Heterogenous response to R-pyocin killing in populations of *Pseudomonas aeruginosa* sourced from cystic fibrosis lungs

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Abstract

Bacteriocins are proteinaceous antimicrobials produced by bacteria which are active against other strains of the same species. R-type pyocins are phage tail-like bacteriocins produced by *Pseudomonas aeruginosa.* Due to their anti-pseudomonal activity, R-pyocins have potential as therapeutics in infection. *P. aeruginosa* is a Gram-negative opportunistic pathogen and is particularly problematic for individuals with cystic fibrosis (CF). *P. aeruginosa* from CF lung infections develop increasing resistance to antibiotics, making new treatment approaches essential. *P. aeruginosa* populations become phenotypically and genotypically diverse during infection, however little is known of the efficacy of R-pyocins against heterogeneous populations. R-pyocins vary by subtype (R1-R5), distinguished by binding to different residues on the lipopolysaccharide (LPS). Each type varies in killing spectrum, and each strain produces only one R-type. To evaluate the prevalence of different R-types, we screened *P. aeruginosa* strains from the International *Pseudomonas* Consortium Database (IPCD) and from our biobank of CF strains. We found that (i) R1-types were the most prevalent R-type among strains from respiratory sources and (ii) isolates collected from the same patient have the same R-type. We then assessed the impact of diversity on R-pyocin susceptibility and found a heterogenous response to R-pyocins within populations, likely due to differences in the LPS core. Our work reveals that heterogeneous populations of microbes exhibit variable susceptibility to R-pyocins and highlights that there is likely heterogeneity in response to other types of LPS-binding antimicrobials, including phage.

Importance

R-pyocins have potential as alternative therapeutics against *Pseudomonas aeruginosa* in chronic infection, however little is known about the efficacy of R-pyocins in heterogeneous bacterial

47 populations. *P. aeruginosa* is known to become resistant to multiple antibiotics, as well as evolve
48 phenotypic and genotypic diversity over time; thus it is particularly difficult to eradicate in chronic
49 cystic fibrosis (CF) lung infections. In this study, we found that *P. aeruginosa* populations from
50 CF lungs maintain the same R-pyocin genotype but exhibit heterogeneity in susceptibility to R-
51 pyocins from other strains. Our findings suggest there is likely heterogeneity in response to other
52 types of LPS-binding antimicrobials, such as phage, highlighting the necessity of further studying
53 the potential of LPS-binding antimicrobial particles as alternative therapies in chronic infections.
54

55 **Introduction**

56 Pyocins are narrow-spectrum antimicrobials, specifically produced by *Pseudomonas aeruginosa,*
57 that have antimicrobial activity against members of the same species (1-4). *P. aeruginosa* produces
58 three types of pyocin, referred to as S-pyocins, F-pyocins, and R-pyocins (1-3). The focus of this
59 work is R-pyocins, which are narrow-spectrum, phage-tail-like bacteriocins that vary by subtype
60 (types R1-R5) (2, 3, 5-11). Each *P. aeruginosa* strain uniquely produces only one of these R-
61 pyocin types (2, 3). As the variable C-terminus region of the tail fibers of sub-types R2-R4 are
62 approximately 98% similar in amino acid sequence, they are often grouped together under the R2
63 subtype (12-14). R-pyocin subtype and specificity are conferred by differences in the "foot" of the
64 tail fiber (Fig. 1), which is believed to bind to specific residues on the lipopolysaccharide (LPS)
65 decorating the outer membrane of Gram negative bacteria (12-26). A strain may be susceptible or
66 resistant to any variation of the different R-types (2, 3, 14, 25, 27-29) .
67

68 R-pyocins are similar to phage tails of the *Myoviridae* phage family, both in structure and in killing
69 mechanism (2, 3, 14-17, 24, 27). The proposed mode of action is through the foot of the R-pyocin

70 tail fiber recognizing a receptor on the LPS; binding to the target cell triggers the sheath to contract,
71 pushing the tail spike and core into the outer membrane (1-3, 12, 14, 15, 22-27, 29-33). The
72 puncture causes membrane depolarization, inhibiting active transport and eventually leading to
73 cell death (32-36). Several monosaccharide residues on the outer core of the LPS have been
74 proposed to confer susceptibility and specificity to some of the R-pyocin types (24, 25), however,
75 only the receptor of the R3-type pyocin has been clearly defined to be the Glc^{IV} terminal glucose
76 on the outer core of the LPS (26). R-pyocins have previously been explored as potential
77 therapeutics (12, 13, 37, 38), but little is known about their binding to target cells, or their killing
78 efficacy in heterogeneous *P. aeruginosa* populations.
79
80 *P. aeruginosa* is found in a number of human infections and is the leading cause of morbidity and
81 mortality in individuals with cystic fibrosis (CF). Once established in the CF lung, single-strain
82 populations become heterogeneous - evolving genotypic and phenotypic diversity during the
83 course of infection (39-49). *P. aeruginosa* possesses many intrinsic antibiotic resistance
84 mechanisms, and isolates from CF lung infections are known to become increasingly resistant to
85 multiple antibiotics over time (4, 42, 50-56). It is also known that isolates sourced from clinical
86 populations of *P. aeruginosa* can exhibit considerable variation in susceptibility to antibiotics (44,
87 49, 54, 57-59), however heterogeneity in R-pyocin susceptibility, or susceptibility to other types
88 of bacteriocins have not been explored. Here we determined that R1-type pyocins were the most
89 prevalent genotype among clinical strains, while a large proportion of strains are untypeable by
90 classical R-pyocin typing methods. We found that multiple isolates from diverse, clonal
91 populations of *P. aeruginosa* have the same R-pyocin genotype and that R-pyocin type does not
92 change across populations collected over a 2-year period from a single patient. Moreover, we

93 observed heterogeneity in R-pyocin susceptibility within a population of *P. aeruginosa.* This
94 heterogenous response is likely due to differences in the LPS core composition among isolates,
95 supporting evidence from other research groups that the LPS core contains the receptors for R-
96 pyocins. Our findings highlight that diverse *P. aeruginosa* populations exhibit within-population
97 heterogeneity in R-pyocin susceptibility, suggesting there is likely also heterogeneity in response
98 to other types of LPS-binding alternative therapies, such as phage.
99

100 **Results**

101 **R1 genotype is the most prevalent R-pyocin type in clinical *P. aeruginosa* strains.** To
102 determine the prevalence of different R-pyocin subtypes in *P. aeruginosa* isolates sourced from
103 CF infections, we conducted an *in silico* screen to determine R-pyocin genotypes of *P. aeruginosa*
104 isolates publicly available through the International *Pseudomonas* Consortium Database (IPCD)
105 (60). The IPCD is a repository of *P. aeruginosa* isolates collected from a variety of sources (with
106 an emphasis on CF), created to facilitate metadata analyses specifically to improve prognostic
107 approaches for CF treatment (60). By using the BLAST algorithm from NCBI, we screened 852
108 strains from the database for nucleotide sequence homology to R1-, R2-, or R5-pyocin tail fiber
109 genes. As subtypes R2-R4 are highly similar, we considered these three subtypes as one larger R2-
110 subtype for this analysis (12-15). Out of the 852 isolates in the database, we could not conclusively
111 type 303 strains, and further analysis is necessary to determine if these strains are lacking R-pyocin
112 genes entirely, if the tail fiber genes are incomplete, or if there are potentially other subtypes of R-
113 pyocin among these strains. It is important to note that untyped isolates are not able to be
114 categorized into one of the three major subtypes of R-pyocin through classical typing methods,

115 and that typing alone does not have implications on whether a strain produces functional R-pyocins
116 or not.
117
118 Of the 852 strains in the database, we categorized 448 strains as isolated from human "respiratory"
119 sources; these include *P. aeruginosa* isolated from throat, CF, bronchiectasis, sputum, sinus, and
120 nasopharynx. Among the 448 respiratory isolates, we typed 144 (32.14%) strains as R1-type, 76
121 (16.96%) as R2-type, and 43 (9.6%) as R5-type pyocin producers. The remaining 185 (41.29%)
122 of strains were untypeable. Fig. S1 depicts the distribution of R-types for each source; more
123 information regarding the categorization and typing of strains from the IPCD can be found in
124 Supplementary Dataset 1. Due to the curation and labeling of the strain information in the database,
125 we were not able to precisely distinguish all CF strains from strains isolated from other respiratory
126 infections, however is it likely that many of the samples labeled by anatomical respiratory sources
127 are from CF patients, given the research areas of the curating research groups. The distribution of
128 R-pyocin types among the typeable respiratory isolates shows that (i) R1-pyocin producers are
129 likely more prevalent in CF than the other R-pyocin subtypes and (ii) a large proportion of isolates
130 are untypeable by classical typing methods (Fig. 2A).
131
132 **R-pyocin type does not vary within clonal CF populations of *P. aeruginosa*.** Through a
133 collaboration with Children's Healthcare of Atlanta and the CF Discovery Core at Emory
134 University, we collected whole populations of *P. aeruginosa* from fresh CF sputum samples of
135 patients with chronic lung infections. Using multiplex PCR (Table S1), we R-pyocin typed isolates
136 from 19 of these populations (183 isolates total) collected from 8 different patients over a 2 year
137 period (Supplementary Dataset 2). In our cohort, the R1 genotype was the most prevalent R-pyocin

138 type found among CF patients. We found that while R-pyocin type does not change within a
139 population or over longitudinal collections from the same patient, different patients from the same
140 clinic may be infected with *P. aeruginosa* containing different R-types. Out of the 8 patients we
141 typed, 5 of these patients were chronically infected with an R1-pyocin producing strain, 2 patients
142 were infected with untypeable populations, and 1 patient was infected with an R2-pyocin strain
143 (Fig. 2B). It appeared that in the time frame sampled (over a 2-year period) there were no strain
144 switching events within these patients. We did not find any populations containing the R5-pyocin
145 type in our biobank.
146
147 **R-pyocin-mediated killing varies among heterogeneous *P. aeruginosa* populations.** Previous
148 work has shown that isolates from within the same population have diverse antibiotic resistance
149 profiles (54), but diversity in R-pyocin susceptibility has yet to be evaluated within whole
150 populations of *P. aeruginosa*. We chose three *P. aeruginosa* populations from our biobank, each
151 from a different CF patient, that exhibited diversity in growth rate (Fig. 3A) and morphology (Fig.
152 3B). Populations from Patient 1 and Patient 2 were untypeable (UT), while the population from
153 Patient 3 was determined to consist of R1-pyocin producers (Supplementary Dataset 2).
154 Preliminary experimentation suggested that these populations each exhibited some susceptibility
155 to R2-pyocins. To assess heterogeneity in susceptibility to R-pyocins within these populations, we
156 used partially fractionated R2-pyocin cell lysate from PAO1. To show that other pyocin-associated
157 lytic enzymes such as holin or lysin are not responsible for the bactericidal activity against other
158 strains, we included lysates extracted from an isogenic PAO1 R-pyocin mutant lacking the tail
159 fiber gene and chaperone (PAO1ΔR), which produces nonfunctional partial pyocins. This strain
160 has an intact lysis cassette, holin genes, and *Pf4* prophage, however filamentous prophage has

161 previously been shown to be deactivated by chloroform (61-63), which is used during the R-pyocin
162 isolation process to lyse the cells.
163
164 By comparing growth (optical density) of *P. aeruginosa* isolates with and without R2-pyocins
165 added to liquid culture, we found that across patients, each population as a whole exhibited
166 different levels of susceptibility. We also found that within each population, there was
167 heterogeneity in susceptibility among the isolates (Fig. 3C). In *P. aeruginosa* populations sourced
168 from Patient 1 and Patient 2, all 20 isolates were susceptible to R2-pyocins, however the response
169 varied approximately 10-fold between individual isolates. In Patient 3, the response ranged from
170 susceptible to completely resistant. This finding demonstrates that even within one population of
171 *P. aeruginosa* producing the same R-pyocin type, there is diversity in susceptibility to R-pyocins
172 of other types.
173
174 **There is significant heterogeneity in R-pyocin susceptibility within a *P. aeruginosa***
175 **population.** To further distinguish differences in susceptibility between isolates, we chose three
176 isolates from the R1-pyocin producing population of Patient 3 for further analysis. We first tested
177 the three isolates for susceptibility to R-pyocins from several R2-pyocin producing strains by
178 visualizing killing activity with a standard, soft-agar overlay spot assay (Fig. 4A). We used R2-
179 pyocin containing lysates from the standard laboratory strain PAO1 and a PAO1 R-pyocin null
180 mutant lacking a functional R-pyocin (PAO1ΔR), as well as a previously described CF isolate
181 A018 (also a R2-pyocin producer) and a corresponding R-pyocin null mutant (25, 64, 65). The
182 spot assay indicated that Isolate 1 from Patient 3 was resistant to the R2-pyocins of PAO1 and

183 A018, while Isolates 2 and 3 were susceptible (depicted by the zones of inhibition where the R2-
184 pyocin lysates were spotted) (Fig. 4A).
185
186 We next measured R2-pyocin susceptibility of the three isolates with lysates from PAO1 in liquid
187 culture, and measured Log_2 fold-change of colony forming units (CFU) after 4 hours of growth to
188 compare cell density with and without R-pyocin-containing lysates. Through this assay, we
189 confirmed that Isolates 2 and 3 from Patient 3 are susceptible to R2-pyocins, while Isolate 1 is
190 resistant (Fig. 4B). We confirmed in both planktonic growth and in an agar overlay that within a
191 single population, two isolates were susceptible to R2-pyocins while one isolate was resistant,
192 indicating that *P. aeruginosa* isolates of the same R-pyocin type and from the same population can
193 exhibit different susceptibilities to other R-pyocins.
194
195 **R-pyocin susceptibility of isolates is dependent on the LPS core structure and not alginate**
196 **production.** As there was diversity in colony morphology and mucoidy among the *P. aeruginosa*
197 population of Patient 3, we quantified the alginate production of Isolates 1-3 from Patient 3 to
198 determine if alginate production may influence the susceptibility differences among these isolates.
199 We found that only Isolate 3 (R2-susceptible) produced alginate while Isolate 2 (R2-susceptible)
200 and Isolate 1 (R2-resistant) did not produce alginate (Fig. 5A), suggesting that alginate production
201 did not influence R2-pyocin susceptibility between the three isolates.
202
203 As LPS has been shown to be involved in the binding of R-pyocins to target cells (1-3, 12, 14, 15,
204 22-27, 29-34), we further characterized Isolates 1-3 from Patient 3 by extracting and visualizing
205 major LPS components via gel electrophoresis and staining. All three isolates showed differences

206 in O-specific antigen (OSA, B-band) and common antigen (CPA, A-band) presentation, but a
207 notable difference was that the R2-resistant Isolate 1 possessed a LPS core band that migrated
208 differently from the susceptible isolates (Fig. 5B). Along with an altered LPS core, Isolate 1 lacks
209 both the A-band and B-band. We found that Isolate 2 lacks A-band, presented some low molecular
210 weight B-band, and had a normal core, while Isolate 3 also had a normal core and only presented
211 A-band. This variation in LPS phenotypes among isolates within a population is in agreement with
212 previous studies that have shown LPS mutations frequently arise in the CF environment, both *in*
213 *vivo* and *in vitro* (66-75).
214
215 **WGS sequencing of isolates reveals possible SNVs responsible for R-pyocin resistance.** We
216 obtained whole genome sequences of Isolates 1-3 from Patient 3 to determine the strain type and
217 serotype. We found that all three isolates are of the multi-locus sequence type (MLST) 2999,
218 suggesting they are derived from the same originating infecting strain. The serotype of each isolate
219 was predicted to be O6, indicating that the isolates all carried the O6 gene cluster (encoding genes
220 for synthesis of the variable O-specific antigen or B-band), and that serotype alone is likely not
221 responsible for the differences in R-pyocin susceptibility. We found that all three isolates shared
222 30,127 single nucleotide variants (SNVs) when mapped against a PAO1 reference genome (Fig.
223 6A). We found 232 SNVs unique to the R2-resistant Isolate 1 from Patient 3, when mapped to
224 PAO1 and compared to SNVs of the two susceptible isolates from Patient 3 (Fig. 6B). Of the 232
225 SNVs unique to Isolate 1, 169 SNVs were non-synonymous (Supplementary Dataset 3).
226
227 Given the high number of SNVs and stark diversity between the isolates, through genetic analysis
228 we were able to identify several candidate SNVs potentially involved in the R-pyocin resistant

229 phenotype of this isolate, though it could be any number of these mutations in combination that
230 play a role in the R-pyocin resistant phenotype we observed. Noteable candidate SNVs unique to
231 Isolate 1 included a C151T (Arg51Trp) missense variant at position 833604 in the *mucC* gene, a
232 G710A (Arg237His) missense variant at position 1199237 of the *roeA* gene, and a T1169C
233 (Leu390Pro) missense variant at position 3968193 in the *algK* gene. Each of these genes are
234 involved in alginate production or polysaccharide biosynthesis but may indirectly influence LPS
235 biosynthesis or presentation. However, when we complemented each of these genes (using the
236 gene sequences from PAO1) into Isolate 1, there were no differences to R2-pyocin susceptibility;
237 the complemented strains of Isolate 1 remained resistant to R2-pyocins (Fig. S2A) and did not
238 alter LPS presentation (Fig. S2B).
239

240 **Discussion**

241 Chronic infections found in CF lungs are debilitating and often lethal. *P. aeruginosa* is a common
242 pathogen found in CF lungs and is highly resistant to multiple classes of antibiotic (4, 39-56),
243 making the development of new and alternative therapies critical. Bacteriocins have long been
244 considered in food preservatives as bio-controlling agents and also as therapeutics (76-82). R-
245 pyocins have potential as an anti-pseudomonal therapeutic, but little is known about their
246 antimicrobial efficacy against the heterogeneous *P. aeruginosa* populations found in CF lungs. We
247 evaluated R-pyocin type and susceptibility among *P. aeruginosa* isolates sourced from CF
248 infections and found that (i) R1-pyocins are the most prevalent R-type among respiratory infection
249 and CF strains; (ii) isolates from *P. aeruginosa* populations collected from the same patient have
250 the same R-pyocin type; (iii) there is heterogeneity in susceptibility to R-pyocins within *P.*

251 *aeruginosa* populations and (iv) susceptibility is likely driven by diversity of LPS phenotypes
252 within clinical populations.
253
254 We first assessed the prevalence of the different R-pyocin subtypes in *P. aeruginosa* isolates
255 sourced from different environments by screening for R-pyocin genotypes of *P. aeruginosa*
256 isolates through the International Pseudomonas Consortium Database (IPCD) and our biobank of
257 CF isolates (60). We found that R1-pyocin producers make up the majority of typeable CF strains
258 from both sources. This suggests that many CF infections may initially be colonized with R1-
259 producing ancestor strains, or that there is a possible benefit to producing R1-type pyocins during
260 strain competition in early stages of infection. Our findings agree with previous work, as R1-
261 producers have been shown to be the most prevalent subtype of CF isolates evaluated in a separate
262 study which typed 24 *P. aeruginosa* CF isolates (62.5% of which were R1-producers) (64). We
263 found a large number of strains from the IPCD that could not be typed by our screen using classical
264 R-pyocin typing methods. Another group evaluating R-pyocin susceptibility among 34 CF isolates
265 found that 23 (68%) of the tested isolates did not produce R-pyocins or could not be typed (28).
266 Other work has also shown that CF isolates of *P. aeruginosa* exhibited loss of pyocin production
267 when compared to environmental strains (83). This suggests that CF isolates of *P. aeruginosa* may
268 lose the ability to produce R-pyocins later in chronic infection, however further study is necessary
269 to explore the potential fitness advantages of this evolutionary trajectory.
270
271 We are only just beginning to understand how heterogeneous *P. aeruginosa* populations impact
272 upon treatment and disease outcomes. Previous studies have R-pyocin typed *P. aeruginosa* from
273 CF patients and compared R-types from longitudinal collections, only evaluating single isolates

274 from each patient (28, 64, 83). The typing of single isolates, however, does not consider the
275 genotypic and phenotypic diversity of *P. aeruginosa* populations, which evolve over the course of
276 chronic infection (39-49). Out of the *P. aeruginosa* populations collected from eight CF patients
277 we found primarily R1-producing or untypeable strains. We also found that all of the isolates in
278 each population consistently exhibited the same R-pyocin genotype over a 2-year period. A
279 previous study typed single isolates yearly for 8 years and found that R-pyocin type could change
280 over the course of a patient's infection, but strain typing also showed that these patients were often
281 infected with different strains during those events, suggesting that the observed changes in R-
282 pyocin type were likely due to transient strains (28). Several studies have shown that diverse
283 populations of *P. aeruginosa* from chronic infections show heterogeneity in susceptibility to a
284 number of antibiotics, complicating susceptibility testing and treatments (39, 44, 54, 57-59).
285 Though heterogeneity did not influence R-pyocin genotypes within our CF populations, we found
286 that diverse populations of *P. aeruginosa* from chronic CF lung infections exhibit heterogeneity
287 in susceptibility to R-pyocins. Specifically we found that (i) each population of *P. aeruginosa* as
288 a whole exhibited differential levels of susceptibility and (ii) within each population, there was
289 heterogeneity in susceptibility among individual isolates.
290
291 Previous studies have shown the effect of various LPS mutations on R-pyocin susceptibility in
292 laboratory strains PAO1 and PAK (24-26), however, to our knowledge, our work is the first to
293 examine LPS phenotypes associated with R-pyocin susceptibility and resistance in many different
294 clinical isolates of multiple *P. aeruginosa* populations sourced from chronic CF lung infections.
295 We found that differences in the core of the LPS appear to be correlated with the differences in
296 susceptibility and resistance to R-pyocins among the isolates from one patient; specifically, that a

297 resistant CF isolate produces an altered LPS core. Our findings are in agreement with recent work
298 demonstrating heterogenous resistance mechanisms and persistence against tailocin killing by
299 alterations of the LPS and associated glycosyltransferases in *Pseudomonas syringae* (84). It is
300 unknown if the altered LPS core phenotype we have found is commonly identified in CF *P.*
301 *aeruginosa* populations, or if there is a fitness benefit it confers during infection. During initial
302 colonization and strain establishment in the early stages of infection, the LPS (and therefore R-
303 pyocin susceptibility) may play a role in antagonistic interactions between competing strains and
304 ultimately determine the dominating strain.
305
306 While investigating possible mechanisms for the variable R2-pyocin susceptibility of Isolates 1-3
307 from Patient 3, we showed that serotype, strain type, and alginate production were not responsible
308 for the resistance in Isolate 1. When we probed the genomes of three isolates from a single clinical
309 population exhibiting heterogenous susceptibility to R2-pyocins, we identified several candidate
310 SNVs in *mucC*, *roeA,* and *algK,* unique to the resistant isolate; however ultimately these genes did
311 not effect R2-pyocin resistance or LPS presentation when complemented. All three genes are
312 involved with the alginate production pathway or other extracellular polysaccharides (41, 85-98),
313 known to be closely intertwined with various LPS synthesis and secretory pathways; thus it is
314 possible that any combination or all three variants combined may play a role in indirectly impeding
315 presentation of various portions of the LPS or core residues. As there are many regulatory
316 mechanisms and biosynthetic genes still yet to be understood that are involved in LPS synthesis
317 and presentation, it is also possible that there is a mechanism at play that has yet to be described.
318

319 Overall, our findings highlight that clinical populations of *P. aeruginosa* exhibit a heterogenous
320 response to R-pyocins and that this likely extends to any antimicrobial that utilizes the LPS. A
321 number of LPS- and saccharide-binding phage have been identified and tested as antimicrobials
322 against *P. aeruginosa* (24, 99-105) and other types of LPS-specific antimicrobials, including R-
323 pyocins, are also being considered as treatments (12, 13, 37, 38, 106, 107). Our work implies that
324 treatment with alternative therapies utilizing the LPS may not eradicate strains within infections
325 completely, potentially leading to highly resistant isolates taking over. This reiterates the
326 importance of assessing multiple, diverse isolates from populations of *P. aeruginosa* rather than
327 taking a single isolate from a population. To better understand how R-pyocins and other LPS-
328 binding antimicrobial therapies can be utilized for alternative treatments, it is crucial to know more
329 about the LPS, core phenotypes, and how they are evolving in *P. aeruginosa* populations during
330 infection, to better understand the resulting impact on the potential efficacy of these antimicrobials.
331

332 **Materials and Methods**

333 **Bacterial strains, media, and culture conditions.** Expectorated sputum samples for this study
334 were collected from adult CF patients through Emory–Children's Center for Cystic Fibrosis and
335 Airways Disease Research by the Cystic Fibrosis Biospecimen Laboratory with IRB approval
336 (Georgia Tech approval H18220). *P. aeruginosa* populations were collected from each sputum
337 sample using selective media (Pseudomonas Isolation Agar, Sigma-Aldrich) before isolating
338 single colonies for further characterization. In total, 204 isolates from our biobank, sourced from
339 CF chronic lung infections, were studied. All *P. aeruginosa* isolates, laboratory and indicator
340 strains used in this work are listed and described in Supplementary Dataset 2. All bacterial cultures
341 were grown in lysogeny broth (LB) medium at 37°C with 200rpm shaking agitation. Standard

342 genetic techniques were used for construction of *P. aeruginosa* mutants. The construction of
343 plasmids and generation of mutants can be found in more detail in Text S1 of the supplemental
344 material. All plasmids and primers used for mutant generation can be found in Table S1.
345
346 **Assessing colony morphology diversity in clinical isolates.** To evaluate the diversity in colony
347 morphologies between *P. aeruginosa* clinical isolates, we used a Congo Red-based agar media
348 (1% agar, 1×M63 salts (3 g of monobasic KHPO4, 7 g of K2PO4, and 2 g of NH4.2SO4, pH
349 adjusted to 7.4), 2.5 mM magnesium chloride, 0.4 mM calcium chloride, 0.1% casamino acids,
350 0.1% yeast extracts, 40 mg/l Congo red solution, 100 µM ferrous ammonium sulfate, and 0.4%
351 glycerol) (108). Isolates were grown overnight (16-18 hours) in LB medium at 37°C with 200rpm
352 shaking agitation before spotting 10 µL onto the plates. We incubated the plates overnight at 37°C,
353 and for a further 4 days at 22°C. The colonies were imaged with an Epson scanner at 800 dpi.
354
355 **R-pyocin typing strains of the IPCD.** Previously characterized *P. aeruginosa* strains were
356 selected for query sequences for each R-type: PAK an R1, PAO1 an R2, and E429 an R5 (25). As
357 the R-pyocin tail fiber sequences of PAO1 (NP_249311.1) (109-111) and PAK (Y880_RS29810;
358 accession GCA_000568855.2) (112) are annotated, the corresponding nucleotide sequences were
359 downloaded in FASTA format from NCBI. The E429 (R5) tail fiber sequence was identified from
360 previous work (25) and downloaded from NCBI by using the PAO1 tail fiber sequence (PA0620)
361 to BLAST against the E429 genome (60). NCBI's BLAST+ was also used to determine percent
362 identity between the whole tail fiber sequences of each strain/R-type pair-wise, in order to
363 determine the variable region of the tail fiber gene to use for typing. Homology information,
364 percent identity, query coverage and parameters can be found in Supplementary Data 1 (113). A

365 detailed description of R-pyocin typing the IPCD can be found in Text S1 in the supplemental
366 material.
367
368 **PCR conditions and R-pyocin typing.** A detailed description of R-pyocin typing the biobank of
369 CF populations can be found in Text S1 of the supplemental material. R-pyocin typing primer
370 details and product sizes are listed in Table S1.
371
372 **Expression and extraction of R-pyocins.** Expression of R2-pyocins used in the susceptibility
373 assays were induced and pyocins extracted from PAO1 with a modified method from published
374 work (12) and can be found in detail in text S1 of the supplemental material.
375
376 **Spot assay for R-pyocin activity.** Overnight cultures of clinical *P. aeruginosa* isolates were used
377 to inoculate 4 mL of cooled soft top (0.7%) agar at an optical density at 600 nm (OD_{600nm}) of 0.01.
378 For complemented strains, 4µL of Chromomax isopropyl β-D-1-thiogalacto-pyranoside
379 (IPTG)/X-Gal solution (Fisher Scientific) was added per 1mL of soft agar for induction of gene
380 expression. This mixture was poured onto LB agar plates for an overlay of the indicator strain. R2-
381 pyocin lysates extracted from PAO1, A018, and respective R-pyocin null mutants and were
382 vortexed before spotting 5 µL of each lysate onto the soft-top overlay indicator strains. Spots were
383 allowed to dry before plates were incubated at 37°C overnight. Clear zones of growth inhibition
384 indicated R-pyocin dependent activity against the indicator strains. Spot assays for R-pyocin
385 susceptibility were conducted in triplicate.
386

387 **Microtiter plate method for R-pyocin activity.** R2-pyocin susceptibility was measured by
388 changes optical density under conditions with and without added R2-pyocins, using a 96-well
389 microtiter plate. A detailed description of the microtiter plate method can be found in Text S1 of
390 the supplemental material.
391
392 **Alginate Isolation and Quantification**. Alginate was isolated and quantified by carbazole
393 methods described previously (114, 115) and described in detail in Text S1 of the supplemental
394 material.
395
396 **Statistical analysis.** Data analysis was performed using Prism (Graphpad software, version 9).
397 Population heterogeneity experiments were normalized to R-pyocin null mutant lysate controls for
398 fold-change, and analyzed with Prism. CFU data was analyzed for four isolates by normalizing
399 CFU measurements at t=0 hours and t=4 hours for cultures with and without R-pyocin lysates to
400 compare $\log_2$ fold-change between treatments for each isolate. Statistical significance between
401 treatments for each isolate was measured with a paired, parametric Student's T-test. Differences
402 across all three isolates with the R-pyocin treatments and alginate production were analyzed using
403 a one-way analysis of variance (ANOVA) with Tukey's multiple comparisons analysis in Prism.
404 Fig. 3C and Fig. 5A show mean values of three biological replicates for each individual isolate,
405 while Fig. 4B shows four biological replicates (averaged from technical replicates) as individual
406 data points. Error bars represent standard error (SEM) and significance is denoted as follows: **,
407 $P = 0.005$.
408

409 **LPS extraction and characterization.** LPS of bacterial cultures were isolated and visualized as
410 described by previous methods (116) and described in detail in Text S1 of the supplemental
411 material.
412
413 **Whole genome sequencing analysis.** A detailed description of genomic DNA preparation,
414 sequencing, and analysis can be found in Text S1 of the supplementary material. The full list of
415 SNVs unique to each isolate can be found in Supplementary Data 3.
416
417 **Data Availability.** All sequences were deposited in the National Center for Biotechnology
418 Information's SRA database under the accession number PRJNA67996 (117). Raw data and code
419 have been made available in the Dryad Digital Repository:
420 https://doi.org/10.5061/dryad.573n5tb67 (118).
421
422 **Supplemental Material**
423 Table S1. Primers and plasmids used in this study.
424 Supplementary Dataset 1. Detailed description of bacterial strains used in this study.
425 Supplementary Dataset 2. Detailed results of R-pyocin typed IPCD strains.
426 Supplementary Dataset 3. Detailed description of whole genome sequencing parameters and SNV
427 lists.
428 Text S1. Detailed description of materials and methods.
429 Text S2. Supplemental references.
430

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437 M.M., J.T. and S.P.D. conceived the study and wrote the manuscript. J.T. generated the PAO1ΔR
438 mutant strain. M.M. performed the experimental work and analyzed data.
439

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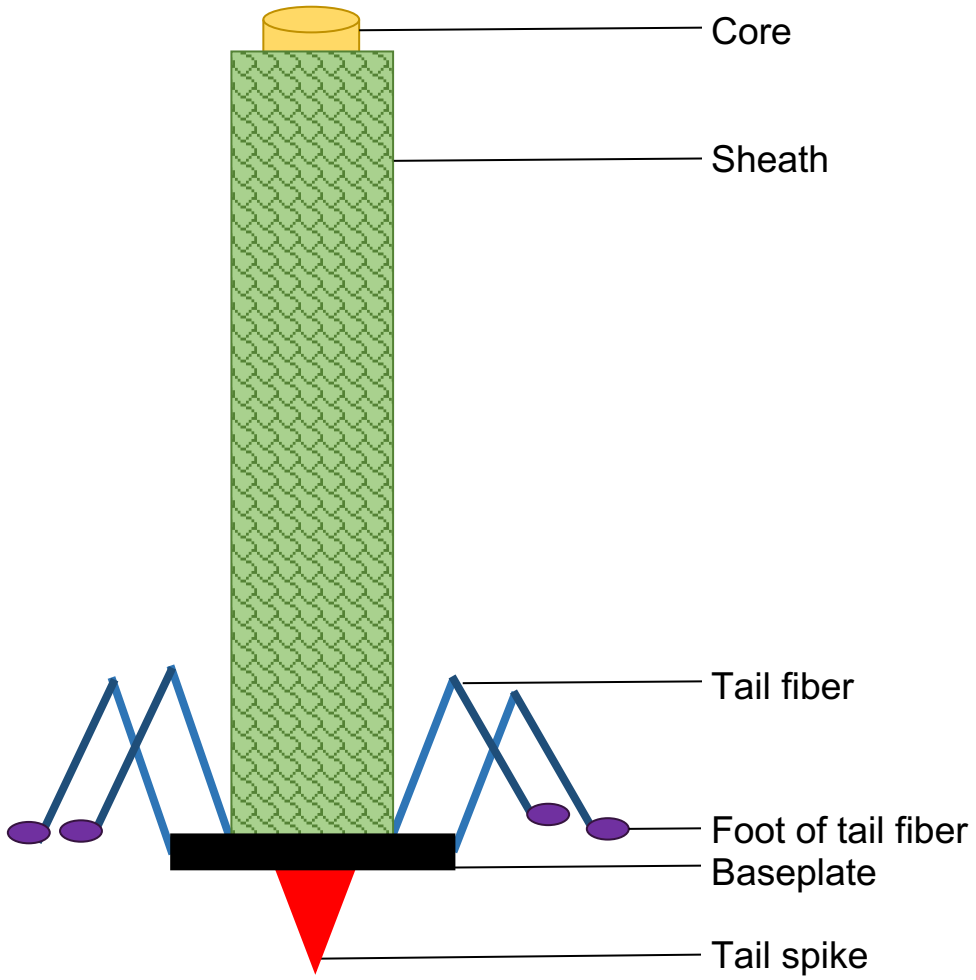
Figure 1



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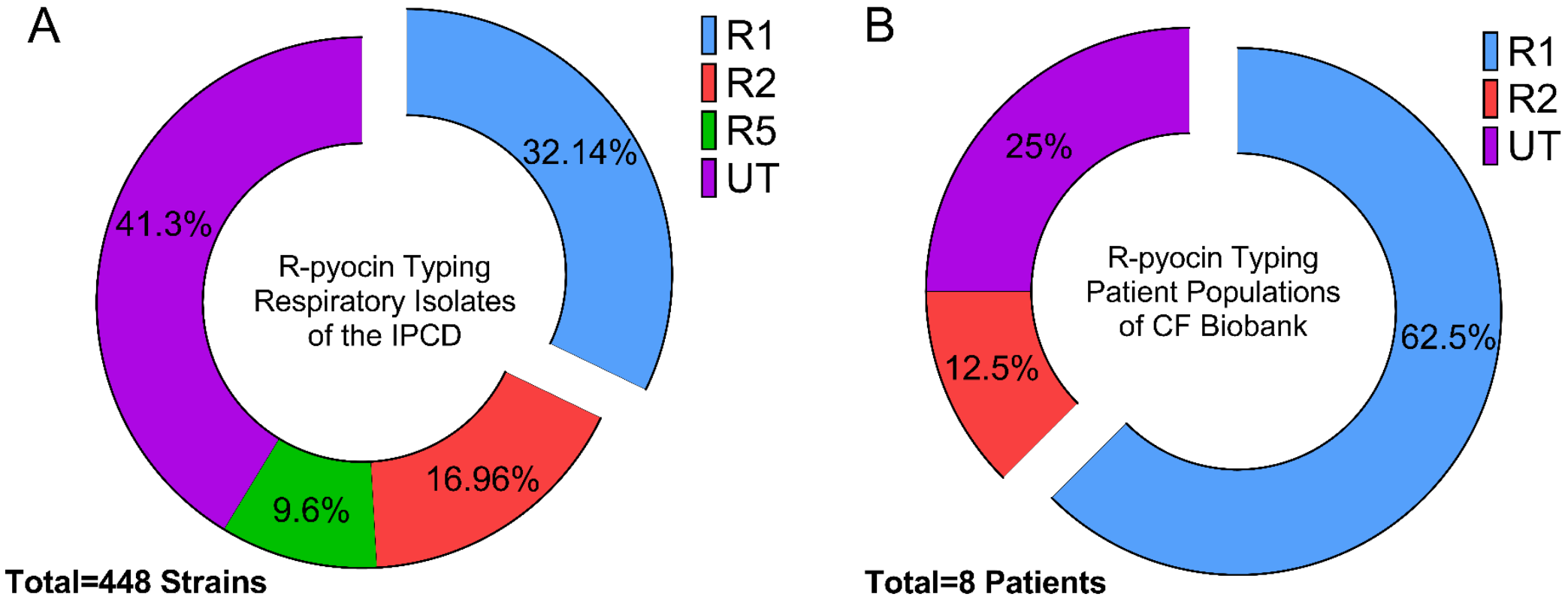

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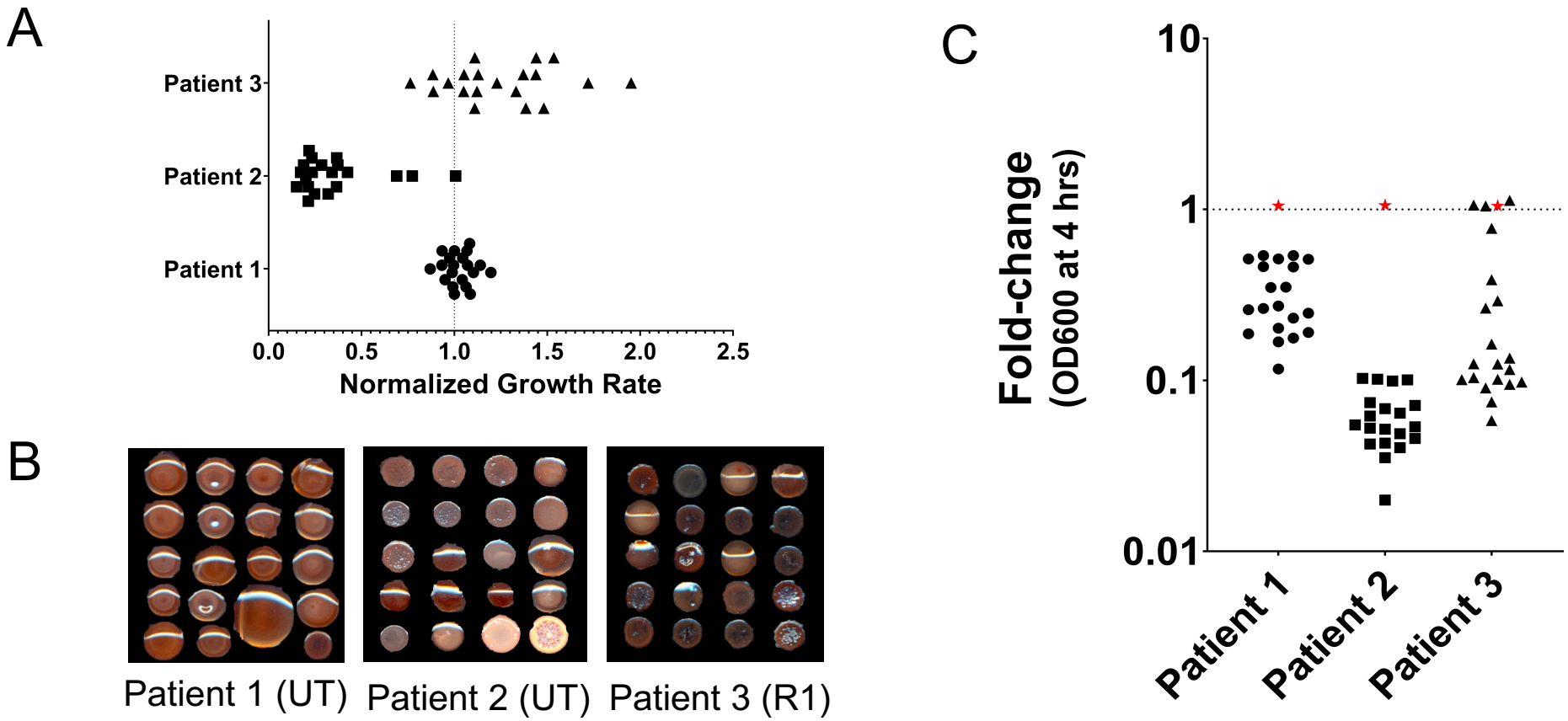

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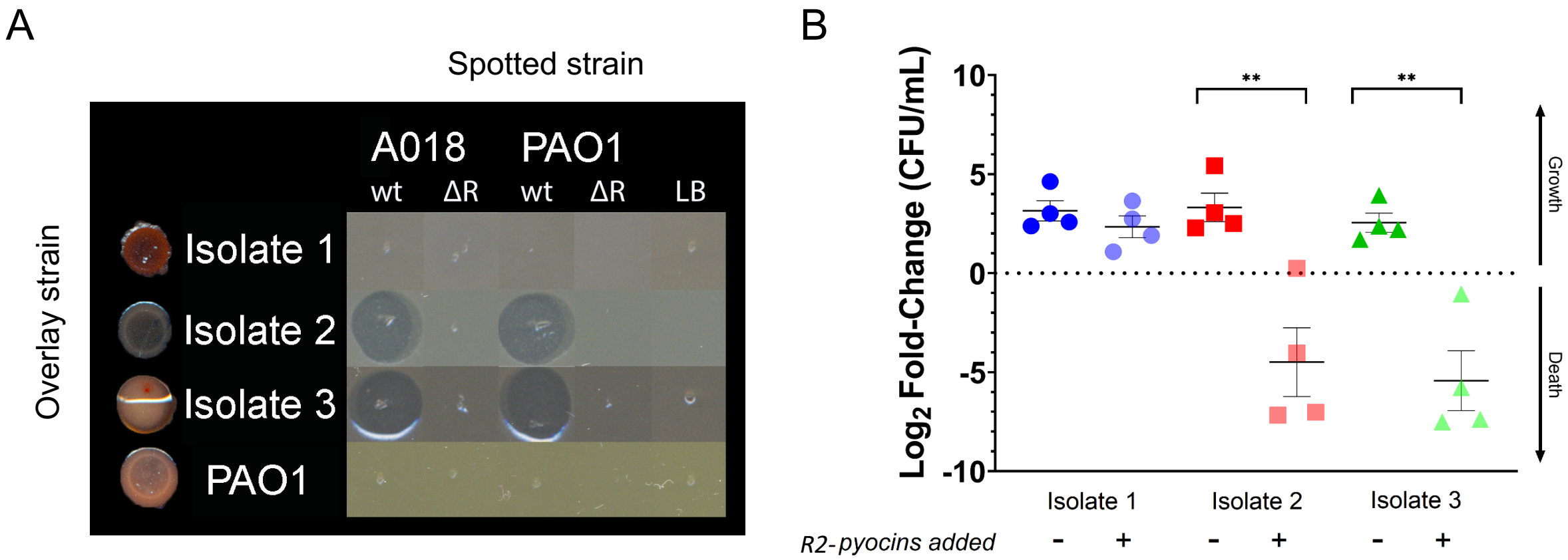

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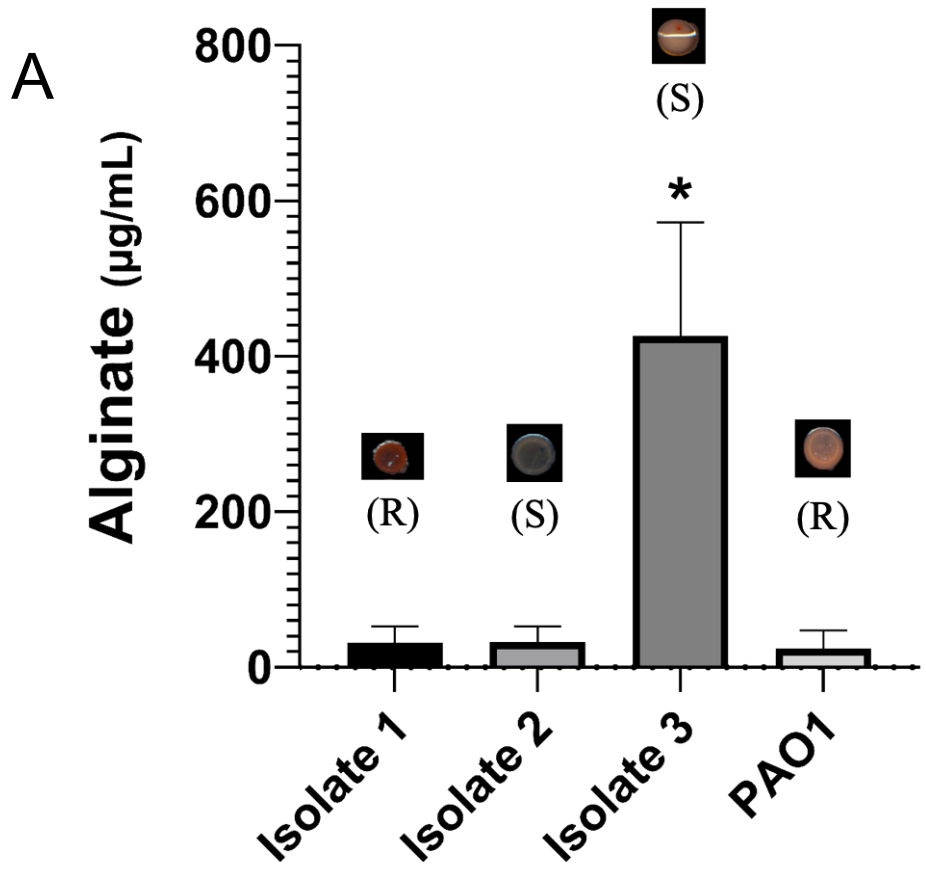

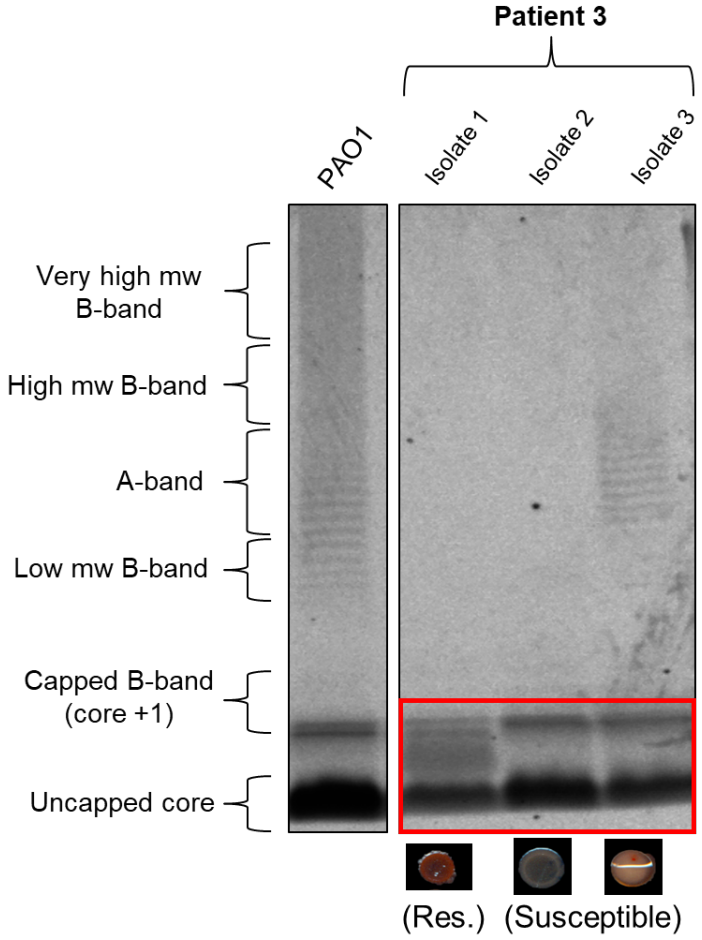

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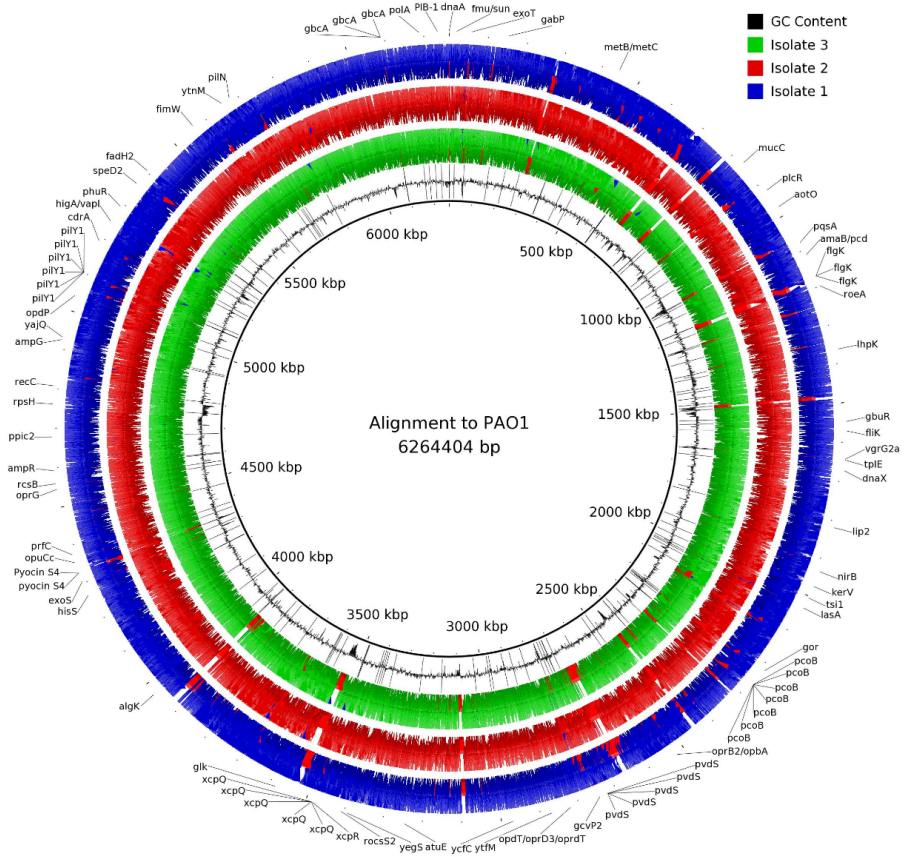

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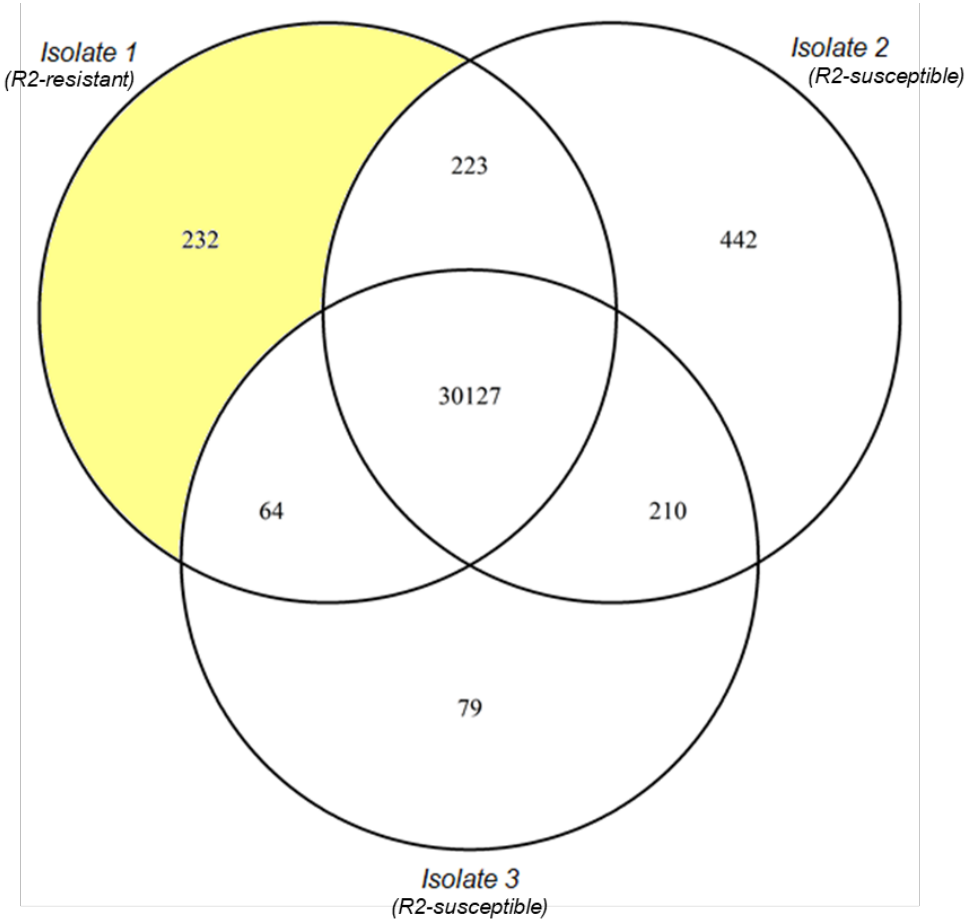

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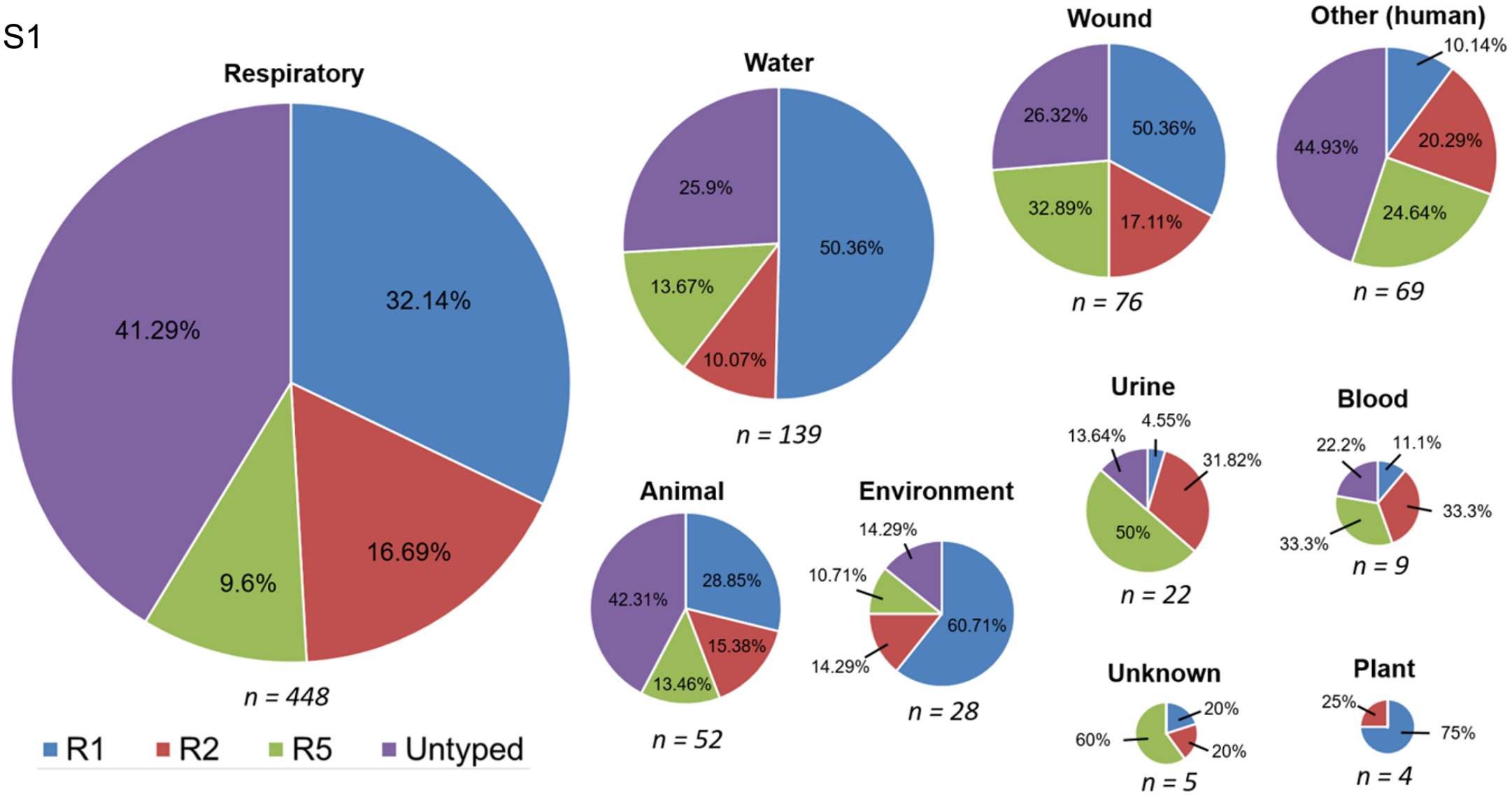

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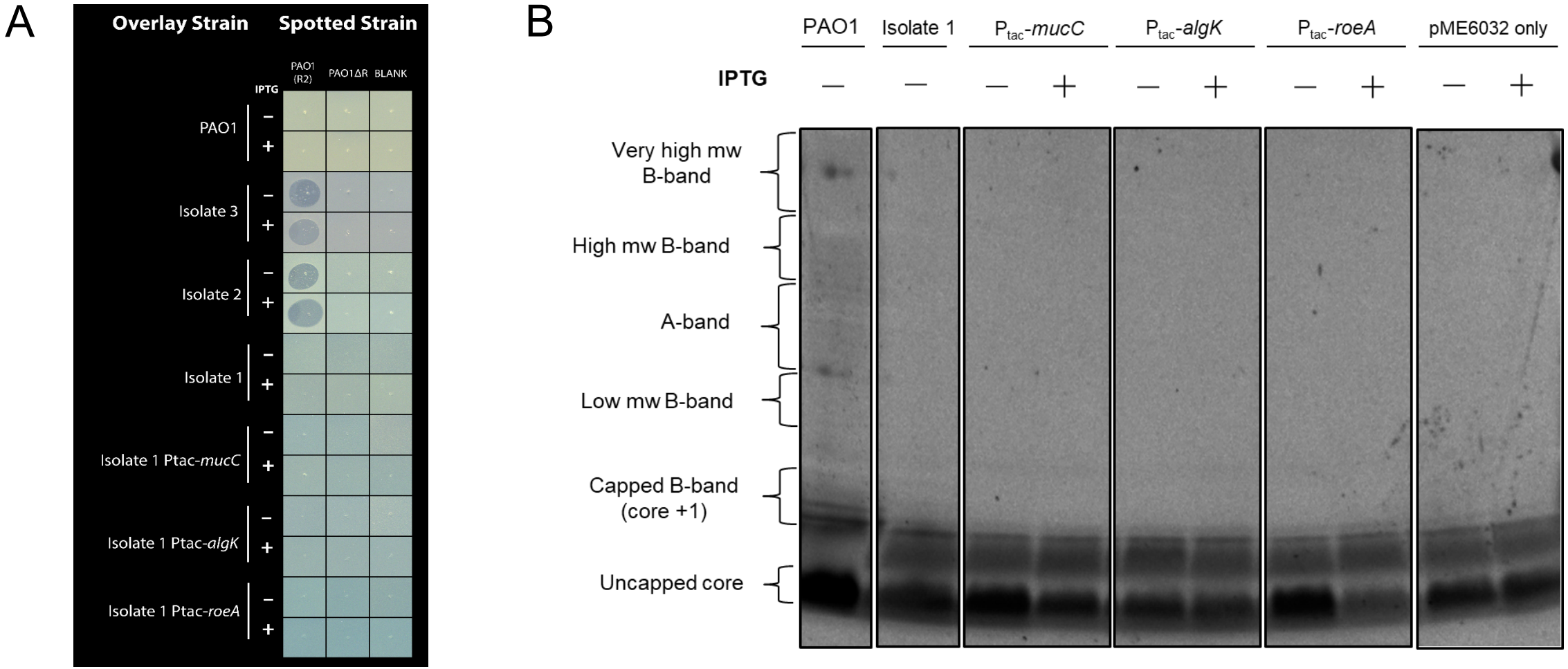

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