

1 **Alginate-regulating genes are identified in the clinical cystic fibrosis**
2 **isolate of *Pseudomonas aeruginosa* PA2192**

3

4 Brett Colbert¹, Hansi Kumari², Ana Piñon¹, Lior Frey¹,
5 Sundar Pandey¹, and Kalai Mathee^{2*}

6

7 ¹ Department of Biological Sciences, College of Arts and Sciences, Florida International
8 University, Miami, FL, USA

9 ² Department of Human and Molecular Genetics, Herbert Wertheim College of
10 Medicine, Miami, FL, USA

11

12 ***Corresponding Author:**

13 Kalai Mathee, Ph.D.

14 Department of Human and Molecular Genetics

15 Herbert Wertheim College of Medicine

16 Florida International University

17 Miami, FL 33199

18 Tel. No: 305 348 0628, Fax: 305 348 2913, Email: matheelabfor65roses@gmail.com

19

20 **Running Title:** Alginate Regulation in *Pseudomonas aeruginosa* PA2192

21

22 **Key Words:** Sigma factor, anti-sigma factor, cystic fibrosis

23

ABSTRACT

24 Cystic fibrosis (CF) is a genetic disorder that leads to a buildup of mucus in the
25 lungs ideal for bacterial colonization. When *Pseudomonas aeruginosa* enters the
26 CF lung, it undergoes a conversion from nonmucoid to mucoid; colonization by a
27 mucoid strain of *P. aeruginosa* greatly increases mortality. The mucoid
28 phenotype is due to the production of alginate. The regulator of alginate
29 production is the AlgT/U sigma factor. The observed phenotypic conversion is due
30 to a mutation in the *mucA* gene coding for an anti-sigma factor, MucA, which
31 sequesters AlgT/U. This mucoid phenotype is unstable when the strains are
32 removed from the lung as they acquire second-site mutations. This *in*
33 *vitro* reversion phenomenon is utilized to identify novel genes regulating alginate
34 production. Previously, second-site mutations were mapped to *algT/U*,
35 *algO*, and *mucP*, demonstrating their role in alginate regulation. Most of these
36 studies were performed using a non-CF isolate. It was hypothesized that second
37 site mutations in a clinical strain would be mapped to the same genes. In this
38 study, a clinical, hyper-mucoid *P. aeruginosa* strain PA2192 was used to study the
39 reversion phenomenon. This study found that PA2192 has a novel *mucA* mutation
40 which was named them *mucA180* allele. Twelve colonies were sub-cultured for
41 two weeks without aeration at room temperature in order to obtain nonmucoid
42 **suppressors of alginate production** (*sap*). Only 41 *sap* mutants were stable for
43 more than 48 hours — a reversion frequency of 3.9% as compared to ~90% in
44 laboratory strains showing that PA2192 has a stable mucoid phenotype. This

45 phenotype was restored in 28 of the 41 *sap* mutants when complemented with
46 plasmids harboring *algT/U*. Four of the *sap* mutants are complemented
47 by *algO*. Sequence analyses of the *algT/U* mutants have found no mutations in
48 the coding region or promoter leading to the hypothesis that there is another, as
49 yet unidentified mechanism of alginate regulation in this clinical strain.

50

INTRODUCTION

51 Cystic fibrosis (CF) is the most common cause of death due to genetic disorder
52 (1). CF is an autosomal recessive disorder caused by a mutation in the cystic
53 fibrosis transmembrane conductance regulator (*cftr*) gene (2-4). The most
54 common CF mutation (~90%) is the $\Delta F508$ which is the deletion of three
55 nucleotides leading to the loss of a phenylalanine in the 508 position, and thus, a
56 defective protein (3). A wide range of other mutations are possible that lead to
57 either impaired function or total loss of activity (5).

58 Ordinarily, CFTR functions as anion transporter (6). When mutated, normal anion
59 flow is restricted (7) and mucus accumulates in the CF lung; resulting pulmonary
60 failure is the foremost killer of CF patients (8, 9) The mucus buildup provides a
61 breeding ground for many pathogenic bacterial species especially *S. aureus*, *H.*
62 *influenzae*, *Pseudomonas aeruginosa*, and *Burkholderia cenocepacia* (10); the
63 relative population of each species fluctuates over the life of the individual (11).
64 The pathogen that rises to prominence over the life of a CF patient and is the
65 leading cause of mortality is *P. aeruginosa* (11, 12).

66 *P. aeruginosa* expresses a multitude of virulence factors (13). The major
67 contributor to *P. aeruginosa* virulence in patients with CF is its ability to change
68 from the standard, non-mucoid form to the mucoid form (14, 15). Mucoid *P.*
69 *aeruginosa* is considered highly virulent because patients show poor clinical
70 outcome despite having a heightened immune response (14, 15). The mucoid

71 phenotype is a result of the production of a complex polysaccharide called
72 alginate (16).

73 Alginate protects *P. aeruginosa* from phagocytosis, antibiotics, oxygen radicals,
74 and the host immune response (17-23) (Leid et al., 2005). The importance of
75 alginate in the virulence of *P. aeruginosa* has also been demonstrated in mouse
76 models (24, 25). In mice, an alginate-overproducing strain causes aggressive
77 polymorphonuclear leukocyte (PMN) infiltration- similar to human infection- and
78 causes inefficient pulmonary clearance. A protracted lung infection has the
79 potential to spread to other organs such as the spleen. These properties suggest
80 that alginate is an important virulence factor.

81 Alginate biosynthesis comes at a high metabolic cost, and thus is tightly regulated
82 (Figure 1) by an intricate system of periplasmic and inner membrane proteins (26-
83 29). The primary regulatory unit of alginate production is a five-gene operon
84 containing *algT/U-mucA-mucB-mucC-mucD* (30). The first gene of this operon,
85 *algT/U*, codes for a sigma factor able to bind to RNA polymerase (RNAP), guiding
86 it to transcribe the genes necessary for alginate production (31, 32).

87 Under normal circumstances, *P. aeruginosa* is non-mucoid, as is the case with the
88 prototypic reference strain, PAO1 (33). Upon colonizing the CF lung, *P. aeruginosa*
89 must confront the host immune system and antibiotics. The typical response is to
90 convert to a mucoid phenotype by producing alginate. This is commonly
91 accomplished by mutating *mucA* which codes for the anti-sigma factor to AlgT/U

92 (34). Ordinarily, MucA sequesters AlgT/U to the inner membrane, preventing it
93 from directing RNAP; however, when *mucA* is mutated, AlgT/U is left free to guide
94 RNAP (Figure 1) to transcribe the genes needed for alginate biosynthesis (27, 35).
95 The most common *mucA* mutation (~85%) found in clinical, mucoid strains of *P.*
96 *aeruginosa* is the *mucA22* allele which is the deletion of a single G in a string of
97 five Gs resulting in a frameshift mutation and premature stop codon (34, 35).

98 Since alginate production is metabolically expensive, mucoid strains revert to a
99 non-mucoid phenotype when isolated from the lung and cultured *in vitro* (Figure
100 2), especially when grown at low oxygen levels (36). The isolates maintain the
101 original *mucA* mutation but revert to Alg⁻ by mutating at another gene, a second
102 site crucial to alginate biosynthesis (32, 37). This has proven to be a highly
103 advantageous phenomenon when it comes to determining novel genes involved
104 in alginate regulation. Several studies have utilized this *in vitro* reversion
105 phenomenon to map second-site mutations to genes coding for the sigma factor
106 AlgT/U (31, 32, 37), a putative periplasmic protease AlgO (37, 38), and an inner
107 membrane protease MucP (Delgado *et al.*, submitted).

108 The study by DeVries *et al.*, (1994) used the mucoid CF isolate FRD1, but the great
109 caveat tied to many of these other studies is that they were carried out in a
110 laboratory-generated strain, PDO300 (22). This form is an isogenic derivative of
111 PAO1 with the addition of the *mucA22* allele in order to imitate clinical isolates

112 (22). The potential issue with using PDO300 is that laboratory strains cannot
113 faithfully mirror the real-world pathogenesis of a clinical isolate (39).

114 The present study was undertaken to map the location of second-site mutations
115 in a clinical isolate. The strain utilized is *P. aeruginosa* 2192 which was isolated from
116 a CF patient in Boston who passed away from the infection (40). *P. aeruginosa*
117 2192 produces about 60% more alginate than PDO300 (Delgado *et al.*, submitted)
118 and is far more stable in its mucoid phenotype (this study). We hypothesized that
119 the non-mucoid revertants of *P. aeruginosa* 2192 would harbor second-site
120 mutations in *algT/U*, *algO*, and *mucP* while maintaining the original *muca*
121 mutation. This would demonstrate the role of these genes in *P. aeruginosa* 2192
122 alginate regulation.

123

MATERIALS AND METHODS

124 **Bacterial Strains**

125 The *P. aeruginosa* and *Escherichia coli* strains used in this study are listed in
126 Table 1. The *E. coli* strains were grown on Luria-Bertani (LB) media supplemented
127 with tetracycline (Tc) at 20 µg/ml and ampicillin (Ap) at 50 µg/ml. *Pseudomonas*
128 *aeruginosa* strains were grown on LB or LB/PIA plates, which is a 1:1 mixture of LB
129 and *Pseudomonas* isolation agar. These were supplemented with Tc at 100 µg/ml
130 or carbenicillin (Cb) at 150 µg/ml when appropriate.

131 **Isolation of *sap* mutants**

132 The parent strain used to isolate **s**uppressor of **a**lginate **p**roduction (*sap*)
133 mutants was *P. aeruginosa* 2192, a hypermuroid, clinical CF isolate possessing a
134 *mucA* mutation (Mathee *et al.*, 2008, Delgado *et al.*, Submitted). *P. aeruginosa*
135 2192 was plated and grown overnight on LB/agar plates. Twelve muroid colonies
136 were selected and inoculated into separate tubes containing 5 ml of LB nutrient
137 broth. These were serially cultured at 25°C without aeration for two weeks.
138 Dilutions of each of the 12 cultures were plated daily for single colonies. The 127
139 *sap* mutant colonies were frozen in 1:1 culture/skim milk at -80°C for further analysis
140 (Table 1). At the end of two weeks, all *sap* mutants were re-streaked on to LB/PIA
141 plates and incubated at 37°C for 24 hours followed by 25°C for 24 more hours.
142 Only the 41 *sap* mutants that maintained a non-muroid phenotype after 48 hours
143 during this secondary screening were used in subsequent analyses.

144 **Complementation assays**

145 Complementation of the *sap* mutants was accomplished by a modified tri-
146 parental mating protocol (41) developed during this study. The donor *E. coli* strain
147 containing the plasmid of interest was crossed on an LB plate with the two helper
148 *E. coli* strains, pRK600 and pRK2013 (Table 1), and the recipient, in this case, the
149 *sap* mutants. The following day, the mating conglomeration was homogenized in
150 LB broth, diluted, and plated on selective media (LB/PIA supplemented by Tc 100
151 µg/ml or Cb 150 µg/ml). Colonies were checked for a mucoid or non-mucoid
152 phenotype at 24 and 48 hours. Each of the *sap* mutants was complemented with
153 pCD100 (Tc resistant) which contains the *algT/U-mucA22-mucB-mucC-mucD*
154 operon (37). Those that saw a reversion to a mucoid phenotype were then
155 complemented by pJG293 (Tc resistant) which contains *algT/U* alone (37). The
156 remaining *sap* mutants that were not complemented by *algT/U* were conjugated
157 with a plasmid containing *algO* (pAlgO), and another harboring *mucP* (pMucP).

158 **Genomic DNA isolation**

159 Genomic DNA was isolated from each of the *sap* mutants following a standard
160 phenol-chloroform protocol (42). Briefly, 1 ml of an overnight culture was pelleted
161 and mixed with lysozyme and proteinase K. After incubation at 37°C for 30
162 minutes, a 1:1 mixture of phenol:chloroform was added and vortexed to
163 homogeneity. After centrifugation, the top layer containing the genomic DNA
164 was removed and mixed with ethanol to precipitate the DNA and pelleted by

165 centrifugation at 16,000 xg for two minutes. The ethanol was decanted and the
166 DNA was resuspended in water and stored at -20°C.

167 **PCR amplification**

168 Primers for polymerase chain reaction (PCR) were designed for *algT/U*, *mucA*,
169 *algO*, and *mucP* (Table 2; Integrated DNA Technologies Inc., Coralville, IA). Each
170 set of primers was designed to fall about 100 bp up and downstream of the gene
171 so that subsequent DNA sequencing would not cut off the beginning and end of
172 each gene.

173 The *mucA* gene of each of the *sap* mutants as well as the gene identified in the
174 complementation assay as containing a potential mutation were PCR amplified
175 for sequencing. Two and a half microliters of genomic DNA was mixed with 1 µl of
176 each primer (Table 2), 5 µl of bufferxII, 0.5 µl of HiFi Taq polymerase (Invitrogen,
177 Carlsbad, CA) and the volume was made up to 50 µl with water. The mixture was
178 amplified in the thermocycler with the following program: 95°C for 5 min; 95°C 30
179 sec, 61°C for 30 sec, 72°C for a time dependent on the amplicon size (1 min/kb)
180 repeated for 30 cycles; 72°C for 10 min; hold at 4°C for further analysis. Products
181 were run on a 2% agarose gel to verify amplification. PCR products were then
182 cleaned according to a kit and standard protocol (Promega, Madison, WI).

183

184

185 **DNA sequencing**

186 Samples were sent to GeneWiz Inc. (Plainsfield, NJ) for sequencing. The *mucA*
187 gene and the complementing genes of each *sap* mutants were sequenced.
188 Samples were prepared according to the company's requirements. Each tube
189 sent out was premixed with 5 µl of 5 µM primer (forward and reverse separately)
190 and 40 ng of the PCR product.

191 **Sequence analysis**

192 Sequences were aligned using NCBI Blast, ClustalΩ, T-Coffee, LaserGene
193 software, and Boxshade against the *P. aeruginosa* 2192 wildtype sequence (43,
194 44) to check for mutations (45-48).

195

196

RESULTS

197

198 **Isolation of *sap* mutants**

199 The clinical isolate *P. aeruginosa* 2192 was grown for two weeks at 25°C without
200 aeration. A total of 1058 colonies were analyzed during that period. Of these, 127
201 (12%) were nonmucoid and considered *sap* mutants. These were then plated on
202 PIA media and allowed to grow for 48 hours to verify stability. Only 41 (3.8%)
203 retained the Alg⁻ phenotype. Of these, 39 *sap* mutants produce alginate when
204 the cells are at high density on a plate and they remain completely nonmucoid
205 when they are single colonies. Two, *sap8* and *sap20*, remain completely
206 nonmucoid indefinitely, whether in a dense community or single colony.

207 **Complementation assays**

208 Each of the *sap* mutants was complemented with genes previously identified as
209 common second-site mutations (32, 37). Twenty-eight *sap* mutants (68%) were
210 complemented by pCD100 containing the whole *algT/U* operon. These were also
211 complemented by pJG293 containing *algT/U* alone (Table 1).

212 The mucoid phenotype was restored in 11 *sap* mutants (17%) when
213 complemented by *mucP*. Similarly, *algO* successfully complemented four (10%)
214 of the mutants that were also complemented by *mucP*.

215 Two *sap* mutants, *sap8* and *sap20*, were not complemented by any of the
216 previously identified genes. When pCD100 (37) was introduced, the two mutants

217 failed to grow. In the presence of pJG293 (37), the strains grew and were
218 nonmucoid.

219 **Sequencing *mucA***

220 *P. aeruginosa* 2192 contains a mutation in the anti-sigma factor *mucA* which
221 results in constitutive alginate production. To determine the exact mutation, the
222 *mucA* of *P. aeruginosa* 2192 was aligned with PAO1, the common reference strain
223 which has no mutation, and PDO300, the laboratory-generated strain containing
224 a *mucA22* allele. *P. aeruginosa* 2192 was seen to have A343G resulting in a silent
225 mutation, and G539T leading to a stop codon at the 180th position of the protein
226 (Figure 4). The *mucA* genes of 10 of the *sap* mutants were aligned to *P.*
227 *aeruginosa* 2192 and shown to possess the original mutations (Figure 4).

228 **Analysis of *algT/U*, *algO*, *mucP* in *P. aeruginosa* 2192 and *sap* mutants**

229 ***algT/U*:** Alignment has shown that the *algT/U* ORF and promoter sequence is
230 conserved between PAO1 and *P. aeruginosa* 2192 (data not shown). The *sap*
231 mutants are also mutation free.

232 ***algO*:** The *P. aeruginosa* 2192 *algO* sequence shows nine SNPs which all result in
233 silent mutations. The *sap* mutants are yet to be sequenced.

234 ***mucP*:** *mucP* of *P. aeruginosa* 2192 has two SNPs relative to PAO1; one relays a
235 silent mutation, and the other a change of an alanine to a valine in the 313. This
236 change is to an amino acid of similar functional group, and thus it is hypothesized

237 that the function is conserved. The *sap* mutants have not been sequenced as of
238 yet.

239

240
241
242
243
244
245
246
247
248
249
250

251

252
253
254
255
256

257
258
259
260
261

DISCUSSION

Laboratory generated strains have been immensely useful in scientific research and have driven the depth of our knowledge to where it is today. However, laboratory strains, such as PDO300, will always fall short of perfectly mimicking the real-world pathogenesis of clinical isolates (39). This project has certainly confirmed the importance of utilizing a strain isolated directly from the lungs of a patient who passed away from the infection. It has also made the research eminently personal. The present study was designed to confirm the conclusions about alginate regulation drawn from studies using PDO300, as well as investigate the novelty and peculiarity of *P. aeruginosa* 2192.

***P. aeruginosa* 2912 shows a hyperstable mucoid phenotype**

Non-mucoid variants of *P. aeruginosa* 2192 were isolated in the same way as previous studies that utilized PDO300 as the parent strain (37). Studies using PDO300 saw a 90% reversion to *sap* mutants after just 48 hours of culturing at 25°C without aeration (37). In contrast, PA2192 took two weeks under the same conditions to yield even a 3.8% reversion that could be utilized in further analyses. This extended time needed to isolate non-mucoid variants is unique to *P. aeruginosa* 2192 when compared with another clinical strain as well. One study obtained mutants from FRD1, a CF isolate, in 24 hours under the same conditions (32). When compared with the PDO300 and FRD1 studies, *P. aeruginosa* 2192 has a hyperstable mucoid phenotype since it took seven and fourteen times longer

262 before any non-mucoid colony was isolated. It remains to be seen what the
263 contributing factors to this hyperstability are, including the chemical makeup of
264 *P. aeruginosa* 2192 alginate when compared with PDO300. It is interesting to
265 speculate that the hyperstability is directly related to the clinical virulence of *P.*
266 *aeruginosa* 2192.

267 ***P. aeruginosa* 2192 possesses a novel *mucA* mutation**

268 Sequence analysis of *mucA* shows that PA2192 does not have the common
269 *mucA22* allele (22) (Figure 4). Instead, it has a previously undocumented *mucA*
270 mutation, which has been named the *mucA180* allele. Alginate production is
271 frequently accomplished in clinical isolates by mutating the *mucA* anti-sigma
272 factor (34). The most common mutation is the *mucA22* allele possessed by ~85%
273 of mucoid *P. aeruginosa* strains (34, 35). It was in this light that PDO300 was
274 constructed from PAO1 with the *mucA22* allele to imitate clinical, mucoid strains
275 (22). The majority of *mucA* mutations are toward the 3' end of the sequence (24,
276 49, 50). This results in an altered or truncated C-terminus of MucA that is the end
277 that protrudes from the inner membrane into the periplasm (Figure 4) and
278 interacts with MucB; as a result, MucB binding is reduced or inhibited altogether
279 (50-52). Without MucB binding, MucA is destabilized, and the AlgT/U sigma factor
280 is released resulting in alginate production (50).

281 It is hypothesized that MucA180 also has a reduced interaction with MucB due to
282 protein truncation. Further experimentation with a yeast two-hybrid system is

283 required to demonstrate this. It could be accomplished by designing a MucA180
284 bait protein and a MucB fish protein. When compared to wildtype MucA, the
285 MucA180 two-hybrid system should show reduced transcription of the reporter
286 gene.

287 ***P. aeruginosa* 2192 *algT/U* shows no mutation**

288 The *mucA* sequence alignment of the non-mucoid variants revealed that they
289 maintain the *mucA180* allele. Thus, the loss of alginate production is not the result
290 of a true reversion that has restored the function of MucA, but is due to a second-
291 site mutation, as was hypothesized.

292 Complementation assays have shown that a majority (68%) of the second-site
293 mutations may be mapped to *algT/U*. Interestingly, no mutation was detected in
294 the *algT/U* open reading frame (ORF) or in the promoter region upstream of the
295 ORF.

296 This suggests that there may be a novel mechanism of alginate regulation in *P.*
297 *aeruginosa* 2192 that is bypassed when AlgT/U is overexpressed. Further studies
298 will investigate this possibility. This does not rule out the possibility that this set of
299 revertants may harbor an entirely novel mutation.

300 ***mucP* and *algO* are involved in *P. aeruginosa* 2192 alginate regulation**

301 This study also found that *mucP* was able to restore the Alg⁺ phenotype of 17% of
302 the non-mucoid variants, indicating that second-site mutations in *mucP* were the

303 second most common mode of alginate repression in *P. aeruginosa* 2192. The
304 third most common second-site mutation in alginate suppression is in *algO* (10%).
305 Moreover, all the non-mucoid variants that were complemented by *algO* were
306 also complemented by *mucP*, suggesting that *algO* mutations can be bypassed
307 in *P. aeruginosa* 2192 by increasing the *mucP* copy number as previously
308 demonstrated (Delgado *et al.*, 2018, Submitted). The exact function of AlgO has
309 not been elaborated as yet but presumed to be a periplasmic protease (37). The
310 *mucP* and *algO* genes in the *sap* mutants are being sequenced to confirm the
311 mutations and find the exact region of the proteins that is mutated.

312 **Uncomplemented non-mucoid variants possess novel second-site mutations**

313 Two of the non-mucoid variants of *P. aeruginosa* 2192 (*sap2192-8* and *sap2192-*
314 *20*; Table 1) were not complemented by any of the genes previously identified as
315 liable to second-site mutations. Interestingly, these two also remain completely
316 nonmucoid indefinitely while the others begin producing alginate after 48 hours.
317 This indicates that these possess mutations in one or more novel genes that
318 previously have not been identified as involved in alginate regulation. The *mucA*
319 of these mutants did not possess true reversions and restoration of function. The
320 mutants will be complemented with a previously constructed *P. aeruginosa*
321 cosmid library (37) to identify any novel mutations elsewhere on the chromosome.
322 These two mutants prove fatal when the *algT/U* operon was introduced on
323 pCD100. It is hypothesized that either these two do not take up the plasmid for

324 some reason, and thus are killed on the selective media, or the novel mutation,
325 which is yet to be identified, will be able to explain this highly unusual
326 phenomenon.

327 **Conclusion**

328 Care for CF patients over the last thirty years has dramatically improved. Life
329 expectancy has risen from 18 years in the 1980's to nearly 50 today (9). *P.*
330 *aeruginosa*, a ubiquitous bacterium, is devastatingly efficient as a CF pathogen
331 (53). The most indicative factor pointing towards poor patient outcome is the
332 production of alginate by the colonizing strain (54). As of yet, there is no effective
333 anti-alginate therapy. Understanding all variables of *P. aeruginosa* alginate
334 regulation and synthesis is inseparable from combating this deadly pathogen.
335 The present study sought to contribute to this end by investigating regulatory
336 genes involved in the mucoid to non-mucoid reversion of the clinical strain *P.*
337 *aeruginosa* 2192

338 **Acknowledgements**

339 We thank members of the Mathee lab for their valuable insights. This research was
340 supported by NIH-National Institute of Allergy and Infectious Diseases (NIAID)
341 1R15AI111210 (to KM and HK), and NIH-National Institute of General Medical
342 Sciences (NIGMS) T34 GM08368 (to LF). The funders had no role in study design,

343 data collection and analysis, decision to publish, or preparation of the
344 manuscript.

345 **Conflicts of interest**

346 There are no conflicts of interest.

347

348 **Ethical Statement**

349 Not applicable.

350
351
352
353
354
355
356
357
358
359
360
361
362
363
364
365
366
367
368
369
370
371
372
373
374

Bibliography

1. **FitzSimmons SC**. The changing epidemiology of cystic fibrosis. *The Journal of pediatrics*. 1993;122(1):1-9.
2. **Rommens JM, Iannuzzi MC, Kerem B, Drumm ML, Melmer G, Dean M, et al**. Identification of the cystic fibrosis gene: chromosome walking and jumping. *Science*. 1989;245(4922):1059-65.
3. **Kerem B**. Identification of the cystic fibrosis gene: genetic analysis. *Trends in Genetics*. 1989;5:363.
4. **Riordan JR, Rommens JM, Kerem B-s, Alon N, Rozmahel R**. Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science*. 1989;245(4922):1066-10722.
5. **Tsui LC**. Mutations and sequence variations detected in the cystic fibrosis transmembrane conductance regulator (CFTR) gene: a report from the Cystic Fibrosis Genetic Analysis Consortium. *Human Mutation*. 1992;1(3):197-203.
6. **Anderson MP, Gregory RJ**. Demonstration that CFTR is a chloride channel by alteration of its anion selectivity. *Science*. 1991;253(5016):202.
7. **Gadsby DC, Vergani P, Csanády L**. The ABC protein turned chloride channel whose failure causes cystic fibrosis. *Nature*. 2006;440(7083):477-83.
8. **Boat TF, Cheng PW, Iyer RN, Carlson DM, Polony I**. Human respiratory tract secretions: mucous glycoproteins of nonpurulent tracheobronchial secretions, and sputum of patients with bronchitis and cystic fibrosis. *Archives of Biochemistry and Biophysics*. 1976;177(1):95-104.
9. **CFFoundation**. Cystic Fibrosis Foundation Patient Registry. 2015:1-92.
10. **Govan J, Harris G**. *Pseudomonas aeruginosa* and cystic fibrosis: unusual bacterial adaptation and pathogenesis. *Microbiological Sciences*. 1986;3(10):302-6.

- 375 11. **Sibley CD, Rabin H, Surette MG.** Cystic fibrosis: a polymicrobial infectious disease. Future
376 Microbiology. 2006;1(1):53-61.
- 377 12. **Pedersen SS.** Lung infection with alginate-producing, mucoid *Pseudomonas aeruginosa* in
378 cystic fibrosis. Acta Pathologica, Microbiologica et Immunologica Scandinavica Supplementum.
379 1991;28:1-79.
- 380 13. **Moradali MF, Ghods S, Rehm BH.** *Pseudomonas aeruginosa* lifestyle: A paradigm for
381 adaptation, survival, and persistence. Frontiers in Cellular and Infection Microbiology.
382 2017(7):39-45.
- 383 14. **Pedersen S, Højby N, Espersen F, Koch C.** Role of alginate in infection with mucoid
384 *Pseudomonas aeruginosa* in cystic fibrosis. Thorax. 1992;47(1):6-13.
- 385 15. **Højby N.** *Pseudomonas aeruginosa* infection in cystic fibrosis. Relationship between mucoid
386 strains of *Pseudomonas aeruginosa* and the humoral immune response. Acta Pathologica,
387 Microbiologica, et Immunologica Scandinavica. 1974;82(4):551-8.
- 388 16. **Evans LR, Linker A.** Production and characterization of the slime polysaccharide of
389 *Pseudomonas aeruginosa*. Journal of Bacteriology. 1973;116(2):915-24.
- 390 17. **Schwarzmann S, Boring JR.** Antiphagocytic effect of slime from a mucoid strain of
391 *Pseudomonas aeruginosa*. Infection and immunity. 1971;3(6):762-7.
- 392 18. **Govan J, Fyfe JA.** Mucoid *Pseudomonas aeruginosa* and cystic fibrosis: resistance of the
393 mucoid form to carbenicillin, flucloxacillin and tobramycin and the isolation of mucoid variants
394 *in vitro*. Journal of Antimicrobial Chemotherapy. 1978;4(3):233-40.
- 395 19. **Kulczycki LL, Murphy T, Bellanti JA.** *Pseudomonas* colonization in cystic fibrosis. Jama.
396 1978;240:30-4.
- 397 20. **Oliver A, Weir D.** Inhibition of bacterial binding to mouse macrophages by *Pseudomonas*
398 alginate. Journal of clinical & laboratory immunology. 1983;10(4):221-4.

- 399 21. **Hodges NA, Gordon CA.** Protection of *Pseudomonas aeruginosa* against ciprofloxacin and
400 beta-lactams by homologous alginate. *Antimicrobial Agents and Chemotherapy*.
401 1991;35(11):2450-2.
- 402 22. **Mathee K, Ciofu O, Sternberg C, Lindum PW, Campbell JI, Jensen P, et al.** Mucoid
403 conversion of *Pseudomonas aeruginosa* by hydrogen peroxide: a mechanism for virulence
404 activation in the cystic fibrosis lung. *Microbiology*. 1999;145(6):1349-57.
- 405 23. **Leid JG, Willson CJ, Shirliff ME, Hassett DJ, Parsek MR, Jeffers AK.** The
406 exopolysaccharide alginate protects *Pseudomonas aeruginosa* biofilm bacteria from IFN- γ -
407 mediated macrophage killing. *The Journal of Immunology*. 2005;175(11):7512-8.
- 408 24. **Boucher J, Yu H, Mudd M, Deretic V.** Mucoid *Pseudomonas aeruginosa* in cystic fibrosis:
409 characterization of muc mutations in clinical isolates and analysis of clearance in a mouse model
410 of respiratory infection. *Infection and Immunity*. 1997;65(9):3838-46.
- 411 25. **Bragonzi A, Worlitzsch D, Pier GB, Timpert P, Ulrich M, Hentzer M, et al.** Nonmucoid
412 *Pseudomonas aeruginosa* expresses alginate in the lungs of patients with cystic fibrosis and in a
413 mouse model. *The Journal of Infectious Diseases*. 2005;192(3):410-9.
- 414 26. **Mathee K, Kharazmi A, Høiby N.** Role of exopolysaccharide in biofilm matrix formation:
415 the alginate paradigm. *Molecular Ecology of Biofilms*. 2002:1-34.
- 416 27. **Pandey S, Martins KL, Mathee K.** Posttranslational regulation of antisigma factors of RpoE:
417 a comparison between the *Escherichia coli* and *Pseudomonas aeruginosa* systems. *Stress and*
418 *Environmental Regulation of Gene Expression and Adaptation in Bacteria*. 2016:361-7.
- 419 28. **Franklin MJ, Nivens DE, Weadge JT, Howell PL.** Biosynthesis of the *Pseudomonas*
420 *aeruginosa* extracellular polysaccharides, alginate, Pel, and Psl. *Pseudomonas aeruginosa,*
421 *Biology, Genetics, and Host-pathogen Interactions*. 2011(1):49-60.
- 422 29. **Damron FH, Goldberg JB.** Proteolytic regulation of alginate overproduction in *Pseudomonas*
423 *aeruginosa*. *Molecular Microbiology*. 2012;84(4):595-607.

- 424 30. **Chitnis CE, Ohman DE.** Genetic analysis of the alginate biosynthetic gene cluster of
425 *Pseudomonas aeruginosa* shows evidence of an operonic structure. *Molecular Microbiology*.
426 1993;8(3):583-90.
- 427 31. **Deretic V, Schurr M, Boucher J, Martin D.** Conversion of *Pseudomonas aeruginosa* to
428 mucoidy in cystic fibrosis: environmental stress and regulation of bacterial virulence by alternative
429 sigma factors. *Journal of Bacteriology*. 1994;176(10):2773-8.
- 430 32. **DeVries CA, Ohman DE.** Mucooid-to-nonmucooid conversion in alginate-producing
431 *Pseudomonas aeruginosa* often results from spontaneous mutations in *algT*, encoding a putative
432 alternate sigma factor, and shows evidence for autoregulation. *Journal of Bacteriology*.
433 1994;176(21):6677-87.
- 434 33. **Holloway B, Morgan A.** Genome organization in *Pseudomonas*. *Annual Reviews in*
435 *Microbiology*. 1986;40(1):79-105.
- 436 34. **Martin D, Schurr M, Mudd M, Govan J, Holloway B, Deretic V.** Mechanism of conversion
437 to mucoidy in *Pseudomonas aeruginosa* infecting cystic fibrosis patients. *Proceedings of the*
438 *National Academy of Sciences*. 1993;90(18):8377-81.
- 439 35. **Schurr M, Yu H, Martinez-Salazar J, Boucher J, Deretic V.** Control of AlgU, a member of
440 the sigma E-like family of stress sigma factors, by the negative regulators MucA and MucB and
441 *Pseudomonas aeruginosa* conversion to mucoidy in cystic fibrosis. *Journal of Bacteriology*.
442 1996;178(16):4997-5004.
- 443 36. **Ohman DE, Chakrabarty AM.** Genetic mapping of chromosomal determinants for the
444 production of the exopolysaccharide alginate in a *Pseudomonas aeruginosa* cystic fibrosis isolate.
445 *Infection and Immunity*. 1981;33(1):142-8.
- 446 37. **Sautter R, Ramos D, Schneper L, Ciofu O, Wassermann T, Koh C-L, et al.** A complex
447 multilevel attack on *Pseudomonas aeruginosa* *algT/U* expression and *algT/U* activity results in the
448 loss of alginate production. *Gene*. 2012;498(2):242-53.
- 449 38. **Reiling S, Jansen J, Henley B, Singh S, Chattin C, Chandler M, et al.** Prc protease promotes
450 mucoidy in *mucA* mutants of *Pseudomonas aeruginosa*. *Microbiology*. 2005;151(7):2251-61.

- 451 39. **Fux C, Shirtliff M, Stoodley P, Costerton JW.** Can laboratory reference strains mirror ‘real-
452 world’ pathogenesis? *Trends in Microbiology*. 2005;13(2):58-63.
- 453 40. **Mathee K, Narasimhan G, Valdes C, Qiu X, Matewish JM, Koehrsen M, et al.** Dynamics
454 of *Pseudomonas aeruginosa* genome evolution. *Proceedings of the National Academy of Sciences*.
455 2008;105(8):3100-5.
- 456 41. **Walkerpeach CR, Velten J.** Agrobacterium-mediated gene transfer to plant cells: cointegrate
457 and binary vector systems. *Plant Molecular Biology Manual*. 1994(1):33-51.
- 458 42. **Wilson K.** Preparation of genomic DNA from bacteria. *Current Protocols in Molecular*
459 *Biology*. 1987(24):1-5.
- 460 43. Database TPG. The *Pseudomonas* Genome Database - Genome annotation and comparative
461 genome analysis 2017 [Available from: <http://pseudomonas.com/>].
- 462 44. **Winsor GL, Griffiths EJ, Lo R, Dhillon BK, Shay JA, Brinkman FS.** Enhanced annotations
463 and features for comparing thousands of *Pseudomonas* genomes in the *Pseudomonas* genome
464 database. *Nucleic Acids Research*. 2016;44(D1):D646-D53.
- 465 45. NCBI. BLAST: Basic Local Alignment Search Tool 2017 [Available from:
466 <https://www.ncbi.nlm.nih.gov/pubmed/>].
- 467 46. Clustal_Ω. Clustal Omega 2017 [Available from: <http://www.ebi.ac.uk/Tools/msa/clustalo/>].
- 468 47. T-Coffee. T-Coffee Server 2017 [Available from:
469 <http://tcoffee.crg.cat/apps/tcoffee/do:mcoffee>].
- 470 48. BoxShade. BoxShade Server 2017 [Available from:
471 http://www.ch.embnet.org/software/BOX_form.html].
- 472 49. **Pulcrano G, Iula DV, Raia V, Rossano F, Catania MR.** Different mutations in *mucA* gene
473 of *Pseudomonas aeruginosa* mucoid strains in cystic fibrosis patients and their effect on *algU* gene
474 expression. *New Microbiologica*. 2012;35(3):295-305.

- 475 50. **Rowen D, Deretic V.** Membrane to cytosol redistribution of ECF sigma factor AlgU and
476 conversion to mucoidy in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients.
477 *Molecular Microbiology*. 2000;36(2):314-27.
- 478 51. **Goldberg JB, Gorman W, Flynn J, Ohman D.** A mutation in *algN* permits trans activation
479 of alginate production by *algT* in *Pseudomonas* species. *Journal of Bacteriology*.
480 1993;175(5):1303-8.
- 481 52. **Mathee K, McPherson CJ, Ohman DE.** Posttranslational control of the *algT* (*algU*)-encoded
482 sigma²² for expression of the alginate regulon in *Pseudomonas aeruginosa* and localization of its
483 antagonist proteins MucA and MucB (AlgN). *Journal of Bacteriology*. 1997;179(11):3711-20.
- 484 53. **Lyczak JB, Cannon CL, Pier GB.** Establishment of *Pseudomonas aeruginosa* infection:
485 lessons from a versatile opportunist. *Microbes and Infection*. 2000;2(9):1051-60.
- 486 54. **Henry RL, Mellis CM, Petrovic L.** Mucoid *Pseudomonas aeruginosa* is a marker of poor
487 survival in cystic fibrosis. *Pediatric Pulmonology*. 1992;12(3):158-61.
- 488
- 489

490 **Tables**

491 **Table 1:** Strains and primers generated and used in this study.

Strain	Genotype	Phenotype	Source
<i>P. aeruginosa</i>			
sap2192-1	<i>mucA180</i>	Alg ⁻	BC01; This study
sap2192-2	<i>mucA180</i>	Alg ⁻	BC02; This study
sap2192-3	<i>mucA180</i>	Alg ⁻	BC03; This study
sap2192-4	<i>mucA180</i>	Alg ⁻	BC04; This study
sap2192-5	<i>mucA180</i>	Alg ⁻	BC05; This study
sap2192-6	<i>mucA180</i>	Alg ⁻	BC06; This study
sap2192-7	<i>mucA180</i>	Alg ⁻	BC07; This study
sap2192-8	<i>mucA180</i>	Alg ⁻	BC08; This study
sap2192-9	<i>mucA180</i>	Alg ⁻	BC09; This study
sap2192-10	<i>mucA180</i>	Alg ⁻	BC10; This study
sap2192-11	<i>mucA180</i>	Alg ⁻	BC11; This study
sap2192-12	<i>mucA180</i>	Alg ⁻	BC12; This study
sap2192-13	<i>mucA180</i>	Alg ⁻	BC13; This study
sap2192-14	<i>mucA180</i>	Alg ⁻	BC14; This study
sap2192-15	<i>mucA180</i>	Alg ⁻	BC15; This study
sap2192-16	<i>mucA180</i>	Alg ⁻	BC16; This study
sap2192-17	<i>mucA180</i>	Alg ⁻	BC17; This study
sap2192-18	<i>mucA180</i>	Alg ⁻	BC18; This study
sap2192-19	<i>mucA180</i>	Alg ⁻	BC19; This study
sap2192-20	<i>mucA180</i>	Alg ⁻	BC20; This study
sap2192-21	<i>mucA180</i>	Alg ⁻	BC21; This study
sap2192-22	<i>mucA180</i>	Alg ⁻	BC22; This study
sap2192-23	<i>mucA180</i>	Alg ⁻	BC23; This study
sap2192-24	<i>mucA180</i>	Alg ⁻	BC24; This study
sap2192-25	<i>mucA180</i>	Alg ⁻	BC25; This study

sap2192-26	<i>mucA180</i>	Alg ⁻	BC26; This study
sap2192-27	<i>mucA180</i>	Alg ⁻	BC27; This study
sap2192-28	<i>mucA180</i>	Alg ⁻	BC28; This study
sap2192-29	<i>mucA180</i>	Alg ⁻	BC29; This study
sap2192-30	<i>mucA180</i>	Alg ⁻	BC30; This study
sap2192-31	<i>mucA180</i>	Alg ⁻	BC31; This study
sap2192-32	<i>mucA180</i>	Alg ⁻	BC32; This study
sap2192-33	<i>mucA180</i>	Alg ⁻	BC33; This study
sap2192-34	<i>mucA180</i>	Alg ⁻	BC34; This study
sap2192-35	<i>mucA180</i>	Alg ⁻	BC35; This study
sap2192-36	<i>mucA180</i>	Alg ⁻	BC36; This study
sap2192-37	<i>mucA180</i>	Alg ⁻	BC37; This study
sap2192-38	<i>mucA180</i>	Alg ⁻	BC38; This study
sap2192-39	<i>mucA180</i>	Alg ⁻	BC39; This study
sap2192-40	<i>mucA180</i>	Alg ⁻	BC40; This study
sap2192-41	<i>mucA180</i>	Alg ⁻	BC41; This study
PAO1	Prototypic strain	Alg ⁻	BC42; Holloway & Morgan 1986
PA2192	Clinical isolate; <i>mucA180</i>	Alg ⁺	BC43; Mathee et al. 2008
Plasmids			
pRK600	pRK600	Cm ^R (mating helper)	BC44; Heeb et al. 2000
pRK2013	pRK2013	Km ^R (mating helper)	BC45; Figurski & Helinski 1979
pCD100	pRK404; <i>algTmucAmucBmucCmucD</i>	Tc ^R	BC46; Sautter et al. 2012
pJG293	pRK404; <i>algT/U</i>	Tc ^R	BC47; Sautter et al. 2012
pAlgO	pMF54; <i>algO</i>	Ap ^R	BC48; Caballos submitted
pMucP	pMF54; <i>mucP</i>	Ap ^R	BC49; Caballos submitted

492

Primer	Sequence (5' to 3')	Amplicon (bp)
AlgT Fw	TGTTGATAATGTTGGCTCATGCCCCGCATTC	1183
AlgT Rv	AGCGATATCCAGCTTCGGCAGGGTAG	
MucA Fw	AGGACGTAGCGCAGGAAGCCTTCATC	1221
MucA Rv	AAGCTGCCATTGCGCTCGTAGACGAAG	
AlgO Fw	TTCTGCAACAGGTCGGCGCGGTTGAG	2738
AlgO Rv	ACTCCGGGGAGACGTTGAGGAACAGCATG	
MucP Fw	AGCGTGATCCACTCGATGGTGGAC	2212
MucP Rv	GAGCAGGTCTTCCTTGGAATCGCCTTG	
AlgT Seq Fw	ATATCAGAAAGACTCGTGA	Seq. only
AlgT Seq Rv	CATCCGCTTCGTTATCCAT	

493 N.B.: Colored boxes group the *sap* mutants that came from the same parent
 494 colony during the isolation process.

495

496

497 **Figure Legends**

498 **Figure 1:** The *Pseudomonas aeruginosa* alginate regulation pathway. Alginate
499 production is controlled by the sigma factor AlgT/U which is ordinarily bound to
500 the inner membrane by the anti-sigma factor MucA to prevent interaction with
501 RNAP. AlgT/U must be freed from MucA to begin alginate production. When stress
502 is sensed MucE misfolds (1) and induces periplasmic cleavage of MucA by AlgW
503 (2). MucA is also cleaved by MucP (3) on the cytoplasmic end to release AlgT/U.
504 AlgT/U is now free to interact with RNAP and initiate alginate biosynthesis by
505 transcribing the *algD* operon. When MucA is mutated, it is unable to sequester
506 AlgT/U and a mucoid phenotype ensues. Proteins marked with a red star are
507 under investigation in this study. Adapted from Pandey *et al.*, 2016 (27).

508

509 **Figure 2:** The *P. aeruginosa in vitro* reversion phenomenon. Many clinical isolates
510 are mucoid when removed from the lung due to *mucA* mutations. When cultured
511 *in vitro*, they cease producing alginate and become non-mucoid. *mucA* has
512 been seen to remain mutated, and so the reversion is due to second-site
513 mutations in other alginate-regulating genes.

514

515 **Figure 4:** The alignment of MucA. **A.** *mucA* DNA alignment between PAO1,
516 PDO300 (*mucA22*), PA2192, and a sap mutants displaying the differences in *mucA*
517 mutations. Ten *sap* mutants all had the same sequence. PAO1 contains no

518 mutations and is presented as a reference. **B.** Alignment of the respective MucA
519 proteins. **C.** Model of the differences in the MucA protein. MucA22 and MucA180
520 both have a truncated C-terminus in the periplasm. As a result, MucB cannot bind
521 and the protein loses stability, releasing AlgT/U and bestowing the mucoid
522 phenotype.

523 Figures

524

Figure 1

525

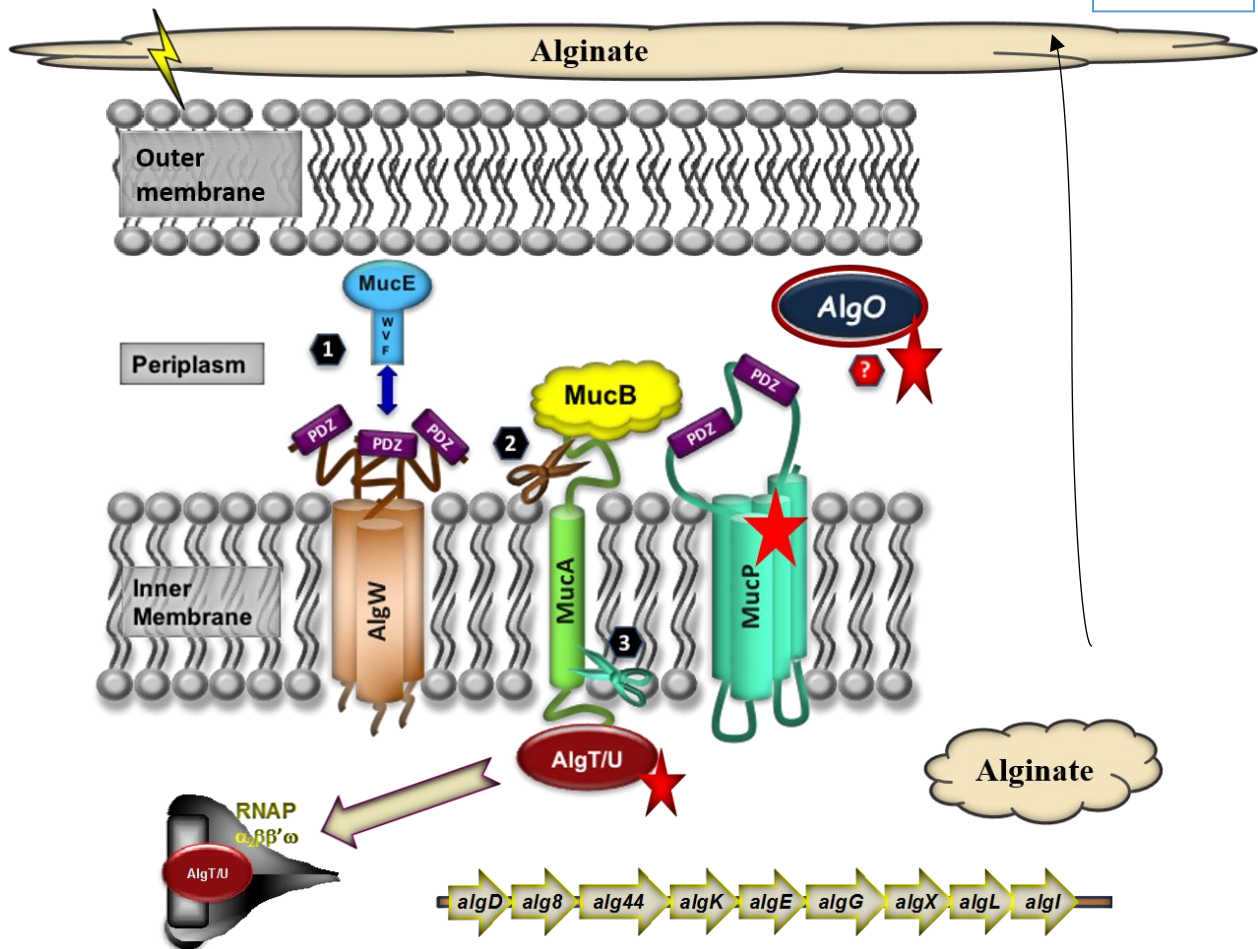


Figure 2

526
527



528

Figure 4

529 **A.**

		1	337	426	534
530	PAO1_mucA	ATGAGTC.....	CCGCAAATGGC.....	GGGGGCGCCGCAGG.....	TACAGAG...
531	PDO300_mucA	ATGAGTC.....	CCGCAAATGGC.....	GGGG- CGCCGCAGG	TACAGAG...
532	PA2192_mucA	ATGAGTC.....	CCGCAG AT GGC.....	GGGGGCGCCGCAGG.....	TACAT AG ...
533	sap1_mucA	ATGAGTC.....	CCGCAG AT GGC.....	GGGGGCGCCGCAGG.....	TACAT AG ...
534			Silent	mucA22	Stop

535 **B.**

		1	139	180
536	PAO1_MucA	MSREA.....	SEEQGAPQVITNSSSSDTRWHEQRLPIYLRQHVQOSAVSGTESALPYARAASLENR	
537	PDO300_MucA22	MSREA.....	SEEQG RRR -----	
538	PA2192_MucA	MSREA.....	SEEQGAPQVITNSSSSDTRWHEQRLPIYLRQHVQOSAVSGT-----	
539	sap1_MucA	MSREA.....	SEEQGAPQVITNSSSSDTRWHEQRLPIYLRQHVQOSAVSGT-----	

540

541

542

543

544

545

