bioRxiv preprint doi: https://doi.org/10.1101/2022.11.22.517596; this version posted November 23, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 **PilD mutant is a new cheater in *Pseudomonas aeruginosa* quorum-sensing evolution**

2 Huifang Qiu[1,2], Weijun Dai[1,2]

3

4 [1]Guangdong Laboratory for Lingnan Modern Agriculture, South China Agricultural University,
5 Guangzhou, 510642, China
6
7 [2]College of Plant Protection, Integrative Microbiology Research Center, South China Agricultural
8 University, Guangzhou, 510642, China
9

10

11

12

13

14

15 Send correspondence to: Weijun Dai

16 Integrative Microbiology Research Center, South China of Agricultural University, Guangzhou,

17 510642, China

18 daiweijun@scau.edu.cn

19
20
21
22
23
24
25
26
27 **Running title**: identification of new cheater mutant
28
29
30
31
32
33
34 **Key words:** quorum sensing, PilD, *Pseudomonas aeruginosa*, evolution

bioRxiv preprint doi: https://doi.org/10.1101/2022.11.22.517596; this version posted November 23, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

35 **Abstract**

36 Pathogen virulence is largely driven by the evolution and adaptation of the genome. LasR is a master
37 regulator of quorum-sensing (QS) system. The QS-inactive LasR-null mutant regains QS activation in
38 a laboratory evolution experiment through obtaining mutations in *mexT*, a gene encoding
39 transcriptional regulator. How the QS-active LasR-MexT mutant continuously evolves is still unknown.
40 Here we passaged the LasR-MexT mutant in casein broth and monitored its evolutionary trajectory. By
41 examining the proteolytic phenotype of isolated single colonies, we found a protease-negative
42 subpopulation arising from the parental population. The whole genome sequencing analysis revealed
43 that these protease-negativ colonies bear mutations in *pilD* gene. In accordance with its role as a
44 prepilin peptidase of type IV system, PilD mutants are defective in twitching motility. In conclusion,
45 we identified a new PilD mutant cheater that evolved from the parental LasR-MexT mutant population.
46 Our work demonstrates that population evolution is highly dynamics, with arising of distinct
47 subpopulations containing different social behaviors.

48

49 **Introduction**

50 *Pseudomonas aeruginosa* is an opportunistic pathogen that causes severe acute and chronic human
51 infections (Gellatly and Hancock, 2013; Klockgether and Tümmler, 2017). A cohort of *P. aeruginosa*
52 virulence factors is under the control of the quorum-sensing (QS) system (Papenfort and Bassler, 2016).
53 QS is a bacterial cell-cell communication system that regulates the expression levels of hundreds of
54 genes in a cell density-dependent manner. *P. aeruginosa* has two acyl-homoserine lactone (AHL) QS
55 systems, Las and Rhl QS systems. LasR is a master regulator of Las QS system. In general, LasR-null
56 mutant results in the inactivation of QS system.

57

58 Intriguingly, many LasR-null *P. aeruginosa* clinical isolates remain QS activity, producing QS
59 molecules and QS-controlled virulence products, such as C4-HSL and pyocyanin (Bjarnsholt et al.,
60 2010; Feltner et al., 2016). Through evolution experiments, mutations in *mexT* gene were found to be
61 responsible for reverting QS activity in a laboratory strain PAO1 LasR-null mutant (Kostylev et al.,
62 2019; Oshri et al., 2018). The *mexT* mutations down-regulates the expression of the MexEF-OprN
63 efflux pump genes, which regulates the QS circuit (Köhler et al., 2001; Tian et al., 2009b).

64

65 To capture the evolutionary trajectory of the QS-reprogramming LasR-null revertant, we evolved a
66 PsdR-LasR-MexT mutant strain in casein broth and monitor the social behavior of isolated single
67 colonies. After one-month continuous passage, protease-negative clones appeared from the parental
68 population. These clones bear mutations in *pilD*, a gene encoding the prepilin peptidase of type IV
69 system. PilD mutants cheat cooperative parental population because they do not produce extracellular
70 proteases. As PilD mutations disrupt the export of many virulence factors as well as the pilus assembly,
71 the arising of PilD mutants expect to adversely affect the whole population virulence.

72

73 **Results**

74 **Screen for protease-negative variants from PsdR-LasR-MexT mutant.**

75 Mutations in *mexT* are known to revert QS activity in the LasR-null mutant of *P. aeruginosa* strain
76 PAO1 (Kostylev et al., 2019; Oshri et al., 2018). To further reveal the evolutionary trajectory of
77 QS-active LasR-null revertants, we evolved the LasR-MexT mutant. As cells evolving in casein broth
78 obtain *psdR* mutations for cellular dipeptide metabolism regulation (Asfahl et al., 2015), we initiated

bioRxiv preprint doi: https://doi.org/10.1101/2022.11.22.517596; this version posted November 23, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

79 the evolution with a constructed triple mutant PsdR-LasR-MexT. Cells were evolved in casein broth,
80 where production of extracellular proteases are necessary for cell growth. Two replicate mutant cells
81 were passaged in casein broth every 5 day and screened for colonies deficient in protease-secretion.
82 After 30-day evolution, protease-negative colonies appeared in one cell line, reaching to about 20% in
83 the evolving population. These colonies exhibited LasR-null-like proteolytic phenotype, suggesting
84 they obtained genomic mutations that impaired the extracellular protease production. These variants
85 utilized the proteases from parent PsdR-LasR-MexT cells, and thus could be defined as cheaters.
86

87 **Identification of mutations responsible for protease-secretion inactivation**

88 To identify mutations responsible for protease-secretion inactivation in those mutant variants, we next
89 selected protease-negative clones for whole genome short read resequencing (WGS). The WGS
90 analysis revealed short fragments of deletions in *pilD* (Table S1), a gene encoding a prepilin peptidase
91 of Type 4 system. No other genomic mutations were identified in tested clones. We next amplified and
92 sequenced fragments across the *pild* coding region in other protease-negative clones. These clones all
93 shared mutations in *pilD*, suggesting mutated *pilD* resulting in protease-secretion inactivation.
94

95 **Phenotypic feature in PilD mutants**

96 To characterize the phenotypic consequences of PilD mutants, we constructed the
97 PsdR-LasR-MexT-PilD mutant. Consistent with the phenotypes of screened colonies,
98 PsdR-LasR-MexT-PilD is protease-deficient (Fig. 1). On the other hand, As PilD is a prepilin peptidase
99 responsible for removing the signal peptide of the prepilin pilA in the type IV system (Pepe and Lory,
100 1998), mutations in *pilD* were expected to paralyze the system. We thus sought out to examine their
101 twitching motilities, a typical feature of type 4 system. As expected, those protease-deficient clones all
102 lacked twitching motilities but not for the parental PsdR-LasR-MexT mutant. In conclusion, our results
103 show that PilD mutant is novel cheater containing a disrupted type IV system.
104

105 **Discussion**

106 In the present study, we characterized the evolutionary trajectory of a QS-active.PsdR-LasR-MexT
107 mutant strain by continuous passage in casein broth. We found that mutant subpopulations arising from
108 parental PsdR-LasR-MexT population contain mutations in *pilD* gene. Similar to the well-known
109 typical cheater LasR mutant (Diggle et al., 2007; Sandoz et al., 2007) , *pilD* mutants do not secret
110 proteases and have to exploit extracellular proteases of neighboring cells. Unlike LasR as a master
111 regulator of QS closely involving in QS circuit (Papenfort and Bassler, 2016), PilD encodes a prepilin
112 peptidase of a type IV system and cleavages the signal peptide of the prepilin pilA prior to pilus
113 assembly (Pepe and Lory, 1998). Therefore, further evolution of a QS-active population appears a
114 novel non-QS cheater independent of QS regulatory pathway.
115

116 The arising of PilD mutant subpopulation may have impacts on the population virulence. PilD controls
117 the export of alkaline phosphatase, phospholipase C, elastase and exotoxin A (Strom et al., 1991).
118 These extracellular products are virulence factors critical for bacterial infection. On the other hand, as
119 cleavage of signal peptide by PilD is necessary for the pilus assembly (Strom et al., 1993), PilD
120 mutations result in type IV-specific defections (Fig. 2). Type IV system in *P. aeruginosa* influences
121 many physiological processes , including adherence to living surfaces, twitching motility, modulation
122 biofilm biosynthesis, DNA uptake and exchange and exoproteins secretion (Giltner et al., 2012). It can

bioRxiv preprint doi: https://doi.org/10.1101/2022.11.22.517596; this version posted November 23, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

123 expect that the growth and development of PilD mutant subpopulation in the whole community would
124 largely determine the population virulence.
125

126 **Materials and methods**

127 **Bacterial strains and growth**

128 The PAO1 strain and the mutant derivatives were grown in Luria Bertani (LB) broth containing 10
129 mg/ml tryptone, 5 mg/ml yeast extract, 10 mg/ml NaCl at 37 ^{o}C or photosynthetic medium (PM)
130 (Kim and Harwood, 1991) supplemented with 1% sodium caseinate (Sigma Aldrich, USA) as the
131 sole carbon source at 37 ^{o}C. Unless otherwise specified, *P. aeruginosa* strains were cultured in
132 14-mm FALCON tubes (Corning, USA) containing 3 mL medium, with shaking (225 RPM) at 37
133 ^{o}C. Escherichia coli was grown in LB broth at 37 ^{o}C.
134

135 **Evolution experiments**

136 Two independent colonies of PsdR-LasR-MexT mutants were separately inoculated into 3 mL
137 LB-Mops broth for overnight culture. Cell evolution was initiated by transferring 50 μl bacteria
138 grown in LB-Mops into 3 mL PM medium in 14-mm FALCON tubes (Corning, USA). Passage
139 was performed every 5 days by exchanging 50 μl bacteria into 3 mL fresh PM medium. To
140 identify protease-positive variants, evolved colonies were isolated on LB agar and spotted onto the
141 skim milk plates at 37 ^{o}C for 18h to monitor the protease-catalyzed zones.
142

143 **Construction of PilD mutant**

144 Full-length of *pilD* gene knocking out was based on the homologous recombination exchange approach
145 as described previously. Briefly, about 500 ~ 1000 bp of DNA flanking the targeted full length of gene
146 of interest were PCR-amplified and cloned into pEXG2 vector (Gentamycin resistance, Gm) with the
147 Vazyme ClonExpress II One Step Cloning kit (Vazyme Biotech, Nanjing, China), generating
148 pEXG-flanking constructs. The pEXG-flanking construct was mobilized into *P. aeruginosa* strain by
149 triparental mating with the help of *E. coli* PRK2013 strain (Kanamycin resistance, Km). Deletion
150 mutants were first selected on Pseudomonas Isolation agar (PIA) containing 100 μg/ul Gm and further
151 selected on LB agar containing 10% sucrose. All mutants were confirmed by PCR amplification and
152 subsequent DNA Sanger sequencing.
153

154 **Skim milk plate assay**

155 Total proteolytic activities of P. aeruginosa strains were evaluated through skim milk assay, in which
156 the tested strains form a zone of clearing on skim milk agar plate. Individual colonies were spotted on
157 the skim milk agar plates (25% LB, 4% skim milk, 1.5% agar). The protease-catalyzed zones were
158 photographed after incubation at 37 ^{o}C for 16h.
159

160 **Twitching motility**

161 Surface-associated twitching motility was assessed via agar stab inoculation in 1% LB agar plate.
162 Colonies were transfer from solid LB agar plate and stab inoculated in the 1% LB agar plate. Twitching
163 motility zones (occurring at the interface of agar/petri plate) were allowed to develop 24 h at 37 ^{o}C,
164 after which the agar was removed. The twitching zones were then visualized by staining of 0.06%
165 Coomassie Brilliant Blue and 10% Acetic Acid for 10 min and washing with distilled water briefly.
166 Skim milk assay

bioRxiv preprint doi: https://doi.org/10.1101/2022.11.22.517596; this version posted November 23, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

167

168 **Funding**

169 This work was supported by the National Natural Science Foundation of China (31771341).

170

171 **References**

172 Asfahl, K.L., Walsh, J., Gilbert, K., and Schuster, M. (2015). Non-social adaptation defers a
173 tragedy of the commons in *Pseudomonas aeruginosa* quorum sensing. *The ISME journal 9*,
174 1734-1746.

175 Diggle, S.P., Griffin, A.S., Campbell, G.S., and West, S.A. (2007). Cooperation and conflict in
176 quorum-sensing bacterial populations. Nature *450*, 411-414.

177 Giltner, C.L., Nguyen, Y., and Burrows, L.L. (2012). Type IV pilin proteins: versatile molecular
178 modules. Microbiology and Molecular Biology Reviews *76*, 740-772.

179 Kim, M.-K., and Harwood, C.S. (1991). Regulation of benzoate-CoA ligase in
180 *Rhodopseudomonas palustris*. FEMS Microbiology Letters *83*, 199-203.

181 Kostylev, M., Kim, D.Y., Smalley, N.E., Salukhe, I., Greenberg, E.P., and Dandekar, A.A.
182 (2019). Evolution of the *Pseudomonas aeruginosa* quorum-sensing hierarchy. Proceedings of
183 the National Academy of Sciences *116*, 7027-7032.

184 Oshri, R.D., Zrihen, K.S., Shner, I., Omer Bendori, S., and Eldar, A. (2018). Selection for
185 increased quorum-sensing cooperation in *Pseudomonas aeruginosa* through the shut-down of
186 a drug resistance pump. *ISME J 12*, 2458-2469.

187 Papenfort, K., and Bassler, B.L. (2016). Quorum sensing signal–response systems in
188 Gram-negative bacteria. Nature Reviews Microbiology *14*, 576.

189 Pepe, J.C., and Lory, S. (1998). Amino Acid Substitutions in PilD, a Bifunctional Enzyme of
190 *Pseudomonas aeruginosa*: EFFECT ON LEADER PEPTIDASE AND
191 N-METHYLTRANSFERASE ACTIVITIES IN VITRO AND IN VIVO. Journal of Biological

bioRxiv preprint doi: https://doi.org/10.1101/2022.11.22.517596; this version posted November 23, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

192 Chemistry *273*, 19120-19129.

193 Sandoz, K.M., Mitzimberg, S.M., and Schuster, M. (2007). Social cheating in *Pseudomonas*
194 *aeruginosa* quorum sensing. Proceedings of the National Academy of Sciences *104*,
195 15876-15881.

196 Strom, M.S., Nunn, D., and Lory, S. (1991). Multiple roles of the pilus biogenesis protein pilD:
197 involvement of pilD in excretion of enzymes from *Pseudomonas aeruginosa*. Journal of
198 Bacteriology *173*, 1175-1180.

199 Strom, M.S., Nunn, D.N., and Lory, S. (1993). A single bifunctional enzyme, PilD, catalyzes
200 cleavage and N-methylation of proteins belonging to the type IV pilin family. Proceedings of
201 the National Academy of Sciences *90*, 2404-2408.

202

203 **Figure legends**

204 **Figure 1. Protease activity of PilD mutants.**

205 Equal numbers of bacteria of indicated strains were spotted on skim milk plates to estimate proteolytic
206 activity. Pictures were taken 24 h later. PsdR-LasR-MexT, triple mutant; PsdR-LasR-MexT-PilD,
207 deletion of PilD from PsdR-LasR-MexT mutant.

208

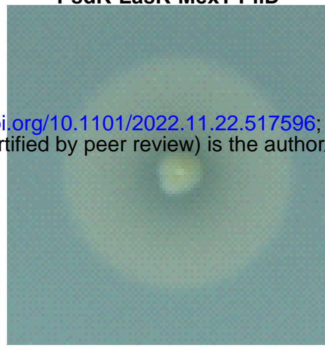
209 **Figure 2. Twitching motilities of PilD mutants.**

210 Twitching motilities of PsdR-LasR-MexT and PsdR-LasR-MexT-PilD mutants were assayed. Thin
211 agar plates (1.0%) were stab inoculated with a toothpick to the bottom of the plate and incubated for 24
212 h at 37 ° C. The twitching zone was visualized by Coomassie Blue staining.

213

214 **Supplementary Tables**

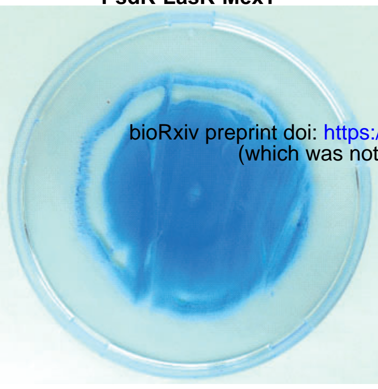
215 **Table S1. Identification of mutations in *pilD*.**



bioRxiv preprint doi: https://doi.org/10.1101/2022.11.22.517596; this version posted November 23, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Fig. 1

PsdR-LasR-MexT



PsdR-LasR-MexT-PilD

bioRxiv preprint doi: https://doi.org/10.1101/2022.11.22.517596; this version posted November 23, 2022. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

Fig. 2