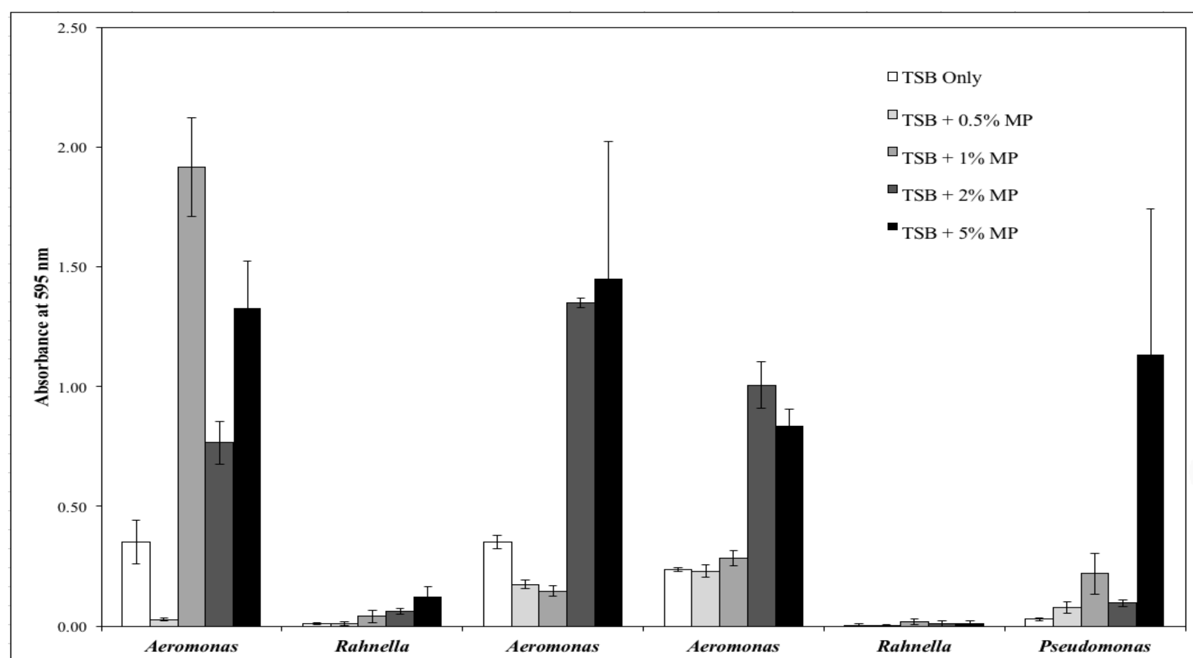

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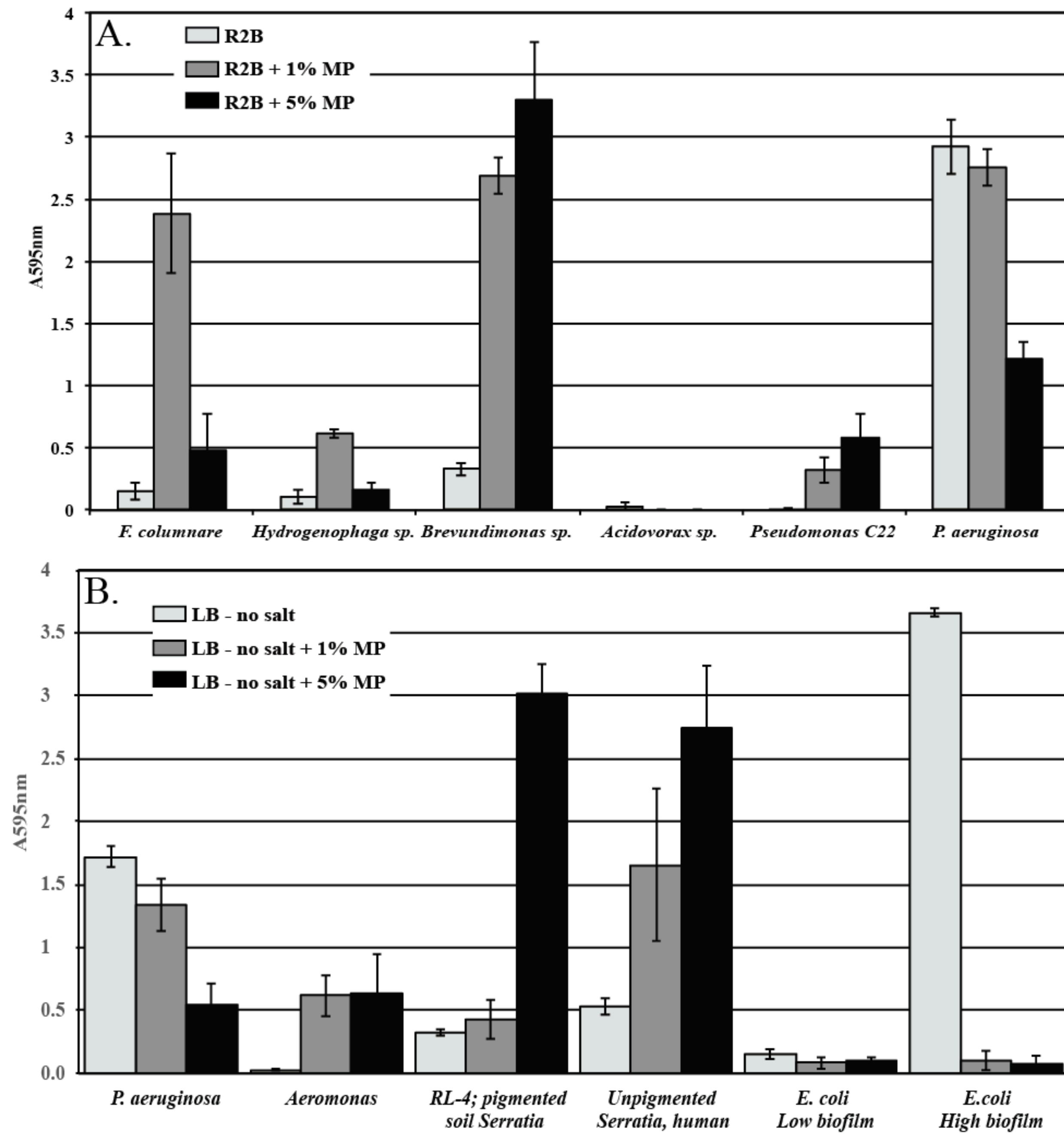

Figure 4

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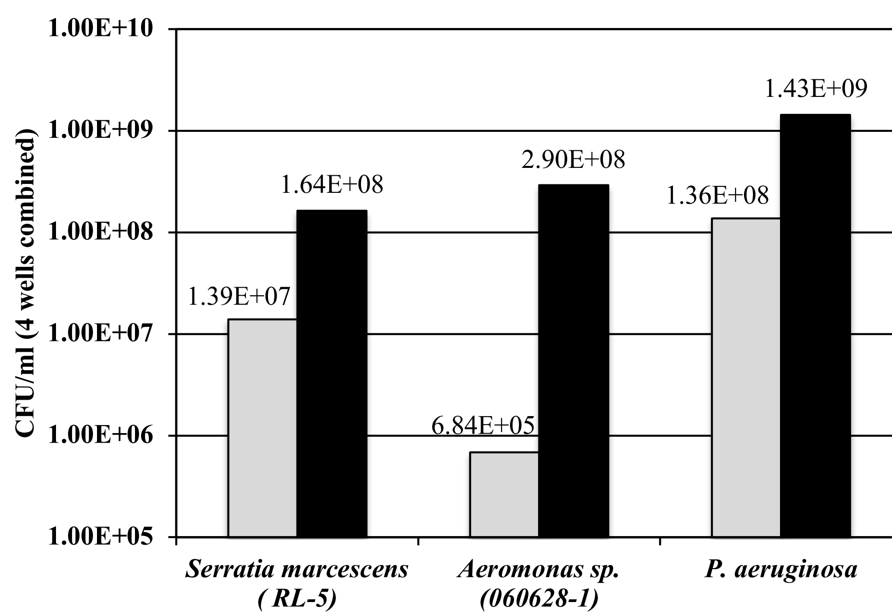

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653 Figure 5
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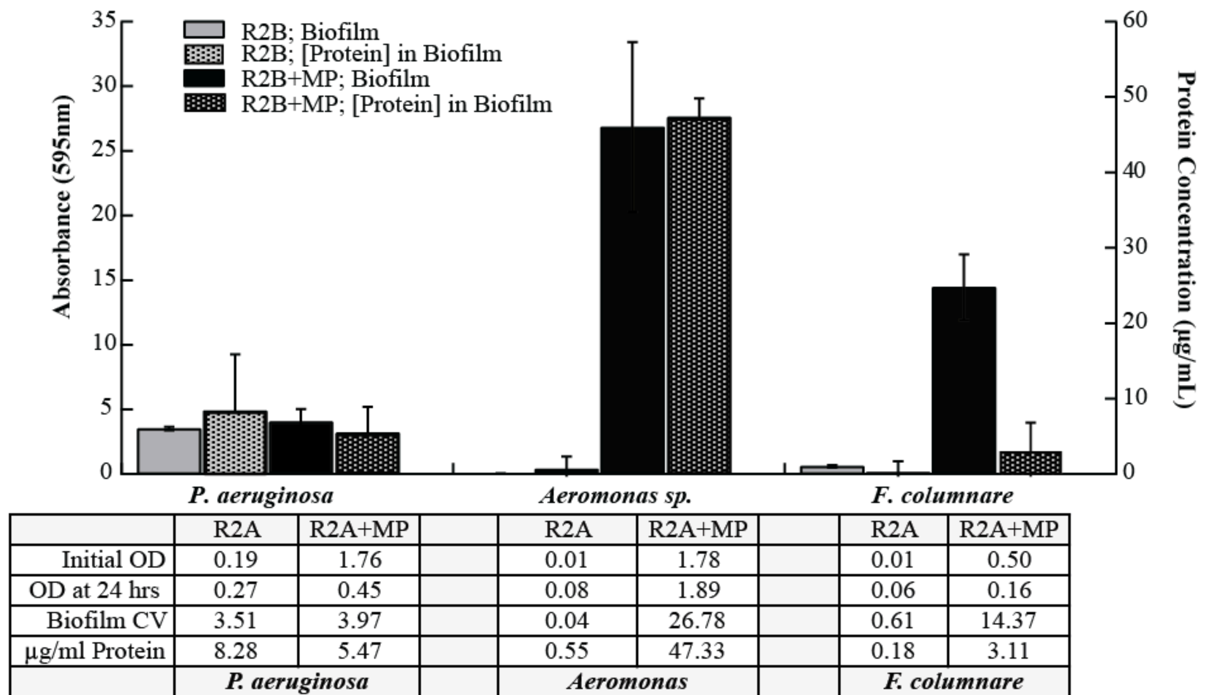

	R2A	R2A+MP		R2A	R2A+MP		R2A	R2A+MP
Initial OD	0.19	1.76		0.01	1.78		0.01	0.50
OD at 24 hrs	0.27	0.45		0.08	1.89		0.06	0.16
Biofilm CV	3.51	3.97		0.04	26.78		0.61	14.37
µg/ml Protein	8.28	5.47		0.55	47.33		0.18	3.11
	P. aeruginosa			***Aeromonas***			***F. columnare***	

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662 Figure 6
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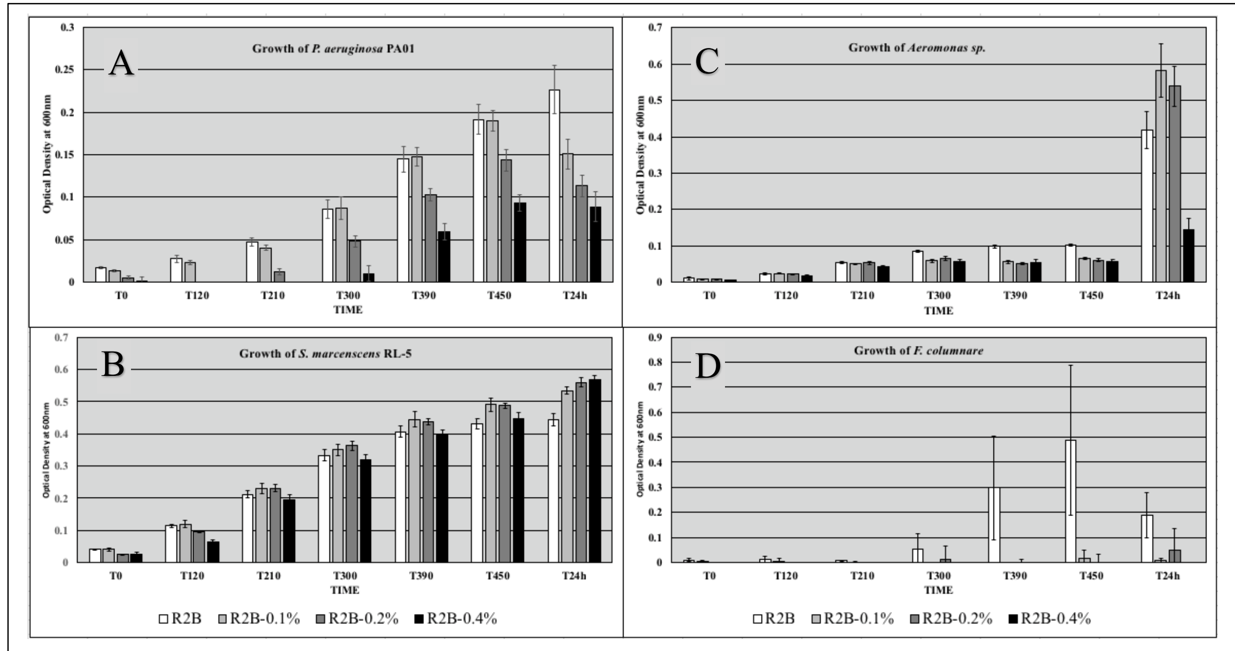

700 Figure 7.

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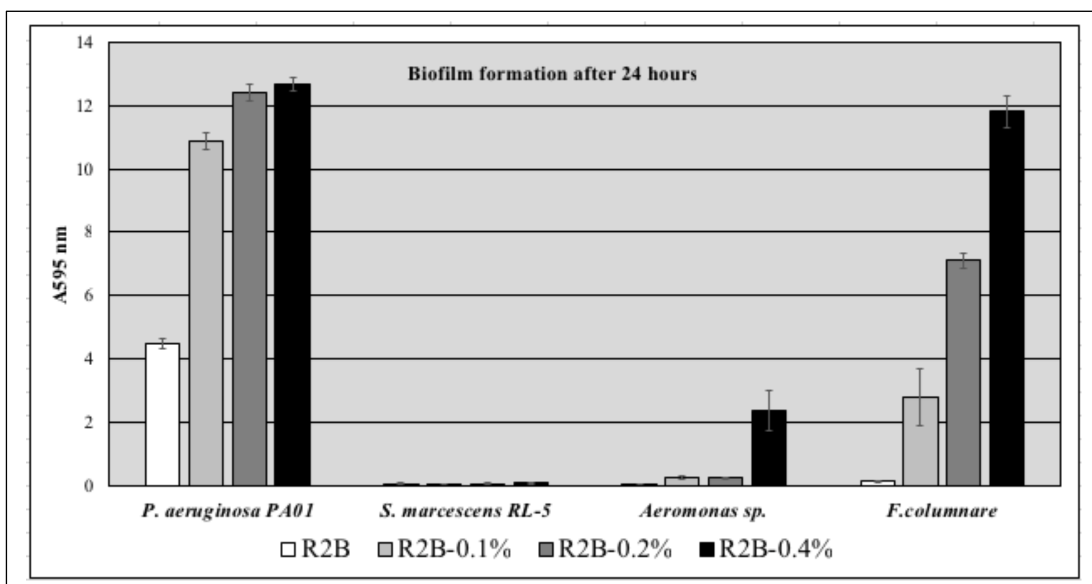

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725 Figure 8.
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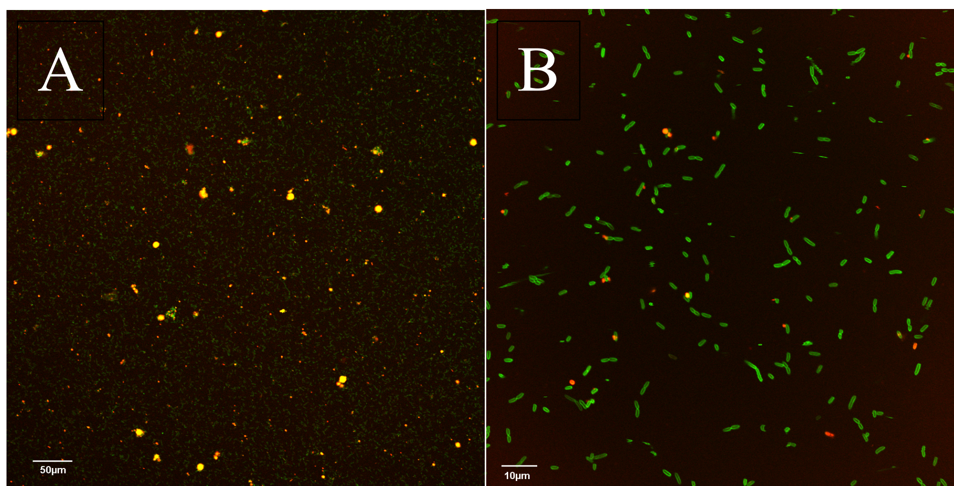

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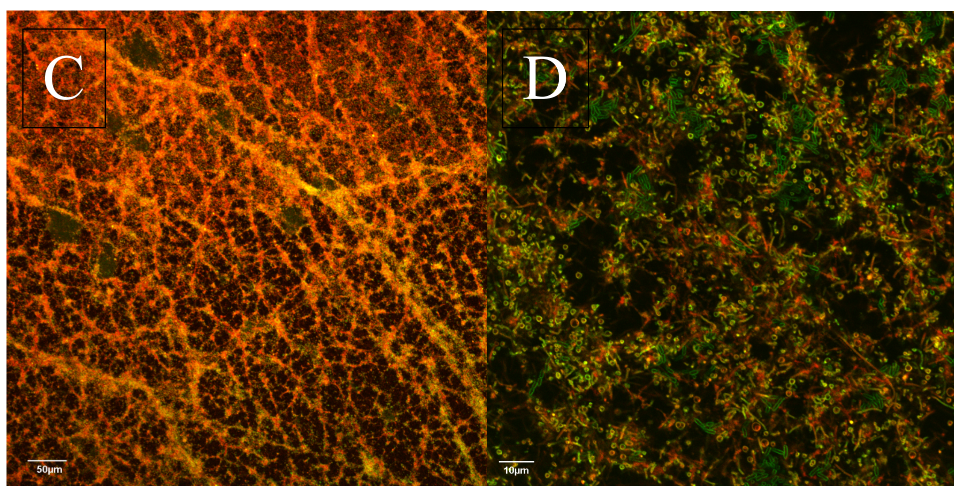

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736 Figure 9
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