1 A multidrug resistant clinical *P. aeruginosa* isolate in the MLST550 clonal
2 complex: uncoupled quorum sensing modulates the interplay of virulence and
3 resistance

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16 Running title: Uncoupled QS modulates virulence in MDR *P. aeruginosa*

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Abstract

Pseudomonas aeruginosa is a prevalent and pernicious pathogen equipped with both extraordinary capabilities to infect the host and to develop antimicrobials resistance (AMR). Monitoring the emergence of AMR high risk clones and understanding the interplay of their pathogenicity and antibiotic resistance is of paramount importance to avoid resistance dissemination and to control *P. aeruginosa* infections. In this study, we report the identification of a multidrug resistant (MDR) *P. aeruginosa* strain PA154197 isolated from a blood stream infection in Hong Kong. PA154197 belongs to a distinctive MLST550 clonal complex shared by two other international *P. aeruginosa* isolates VW0289 and AUS544. Comparative genome and transcriptome analysis of PA154197 with the reference strain PAO1 led to the identification of a variety of genetic variations in antibiotic resistance genes and the hyper-expression of three multidrug efflux pumps MexAB-OprM, MexEF-OprN, and MexGHI-OpmD in PA154197. Yet the strain does not display a metabolic cost and a compromised virulence compared to PAO1. PA154197 produces a substantially higher level of the *P. aeruginosa* major virulence factor pyocyanin (PYO), but a decreased level of pyoverdine and displays decreased biofilm formation than PAO1. Further analysis revealed that the secondary quorum sensing system Pqs which primarily controls the PYO production is hyper-active in PA154197 independent of the master QS systems Las and Rhl. Together, these investigations disclose a unique, uncoupled QS mediated pathoadaptation mechanism in clinical *P. aeruginosa* which may account for the high pathogenic potentials and antibiotics resistance in the MDR isolate PA154197.

43 Introduction

44 *Pseudomonas aeruginosa* is a ubiquitous Gram-negative pathogen that causes a variety of
45 notorious infections in humans such as ventilator-associated pneumonia, lung infections of
46 cystic fibrosis (CF) patients, burn wound infection, and various sepsis syndromes. It is the
47 second leading cause of hospital-acquired infections and is especially problematic in ICUs,
48 where it is the leading cause of pneumonia among pediatric patients and is responsible for a
49 large number of urinary tract (10% in US and 19% in Europe), blood stream (3% in US and 10%
50 in Europe), eye, ear, nose, and throat infections (1-3). Compounding the burden of these
51 infections is the extraordinary capability of the pathogen to develop antibiotic and multidrug
52 resistance (MDR) even during the course of antibiotics therapy. *P. aeruginosa* is one of the
53 “ESKAPE” (*Enterococcus spp.*, *Staphylococcus aureus*, *Klebsiella spp.*, *Acinetobacter*
54 *baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter spp.*) organisms which are recognized
55 by the Infectious Diseases Society of America (IDSA) as an alarming threat to the global public
56 health associated with antimicrobial resistance (AMR). As a consequence, the diseases outcome
57 of the *P. aeruginosa* infections is the complex interplay of the pathogen (its pathogenicity and
58 virulence), hospital environments (antibiotic therapies and the emergence of AMR), and the
59 patient’s conditions (host immune responses) (4-6).

60

61 *P. aeruginosa* is genetically equipped with outstanding intrinsic antibiotic resistance
62 machineries. These include the inducible production of the AmpC cephalosporinase, presence
63 of the housekeeping MexAB-OprM multidrug efflux pump, and the limited permeability of its
64 outer membrane caused by low expression and substrate-specificity of porin proteins (4, 7, 8).
65 In addition, the pathogen has extraordinary capabilities of developing acquired antibiotic

66 resistance. Mutational overexpression of one of at least four efflux pumps, MexAB-OprM,
67 MexCD-OprJ, MexEF-OprN, and MexXY-OprM, encoded in the genome of *P. aeruginosa*
68 often plays an important role in acquired resistance and can lead to clinically significant MDR
69 (9, 10). Among them, the MexAB-OprM pump displays the broadest substrates profile and
70 mutational overexpression of this pump can lead to resistance to all β -lactams (except
71 imipenem), (fluoro)quinolones, tetracyclines, and macrolides in clinics. Similar to the MexAB-
72 OprM pump, overexpression of MexXY is frequent (10-30%) among clinical strains and causes
73 decreased susceptibility to aminoglycosides and cefepime. Overexpression of MexEF-OprN
74 and MexCD-OprJ is less frequent in clinical isolates (<5%) and they mainly affect
75 fluoroquinolones resistance (5, 11). In addition to efflux pump overexpression mediated MDR,
76 *P. aeruginosa* also readily develops resistance to specific class of antibiotics through genetic
77 mutations and acquisition of target or drug inactivation genes. These include overexpression of
78 *ampC* caused by mutations in peptidoglycan-recycling genes *ampD*, *dacB*, or *ampR* that causes
79 resistance to noncarbapenem β -lactams; acquisition of aminoglycosides modification enzymes
80 (AMEs) and ribosomal methyltransferase (Rmts) enzymes which are associated with
81 aminoglycosides resistance; mutations in the DNA gyrase (*gyrA* and *gyrB*) and/or
82 topoisomerase IV (*parC* and *parE*) which results in fluoroquinolone resistance, and mutational
83 repression of the OprD porin which leads to resistance to imipenem (12-15).

84

85 Regardless of the resistance mechanisms involved, the emergence and prevalence of MDR *P.*
86 *aeruginosa* strains continue to rise rapidly world-wide. Although the bacterium displays an
87 overall nonclonal epidemic population structure with most isolates represented by single MLST
88 (multilocus sequence typing) genotypes, MDR or XDR (extensively drug-resistant) *P.*

89 *aeruginosa* isolates display a much lower clonal diversity than the susceptible isolates and
90 recent studies have reported the existence of MDR/XDR global clones disseminated in different
91 hospitals world-wide (11, 16, 17). Inter-patient spread of antibiotic resistant mutations linked to
92 the transmission of epidemic CF strains is also reported. These clones are denominated as
93 international high-risk clones. Among them, the most recognized successful clones are the high
94 risk MDR clones ST111, ST175, and ST235, and the Liverpool Epidemic Strain (LES) ST146,
95 which is the most epidemic clone among CF patients (11). Further deeper analysis of the
96 molecular details of their resistance development and close monitoring of the emerging of new
97 international MDR/XDR high risk clones are of paramount importance to avoid the world-wide
98 dissemination of these clones and to control *P. aeruginosa* infections.

99

100 In addition to their broad and high level of antibiotic resistance, many MDR clones, especially
101 the three major international high risk clones ST111, ST175, ST235, are found to be associated
102 with a defined set of biological markers which include defective motility (swimming, swarming,
103 and twitching), reduced pigment production (pyocyanin and pyoverdine), reduced in vitro
104 fitness, but enhanced biofilm formation (5, 11). These suggest the presence of a fitness trade-off
105 that compromises the pathogenic potentials and virulence of MDR isolates. Several
106 epidemiological survey and molecular evolutionary studies ascribed these phenotypes to
107 quorum sensing (QS) deficiency in these strains (18-21), which is not unexpected since
108 production of many virulence factors, such as exo-proteases, elastase, rhamnolipids, lectin,
109 pyoverdine, pyocyanin, hydrogen cyanide etc. are primarily regulated by QS systems in *P.*
110 *aeruginosa*(1). On the other hand, recent studies also reported antibiotic resistant strains
111 displaying enhanced virulence (17, 22-24), such as *P. aeruginosa* strains lacking the *oprD* porin

(22), implying that certain MDR clones may develop compensatory mutations allowing them to recover their virulence without affecting the level of resistance.

Recently, we surveyed 84 *P. aeruginosa* clinical strains isolated from various of infections including wound (both superficial and deep), urine, ear, pus, drain fluid, and blood in the Queen Mary Hospital, Hong Kong, and identified an MDR isolate PA154197 from a blood stream infection. PA154197 displays an MDR level and profile comparable to the international high-risk clone ST175. Sequencing its genome reveals that it belongs to a distinctive genotype MLST550 which is shared by two additional clinical strains currently available in the database: VW0289 and AUS544. VW0289 and AUS544 were isolated from the sputum of a CF patient in The Netherlands and the bronchial lavage of a CF patient in Australia, respectively. The clonal lineage of PA154197 with these two strains suggests a potential international transmission of this emerging clonal complex. Yet the two CF isolates VW0289 and AUS544 are poorly characterized and little is known about the ST550 clonal lineages. This prompts us to use PA154197 as a prototype to conduct comparative genomic and transcriptomic analysis and understand the resistance development and virulence of the MLST550. Our investigations led to the identification of the adaptive activation of the secondary quorum sensing system Pqs (*Pseudomonas* quinolone system) independent of the primary QS system Las and Rhl which may account for the uncompromised virulence in the MDR strain PA154197. Together, our studies report the emergence of a high-risk clone in clinics and its underlying pathoadaptation mechanism mediated by uncoupled QS.

Results

135 ***PA154197 displays extraordinary antibiotic resistance***

136 We examined the susceptibility of PA154197 to various classes of antibiotics including the
137 common antipseudomonal drugs aztreonam (ATM), imipenem (IPM), ciprofloxacin (CIP),
138 levofloxacin (LVX), amikacin (AMK) etc. by measuring their minimal inhibitory concentration
139 (MIC) values and compared with that for PAO1 (Fig. S1A). We found that PA154197 displays
140 resistance to almost all these different classes of antibiotics compared to PAO1. The only two
141 classes of antibiotics to which it remains susceptible are the aminoglycoside (amikacin and
142 gentamicin) and the cyclic non-ribosomal polypeptides (polymyxin B and E), which meets the
143 XDR criteria (resistance to all but one or two of the eight classes of antipseudomonal drugs)
144 according to Magiorakos et al. (16). To further evaluate its resistance level, we compared the
145 antibiotics susceptibility profile of PA154197 with the three international high-risk clones using
146 the published data (25) and found that PA154197 displays a comparable resistance profile with
147 the high-risk clone ST175 (Fig. S1B), highlighting the high epidemic and risk potential of
148 PA154197.

149

150 ***Sequencing type, phylogenetic position, and genomic islands of PA154197***

151 We then sequenced the genome of PA154197 which resulted in one single circular chromosome
152 with a length of 6,445,239 bp and a GC content of 66.38%. A total number of 5,923 genes are
153 predicted in PA154197 genome which include 5,816 coding gene sequences (CDS), 26 pseudo-
154 genes, 12 rRNA, 65 tRNA and 4 ncRNA (Table 1). Based on PubMLST, PA154197 is assigned
155 to the multilocus sequence typing (MLST) 550, a clonal complex which is currently shared by
156 two additional clinical isolates, VW0289 and AUS544 from the CF patients in the Netherland
157 and Australia, respectively (Fig. S2A), but the complete genome sequences of these two strains

158 are unavailable. PAST analysis revealed that PA154197 belongs to the Serotype O9 (26) (Table
159 1). Analyzing its accessory genome identified 21 genome islands (GIs) including 3 prophages
160 (Table S1). In addition to hypothetical proteins, a large number of metabolic enzymes such as
161 oxidoreductase, oxygenase and halogenase, as well as the AraC and LysR type transcription
162 regulators are encoded in these GIs, implying the potential roles of the accessory genome of
163 PA154197 in the adaptation and fitness of the bacterium.

164 To examine the phylogenetic relationship of PA154197 with other *P. aeruginosa* strains, we
165 constructed a phylogenetic tree based on 140,634 SNPs generated from 150 *P. aeruginosa*
166 genomes which include all publicly-available complete genomes (78 as of October 2017) and
167 representative non-complete genomes. As shown in Fig. S2B, PA154197 exhibits a distinct
168 phylogenetic position from other genomes available in the database. This may indicate its
169 unique virulence and pathogenic potentials.

170

171 ***Antibiotic resistance genes and mutational changes therein***

172 Using Resistance Gene Identifiers (RGI), we then identified antibiotic resistance genes in
173 PA154197 against the Comprehensive Antibiotic Resistance Database (CARD) (27), and
174 examined genomic variations including mutations, deletions and indels in these genes with the
175 genome of PAO1 as the reference (Table 2). It was revealed that PA154197 contains a large
176 number of mutations associated with antibiotic resistance genes, including both previously
177 reported genetic variations and those newly identified in PA154197. Several well established
178 mutations include an 8-bp deletion in the *mexT* gene which leads to up-regulation of the
179 MexEF-OprN efflux pump and down-regulation of the OprD porin, and consequently resistance
180 to fluoroquinolones and imipenem, respectively (28-30); the T105A substitution in AmpC,

181 which causes resistance to non-carbapenem β -lactams (31), and the T83I substitution in the so
182 called quinolone resistance-determining region (QRDR) of GyrA, conferring quinolone
183 resistance (32). Another genomic variation commonly found to cause resistance in many *P.*
184 *aeruginosa* isolates is the non-synonymous mutations in the TetR transcriptional regulator NalC
185 which often leads to aztreonam resistance (33). Three non-synonymous mutations, G71-E,
186 E153-Q, S209-R, are identified in the *nalC* gene in PA154197 (Table 2).

187

188 New genomic variations which have not been reported in other strains but potentially lead to
189 antibiotic resistance is also identified in PA1541197, such as a 6-bp deletion (corresponding to
190 the deletion of E959 and S969) in the C-terminal of GyrA and a E76-*(stop codon) mutation in
191 the *mexR* gene which will lead to pre-mature termination of MexR at E76 and potentially de-
192 repression of the MexAB-OprM major efflux pump, causing multidrug resistance(34).
193 Mutations that potentially de-repress or induce the expression of several other efflux pumps are
194 also identified in the corresponding transcription regulators or the promoter region of the efflux
195 genes. For example, a single point mutation H398R is identified in *parS* gene which regulates
196 the expression of the *mexEF-oprN* efflux pump (35). A 5-nucleotides alteration in the promoter
197 region (-1 to -250bp) of the *mexGHI-opmD* operon is present in PA154197 compared with that
198 in PAO1, which may lead to the expression change of the MexGHI-OpmD efflux pump. A
199 complete summary of the identified genomic variations in all the antibiotic resistance genes is
200 provided in Table 2.

201

202 ***Expression of the resistance genes and the functional genome of P. aeruginosa PA154197***

203 To examine the resistance mechanisms of PA154197 especially the expression levels of the

204 resistance genes identified, we performed a comparative transcriptome analysis on PA154197
205 and the reference strain PAO1 using RNA-seq. Among the 5,543 orthologous genes identified
206 in the two strains using progressiveMauve (36), differential expression of 3,148 genes are
207 observed and their expression levels are compared using DESeq (37) (Fig. 1 and Table S2).
208 Notably, among the genes which display significantly higher expression levels in PA154197
209 than in PAO1, those encoding four efflux pumps, such as *mexEF-oprN* (PA2493-PA2495),
210 *mexRAB-oprM* (PA0424-PA0427), *mexGHI-opmD* (PA4205-PA4208), and *mexKJL* (PA3676-
211 PA3678) are observed (Fig. 1 & Fig. 2A), consistent with the MDR profile of PA154197.
212 Genes that confer resistance to specific antibiotics, such as that of *ampC*, *gyrA*, *gyrB*, and *parC*,
213 *parE*, are not differentially expressed in the RNA-Seq analysis, suggesting that over-expression
214 of several multidrug efflux pumps may play a major role in the MDR development in
215 PA154197. COG functional distribution analysis of the differentially expressed genes also
216 reveals the enrichment of efflux pump genes among all the genes which are expressed at a
217 higher level in PA154197 than in PAO1 (Fig. S3).
218 To verify the relative expression levels of these genes in the two strains, we conducted RT-
219 qPCR analysis. As shown in Fig. 2B, consistent with the RNA-Seq data, the MexEF-OprN
220 efflux system displayed the most significant higher expression (265-574 fold) in PA154197
221 than in PAO1, followed by the MexGHI-OpmD (8.5-41.9 fold) and the MexAB-OprM (4.6-11
222 fold) efflux system. On the other hand, another efflux system which over-expression is
223 associated with aminoglycosides resistance, the MexXY-OprM system, did not show increased
224 relative expression in PA154197 in either RNA-Seq or the RT-qPCR analysis, consistent with
225 the fact that PA154197 remains susceptible to the aminoglycoside antipseudomonal drug
226 amikacin and gentamicin (Fig. S1). RT-qPCR analysis also revealed the decreased expression

227 of another resistance gene *oprD* in PA154197 which serves as the entry portal of imipenem (38),
228 consistent with the observed imipenem resistance (MIC as 8µg/ml) in this strain.

229

230 ***Virulence of P. aeruginosa PA154197***

231 Our comparative transcriptome analysis also reveals a significantly higher expression of several
232 virulence genes in PA154197 than in PAO1 (Fig. 1 and Table S2). These include the type III
233 (*psc* genes) and the type VI (*tss* and *hcp-1* genes) secretion systems, pyocyanin production (*phz*
234 genes), the PQS quorum sensing system (*pqs* genes), and a series of Fe and heme acquisition
235 genes (*fep* and *has* genes). On the other hand, several genes involved in biofilm formation (such
236 as *psl*, *alg*, *lecA*), motility, pili and fimbrial assembly proteins (such as *cup* genes), and
237 pyoverdine production (*pvd* genes) are expressed at a lower level in PA154197 than in PAO1.

238 To examine the virulence factors production and the virulence of PA154197, we examined
239 pyocyanin (PYO) and pyoverdine production, biofilm formation, swimming and swarming
240 motilities and infection virulence of PA154197 and compared with that of PAO1. As shown in
241 Fig. 3, PA154197 cells produce a significantly higher level of PYO than PAO1 cells (Fig. 3A),
242 which may be responsible for the uncompromised virulence of the strain as observed in the
243 *Caenorhabditis elegans* infection assay (Fig. 3E). In contrast, PA154197 displays reduced
244 pyoverdine production compared to PAO1 (Fig. 3B), suggesting that the strain may utilize other
245 components and pathways rather than pyoverdine for iron acquisition (39). Consistent with the
246 lower expression of biofilm genes in PA154197 than in PAO1, PA154197 displays a reduced
247 biofilm formation capability as examined by the crystal violet staining (Fig. 3C). In terms of the
248 motility of the bacterium, PA154197 displays a comparable swimming activity with that of
249 PAO1, but a defective swarming motility (Fig. 3D) which is consistent with the lower

250 expression of the pili and fimbria assembly genes in this strain. To evaluate the in vivo
251 virulence of PA154197, we conducted *Caenorhabditis elegans* fast killing and slow killing
252 assays which are established infection models to evaluate the cytotoxicity and pathogenicity of
253 *P. aeruginosa*, respectively(40). We found that the two strains display a comparable
254 cytotoxicity and pathogenicity (virulence) (Fig. 3E) to the *C. elegans* host. Together, these
255 studies indicate that PA154197 over-expresses a subset of virulence factors and does not
256 display a compromised virulence as reported for many other resistant strains.

257

258 ***The Pqs quorum sensing system is activated in PA154197 independent of the primary QS***
259 ***system Las and Rhl***

260 It is known that three hierarchically organized QS systems, Las, Rhl, and Pqs, regulate the
261 production of an arsenal of virulence factors in *P. aeruginosa* with each being primarily
262 associated with the production of virulence factors elastase (encoded by *lasB* gene),
263 rhamnolipid (encoded by *rhlA* gene) and pyocyanin (encoded by *phz* genes), respectively (41,
264 42) (Fig. 4A). Among them, the Las system proceeds the Rhl and Pqs systems and is the master
265 regulator of the QS circuit. Several previous epidemiological survey and molecular
266 evolutionary studies have identified *lasR* null and mutant variants in clinical isolates and
267 ascribed the attenuated virulence of the isolates to an overall QS deficiency caused by the
268 inactivation of the master regulator Las (43, 44). However, PA154197 does not display an
269 uncompromised virulence as most of the resistant strains, and comparative genomics did not
270 reveal mutations in QS regulatory genes *lasR*, *rhlR*, and *pqsR*. Nonetheless, to explore whether
271 the uncompromised virulence PA154197 is related to QS, we examined the RNA-Seq
272 transcription levels of the QS systems and genes regulated by each of the QS systems,

273 especially virulence genes. Surprisingly, we found that while expression of the *lasRI* and *rhlRI*
274 in PA154197 is undetectable, expression of the genes encoding the secondary QS system *pqsA*-
275 *E* is very high and is among the top three most significantly expressed (Log_2 RPKM abundance
276 in a range of 4.1 – 5.4) gene operon in PA154197 than in PAO1 (Fig. 4A). Consistently, genes
277 primarily controlled by the Las and Rhl systems, such as *toxA*, *lasB*, *lecA* are expressed in a
278 lower level in PA154197 than in PAO1, whereas genes controlled by Pqs, such as *phZ* genes,
279 *pqsA-E*, and *phnAB* are expressed in a significantly higher level in PA154197 than in PAO1
280 (Fig. 4A and Table S2). RT-qPCR analysis confirmed this observation (Fig. 4B). These data
281 suggest that the secondary QS system Pqs is activated and expressed independent of the
282 primary QS systems Las and Rhl in PA154197, which may account for the hyper-production of
283 a subset of virulence factors and consequently its uncompromised virulence.

284

285 Discussion

286 As a successful and ubiquitous pathogen, *P. aeruginosa* is equipped with extraordinary
287 machineries to adapt to the host environments and antibiotic therapies to survive and
288 disseminate. As a result, the disease development caused by *P. aeruginosa* infections is driven
289 by several dynamic factors, including bacterial pathogenesis, selective forces resulting from the
290 antimicrobial interventions, and the fitness costs of resistance development. It is generally
291 recognized that while acquisition of antibiotic resistance mechanisms confers selective
292 advantages in the presence of antimicrobial therapies, expressing and maintenance of the
293 resistance determinants often incurs metabolic costs to the pathogen and consequently
294 compromises its fitness and pathogenicity potentials (5). How *P. aeruginosa* reconciles this
295 scenario and succeeds in the dynamic cycle of infection and dissemination is not known. In this

296 study, we identify and utilize an MDR *P. aeruginosa* isolate PA154197 which displays a
297 comparable MDR profile to the epidemic high-risk clone ST175 but does not exhibits
298 compromised virulence as a paradigm to examine the underlying pathoadaptive mechanisms.
299 Comparative transcriptome and RT-qPCR analysis reveals an uncoordinated, significant
300 activation of the Pqs quorum sensing system and its regulated virulence genes in PA154197
301 independent of the primary QS Las and Rhl systems, providing a compensatory mechanism for
302 virulence factor production without the activation of the master and primary QS systems Las
303 and Rhl, as well as the hundreds of genes regulated by them. A schematic diagram to illustrate
304 this pathoadaptive mechanism is shown in Fig. 5.

305

306 Several previous studies reported QS deficiency in antibiotic resistant clinical isolates and
307 attributed the attenuated virulence of the strains to this mechanism, as these mutants are
308 proposed to be social cheaters that exploit shared QS products without incurring metabolic costs
309 to themselves (18-21). However, majority of these studies were based on comparative genomic
310 analysis to identify mutations in the QS system genes, and the identified variations were mainly
311 found in the master QS regulator LasR, including both *lasR*-null and various *lasR* point
312 mutations. Consistently, the identified strains often display an overall QS-inactivation
313 phenotype, such as decreased production of all the major QS regulated virulence factors
314 including elastase, rhamnolipids, pyoverdine, pyocyanin. Different from these *lasR* defective
315 isolates, PA154197 produces a significantly higher amount of PYO, a major virulence factor
316 secreted by *P. aeruginosa* (45), and other Pqs regulated gene products than PAO1, while a
317 lower level of Las and Rhl regulated virulence factors and genes than in PAO1. This uncoupled
318 activation of Pqs system ensures the production of a major virulence factor PYO with minimal

319 metabolic burden, thus is beneficial to the fitness and virulence of the pathogen. How *P.*
320 *aeruginosa* achieves this differential activation is not known currently. We compared the
321 promoter regions of the *lasRI*, *rhlRI*, *pqsR*, and *pqsA-E* operons with the corresponding
322 sequences in PAO1, but did not find any nucleotide alternations that may account for the
323 differential activation of *pqs* genes. Comparative genomics analysis revealed a point mutation
324 Q98P in PA154197 LasR which is located at the $\alpha 5$ of the N-3-oxo-dodecanoyl-homoserine
325 lactone binding domain of the protein but the residue is not located in the binding pocket (46).
326 Whether this point mutation leads to differential activation of the Pqs system warrants further
327 investigations. Notably, Las independent activation of a downstream QS system was also
328 reported in a collection of *P. aeruginosa* isolates from CF patients in which half of the *lasR*-null
329 strains were found to retain the RhlR activity (20). Hence, adaptable QS hierarchy which
330 uncouples a downstream QS system from the master regulator LasR may represent an emerging
331 compensatory mechanism that facilitates the fitness, virulence, and persistence of *P. aeruginosa*
332 in the host settings. In PA154197, at least one downstream physiological process mediated by
333 the uncoupled QS, production of the virulence factor PYO, is observed. This process might be
334 further facilitated in PA154197 by the over-production of the MexGHI-OpmD efflux pump (Fig.
335 2B) in the strain which has been shown to dedicate to the export of 5-methylphenazine-1-
336 carboxylate, an intermediate in PYO biosynthetic pathway, and promote PYO production (47).

337

338 With the increasing frequency of detecting the MDR *P. aeruginosa* isolates from various
339 sources, it is recognized that the resistance traits of a strain are often the result of a complex
340 interaction of several cellular processes and no individual mutation or resistance gene is
341 sufficient to confer clinically significant resistance (48, 49). In this study, we identified multiple

342 genetic variations potentially associated with the resistance development in PA154197. These
343 include both machineries which confer resistance to a diverse class of antibiotics and hence
344 causing MDR(4), such as over-expression of multidrug efflux pumps, and genes which
345 mutations result in resistance to specific classes of antibiotics, such as mutations in *ampC* and
346 *gyrA*. Transcriptome and RT-qPCR confirmed the hyper-expression of three multidrug efflux
347 pumps, MexAB, MexEF, and MexGHI in PA154197, but the expression of *ampC* and *gyrA* was
348 found to be in a similar level to that in PAO1. Whether these two genes contribute to the profile
349 and level of resistance exhibited in PA154197 remains further molecular validation. Notably,
350 both the T105A mutation in AmpC and T83I mutation in GyrA are the well characterized
351 genetic variants associated with antibiotic resistance, which have been termed as the PDC-3
352 type AmpC variant (31) and the quinolone resistance-determining region (QRDR) hotspot
353 mutation, respectively (14). However, these two mutations are also identified in other *P.*
354 *aeruginosa* isolates we surveyed such as PA150577 which does not display resistance to β -
355 lactams or fluoroquinolones (data not shown). Hence, the observed resistance to
356 antipseudomonal aztreonam (monobactam) and imipenem (carbapenem) in PA154197 may not
357 be due to the T105A mutation in AmpC, and the observed resistance to ciprofloxacin and
358 levofloxacin (fluoroquinolone) may not be due to the T83I mutation in GyrA. In addition,
359 acquiring additional and extended-spectrum β -lactamase on mobile genetic elements represents
360 another common mechanism of β -lactam resistance in *P. aeruginosa* (50), but no plasmid
361 encoding β -lactamases are identified in PA154197. Indeed, it has been proposed that genetic
362 background of the drug resistant strains influences the epistatic interactions of the various
363 resistant determinants and the resistance readout (51). This highlights the intricacy of the
364 mechanisms that underlie resistance development of MDR strains and our ongoing targeted

molecular investigations and evolutionary trajectory analysis may provide mechanistic insight into these processes.

Materials and methods

Minimum inhibitory concentration measurements

MICs were measured following the standard protocol from ASM with slight modification (52). Single fresh colonies of PA154197 and PAO1 were inoculated in Mueller Hinton (MH) broth overnight at 37 °C with 220-rpm agitation. The resulting cell culture was diluted and distributed to the wells of 96-well plate with a final cell density as 5×10^5 CFU/ml. Selected antibiotics were added to the wells with concentration ranging from 0.25 to 128 µg/ml. Plates were incubated at 37 °C for 16-20 h. MIC values were determined as the lowest concentration of antibiotics that completely inhibit growth of bacteria as detected by the unaided eye. Antibiotic susceptibility profiles of the strains indicated are displayed in color scheme with low susceptibility (high MIC values) in red color and high susceptibility (low MIC values) in blue color. The color scheme is constructed using background filling application in the Excel of MS Office.

Genomic DNA extraction

Extraction of the genomic DNA of PA154197 was performed following the description in a previous study (53). Briefly, PA154197 was cultivated in Luria-Bertani (LB) broth overnight with shaking (220 rpm) at 37 °C. Bacterial cells were harvested from 1 ml culture via centrifugation at 10,000 rpm for 10 minutes. Genomic DNA was extracted using the QIAamp

388 DNA Mini Kit following the manufacturer's instructions (Qiagen, Hilden, Germany). The
389 concentration and quality of genomic DNA was determined by NanoDrop and agarose (0.8%)
390 gel electrophoresis.

391

392 ***Genome sequencing and annotations***

393 Genome sequencing of *P. aeruginosa* PA154197 was conducted on the Illumina NextSeq (300
394 Cycles) PE150 High Output Flow Cell platform in Georgia Genomics Facility at University of
395 Georgia, USA. SPAdes (54) was used to assemble the reads after removal of adapter, primers,
396 and low quality bases using Trimmomatic (55). The initial assembly of PA154197 genome
397 yielded 21 contigs. The 21 contigs were aligned against the reference genome of PAO1 and
398 ATCC 27853 and gaps were filled using Sanger sequencing to generate the complete genome
399 sequence of PA154197. Gene calling and annotation were carried out using the National Center
400 for Biotechnology Information (NCBI)'s Prokaryotic Genome Annotation Pipeline 2.0 (PGAP)
401 (56).

402

403 ***Sequence type and phylogenetic analyses***

404 The multiple locus sequence type (MLST) and serotype of PA154197 was predicted based on
405 the PubMLST database (www.pumlst.org) and the *Pseudomonas aeruginosa* serotyper (PAst)
406 tool (26).

407

408 To carry out the phylogenetic analysis, we downloaded all available complete genomes of *P.*
409 *aeruginosa* (one representative per distinct phylogenetic group) from NCBI GenBank (78
410 genomes by Oct 2017) (Table S3, for reviewers' information only) and selected 72

411 representative non-complete genomes that are distributed in the 32 phylogenetic groups as
412 defined in the NCBI. Single nucleotide polymorphisms (SNPs) were collected using Parsnp
413 with default parameters (57) and that of PAO1 served as the reference. SNPs were then used to
414 build a maximum likelihood tree in MEGA (58) with the following parameters: Tamura-Nei
415 substitution model, gamma rate distribution among sites, Nearest-Neighbor-Interchange for tree
416 inference options, a bootstrap value 100, and initial tree was generated using the Neighbor
417 Joining method. Variants were called using the Harvest tools (57) and annotated using SnpEff
418 (59).

419

420 *Identifications of antibiotic resistance genes (ARG) and mutations therein*

421 Resistance Gene Identifiers (RGI) from the Comprehensive Antibiotic Resistance Database
422 (CARD) (27) were used to identify antibiotic resistance genes in PA154197 using “Perfect and
423 Strict hits only” parameter. BLAST of all genes from the present study against the antibiotic
424 resistance gene database of ResFinder (version 2.1) (60) and BLAST against a database of
425 antibiotic resistance genes curated by ourselves based on previous reports (61) were also
426 conducted.

427

428 Variants of all predicted antibiotic resistance genes were collected from the annotation with
429 SnpEff (59) using PAO1 as the reference. SNPs that cause non-synonymous mutations and gaps
430 less than 6 bp were also identified and summarized.

431

432 *RNA-seq*

433 RNA extraction, quality control, and RNA-Seq were performed in PA154197 and the reference

434 strain PAO1 with three biological replicates following our previous descriptions (53). Stranded
435 libraries for all RNA samples were constructed using Kapa Biosystems RNA library
436 preparation chemistry in Georgia Genomics Facility at University of Georgia.

437

438 Orthologous genes between PAO1 and PA154197 were obtained by comparison using
439 *progressiveMauve* with default settings (36) and were employed in the following RNA-seq
440 analysis. RNA-seq reads were pre-processed for quality control using Trimmomatic (55) and
441 were then mapped to the reference genomes of PAO1 and PA154197 respectively, using
442 Stampy (to speed up the alignment process, alignment was initially conducted using BWA-
443 MEM with default parameters (35) then passed to Stampy with the flags --bamkeepgoodreads -
444 M) (62). SAMtools and BamTools were used for format conversions, statistics, and quality
445 assessment and control (63). The Integrative Genomics Viewer was used to visually inspect
446 mapping quality (64). Fragments counting per genomic features (genes) were performed using
447 featureCounts (*featureCounts -R -M -Q 10 -p -P -s 2 -t gene -g locus_tag --largestOverlap*) (65).
448 Reads that mapped with MAPQ scores below 10 were removed. Enforcing a MAPQ score
449 below 10 also excludes multi-mapped reads albeit the percentage of this category is low (data
450 not shown). DESeq was employed to analyze differentially expressed genes. Selective genes
451 with high expression levels in PA154197 were verified using RT-qPCR. Primers used in the
452 present study are listed in Supplementary Table S4.

453

454 ***Motility assays***

455 The assays were performed as previously described with slight modifications(66). (1)

Swimming motility. The semi-solid motility plates were prepared by mixing the LB broth with 0.25% (wt/vol) agarose. Overnight cultures of PAO1 and PA154197 were sub-cultured (1:200 dilution) into LB broth. When OD₆₀₀ reached 0.1-0.2, 2 µl of cell was spotted at the center of a freshly prepared semi-solid swimming plate. The plates were dried at room temperature for 1 h and subsequently were incubated at 37 °C for 8 h. Swimming motility was evaluated by measuring the diameter of the covered areas. All assays were conducted in triplicates. (2)

Swarming motility. The motility plates were prepared by mixing the M8 broth [21.1 mM Na₂HPO₄, 11 mM KH₂PO₄, 42.8 mM NaCl, 9.3 mM NH₄Cl, 1 mM MgSO₄, 0.2% glucose, 0.2% casamino acids, pH 6.5] with 0.5% (wt/vol) agarose. 2 µl of sub-cultures of PAO1 and PA154197 (OD₆₀₀ of 0.1-0.2) as mentioned above was spotted at the center of a pre-dried swarming plate. The plates were incubated at 37°C, and images of the developed swarms were recorded at 16-48 h. All assays were conducted in triplicates.

Pyocyanin production assay

Pyocyanin production was examined according to a protocol described previously with modification (53). 1 ml of the bacterial culture was subject to centrifugation at 13,000 rpm for 5 min. The supernatant was collected and extracted with 600 µl chloroform following vortex for 10 s twice. After centrifugation at 13,000 rpm for 5 min, the chloroform phase was transferred into a clean tube, and subsequently mixed with 0.5 ml 0.2 M HCl followed by gentle shaking to transfer the pyocyanin to the aqueous phase. The concentration of PYO was determined by measuring the absorbance of the aqueous phase at 510 nm.

Biofilm assay

479 Crystal violet staining method was used to determine biofilm formation of PAO1 and
480 PA154197 with slight modifications (67). Overnight cultures of *P. aeruginosa* cells were
481 inoculated into 0.5 ml of LB broth in 5 mL round bottom polypropylene Falcon tubes (final cell
482 density as 0.1) and incubated statically at 37 °C for 24 or 48 h to allow biofilm formation. After
483 removing the medium, the biofilms formed at the bottom of the tubes were gently washed with
484 phosphate buffered saline (PBS) three times to remove residual planktonic cells. The tubes were
485 then air-dried and stained with 0.1% crystal violet for 15 min at room temperature. After
486 washing the tubes three times with sterile distilled water to remove excess dye molecules,
487 biofilms were dissolved in 150 µl of 30% acetic acid and the optical density (OD) was
488 measured at 595 nm using a 96-well plate spectrometer reader. All experiments were performed
489 in triplicates.

490

491 ***Nematode killing assay***

492 To examine the virulence and pathogenesis of PA154197 compared with PAO1, fast and slow
493 killing kinetics of *Caenorhabditis elegans* (*C. elegans*) were performed with slight modification
494 (68). (1) Fast killing assay. Preparation of the killing plates can be divided into two steps. i) 100
495 µL of overnight culture of PA154197 or PAO1 were spread onto 3.5 cm LB agar plates and
496 incubated at 25°C for 2 days to produce the toxins. ii) Lawn of PA154197 or PAO1 was scraped
497 from LB agar plates containing the toxins completely using a sterile L spreader. *C. elegans*
498 strain N2 were synchronized by isolating eggs from gravid adults and plating eggs on to lawns
499 of *E. coli* OP50 on NGM [0.25% peptone, 0.3% NaCl, 2% agar, 5 µg/ml cholesterol, 1 mM
500 MgSO₄, and 25 mM KH₂PO₄ (pH 6)] agar plates followed by incubation at 25°C for 48-52 h to
501 reach the L4 stage. Each fast killing plate was seeded with 20-30 worms. Plates were incubated

at 25°C and scored every 4-6 h. A worm was considered to be dead when it no longer responded to touch. (2) Slow killing assay. The procedure was similar as fast killing assay except the preparation of killing plates. For slow killing plates, lawn of PA154197 or PAO1 was not moved away. All experiments were performed in triplicates.

Pyoverdine (PVD) production assay

The siderophore pyoverdine is observed under UV light as a fluorescent zone around the colony (69). Fresh single colonies of PA154197 and PAO1 were inoculated in LB medium overnight at 37 °C with 220-rpm agitation. Following diluting overnight culture to 1×10^9 CFU/ml with fresh LB medium, 10 µl cell suspension was then spotted on a LB agar plate. The plate was incubated at 37 °C for 16-20 h. Fluorescent PVD was visualized and imaged under UV light.

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525 The authors do not have conflict of interests to declare.

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- 728

729 **Figures legends:**

730 **Figure 1.** The genome wide transcriptomic profile of *P. aeruginosa* PA154197 and PAO1.
731 Orange dots indicate genes with higher relative expression levels in PA154197 than in PAO1
732 and blue dots represent genes with higher relative expression level in PAO1 than in PA154197.
733 The black dashed lines represent 4 fold (value of $\log_2 > 2$) changes in expression. Genes and
734 operons with distinctive expression patterns in the two strains are indicated. Among them,
735 antibiotic resistance genes are highlighted in red, genes encoding virulence factors are
736 highlighted in green (expressed in a higher level in PA154197 than PAO1) or purple (expressed
737 in a higher level in PAO1 than in PA154197). X-axis represents the gene locus with PAO1
738 genome as reference.

739 **Figure 2.** Several multidrug efflux pump genes are over-expressed in *P. aeruginosa* PA154197.
740 **A.** RPKM abundance of the major efflux pump genes in PA154197 and PAO1 calculated from
741 the RNA-Seq data. **B.** RT-qPCR analysis of the expression of selective efflux genes and other
742 genes associated with resistance to specific classes of antibiotics.

743 **Figure 3.** Virulence and production of several virulence factors in PA154197 and PAO1. **A.**
744 Pyocyanin production in PA154197 and PAO1 cells cultured in both LB broth and agar. **B.**
745 Pyoverdine production examined under UV light. **C.** Biofilm formation examined by the crystal
746 violet stain. **D.** Swimming and swarming motilities examined in the corresponding specific agar
747 plates. **E.** Virulence of PA154197 and PAO1 measured in a *C. elegans* infection model. N2 *C.*
748 *elegans* grown to L4 stage adults were infected with PA154197 or PAO1 for 24hrs. Survival of
749 *C. elegans* were monitored under the light microscope and recorded.

750 **Figure 4.** Hyper-activation of the PQS quorum sensing system independent of the master
751 system Las and Rhl in PA154197. **A.** Schematic diagram of the three quorum sensing systems
752 in *P. aeruginosa*, Las, Rhl, and PQS, and their selective regulon. Relative expression of the
753 genes in the Las, Rhl, and PQS regulon is depicted in color scheme with the genes expressed at
754 a lower level in PA154197 than in PAO1 in red color and those expressed at a higher level in
755 PA154197 than in PAO1 in blue color. RPKM abundance of the genes calculated from the
756 RNA-Seq data are indicated. **B.** RT-qPCR of selective genes belonging to the Las, Rhl, and Pqs
757 regulon, respectively.

758 **Figure 5.** Schematic model to show the pathoadaptation mechanism adopted by PA154197 to
759 modulate its antibiotic resistance and virulence. In the high virulent, susceptible strains, the
760 three quorum sensing (QS) systems Las, Rhl, and Pqs mediate the production of a variety of
761 virulence factors and a high virulence in the cell. Antibiotic resistant strains with acquired
762 resistance determinants often carry mutations in the master QS regulator Las to downregulate
763 the QS system and virulence. Strains with pathoadaptive strategy such as PA154197 uncouple
764 the activation of the secondary QS system Pqs from Las and Rhl to maintain an uncompromised
765 virulence with simultaneous production of its resistance traits.

766 **Table 1** The genomic features of *P. aeruginosa* PA154197.

767 **Table 2** Mutational changes of genes conferring antibiotic resistance and their expression levels
768 in PA154197 relative to that in PAO1.

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A

		PA154197	PAO1
<i>mexEF-oprN</i>	<i>mexS</i>	8.79	8.84
	<i>mexT</i>	6.68	7.34
	<i>mexE</i>	8.31	2.82
	<i>mexF</i>	7.55	2.40
	<i>oprN</i>	7.36	2.13
<i>mexAB-oprM</i>	<i>mexR</i>	8.36	7.24
	<i>mexA</i>	11.03	9.00
	<i>mexB</i>	10.66	8.57
	<i>oprM</i>	10.85	8.85
<i>mexGHI-opmD</i>	<i>mexG</i>	10.53	8.29
	<i>mexH</i>	9.44	7.05
	<i>mexI</i>	8.31	5.86
	<i>opmD</i>	8.11	5.76
<i>mexXY-oprM</i>	<i>mexX</i>	3.50	4.69
	<i>mexY</i>	3.32	4.64
	<i>mexZ</i>	5.69	7.41
<i>mexCD-oprJ</i>	<i>nfxB</i>	5.97	6.38
	<i>mexC</i>	3.67	3.01
	<i>mexD</i>	2.67	3.40
	<i>oprJ</i>	2.44	3.39
<i>mexJKL</i>	<i>mexJ</i>	7.30	5.86
	<i>mexK</i>	7.08	5.36
	<i>mexL</i>	6.94	7.41

-1.56
11.03

Table 1 The genomic features of *P. aeruginosa* PA154197.

Features	Total Length	% GC	Genes	Proteins	rRNA	tRNA	ncRNA	Pseudo	CRISPR	Prophages	Serotype
	(bp)	Content						Genes	Arrays		
PA154197	6,445,239	66.4%	5,923	5,816	12	65	4	26	3	3	O9
PAO1	6,264,404	66.6%	5,697	5,572	13	63	4	19	0	2	O5

Table 2. Mutational changes of genes conferring antibiotic resistance and their expression levels in PA154197 relative to that in PAO1.

Genes	Non-synonymous/Indel mutations in encoding region	Fold Change (PA154197/PAO1)	Log2 Fold Change (PA154197/PAO1)
<i>mexS</i>	D249-N	2.61	1.38
<i>mexT</i>	Del (8bp:237-244),F172-I,G301-C	1.52	0.60
<i>mexE</i>	N/A	122.10	6.93
<i>mexF</i>	N/A	97.54	6.61
<i>oprN</i>	N/A	102.94	6.69
<i>mexG</i>	N/A	12.55	3.65
<i>mexH</i>	N/A	13.91	3.80
<i>mexI</i>	A782-E, A936-T	14.58	3.87
<i>opmD</i>	N/A	13.64	3.77
<i>armR</i>	S21-T,Y32-C	2.87	1.52
<i>mexR</i>	E76-*	5.82	2.54
<i>mexA</i>	N/A	11.08	3.47
<i>mexB</i>	I1001-T	11.49	3.52
<i>oprM</i>	N/A	10.84	3.44
<i>mexX</i>	Q4-H,K329-Q,L331V,W358-R	-	-
<i>mexY</i>	T543-A,L984-F	-	-
<i>mexZ</i>	N/A	-	-
<i>mexC</i>	P57-S,R76-Q,H310-R,S330-A,A378-T ,P383-S,A384-V	4.14	2.05
<i>mexD</i>	T87-S,A155-T,E257-Q,E314-D,V434-A,N669-D,S685-G,I703-V,S845-A,S915-A,I982-V,K1031-R,S1040-T	1.64	0.72

<i>oprJ</i>	T376-S,K413-M	-	-
<i>mexJ</i>	F13-L,A314-P	7.24	2.86
<i>mexK</i>	I21-L,K694-R,A841-T	8.93	3.16
<i>mexL</i>	S4-P	1.96	0.97
<i>muxA</i>	T261-A	-	-
<i>muxB</i>	N/A	-	-
<i>muxC</i>	N/A	-	-
<i>opmB</i>	N/A	-	-
<i>mexP</i>	N/A	2.05	1.03
<i>mexQ</i>	I294-V,V384-I,G505-D,V517-A,R656-K,D722-E	-	-
<i>opmE</i>	S46-G,I50-V,S175-T,W354-R	-	-
<i>oprD</i>	D43-N,S57-E,S59-R,E202-Q,I210-A,E230-K,S240-T,N262-T,A267-S,A281-G,K296-Q,Q301-E,R310-G,V359-L,Del(6bp:1132-1137),M372-V,S373-D,D374-S,N375-S,N376-S,V377-S,K380-Y,N381-A,Y382-G,G383-L	0.64	-0.65
<i>ampC</i>	T105-A	0.54	-0.88
<i>ampR</i>	N/A	-	-
<i>gyrA</i>	T83-I, Del(6bp:2734-2739)	-	-
<i>gyrB</i>	N/A	-	-
<i>parC</i>	N/A	-	-
<i>parE</i>	N/A	1.79	0.84
<i>parS</i>	H398R	1.55	0.64
<i>parR</i>	N/A	1.53	0.61
<i>mvaT</i>	N/A	0.71	-0.48

<i>poxB</i>	N/A	0.17	-2.55
<i>nalC</i>	G71-E,E153-Q,S209-R	1.33	0.42
<i>nalD</i>	N/A	-	-
<i>aph</i>	A14-S	-	-
<i>amgS</i>	I260-V,G288-D	-	-
<i>spuF</i>	P276-S	-	-
<i>fusA1</i>	I186-V	1.85	0.89
<i>fusA2</i>	S176-A,A690-S,G695-A	0.15	-2.78

Notes: N/A, no non-synonymous mutation occurred; Ins: insertion; Del: deletion; “-” indicates not significant difference. “*”: stop codon.