

1 **TITLE: Prevalence and impact of *oprD* mutations in *Pseudomonas aeruginosa* strains in**
2 **cystic fibrosis**

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44 **Running title:** *P. aeruginosa* AMR and *oprD* sequences

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46 **Character count:** 59,898

47

48

49 **ABSTRACT**

50 Defective OprD porins contribute to carbapenem resistance and may be important in
51 *Pseudomonas aeruginosa* adaptation to cystic fibrosis airways. It is unclear whether *oprD*
52 mutations are fixed in populations of shared strains that are transmitted between patients or
53 whether novel variants arise during infection. We investigated *oprD* sequences and
54 antimicrobial resistance of two common Australian shared strains, constructed *P. aeruginosa*
55 mutants with the most common *oprD* allelic variants and compared characteristics between
56 patients with or without evidence of infection with strains harbouring these variants. Our data
57 show that three independently acquired nonsense mutations arising from a 1-base pair
58 substitution are fixed in strain sub-lineages. These nonsense mutations are likely to contribute
59 to reduced carbapenem susceptibility in the sub-lineages without compromising *in vitro*
60 fitness. Not only was lung function worse among patients infected with strains harbouring the
61 nonsense mutations than those without, but they also had an increased hazard rate of lung
62 transplantation/death. Our findings further highlight that understanding adaptive changes may
63 help to distinguish patients with greater adverse outcomes despite infection with the same
64 strain.

65

66 **Key words:** antimicrobial resistance / carbapenems / cystic fibrosis / *oprD* / *Pseudomonas*
67 *aeruginosa*

68

69 INTRODUCTION

70 Cystic fibrosis (CF) is a life-shortening autosomal recessive disease resulting in progressive
71 lung damage (Elborn, Ramsey et al., 2016). Lower airway infection with *Pseudomonas*
72 *aeruginosa* is common, increases with age and, when chronic infection is established, is
73 associated with increased antimicrobial resistance (AMR) (Murray, Egan et al., 2007).
74 Although diverse *P. aeruginosa* genotypes are ubiquitous in the natural environment, some
75 people with CF are infected with clonally-related strains (“shared stains”), which often
76 display AMR to multiple drugs (Parkins, Somayaji et al., 2018). Shared strains have been
77 identified nationally in Australia with three genotypes prominent: AUST-01, AUST-02 and
78 AUST-06 (the latter two are particularly common in the state of Queensland, Australia)
79 (Kidd, Ramsay et al., 2013).

80

81 Adaptation of *P. aeruginosa* to survive and persist in the CF lower airway microenvironment
82 occurs in the setting of long-term antibiotic selective pressure with the occurrence of
83 substantial intra-strain phenotypical and genotypical population diversity. Genome
84 sequencing studies have demonstrated that certain loci are more prone to mutation than others
85 (e.g. *mexZ*, *gyrA*) (Dettman, Rodrigue et al., 2013, Diaz Caballero, Clark et al., 2015, Marvig,
86 Dolce et al., 2015a, Marvig, Johansen et al., 2013, Marvig, Sommer et al., 2015b, Mowat,
87 Paterson et al., 2011, Smith, Buckley et al., 2006, Williams, Evans et al., 2015). However,
88 there is limited information on whether mutations in AMR-related loci are fixed within
89 independent shared strain genotypes or whether novel variants are generally observed in
90 individual patient isolates, suggesting parallel evolution. Identifying differences in adaptive
91 changes in *P. aeruginosa* may help to predict people with CF at the greatest risk of lung

92 function deterioration (Hoffman, Kulasekara et al., 2009, Li, Kosorok et al., 2005, Mayer-
93 Hamblett, Rosenfeld et al., 2014, Tai, Bell et al., 2015a).

94 We have performed a detailed analysis of *oprD* encoding an outer membrane porin, OprD
95 (Porin D or Protein D2 [(Winsor, Griffiths et al., 2016)]), and AMR in a longitudinal
96 collection of shared strains from patients with CF. Polymorphisms in *oprD* have been
97 detected frequently within, and between, strains in many studies indicating their potential
98 importance in adaptation of *P. aeruginosa* to the CF airways (Greipel, Fischer et al., 2016,
99 Kos, Deraspe et al., 2015, Marvig et al., 2013, Marvig et al., 2015b, Wee, Tai et al., 2018).
100 OprD facilitates diffusion of substrates, such as carbapenems and basic amino acids, across
101 cell membranes. It is well recognised that decreased expression or mutational inactivation of
102 *oprD* is an important AMR mechanism (Courtois, Caspar et al., 2018, Jaillard, van Belkum et
103 al., 2017, Kos et al., 2015, Strateva & Yordanov, 2009). It has been shown that virulence is
104 enhanced in *P. aeruginosa* lacking OprD (Skurnik, Roux et al., 2013), demonstrating that
105 mutations at this locus could potentially have consequences for *in vivo* pathogenicity.

106

107 In this study we hypothesised that commonly encountered shared *P. aeruginosa* strains
108 derived from patients with CF harbour loss-of-function *oprD* mutations that: (1) contribute to
109 reduced susceptibility to carbapenems, and (2) could be used as genetic markers to identify a
110 subset of patients at higher risk of an adverse clinical course.

111 **RESULTS**

112 ***In silico* analysis of *oprD* sequences**

113 The phylogenetic relationship of *oprD* variants is shown in Fig 1A. The wild-type variant of
114 AUST-02 and AUST-06 (1326-bp) is denoted as the variant that is closest to the base of each
115 *oprD* clade. These are highly conserved with only 2 nucleotide differences between the
116 lineages at nucleotide positions 807 (T or C) and 1065 (C or G); their protein sequences are
117 identical (441 amino acids). Fourteen of 114 (12%) isolates had the denoted wild-type
118 variant.

119

120 Conspicuous loss-of-function mutations in *oprD* caused by a 1-bp substitution or a 1-bp
121 insertion or deletion (indel) occurred multiple times in the strain collection. Notably, three
122 independent nonsense mutations caused by a 1-bp substitution were identified in 79/114
123 (69%) isolates from 32/50 (64%) patients; two mutations were exclusive to the AUST-02
124 genotype (*oprD* c.526G>T or *oprD* c.748C>T) and one exclusive to AUST-06 (*oprD*
125 c.956C>A). These SNPs were predicted to result in a truncated protein sequence *in silico*,
126 with *oprD* c.526G>T (p.E176X), c.748C>T (p.Q250X), and c.956C>A (p.S319X) leading to
127 truncation at amino acids 175, 249 and 318, respectively. All AUST-02 isolates carrying the
128 *oprD* c.526G>T (p.E176X) variant belong to the M3L7 sub-type, described previously
129 (Sherrard, Tai et al., 2017, Tai et al., 2015a, Wee et al., 2018).

130

131 Seven independent 1-bp indels resulting in a shift in the reading frame were also identified in
132 a smaller proportion of isolates ($n=20/114$; 18%) from 10/50 (20%) participants; each of
133 these belong to the AUST-06 genotype. Six indels were predicted *in silico* to result in a
134 truncated protein sequence, with the remaining indel extending the protein length by 31
135 amino acids to 472 amino acids.

136

137 Only two isolates had acquired additional missense mutations AUS925 (*oprD*
138 c.464_465TG>AA, p.L155Q) and AUS899 (*oprD* c.1174A>C, p.N392H), which were each
139 predicted computationally to affect protein function (Choi, Sims et al., 2012). One isolate
140 (AUS901) had an additional synonymous mutation (*oprD* c.1206T>G). Finally, the *oprD*
141 variant of one AUST-02 isolate (AUS426) was disrupted by an insertion sequence (IS) of the
142 IS3 family that is 100% identical to that found in a sequenced plasmid (GenBank:
143 JX469825.1).

144

145 Participants with >1 isolate sequenced, were generally infected with a strain that had an
146 identical *oprD* variant ($n=14/18$; 78%). However, four participants (Patient-11, Patient-17,
147 Patient-24 and Patient-37), had multiple *oprD* variants among their sequenced isolates (Fig
148 1). Based on the core genome phylogeny of AUST-02 and AUST-06 (Fig 1B), nearly all
149 *oprD* variants emerge from monophyletic clades, which suggests that lineages with specific
150 *oprD* variants pre-existed in the population before spreading between patients. However, we
151 also observed in isolates from Patient-11 and Patient-37, a scenario where multiple *oprD*
152 variants had arisen independently from the same lineage (Fig 1B).

153

154 **AMR of clinical isolates**

155 There were no statistical differences in MICs of the six anti-pseudomonal antimicrobials
156 tested between the AUST-02 ($n=63$) and AUST-06 ($n=51$) genotypes (Fig S1A).

157

158 Given the prevalence of isolates with a nonsense mutation arising from a 1-bp substitution
159 ('target SNPs'; $n=79$), we decided to focus on characterising their AMR patterns compared to
160 isolates with other ('indel') *oprD* mutations ($n=21$; frameshift caused by 1-bp indel or

161 disruption by IS) or the denoted wild-type variant ($n=14$). MIC comparisons are shown in Fig
162 S1B. Statistically significant differences in MICs were apparent for all antimicrobials except
163 colistin to which 99% of isolates were susceptible (Table I). Isolates with a variant containing
164 a target SNP (100%) or an indel (95%) were highly resistant to imipenem (MIC_{50} : >32 mg/L)
165 whereas only one isolate with the wild-type variant (MIC_{50} : 1.5 mg/L) was resistant (Table I).
166 Those with a target SNP also demonstrated greater resistance and had statistically higher
167 MICs for meropenem (all $p<0.001$), ceftazidime ($p=0.04$ or $p<0.001$) and ticarcillin-
168 clavulanic acid (all $p<0.001$) relative to isolates with other variants (Table I). Isolates with a
169 target SNP had higher MICs for tobramycin ($p=0.006$) compared to isolates with an indel.

170

171 Finally, to indicate if clinical isolates with target SNPs (*oprD* c.526G>T, p.E176X; *oprD*
172 c.748C>T, p.Q250X; *oprD* c.956C>A; p.S319X) had similar AMR profiles, their MICs were
173 compared. Although MICs did not differ for the carbapenems, they varied statistically for the
174 remaining four antimicrobials tested (Fig S1C).

175

176 **Allelic replacement of *oprD* in PAO1**

177 Next, we investigated the specific contribution that each target SNP had on carbapenem
178 resistance profiles using PAO1, which is susceptible to imipenem (MIC 1 mg/L) and
179 meropenem (MIC 0.38 mg/L).

180

181 Introduction of the observed 1-bp substitutions into *oprD* of PAO1 *via* allelic replacement led
182 to imipenem resistance for all isogenic mutants (PAO1-OprD^{E176X}, 32 mg/L; PAO1-
183 OprD^{Q250X}, 24 mg/L; PAO1-OprD^{S319X}, 32 mg/L) and increased the meropenem MIC by ~2-3
184 doubling dilutions (PAO1-OprD^{E176X}, 3 mg/L; PAO1-OprD^{Q250X}, 2 mg/L; PAO1-OprD^{S319X},
185 1.5 mg/L).

186 The meropenem MICs are much lower than the MICs seen in the clinical isolates (MIC₅₀:
187 >32 mg/L). All complemented mutants had MICs at wild-type PAO1 levels (Table II).
188 Furthermore, there were no apparent differences in the susceptibility of each isogenic mutant
189 to ceftazidime, ticarcillin-clavulanate, tobramycin and colistin compared to the wild-type
190 strain (Table II).

191

192 It was predicted *in silico* that each of the target SNPs would lead to a truncated protein of
193 between 175 and 318 amino acids. A global data-dependent liquid chromatography-mass
194 spectrometry analysis found no evidence of OprD protein in the PAO1-OprD^{E176X}, PAO1-
195 OprD^{Q250X}, and PAO1-OprD^{S319X} isogenic mutants. In contrast, peptides spanning both N-
196 and C-terminal regions of the OprD protein were detected in PAO1 and all of the
197 complemented mutants. Targeted mass spectrometry using parallel reaction monitoring
198 (PRM) analysis confirmed these results using tryptic peptides that were unique to OprD when
199 compared to the background proteome of PAO1. Finally, *in vitro* growth was not affected by
200 the introduction of the target *oprD* SNPs into the chromosome of PAO1 (Fig S2).

201 **Clinical course**

202 Variant-specific PCR indicated that 434/890 (49%) isolates from 149 participants had one of
203 the target SNPs in *oprD* (Fig 2).

204

205 Overall, 83/149 (56%) participants were infected with ≥ 1 isolate carrying a target SNP in
206 *oprD* (Fig 2); whereas the remaining participants (control subjects) had infection with
207 isolates with other *oprD* variants. Specifically, 101/569 (18%; 22 participants) and 154/569
208 (27%; 27 participants) AUST-02 isolates tested positive for the *oprD* c.526G>T (p.E176X)
209 and *oprD* c.748C>T (p.Q250X) variants, respectively. All AUST-02 isolates positive for the
210 *oprD* c.526G>T (p.E176X) variant were confirmed as belonging to the M3L7 sub-type by
211 PCR. More than half ($n=179/321$, 56%; 41 participants) of AUST-06 isolates tested positive
212 for the *oprD* c.956C>A (p.S319X) variant. Seven (5%) participants with >1 isolate had
213 multiple target variants detected in concurrent or longitudinal isolates.

214

215 Cases ($n=82$, one case lacked clinical data and was excluded from the analysis) and control
216 ($n=66$) subjects were similar with respect to age, sex, CFTR function, chronic/type of shared
217 strain infection status and co-morbidities. The odds of being a case was reduced with higher
218 lung function (OR, 0.97; 95% CI, 0.96-0.99; $p<0.001$) and increased with a greater number of
219 hospital days (OR, 1.01, 95% CI, 1.00-1.03; $p=0.010$). Although not statistically significant,
220 the odds of being a case was reduced with increasing BMI (OR, 0.92, 95% CI, 0.83-1.02;
221 $p=0.11$). Amongst long-term treatments prescribed, inhaled colistin significantly increased
222 the odds of being a case (OR, 2.48, 95% CI, 1.22-5.04; $p=0.010$) compared to non-inhalation
223 as did tobramycin although it was not significant (OR, 2.13, 95% CI, 0.84-5.43; $p=0.11$)
224 (Table III). After controlling for all these factors in a multivariable logistic regression, only
225 lung function remained statistically significant.

226

227 Furthermore, 45/148 (30%; one participant lacked clinical data and was excluded from the
228 analysis) participants were deceased ($n=15$) or had received a lung transplant ($n=30$) by the
229 censor date (Table SI). The type of shared strain infection was not found to be predictive of
230 transplant or death ($p=0.77$). However, being a case had a higher risk of transplant or death
231 (HR, 2.44; 95% CI, 1.27-4.68) compared to being a control subject.

232

233 **DISCUSSION**

234 The emergence of AMR in bacterial pathogens is a foremost concern for clinical management
235 of people with CF, especially as longevity is increasing, and complex antimicrobial treatment
236 regimens are a mainstay of routine care (Waters, Kidd et al., 2019). This study focused on
237 carbapenems, which are fundamental antimicrobials used intravenously to treat pulmonary
238 exacerbations but are often reserved for those with advanced disease or where there is a
239 suboptimal clinical response to other β -lactam antibiotics. Therefore, they are a critical class
240 of antimicrobials to manage the most vulnerable patients. Nevertheless, reduced
241 susceptibility of *P. aeruginosa* to carbapenems is reported, including in our Queensland CF
242 population (Smith, Ramsay et al., 2016, Tai, Kidd et al., 2015b); but the underlying resistance
243 mechanisms and the potential clinical implications of their identification are less clear.

244

245 In our initial work, we performed a targeted analysis of the *oprD* locus of two major
246 Australian shared strains. We found that both strains frequently carry *oprD* variants,
247 supporting previous studies that identified this gene as a common target for mutation during
248 persistent infection of the CF airways (Greipel et al., 2016, Marvig et al., 2015b). The *oprD*
249 variants identified were not shared by AUST-02 and AUST-06, providing no current
250 evidence of DNA transfer of a variant between the genotypes, even though some patients

251 may be concurrently infected with both strains (Tai, Sherrard et al., 2017). Furthermore,
252 rather than identifying many novel *oprD* variants that were generally exclusive to individual
253 patient isolates, we repeatedly identified three specific variants in isolates from multiple
254 patients, which were characteristic of monophyletic strain sub-lineages (Fig 1B). Each variant
255 was truncated due to a nonsense mutation caused by a SNP. We hypothesise that these SNPs
256 became fixed in the sub-lineages following their divergence from all other AUST-02/AUST-
257 06 strains and these sub-lineages were subsequently disseminated between patients, possibly
258 from patient socialisation or via the aerosol mode of transmission (Knibbs, Johnson et al.,
259 2014, Ojeniyi, Frederiksen et al., 2000). This is supported by finding that all AUST-02
260 isolates positive by PCR for the *oprD* c.526G>T (p.E176X) variant were confirmed as
261 belonging to the recently characterised AUST-02 sub-lineage, M3L7, and provides further
262 evidence that this mutation is exclusive to the lineage (Wee et al., 2018). In addition,
263 introduction of the various SNPs of interest into *oprD* of PAO1 had no impact on *in vitro*
264 growth suggesting that these mutations may be acquired without a cost to fitness. Acquiring
265 mutations without a fitness cost may further substantiate the likelihood of clinical strains
266 carrying these *oprD* variants successfully spreading and establishing new infections in the CF
267 patient population (MacLean & San Millan, 2019).

268

269 Interestingly, we also observed two cases where a single AUST-06 lineage acquired two or
270 three different *oprD* variants within a patient (Fig 1B). It is unclear whether these variants
271 were already present in the AUST-06 population before the patient was infected or whether
272 each variant arose independently during the course of infection.

273

274 It is reported that reduced expression or inactivation of OprD constitutes a major resistance
275 mechanism to carbapenems (Courtois et al., 2018, Li, Luo et al., 2012, Llanes, Pourcel et al.,

276 2013) and in keeping with this, our analysis revealed that 99% of imipenem-resistant isolates
277 harboured *oprD* with either a nonsense or indel mutation or on one occasion, disruption by an
278 insertion sequence (MIC₅₀: >32mg/L). Conversely, a full-length coding sequence was
279 associated with much lower MICs (MIC₅₀: 1.5mg/L). One clear discrepancy was a single
280 isolate (AUS396) carrying a 1-bp insertion in *oprD* between nucleotides at positions 1199
281 and 1200, which was susceptible to imipenem (MIC: 2mg/L). This frameshift mutation was
282 predicted to extend the length of the protein and indeed an earlier study found that isolates
283 carrying additional amino acids at the C-terminus of OprD remained susceptible to
284 carbapenems (Ocampo-Sosa, Cabot et al., 2012). However, three other isolates (AUS886,
285 AUS916, AUS918) carrying the same 1-bp insertion as AUS396 showed resistance to
286 imipenem (MICs: >32mg/L). This highlights the complexity of deciphering the development
287 of AMR and the need for studies characterising the impact of individual and combinations of
288 chromosomally acquired mutations.

289

290 The impact of the various mutations on porin integrity is influenced by their position in the
291 protein sequence (Epp, Kohler et al., 2001, Huang, Jeanteur et al., 1995, Ocampo-Sosa et al.,
292 2012). We determined the effect of the three most common *oprD* SNPs in our strain
293 collection using site-directed mutagenesis in the reference strain, PAO1. Our results showed
294 imipenem resistance and an apparent loss of the porin for all three truncations (p.E176X,
295 p.Q250X, p.S319X). Although 97% of clinical isolates with the target SNPs also had a
296 meropenem MIC >32mg/L, we found that in our isogenic mutants the target SNPs increased
297 the meropenem MIC only by several fold to a maximum of 3 mg/L. It is recognised that
298 mutations in *oprD* alone do not cause meropenem resistance (Strateva & Yordanov, 2009).
299 The target *oprD* variants are likely to contribute to reduced susceptibility to meropenem in
300 the clinical isolates and clinical resistance may be a polygenic trait. More genetically diverse

301 collections of isolates with meropenem resistance are needed to identify the genes that are
302 significantly associated with this phenotype. Additionally, the SNPs did not impact the MICs
303 of other β -lactam antibiotics, tobramycin or colistin confirming that these mutations
304 specifically affect the activity of carbapenem antibiotics.

305

306 Based on our prior genome sequencing studies of the M3L7 sub-lineage (Sherrard et al.,
307 2017, Wee et al., 2018), it is likely that the individual clinical isolates included in the current
308 study harbor a multitude of concurrent mutational mechanisms to achieve the high-level
309 resistance observed to carbapenems and the other antimicrobials studied. Carbapenem
310 resistance can arise from the production of antibiotic-inactivating carbapenemases; but recent
311 studies failed to detect acquired carbapenemase encoding genes in CF *P. aeruginosa* isolates
312 suggesting that, to date, this mechanism is uncommon in CF (Chalhoub, Saenz et al., 2016,
313 Courtois et al., 2018, Mustafa, Chalhoub et al., 2016, Tai et al., 2015b). Upregulation of
314 multi-drug efflux pumps and de-repression of the cephalosporinase, AmpC, harbouring
315 spectrum-extending mutations may also contribute to increased MICs (Rodriguez-Martinez,
316 Poirel et al., 2009, Strateva & Yordanov, 2009). We previously identified mutations in
317 various efflux pump regulators (e.g. *mexZ*, *nalC*, *nalD*) and pump proteins (e.g. *mexX*, *mexY*)
318 and *ampC* which might also contribute to carbapenem resistance in M3L7 (Sherrard et al.,
319 2017). It was proposed that inactivation of OprD could be the primary resistance mechanism
320 induced by carbapenem selective pressure with further mechanisms acquired thereafter (Li et
321 al., 2012).

322

323 The AUST-02 and AUST-06 genotypes, have been associated with increased treatment
324 requirements compared to unique *P. aeruginosa* genotypes (Kidd et al., 2013). Prior studies
325 have also identified that adaptive mutations in specific loci, including in *mucA* or related

326 genes that lead to mucoidy or loss-of-function mutations in the transcriptional regulator of
327 quorum sensing *lasR* were associated with accelerated decline in lung function or disease
328 progression (Hoffman et al., 2009, Li et al., 2005). In our current work, we identified a subset
329 of people (>50%) infected with AUST-02/AUST-06 with poorer clinical outcomes based on
330 detection of specific *oprD* variants. This finding extends our previous work that identified a
331 small group of adults infected with M3L7 who had a greater 3-year risk of lung
332 transplantation/death (Tai et al., 2015a) and further highlights the heterogenous clinical
333 course of people despite infection with clonally-related strains. When we investigated the
334 characteristics of people who had acquired strains carrying the target *oprD* SNPs in our
335 multivariable model, lower forced expiratory volume in one-second %predicted was
336 potentially important. As we are unable to precisely determine when each person first became
337 infected with these strains, it is unknown whether they contribute to lung function decline or
338 alternatively, if certain individuals were at greater risk of their acquisition e.g. from spending
339 more time in hospital; a statistically significant variable in the univariable analysis.
340 Moreover, we found a relationship between people infected with a strain carrying a target
341 *oprD* SNP and subsequent increased hazard rate of lung transplantation or death; but this also
342 does not establish causality. Other studies of
343 *P. aeruginosa* blood stream infections have reported that loss of OprD is associated with
344 mortality (Fluit, Rentenaar et al., 2019, Yoon, Kim et al., 2019).

345

346 A recent study showed that *P. aeruginosa* PA14 transposon *oprD* mutants were carbapenem
347 resistant, and also had enhanced serum resistance and survival in a low pH environment and
348 cytotoxicity against phagocytes in a murine model, which possibly occurred *via* alteration of
349 expression of other genes (Skurnik et al., 2013). Therefore, further work is required to
350 characterise if the target *oprD* SNPs identified in our study also lead to the emergence of

351 more virulent CF sub-lineages with enhanced survival in the acidified airway surface liquid
352 that is characteristic of the CF lungs (Pezzulo, Tang et al., 2012).

353

354 Limitations of this study include the small number of isolates that were available per person,
355 especially as infection with multiple strain types and co-existence of divergent strain sub-
356 lineages is recognised (Diaz Caballero et al., 2015, Tai et al., 2017, Williams et al., 2015).

357 Therefore, we may have underestimated the proportion of people infected with strains
358 carrying the target variants. In addition, the variant-specific PCRs were specific to the SNPs
359 of interest and therefore, only have a potential prognostic value to patient populations where
360 AUST-02 and AUST-06 genotypes have been identified. Furthermore, it is likely that the
361 proportion of isolates, which were negative for the target SNPs, will harbour other loss-of-
362 function *oprD* mutations. If these mutations have similar features to the target SNPs is
363 unclear. It is also unknown if the *oprD* SNPs identified have a therapeutic value in predicting
364 response to pulmonary exacerbation treatment regimens containing carbapenems. Currently,
365 *in vitro* AMR and *in vivo* response to treatment are not well correlated in chronic *P.*
366 *aeruginosa* infection (Somayaji, Parkins et al., 2019). Finally, we were unable to exchange
367 the *oprD* variant carrying a nonsense mutation in AUST-02 or AUST-06 clinical isolates with
368 that of the wild-type variant, as the clinical isolates were insensitive to SacB-mediated
369 sucrose toxicity used for counter selection.

370

371 **CONCLUSIONS**

372 We found that nucleotide substitutions and indels were commonly encountered in the *oprD*
373 gene of two prevalent shared strains in an Australian CF population and included three
374 independently acquired nonsense mutations fixed in different sub-lineages. These nonsense
375 mutations could largely explain imipenem resistance amongst the corresponding CF isolates

376 and may be a marker of worse patient outcomes. Studying genes under strong selection
377 pressure, such as *oprD*, in detail may help improve our understanding of chronic-stage
378 adaptations that contribute to strain transmissibility and greater adverse patient outcomes
379 observed in some people.
380

381 MATERIALS AND METHODS

382 Clinical isolates

383 To account for potential diversity in AMR *in vitro* and *oprD* sequences, two shared strains,
384 AUST-02 and AUST-06, were selected for this study. All isolates were obtained by prior
385 surveillance studies between 2001-2014 (Kidd et al., 2013, O'Carroll, Syrmis et al., 2004,
386 Ramsay, Bell et al., 2016, Sherrard et al., 2017) from single colony sputum cultures and were
387 identified as AUST-02 or AUST-06 *via* ERIC-PCR and/or iPLEX20SNP genotyping (Kidd et
388 al., 2013, Kidd, Ramsay et al., 2015, O'Carroll et al., 2004, Ramsay et al., 2016, Sherrard et
389 al., 2017, Syrmis, Kidd et al., 2014). Isolates are held in the QIMR Berghofer Pseudomonas
390 Biobank.

391

392 Ethics approval

393 Ethics approval was granted under HREC/07/QRCH/9 and HREC/13/QPCH/127 by The
394 Prince Charles Hospital (TPCH) Human and Research Ethics Committee. All participants
395 provided written, informed consent/assent.

396

397 Whole genome analysis

398 Genomic DNA from 63 AUST-02 isolates (including 37 isolates of the AUST-02 strain sub-
399 type, M3L7; PRJEB14781, PRJEB14771, PRJEB21755) and 51 AUST-06 isolates
400 (PRJEB35026, PRJNA325248) were utilised (Freschi et al., 2019, Sherrard et al., 2017, Tai
401 et al., 2015a, Wee et al., 2018). These isolates originated from the sputum of 50 participants
402 (10 aged <18-years) attending three CF centres in Brisbane (see Table SII for further details).
403 One isolate was sequenced from 32 participants with a median of three (range, 2-17) isolates
404 sequenced from the remaining 18 participants. Two participants had both strains identified

405 contemporaneously (Patient-37 & Patient-38). Whole genome sequences were evaluated for
406 contamination and quality filtered using Neson clip (v0.128) ([https://github.com/Victorian-](https://github.com/Victorian-Bioinformatics-Consortium/nesoni)
407 [Bioinformatics-Consortium/nesoni](https://github.com/Victorian-Bioinformatics-Consortium/nesoni)). Genomes were assembled using Velvet (v1.2.10)
408 (Zerbino & Birney, 2008) and VelvetOptimiser (v2.2.5)
409 (<https://github.com/tseemann/VelvetOptimiser>). Contigs were reordered with PAO1 as the
410 reference using Mauve (v2.4.0) (Darling, Mau et al., 2010). Assemblies were annotated with
411 Prokka (v1.10) with the PAO1 genome as the primary source of annotation (Seemann, 2014).
412

413 Due to the absence of a closely related complete reference genome (less than 20,000 single
414 nucleotide polymorphisms [SNPs]) to AUST-02 and AUST-06 lineages, we first used Parsnp
415 to align the AUST-02 and AUST-06 isolates to PAO1 to determine the approximate position
416 of the root of each lineage (Treangen, Ondov et al., 2014). A multiple genome alignment was
417 then calculated with only genomes from each shared strain. Alignments were filtered for
418 recombination using Gubbins (v2.3.1) and Maximum-Likelihood phylogenies were
419 constructed with IQTREE (v1.6.6) with 1000 bootstrap replicates (Croucher, Page et al.,
420 2014, Nguyen, Schmidt et al., 2015).

421

422 **Identification of *oprD* variants**

423 Gene sequences of *oprD* from the 63 AUST-02 and 51 AUST-06 isolates were then obtained
424 from the whole genome sequences. Sequence similarity to *oprD* from PAO1 (PA0958; 1332-
425 base pairs [bp]) was determined using blastn requiring $\geq 50\%$ of the query sequence to be
426 present. The AUST-02 and AUST-06 *oprD* variants were then aligned using TranslatorX and
427 Mafft (Abascal, Zardoya et al., 2010, Katoh & Standley, 2013). Relationships between the
428 different *oprD* variants were visualised using the Neighbour-Joining algorithm implemented
429 in Jalview (v2.10.4b1) (Waterhouse, Procter et al., 2009) .

430 **Etest® susceptibility testing**

431 Isolates were inoculated onto Mueller-Hinton Agar and minimum inhibitory concentrations
432 (MICs) were obtained by Etest® (BioMérieux) according to the manufacturer's instructions.
433 Isolates were categorised as susceptible, intermediate or resistant (CLSI, 2018). The
434 *P. aeruginosa* ATCC 27853 strain was used for quality control.

435

436 **Site-directed mutagenesis**

437 Bacterial strains and plasmids are described in Table SIII. Strains were cultured in Luria-
438 Bertani broth at 37°C with shaking at 200rpm and supplemented with antibiotics when
439 appropriate: kanamycin 50µg/mL or streptomycin 50µg/mL or 2000µg/mL (Astral
440 Scientific). Polymerase chain reaction (PCR) assays were performed in an Applied
441 Biosystems Veriti Thermal cycler with oligonucleotide primer sequences shown in Table
442 SIV.

443

444 Mutants of *P. aeruginosa* PAO1 (Klockgether, Munder et al., 2010), were constructed by
445 site-directed mutagenesis of chromosomally encoded *oprD*, performed essentially as
446 described by Muhl and Filloux (Muhl & Filloux, 2014); specific details and minor variations
447 are outlined in the supporting information. Plasmid constructs with the SNPs of interest
448 (*oprD* c.526G>T, p.E176X; *oprD* c.748C>T, p.Q250X; *oprD* c.956C>A, p.S319X) were
449 constructed using plasmid pMRS101 (Sarker & Cornelis, 1997). The pMRS101 constructs
450 were mobilised from *Escherichia coli* CC118 λ pir into PAO1 using a triparental mating with
451 *E. coli* DH5α harbouring the helper plasmid pRK2013 (Ditta, Stanfield et al., 1980)
452 according to the method of Sana *et al* (Sana, Laubier et al., 2014). A single homologous
453 recombination event was selected using streptomycin and a second homologous
454 recombination event was counter selected with 5% sucrose was performed using the method

455 described by Muhl and Filloux (Muhl & Filloux, 2014). The disc diffusion method (Thermo
456 Fisher Scientific) was employed to detect resistance to imipenem as a screen for potential
457 isogenic mutants. The presence of the SNPs in the genome of putative PAO1 mutants was
458 confirmed by PCR and DNA sequencing. The confirmed isogenic mutants were named:
459 PAO1-OprD^{E176X}, PAO1-OprD^{Q250X}, and PAO1-OprD^{S319X}.

460

461 Complementation of the PAO1-OprD^{E176X}, PAO1-OprD^{Q250X}, and PAO1-OprD^{S319X} mutants
462 was performed using a pMRS101 plasmid construct with the wild-type *oprD* gene encoded
463 and the same methodologies employed to generate the original mutations. Complemented
464 mutants were named: PAO1-OprD^{E176C}, PAO1-OprD^{Q250C} and PAO1-OprD^{S319C}.

465

466 **Proteomic analysis of OprD from *P. aeruginosa* PAO1**

467 A detailed description of all steps is provided in the supporting information. Briefly, C18
468 reverse phase chromatography was performed on whole protein preparations from the cell
469 pellets of PAO1, each isogenic mutant and its complement. Data-dependent acquisition
470 (DDA) runs were performed to identify sequence coverage of the OprD protein and generate
471 a spectral library and scheduled targeted PRM runs for targeted verification. Protein
472 identification was performed using MaxQuant (v1.6.2.6) (Cox & Mann, 2008, Cox,
473 Neuhauser et al., 2011). The results were visualised using Perseus (Tyanova, Temu et al.,
474 2016). Skyline was used to generate scheduled targeted methods and select precursor-product
475 pairs (transitions) from the DDA dataset (MacLean, Tomazela et al., 2010, Schilling, Rardin
476 et al., 2012).

477

478

479

480 **Growth curve analysis**

481 As an indicator of *in vitro* fitness, the growth of each isogenic mutant was compared to that
482 of PAO1. 200µL of a culture containing 1.0–1.5 x 10⁶ colony-forming units/mL in Luria-
483 Bertani Broth was transferred to a 96-well microtitre plate in triplicate and incubated with
484 shaking at 37°C for 20-hours. Growth was monitored (OD_{620nm}) using a Biotek Powerwave
485 plate reader.

486

487 **Variant-specific PCR**

488 Variant-specific PCR assays were designed to rapidly screen a collection of AUST-02
489 (*n*=569) and AUST-06 (*n*=321) isolates for target *oprD* SNPs. These isolates originated from
490 sputum of 149 participants (median [range], 6 [1-19] isolates/person). Primer sequences are
491 described in Table SV. Each reaction mix contained 10µL Platinum SYBR Green qPCR
492 superMix-UDG (Invitrogen), 1µL each of 10µM sense and anti-sense primer pairs (Sigma-
493 Aldrich), 2µL of isolate DNA (Anuj, Whiley et al., 2009) and nuclease-free water (Qiagen)
494 made up to a total volume of 20µL. The PCR amplification was performed on a Rotor-Gene
495 Q instrument (QIAGEN Pty Ltd) as described in the supporting information.

496

497 **Clinical characteristics**

498 Data were retrieved from medical records, the referring CF centre and/or the Australian CF
499 Data Registry. Age, gender, height, weight, spirometry with percent predicted calculated
500 using the Global Lung Function Initiative equations (Quanjer, Hall et al., 2012), co-
501 morbidities, long-term treatments and infection status with *P. aeruginosa* (Lee, Brownlee et

502 al., 2003, Ramsay, Sandhu et al., 2017) were obtained. Cystic fibrosis transmembrane
503 conductance regulator (CFTR) function was recorded from the genotype as residual, minimal
504 or non-classified (Green, McDougal et al., 2010). Data on patients' hospitalisations in the
505 previous year were collected. The date of death or lung transplantation was recorded, where
506 applicable.

507 **Data analyses**

508 Statistical analyses were generated using IBM SPSS v22. MICs were compared using a
509 Mann-Whitney *U* test or a Kruskal-Wallis test with the Bonferroni adjustment applied.
510 Categorical data were compared using Pearson's Chi-squared test. Based on the outcome of
511 the variant-specific PCRs, participants were categorised as either cases (PCR positive for a
512 target *oprD* SNP) or control subjects (PCR negative) and clinical characteristics of each
513 group were described using descriptive statistics. For cases, data relate to the date of first
514 detection of a strain that tested positive for a target SNP. For controls, data relate to the date
515 of first detection of AUST-02 or AUST-06. Participants ($n=9$) with only one isolate were
516 reported according to that PCR result. Potential predictors of being a case were identified by
517 univariate analysis as having $p \leq 0.15$ and examined in a multivariable logistic regression
518 model using backwards stepwise selection; variables expected to affect risk (age and type of
519 shared strain infection) were retained to control for any potential confounding effects. Prior
520 to their inclusion variables were checked for collinearity. Adjusted odds and 95% confidence
521 intervals are reported. Cox regression was used to assess the association between being a case
522 with time to lung transplantation or death. For patients who had not had a transplant and were
523 still alive, time was censored on the 31st August 2017. The Cox regression model also
524 included age, gender and the type of shared strain infection. The hazard ratio and 95%
525 confidence interval are reported.

526

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533

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542

543 **AUTHOR CONTRIBUTIONS**

544 LJS, BAW, TJK and SCB conceived the study and wrote the first draft of the manuscript.
545 LJS, BAW, CD, KAR, KAD, EB, CEW, KG, HES, DMW, SAB, TJK and SCB
546 designed/performed the experiments, performed analysis, interpreted the results and/or
547 contributed data for this work. All authors contributed to and approved the final version of
548 the manuscript.

549

550 **CONFLICT OF INTEREST**

551 The authors declare that they have no conflict of interest.

552

553 **THE PAPER EXPLAINED**

554

555 **Problem**

556 *Pseudomonas aeruginosa* is the most important pathogen chronically infecting the lungs of
557 people with cystic fibrosis (CF) and cross-infection of strains (shared strains) can occur. *P.*
558 *aeruginosa* is known to develop antimicrobial resistance (AMR) including to carbapenem
559 antibiotics, which are often reserved for people with the most advanced disease. Despite
560 reports of reduced susceptibility of CF *P. aeruginosa* to carbapenems, the underlying
561 resistance mechanisms are less clear. Studies have also shown that as *P. aeruginosa* adapts to
562 persist in the CF lungs during long-term antibiotic treatment, certain genes are more prone to
563 mutation than others. However, there is limited information on whether mutations in AMR-
564 related genes (e.g. *oprD*) are characteristic of particular shared strain genotypes or whether
565 novel variants are generally observed in individual patient isolates. Importantly, there is a
566 paucity of data on whether the chromosomal mutations observed are of any clinical
567 relevance.

568

569 **Results**

570 In this study, we performed a detailed analysis of the *oprD* gene, which is hypothesised to
571 play an important role in adaptation of *P. aeruginosa* to the CF lungs, and AMR in two
572 shared strains that are prevalent in our patient population. We identified commonly occurring
573 *oprD* allelic variants in isolates from multiple patients, that were unique to specific shared-
574 strain lineages suggesting that successful *oprD* alleles emerge and persist in these shared
575 strain populations. By constructing and characterising *P. aeruginosa oprD* mutants, we show

576 that these variants are likely to contribute to reduced carbapenem susceptibility without
577 compromising *in vitro* fitness. We also identified a subset of people infected with the shared
578 strains with poorer clinical outcomes, including an increased hazard rate of death or lung
579 transplantation, based on detection of specific *oprD* variants.

580

581 **Impact**

582 Our findings emphasise the role of *oprD* mutations in carbapenem resistance in the setting of
583 *P. aeruginosa* lung infections in CF. Further our findings highlight the importance of
584 thorough investigations of genes under strong antimicrobial selection pressure to help
585 improve our understanding of (1) chronic-stage *P. aeruginosa* adaptations that may contribute
586 to strain transmissibility and (2) the greater adverse patient outcomes observed in some
587 people with CF despite infection with clonally-related strains.

588

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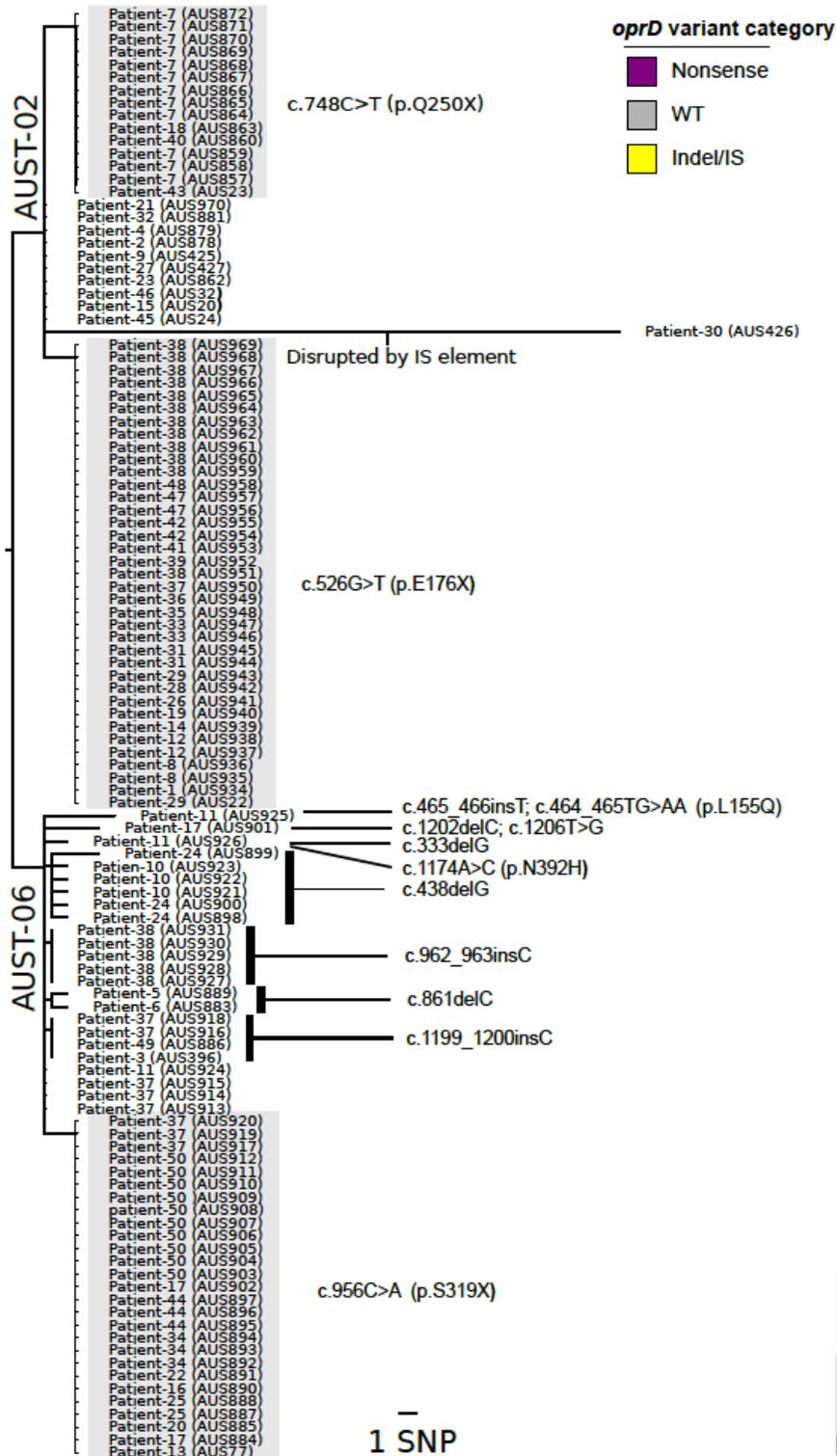
823 **FIGURE LEGENDS**

824 **Fig 1 A** Phylogenetic relationship of AUST-02 and AUST-06 *oprD* variants. Amino acid
825 substitutions shown in parentheses. The Neighbour-Joining algorithm implemented in
826 Jalview was used to visualise relationships between the different variants. The coloured bars
827 on the right-hand side indicate the *oprD* variant categories used to compare AMR results
828 (purple, target SNP i.e. 1-bp substitution leading to nonsense mutation; grey, wild-type
829 sequence; yellow, indel mutation including disruption by IS). **B** Recombination-filtered,
830 Maximum-Likelihood core SNP phylogeny of AUST-02 (left) and AUST-06 (right) isolates
831 encoding the described *oprD* variants. Each variant is given a unique colour (note that one of
832 five isolates with the *oprD* c.438delG variant had acquired an additional *oprD* missense
833 mutation but are denoted by the same colour) in the first column and the patient number is
834 indicated in the 2nd greyscale column. Branches supported by >80% bootstrap are indicated
835 by black circles, branches shown with dotted lines are not to scale. *Definitions*: IS, insertion
836 sequence; WT, wild-type.

837

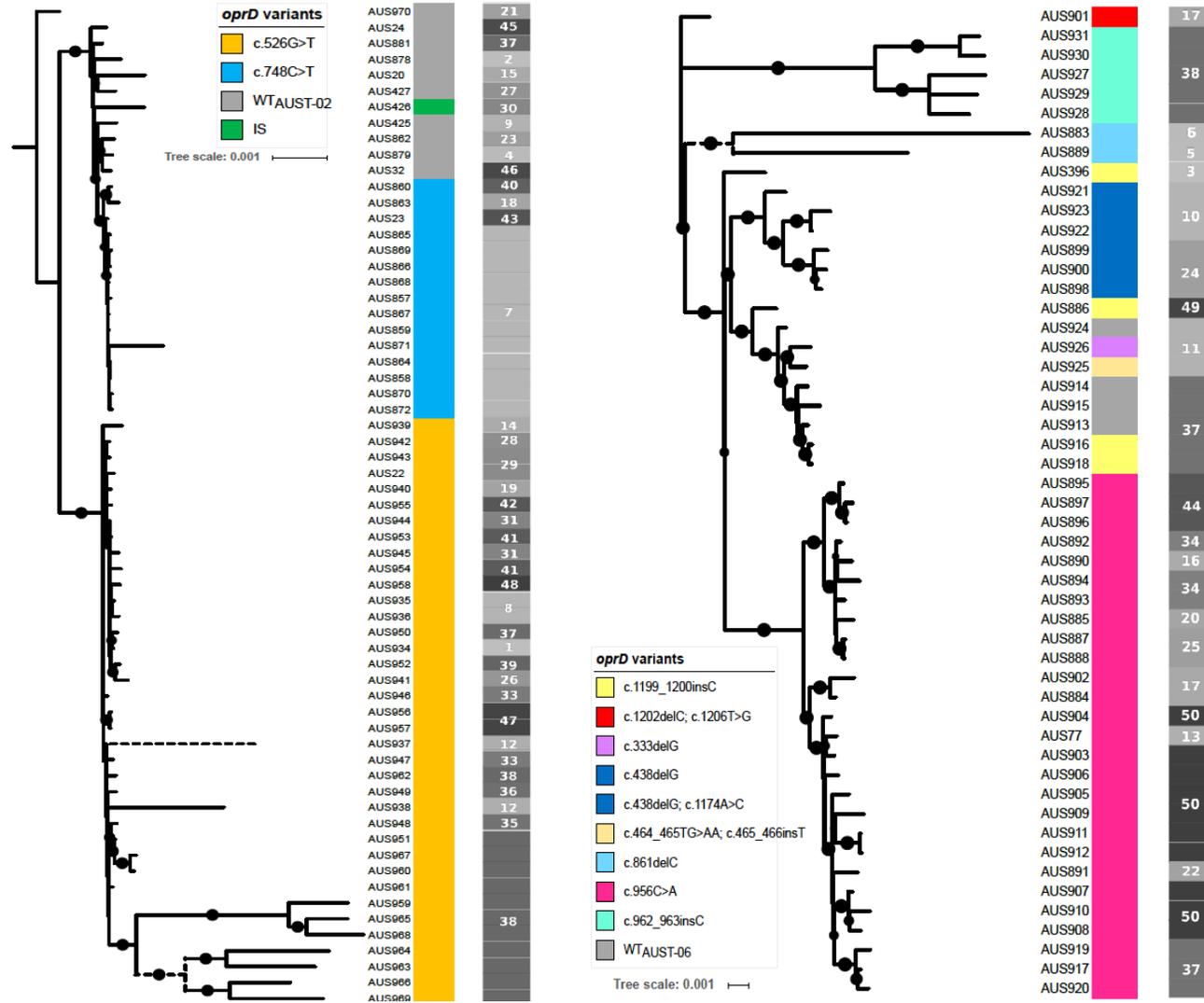
838 **Fig 2** Scheme showing how AUST-02 and AUST-06 isolates were screened for the target
839 *oprD* SNPs. Participants were subsequently stratified according to PCR results: cases,
840 positive for ≥ 1 *Pseudomonas aeruginosa* isolate with a target SNP; control subjects, isolates
841 PCR negative (i.e. infected with isolates with non-target *oprD* variants).

842



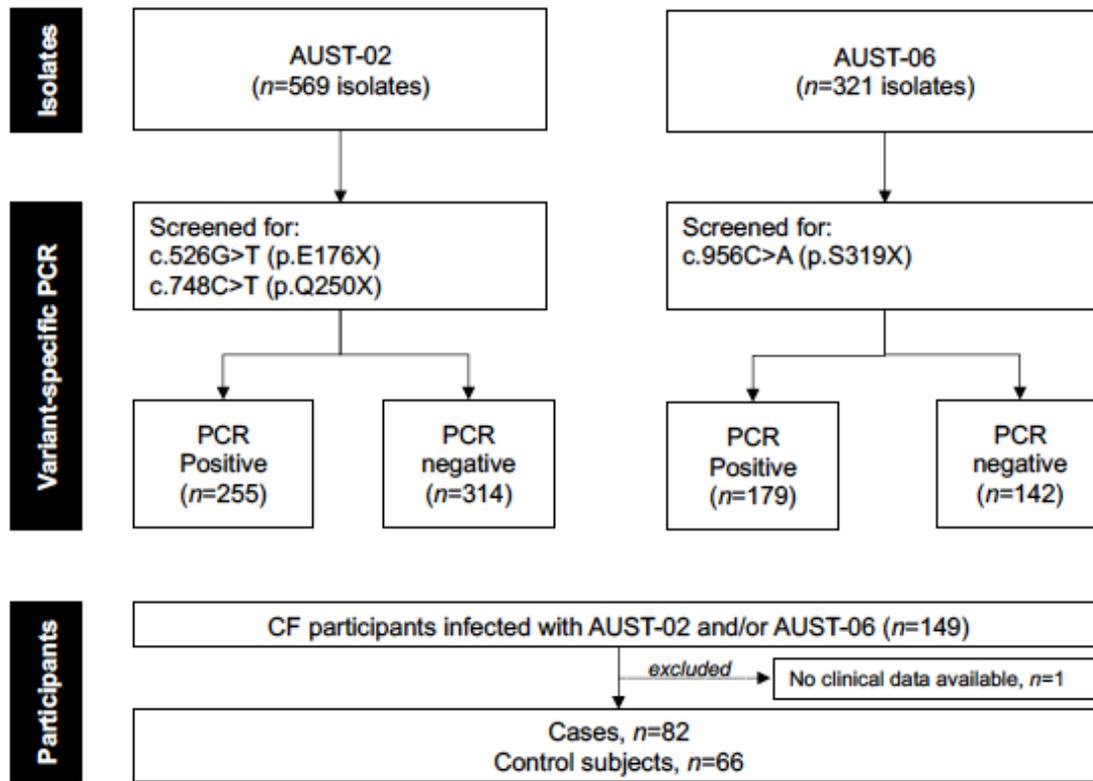
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B



848

849 Fig 2
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851
852

853 **Table I** *In vitro* antimicrobial susceptibilities of isolates stratified by *oprD* variant category.

<i>oprD</i> variant category and antimicrobial agent*	MIC (mg/L)			Percentage of isolates with indicated susceptibility		
	Range	50%	90%	S	I	R
<i>Target SNP (n=79)</i>						
Imipenem	12 to >32	>32	>32	0	0	100
Meropenem	3 to >32	>32	>32	0	2.5	97.5
Ticarcillin-clavulanate	2 to >256	>256	>256	8.9	2.5	88.6
Ceftazidime	1 to >256	>256	>256	17.7	1.3	81.0
Tobramycin	1 to >256	6	>256	40.5	29.1	30.4
Colistin	0.125-1.5	0.5	1	100	0	0
<i>Indel (n=21)</i>						
Imipenem	12 to >32	>32	>32	4.8	0	95.2
Meropenem	0.75 to >32	>32	>32	14.3	9.5	76.2
Ticarcillin-clavulanate	0.75 to >256	>256	>256	33.3	0	66.7
Ceftazidime	0.25 to >256	4	>256	57.1	4.8	38.1
Tobramycin	0.5 to >256	4	64	66.7	19.0	14.3
Colistin	0.125-3	0.75	2	95.2	0	4.8
<i>Wild-type (n=14)</i>						
Imipenem	0.125-6	1.5	3	78.6	14.3	7.1
Meropenem	0.012-2	0.125	1.5	100	0	0
Ticarcillin-clavulanate	0.125 to >256	4	>256	71.4	7.2	21.4
Ceftazidime	0.064-16	1	16	85.7	14.3	0
Tobramycin	0.25 to >256	4	>256	50.0	14.3	35.7
Colistin	0.125-2	0.5	2	100	0	0

854 S, susceptible; I, intermediate; R, resistant

855 **oprD* variant categories: Target SNP, one of three 1-bp substitutions leading to a nonsense mutation; indel, 1-
856 bp insertion or deletion leading to a shift in the reading frame or disruption by an insertion sequence; wild-type,
857 variant that is closest to the base of each *oprD* clade in the phylogeny (Fig 1A).
858

859 **Table II** Susceptibility of PAO1 and mutant clones.

PAO1 strain	<i>oprD</i> variant	Antibiotic MICs (mg/L); median (range)*					
		Imipenem	Meropenem	Ticarcillin-clavulanate	Ceftazidime	Tobramycin	Colistin
Wild-type	Wild-type	1 (1-1.5)	0.38 (0.25-0.5)	12 (12-16)	0.75	0.5 (0.5-0.75)	1.5 (1.5-2)
PAO1-OprD ^{E176X}	c.526G>T (p.E176X)	32 (16-32)	3 (2-4)	12 (12-16)	0.75 (0.5-0.75)	0.5 (0.5-0.75)	1.5
PAO1-OprD ^{E176C}	Wild-type	1	0.25 (0.19-0.25)	12	0.5 (0.5-0.75)	0.5 (0.5-0.75)	1.5 (0.75-2)
PAO1-OprD ^{Q250X}	c.748C>T (p.Q250X)	24 (12-32)	2 (1.5-3)	12	0.5 (0.5-0.75)	0.5	1.5
PAO1-OprD ^{Q250C}	Wild-type	1	0.25 (0.25-0.5)	12 (12-16)	0.75 (0.5-0.75)	0.5 (0.38-0.5)	1.5
PAO1-OprD ^{S319X}	c.956C→A (p.S319X)	32 (24-32)	1.5 (1.5-2)	16 (12-16)	0.5 (0.5-0.75)	0.38	1.5 (1.5-2)
PAO1-OprD ^{S319C}	Wild-type	1 (0.75-1)	0.19 (0.19-0.25)	12 (8-12)	0.5 (0.5-0.75)	0.38 (0.38-0.5)	1.5

860 *Biological replicates in triplicate were performed. If no range shown, then the MICs of replicates were identical

861 **Table III Participant characteristics and their association with being a case.**

Characteristic	Number patients	Cases (n=82) [‡]	Control subjects (n=66) [‡]	Odds ratio (95% CI) [‡]	p
Female; n (%)	148	37 (45.1)	28 (42.4)	1.07 (0.54-2.14)	0.80
<i>CFTR</i> function; n (%)	148				
- Minimal		70 (85.4)	57 (86.4)	1.03 (0.34-3.06)	1.00
- Residual		4 (4.9)	2 (3.0)	0.68 (0.22-2.16)	0.60
- Non-classified		8 (9.7)	7 (10.6)	Reference group	
FEV ₁ % predicted, mean (sd)	144	49.0 (21.8)	64.2 (26.1)	0.97 (0.96-0.99)	<0.001
BMI, median (IQR)	148	19.4 (17.2-22.6)	21.4 (19.3-23.7)	0.92 (0.83-1.02)	0.11
Pancreatic insufficiency; n (%)	148	78 (95.1)	65 (98.5)	0.29 (0.03-2.82)	0.29
<i>Cystic fibrosis-related diabetes</i> ; n (%)	148				
- D+FH		14 (17.1)	12 (18.2)	0.92 (0.38-2.22)	0.84
- IGT		8 (9.7)	5 (7.6)	1.25 (0.37-4.17)	0.72
- Normal GT		60 (73.2)	49 (74.2)	Reference group	
Liver disease; n (%)	148	9 (11.0)	7 (10.6)	1.06 (0.36-3.10)	0.90
Oral azithromycin; n (%)	147	73 (90.1)	54 (81.2)	1.98 (0.73-5.35)	0.18
Inhaled tobramycin; n (%)	147	71 (86.6)	51 (77.3)	2.13 (0.84-5.43)	0.11
Inhaled colistin; n (%)	147	46 (56.8)	24 (36.4)	2.48 (1.22-5.04)	0.010
Inhaled dornase alfa; n (%)	147	60 (74.1)	43 (65.2)	1.51 (0.72-3.16)	0.27
Number of days in hospital, median (IQR)	148	26.5 (10.8-51.8)	8.5 (0.0-16.8)	1.01 (1.00-1.03)	0.010

862 *Definitions:* D + FH, diabetes with fasting hyperglycaemia; IGT, impaired glucose tolerance; Normal GT, normal glucose tolerance; FEV₁ % predicted, forced expiratory
863 volume in the first second percent predicted; BMI, body mass index; *CFTR*, cystic fibrosis transmembrane conductance regulator (*CFTR* function: residual function,
864 harbouring ≥1 allele with Class IV-V mutations; minimal function, harbouring two alleles with Class I-III mutations; non-classified, harbouring two alleles with mutations of
865 unknown function).

866 [‡]Cases, tested positive for ≥1 isolate with a target *oprD* SNP (one case lacked clinical data and was excluded from the analysis); control subjects, tested negative for isolates
867 with a target *oprD* SNP. Data collected relate to the date of first detection of AUST-02 and/or AUST-06 with a target SNP or to the date of first detection of AUST-02 and/or
868 AUST-06 if always tested negative.

869 ^{||}All odds ratios presented are adjusted for age and shared strain infection (AUST-02, AUST-06 or mixed) due to their expected effects on odds of testing positive for an
870 isolate with a target SNP in *oprD*; however, these potential confounders were never statistically significant in any of the models. Odds of being a case compared to being a
871 control.

872 **Bolded text: variables included in the multivariable logistic regression**