# Genomic and phenotypic comparison of environmental and patient-derived isolates of *Pseudomonas aeruginosa* suggest that antimicrobial resistance is rare within the environment

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| **Contribution** | **Author** |
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| Conceptualisation | ILL, WMP, CW, SCB |
| Methodology | ILL, KAR |
| Software | SJTW |
| Validation | KAR |
| Formal Analysis | KAR, SJTW |
| Investigation | KAR, SJTW |
| Resources | ILL |
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**Abstract**

Patient-derived isolates of the opportunistic pathogen *Pseudomonas aeruginosa* are frequently resistant to antibiotics due to the presence of sequence variants in resistance-associated genes. However, the frequency of antibiotic resistance and of resistance-associated sequence variants in environmental isolates of *P. aeruginosa* has not been well studied. Antimicrobial susceptibility testing (ciprofloxacin, ceftazidime, meropenem, tobramycin) of environmental (n=50) and cystic fibrosis (n=42) *P. aeruginosa* isolates was carried out. Following whole genome sequencing of all isolates, twenty-five resistance-associated genes were analysed for the presence of likely function-altering sequence variants. Environmental isolates were susceptible to all antibiotics with one exception, whereas patient-derived isolates had significant frequencies of resistance to each antibiotic and a greater number of likely resistance-associated genetic variants. These findings indicate that the natural environment does not act as a reservoir of antibiotic-resistant *P. aeruginosa,* supporting a model in which antibiotic susceptible environmental bacteria infect patients and develop resistance during infection.

## Introduction

As an environmental bacterium, *Pseudomonas aeruginosa* has been isolated from many different niches including water sources and domestic and health-care settings (1-4). While rarely causing infections in healthy individuals, this opportunistic pathogen can cause a range of infections in people who are immunocompromised or have predisposing conditions such as cystic fibrosis (CF) (1). For adults with CF, *P. aeruginosa* is the most prevalent bacterium causing respiratory infection and *P. aeruginosa* from these infections are frequently resistant to antibiotics, complicating treatment (5). Resistance is associated with variants in key genes that reduce the intracellular concentrations of antibiotics or the affinities of target proteins for antibiotics, relative to antibiotic-susceptible isolates (6). Infection arises from exposure to

63 environmental sources of *P. aeruginosa* included both the natural and health-care environment
64 (1). Epidemiological studies of isolates obtained from high infection risk areas, such as health-
65 care settings, domestic and community areas show that antibiotic resistant *P. aeruginosa* can
66 be present providing a potential reservoir of infectious resistant bacteria (7-18). Acquisition of
67 *P. aeruginosa* from the natural environment, typically during childhood, is also a major source
68 of infection with a number of studies identifying genotypically indistinguishable strains in the
69 natural environment and the respiratory tract of CF patients (3, 19-22). However antibiotic
70 susceptibility of isolates from natural (non-man-made) environmental sources has had very
71 limited studies and so it is not clear whether the natural environment provides a reservoir of
72 antibiotic-resistant *P. aeruginosa* (7, 23, 24). Using a cohort of clinical isolates as a
73 comparator, here we determined the prevalence of antibiotic resistance in *P. aeruginosa* from
74 the natural environment (river), the domestic and community settings (swimming pool and
75 water tank) by assessing antimicrobial susceptibility profiles and allelic variations in resistance
76 genes of *P. aeruginosa*.
77
78 **Materials and Methods**
79 **Isolate Selection and identification**
80 Fifty environmental *P. aeruginosa* isolates obtained from water sources in Queensland,
81 Australia and 42 clinical *P. aeruginosa* isolates obtained from CF patients residing in Australia
82 and New Zealand were examined (Supplementary Table 1). Environmental isolates included
83 those obtained from rivers (n=36), swimming pools (n=13) and one sample from a domestic
84 water tank. Individual multi-locus sequence types (MLST) were present in singleton isolates,
85 expect for sequence types (ST) -155, -179, -266 and -381 which were represented once each in
86 both the CF and environmental isolate cohorts. Isolate identification and MLST typing were
87 confirmed using molecular techniques (https://pubmlst.org/ paeruginosa/) (25-27).

88 **Antimicrobial Susceptibility Testing**

89 The minimum inhibitory concentration (MIC) of ciprofloxacin, meropenem, tobramycin and
90 ceftazidime was determined for all the 92 isolates using ETEST® strips and methodology. The
91 breakpoint was determined according to the Clinical and Laboratory Standards Institute (CLSI)
92 guidelines (https://clsi.org/).

93 **Whole genome sequencing and bioinformatic analysis**

94 DNA was extracted from each strain using the MoBio UltraClean® Microbial DNA isolation
95 kit in accordance with the manufacturer's instructions. Isolates were sequenced to ~50-fold
96 (50x) coverage using illumina MiSeq, 2x150, or 2x300 basepair (bp) paired-end reads. Raw
97 sequence reads were trimmed using Trimmomatic version 0.36 (28). Draft genomes were
98 assembled using SPAdes version 3.12.0 as described previously (29).

99 **Resistance Gene Analysis**

100 Genome assemblies were used for the comparison of 25 well characterised genes associated
101 with antibiotic resistance, with reference sequences sourced from
102 https://www.pseudomonas.com (Supplementary Table 2). Protein Variation Effect Analyzer
103 (PROVEAN) version 1.1 was utilised to identify and analyse genetic variants within the chosen
104 genes (30). Genetic variants (amino acid substitutions) were categorised in accordance to
105 PROVEAN scores, as predicted function-altering variants (PROVEAN score: ≤ -2.5) or
106 variants not predicted to affect function (PROVEAN score: > -2.5). Additionally, each genome
107 was manually screened using tblastn for premature stop codons and deletions which were also
108 classified as function-altering variants (31).

109 ResFinder version 3.1 was used with a prebuilt ResFinder database, including all relevant
110 antibiotics, to determine whether resistance could be affected by acquired genes
111 (https://cge.cbs.dtu.dk/services/ResFinder/).

112 **Statistical analysis**

113 SPSS version 25 was used for statistical analysis. Pearson's chi-square test was used to
114 examine the association between niche and phenotype (susceptible or resistant) and niche and
115 genotype. When more than 20% of the expected values were less than five, Fisher's exact test
116 were used. A *P*-value of <0.05 was considered significant.
117
118 **Results and Discussion**
119 A total of 50 isolates of *P. aeruginosa* from the natural environment and 42 isolates from
120 patients with CF were included in this study. The isolates are genetically diverse and broadly
121 representative of the *P. aeruginosa* species (Supplementary Figure S1).
122 **Environmental isolates are antibiotic susceptible**
123 All 50 environmental isolates were susceptible to ciprofloxacin, meropenem and tobramycin
124 and only one of these isolates was resistant to ceftazidime (Figure 1, Supplementary Table 3).
125 Conversely, a non-susceptible (intermediate or resistant) phenotype was observed for 15 of the
126 42 CF isolates. Specifically, 10%, 17% 19% and 21% of the CF isolates were non-susceptible
127 to tobramycin, meropenem, ceftazidime and ciprofloxacin, respectively (Figure 1,
128 Supplementary Table 4). Overall, the frequency of resistant isolates was significantly less in
129 the environmental cohort than the CF cohort (ceftazidime *P*=0.010, ciprofloxacin *P*=0.001,
130 meropenem *P*=0.003, tobramycin *P*=0.040). Due to limited availability of clinical information
131 we were unable to characterise CF infections as either early/transient or chronic to assess
132 whether resistance is more prevalent in chronically infected patients. However, when we
133 categorised the CF isolates according to patient age (adult ≥18 years; adult n=29, paediatric
134 n=13), isolates from two paediatric patients (15.4%) demonstrated antibiotic resistance
135 compared with isolates from 13 adults (44.8%) (*P*=0.066) (Figure 1). Overall our findings are
136 in agreement with a model of bacterial adaptation during infection leading to increased
137 resistance over time (32). Our results demonstrate that environmental isolates are susceptible

138 to clinically relevant antibiotics and that the environments we tested are not a reservoir for
139 antibiotic resistant isolates.

140 **Environmental isolates have fewer predicted function-altering variants in resistance-**
141 **associated genes**

142 We analysed 25 well characterised resistance-associated genes for the presence of likely
143 function-altering variants that could influence antibiotic susceptibility (Supplementary Table
144 2). The environmental isolates had strikingly fewer predicted function-altering variants than
145 the CF isolates. A total of 75 predicted function-altering variants were identified within 18 of
146 25 resistance genes analysed for the 50 environmental isolates. No predicted function-altering
147 variants were identified in the remaining seven genes (Supplementary Table 3). In this cohort,
148 the predicted function-altering variants are generally not sufficient to confer antibiotic
149 resistance as all except one isolate was fully susceptible to the antibiotics tested. In contrast,
150 110 predicted function-altering variants affecting all 25 resistance-associated genes were
151 identified in the 42 CF isolates. Function-altering variants not analysed by PROVEAN,
152 including premature stop-codons and deletions, were absent in the environmental cohort but
153 were present in nine CF isolates (premature stop-codons n=8, deletions n=2) (Supplementary
154 Table 4). Overall, variants in the environmental cohort had a significantly higher PROVEAN
155 score than those in the CF cohort ($P<0.001$), indicating the presence of fewer amino acid
156 substitutions that may affect function of resistance-associated proteins (Supplementary Figure
157 2).

158 The frequency of function-altering variants within each gene was determined for both cohorts
159 of isolates (Table 1). Statistical differences were noted for 6 genes in the CF cohort with a
160 greater number of function-altering variants being present in *amgS* and *fusA*1 (tobramycin
161 resistance), *gyrA* and *gyrB* (ciprofloxacin resistance) and *mexZ* (broad-spectrum resistance)
162 genes for isolates in the CF cohort, and in *ampE* (β-lactam resistance) for the natural

163 environmental isolates (Table 1). Specific variants present in these genes, such as T83I (*gyrA*),
164 and R504C (*ftsI*) previously associated with resistance were only present in the CF isolate
165 cohort only (33, 34). However, a strong association between individual gene variants and
166 resistance phenotype was not observed (Supplementary Table 4) due to the low numbers of
167 isolates with variants for each gene combined with the multifactorial nature of antibiotic
168 resistance in *P. aeruginosa*.
169 ResFinder analysis assessing the presence of horizontally acquired resistance genes identified
170 a gene *crpP* associated with fluoroquinolone resistance (35). However there was no correlation
171 between ciprofloxacin resistance and the presence of *crpP* for the isolates in this study
172 (Supplementary Table 3 and 4). No other horizontally-transferred resistance genes were
173 identified.
174 In conclusion, our findings show that isolates from *P. aeruginosa* from natural environments
175 have low frequencies of antibiotic resistance, and of genetic variants associated with resistance,
176 compared to isolates from patients with CF. These findings indicate that the natural
177 environment is unlikely to act as a reservoir of antibiotic-resistant *P. aeruginosa*. They are
178 consistent with a model in which patients are infected by antibiotic-sensitive *P. aeruginosa*
179 from the environment which then evolves to become antibiotic-resistant during infection (32).
180

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201 K.A.R, S.J.T-W, W.M.P, B.B, D.W.R and I.L.L have nothing to declare. S.C.B is a Member
202 of Advisory Board, Member of Writing group, Site Principle Investigator, author on several
203 Rempex sponsored studies, a member of advisory boards for Vertex, Abbvie and Galapagos
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207

208 Table 1: Comparison of predicted function altering variants identified in the CF and

209 environmental isolates (n = 92).

| Gene | Predicted function altering variants[$] | | *P*-value† |
|---|---|---|---|
| | Environment (n=50) | CF (n=42) | |
| *amgR* | 0 | 1 | 0.457* |
| *amgS* | 0 | 5 | 0.017* |
| *ampC* | 15 | 17 | 0.293 |
| *ampD* | 9 | 5 | 0.418 |
| *ampDh2* | 1 | 3 | 0.328* |
| *ampDh3* | 6 | 6 | 0.746 |
| *ampE* | 14 | 4 | 0.026 |
| *ampG* | 0 | 1 | 0.457* |
| *ampR* | 7 | 3 | 0.336* |
| *aph3'IIb* | 19 | 19 | 0.204 |
| *ftsI* | 1 | 5 | 0.089* |
| *fusA*1 | 0 | 16 | <0.001 |
| *galU* | 0 | 1 | 0.457* |
| *gyrA* | 1 | 10 | 0.001 |
| *gyrB* | 0 | 6 | 0.007* |
| *mexR* | 1 | 1 | 1.00* |
| *mexZ* | 1 | 14 | <0.001 |
| *mpl* | 1 | 4 | 0.174* |
| *nalC* | 1 | 3 | 0.328* |
| *nalD* | 0 | 3 | 0.091* |
| *nfxB* | 3 | 5 | 0.462* |
| *nuoG* | 3 | 2 | 1.00* |
| *oprD* | 41 | 36 | 0.631 |
| *parC* | 1 | 1 | 1.00* |
| *parE* | 2 | 3 | 0.657* |

210 [$]PROVEAN predicted function-altering variants, premature stop codons and deletions.

211 †Chi-square value; [*]Fisher's Exact Test used in place of chi-square test

212 *P*-value <0.05 is considered significant

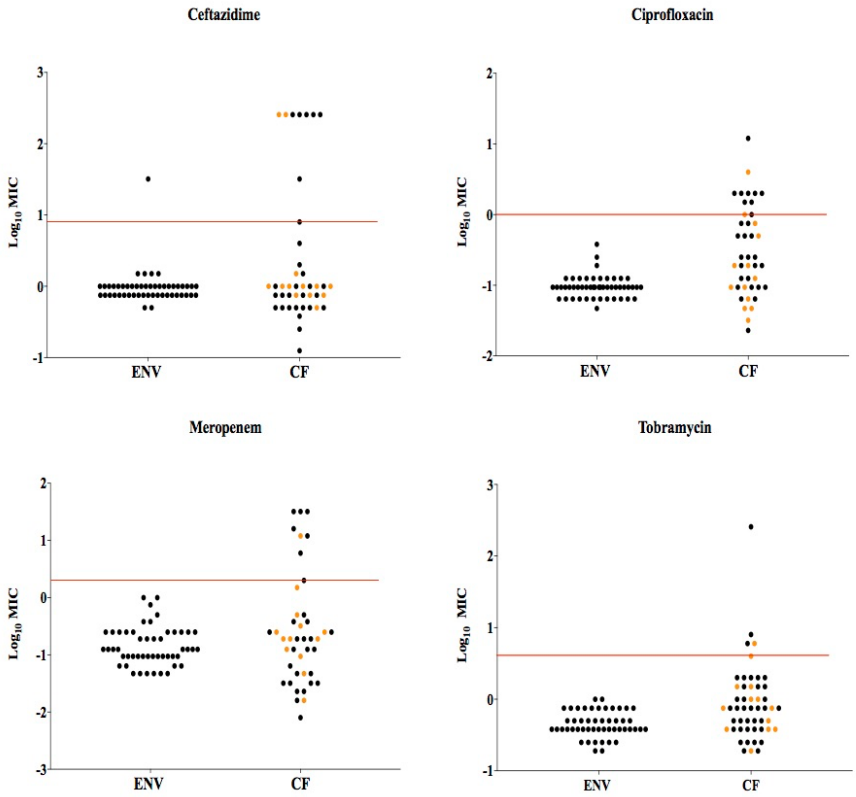


Figure 1: Distribution of antimicrobial susceptibility results of environmental (ENV; n=50) and cystic fibrosis (CF; n=42) isolates for ciprofloxacin, ceftazidime, meropenem and tobramycin. The horizontal red lines represent the antibiotic breakpoint as defined by the CLSI. The CF isolates have been further differentiated into samples collected from adult (black circles) and paediatric (orange circles) patients. The MIC results have been log transformed ($log_{10}$) for ease of graphical representation.

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