# Reduced prevalence of phage defense systems in Pseudomonas aeruginosa strains from cystic fibrosis patients

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# Reduced prevalence of phage defense systems in *Pseudomonas aeruginosa* strains from cystic fibrosis patients

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**ABSTRACT** Cystic fibrosis is a genetic disorder that affects mucus clearance, particularly of the lungs. As a result, cystic fibrosis patients often experience infections from bacteria, which contribute to the disease progression. *Pseudomonas aeruginosa* is one of the most common opportunistic pathogens associated with cystic fibrosis. The presence of *P. aeruginosa* complicates the treatment due to its high antibiotic resistance. Thus, research is ongoing to treat these infections with bacterial viruses instead, known as bacteriophages. Notably, *P. aeruginosa* clinical strains possess a variety of phage defense mechanisms that may limit the effectiveness of phage therapy. In this study, we compared the defense system repertoire of *P. aeruginosa* strains isolated from cystic fibrosis patients with those from non-cystic fibrosis patients. Our findings reveal that *P. aeruginosa* strains isolated from cystic fibrosis patients have fewer phage defense mechanisms per strain than from non-cystic fibrosis patients, suggesting altered phage selection pressures in strains colonizing CF patient lungs.

**IMPORTANCE** Cystic fibrosis patients often experience chronic *Pseudomonas aeruginosa* lung infections, which are challenging to treat with antibiotics and contribute to disease progression and eventual respiratory failure. Phage therapy is being explored as an alternative treatment strategy for these infections. However, assessing strain susceptibility to phage treatment is essential for ensuring efficacy. To address this, we investigated whether CF-associated clinical *P. aeruginosa* strains have a distinct phage defense repertoire compared with those isolated from other lung patients. We observed that CF-associated *P. aeruginosa* strains have significantly fewer phage defenses, possibly affecting the susceptibility of these strains to phage infection.

**KEYWORDS** phage defense systems, *Pseudomonas aeruginosa*, cystic fibrosis, phage therapy, mucus, Zorya, CRISPR-Cas, Wadjet, plasmid-restrictive mechanisms

Cystic fibrosis (CF) is a genetic disorder that affects mucus clearance, especially of the lungs (1). The decrease in mucus clearance creates a breeding ground for opportunistic pathogens, such as bacteria, to colonize the lungs (1). These infections are often chronic, accumulate resistance to antibiotic treatment, and cause major lung damage (1, 2). Over time, this damage results in respiratory failure, the most common cause of death for CF patients (1). Lung infections in CF patients are often caused by strains of *Pseudomonas aeruginosa* (1). Although antibiotic treatments can alleviate patient symptoms and prolong life, they generally fail to fully eradicate *P. aeruginosa* from the lungs (3). Consequently, research has turned to alternative treatment options, including the use of bacterial viruses (phages) (4–8). However, the efficacy of phage-based treatments might also be limited since *P. aeruginosa* strains often possess a variety of phage defense mechanisms that may limit the sensitivity of strains to phages (9). Particularly, a recent study on the abundance of defense systems in clinical isolates of *P. aeruginosa* found that these strains accumulate a large number of defense systems and are less susceptible to

phage infection (9). However, it is unknown if the distribution of phage defense systems varies with the pathogen environment or patient conditions. In this study, we investigated the defense system repertoire of clinical *P. aeruginosa* isolates from mucus-rich lungs of CF patients by comparing them to strains isolated from non-CF lung patients.

We utilized the *Pseudomonas* Genome Database to investigate the defense system repertoire of *P. aeruginosa* isolates from the respiratory system of CF and non-CF (e.g., pneumonia) patients (10). We first compared the prevalence of the phage defense systems and found isolates from CF patients to encode significantly fewer phage defense systems per strain (non-CF: 9.8 vs CF: 6.8; a 30% reduction; Welch two sample *t*-test, t = −9.878, df = 601.37, $P < 2.2e^{-16}$) (Fig. 1A; Fig. S1A) (9). This possibly renders the isolates from CF lungs more susceptible to phages, as we previously observed that *P. aeruginosa* strains, including CF strains, encoding fewer phage defense systems, exhibit greater susceptibility to phages (9). This finding remains valid when only considering lung isolates ($n = 11$, adjusted $R$-squared = 0.37, $P < 0.05$). Interestingly, this reduction in the number of defense systems per strain did not affect the overall diversity of the defense repertoire, where the Shannon index (both: 3.86) and evenness (both: 0.80) remained identical (Table S1). Therefore, we hypothesized that the decrease in defense systems per strain is not the result of a broad, non-specific reduction. Instead, we suspected that the overall reduction is possibly caused by differences in the abundance of a few prevalent defense systems. To assess this, we compared the abundance of each defense system in CF and non-CF lung isolates (Table S1) using a χ test followed by a post-hoc test with false-positive rate (FDR) adjusted *P*-values, considering adjusted *P*-values lower than 0.01 to be significant (Table S2). Additionally, we considered changes greater than twofold ($log_2FC > 1$ or $log_2FC < −1$) as relevant (Table S2). We identified 10 defense systems with considerable changes in abundance (two were more abundant, and eight were less abundant in CF strains; Fig. 1B and C; Fig. S1B to F), but only three of these systems were prevalent enough to potentially affect the overall number of defense systems per strain (present in more than 10%) in either group, all three were more depleted in CF isolates. These defense systems include Wadjet type I (11) (non-CF: 37.8% vs CF:13.4%; $log_2FC = −1.4$), RM type III (non-CF: 23.5% vs CF: 7.7%; $log_2FC = −1.5$) and Zorya type I (12) (non-CF: 24.4% vs CF: 3.4%; $log_2FC = −2.5$). The reduced prevalence of just these three defense systems accounts for one-fifth (344 out of 1786 fewer defense systems than expected: 19.3% of total) of the total reduction in defense systems observed in CF compared with non-CF isolates. Besides these defense systems, CRISPR-Cas type IV-A is also noteworthy for being completely absent in the CF isolates, while present in the non-CF isolates (non-CF: 2.6% vs CF: 0%; $log_2FC = −1.8$). Interestingly, all of the above-mentioned defense systems act on foreign DNA, and notably, two of these defense systems, Wadjet type I and CRISPR-Cas type IV-A, are known to act upon plasmids specifically (11, 13, 14). The reduced presence of plasmid-restrictive mechanisms of these strains may highlight the role of plasmids in CF-infecting strains, potentially facilitating the acquisition of plasmids to gain resistance against antibiotics used for treating CF infections (15, 16). Notably, the reduction in the phage defense repertoire of CF lung isolates is not due to the reduced presence of plasmid-restrictive mechanisms alone, such as Wadjet, RM systems, CRISPR-Cas systems, defense island system associated with restriction-modification (DISARM) (17), and methylation-associated defense system (MADS) (18). Rather the observed reduction remains unaffected by the exclusion of these plasmid-restrictive defenses from the analysis (CF mean: 4.2; non-CF mean: 5.8; Welch two sample *t*-test: $t = 9.1$, df = 680, $P < 2.2e^{-16}$).

It remains unclear whether the reduced prevalence of defense systems in CF isolates was caused by strain selection before or during lung infection in CF patients. As for antibiotic resistance, strains infecting CF lungs adapt over time (1) and could therefore also adapt in the context of phages and other mobile genetic elements such as plasmids. In the CF lung environment, this adaptation seems to involve the loss of phage defense mechanisms or reduction of their expression, possibly because CF lungs are less penetrable to phages due to factors, such as reduced air circulation, a thicker and
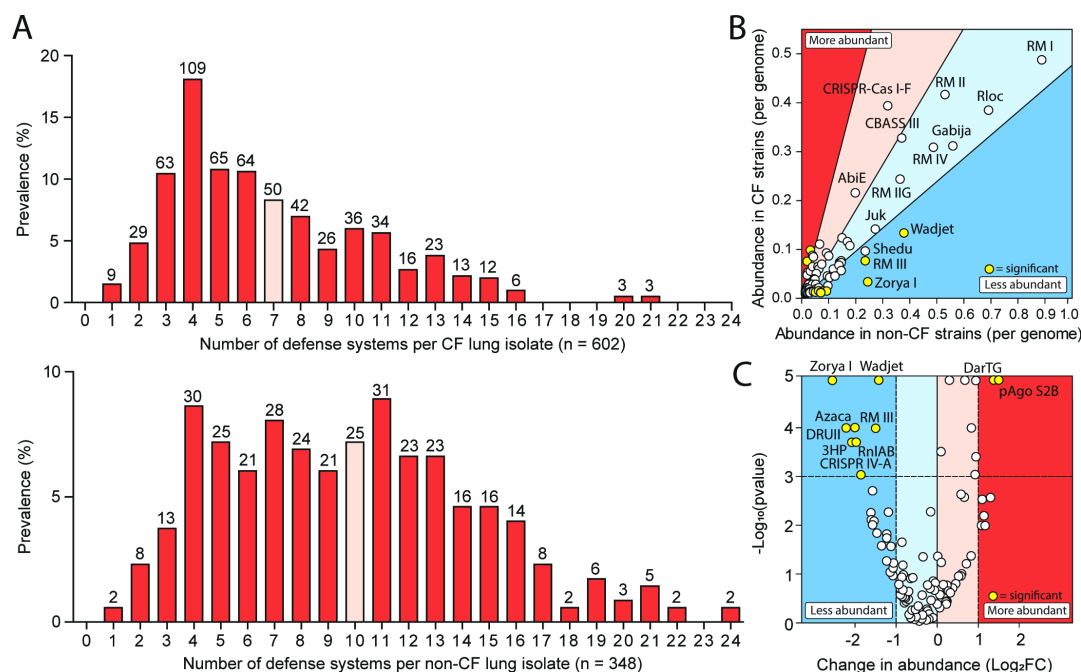
**FIG 1** *Pseudomonas aeruginosa* strains isolated from cystic fibrosis (CF) patient lungs have fewer defense systems. (A) The number of defense systems per *P. aeruginosa* strain isolated from CF lungs (top) and non-CF lungs (bottom). The median number of defense systems per strain is indicated with a lighter red-colored bar. (B) The comparative abundance of individual defense systems in *P. aeruginosa* strains isolated from CF and non-CF lungs. The graph is divided into four sections: red represents a $\log_2FC$ greater than 1, light red indicates a $\log_2FC$ between 0 and 1, light blue corresponds to a $\log_2FC$ between 0 and −1, and blue signifies a $\log_2FC$ less than −1. The significance of the change in abundance was calculated using a $\chi$ test with false-positive rate (FDR) adjusted *P*-values. Significant defense systems (adjusted $P < 0.01$) are indicated as yellow dots. (C) A volcano plot depicting the change in abundance ($\log_2FC$) of defense systems between CF and non-CF isolates as well as the significance of this change.

dehydrated mucus layer, and a higher prevalence of biofilms (1, 19). Supporting this hypothesis, we observed fewer prophages in the genomes of CF lung isolates, suggesting reduced exposure to phage predation (CF: 3.3 vs non-CF: 5.3 prophages per genome; 38% decrease; Welch two sample *t*-test: $t = -12.379$, df = 508.88, $P < 2.2e^{-16}$) (Fig. S1G). Besides the reduced phage defenses of *P. aeruginosa* isolates from CF lung isolates we also observed these strains to have a significantly smaller genome (CF: 6.5 Mb vs non-CF: 6.8 Mb; a 4% decrease; Welch two sample *t*-test: $t = -13.811$, df = 594.47, $P < 2.2e^{-16}$) and to encode fewer genes (CF: 6,053 vs non-CF: 6,298 genes; a 4% decrease; Welch two sample *t*-test: $t = -11.715$, df = 625.07, $P < 2.2e^{-16}$) (Fig. S1H and I). These characteristics reflect a more adapted and specialized genome of *Pseudomonas aeruginosa* strains in the CF lung environment (20–22).

## Conclusion

We observed that *P. aeruginosa* strains isolated from the lungs of CF patients encode a more limited number of phage defense systems compared with strains isolated from non-CF patient lungs. This can be attributed to a reduced abundance of several key phage defense systems, including Wadjet, Zorya type I, and RM type III, which likely results from adaptation to the CF lung environment. This provides a promising perspective that these bacterial strains are more susceptible to phage therapeutic options.

## Method

All complete *P. aeruginosa* genomes were downloaded from the *Pseudomonas* Genome Database on 9 April 2023 ($n = 14,230$). The metadata were utilized to select the strains for the following: Species = *Pseudomonas aeruginosa*, Assembly version status = latest, Host taxonomic name = *Homo sapiens*. A further selection was then made between

isolates from the respiratory system of CF ($n = 602$) and those of non-CF ($n = 348$) (Table S1). The non-CF patients primarily were affected by unspecified respiratory tract infection (73.0%) and ventilator-associated pneumonia (20.1%). Chronic bronchitis and chronic obstructive pulmonary disease (COPD) were less frequently prevalent, 4.9% and 2.0% respectively. Defense systems were detected in *P. aeruginosa* genomes with DefenseFinder v1.3.0 (23) using DefenseFinderModels v1.3.0. Prophages were identified using PhiSpy v4.2.21 (default settings) (24). Differentially abundant defense systems among CF strains compared with non-CF strains were detected using a simulated *P*-value χ test followed by a post-hoc analysis (R-package: chisq.posthoc.test v0.1.2) (25) with false discovery rate (FDR) adjusted *P*-values. An adjusted *P*-value lower than 0.01 was considered significant. Moreover, a $\log_2$FC of at least (–)1 was used as an additional threshold. Visualization of this analysis was done using a volcano plot created by the R-package EnhancedVolcano v1.24.0 (26).

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Daan F. van den Berg, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review and editing | Stan J. J. Brouns, Conceptualization, Funding acquisition, Resources, Supervision, Writing – original draft, Writing – review and editing

## ADDITIONAL FILES

The following material is available online.

### Supplemental Material

**Fig. S1 (mBio03548-24-s0001.tif).** Comparison of the phage defense system repertoire in *Pseudomonas aeruginosa* strains isolated from cystic and non-cystic fibrosis lungs.
**Supplemental legends (mBio03548-24-s0002.docx).** Legends for Figure S1 and Tables S1 and S2.
**Table S1 (mBio03548-24-s0003.xlsx).** Summary table of the *Pseudomonas aeruginosa* strains and additional information, including the defense system repertoire.

**Table S2 (mBio03548-24-s0004.xlsx).** The relative fold changes in defense system prevalence in *Pseudomonas aeruginosa* strains isolated from CF lungs compared with those from non-CF lungs and statistical results of the post-hoc chi-square test.

## REFERENCES

1. Malhotra S, Hayes D, Wozniak DJ. 2019. Cystic fibrosis and *Pseudomonas aeruginosa*: the host-microbe interface. Clin Microbiol Rev 32:e00138-18. https://doi.org/10.1128/CMR.00138-18
2. Bonyadi P, Saleh NT, Dehghani M, Yamini M, Amini K. 2022. Prevalence of antibiotic resistance of *Pseudomonas aeruginosa* in cystic fibrosis infection: a systematic review and meta-analysis. Microb Pathog 165:105461. https://doi.org/10.1016/j.micpath.2022.105461
3. Jackson L, Waters V. 2021. Factors influencing the acquisition and eradication of early *Pseudomonas aeruginosa* infection in cystic fibrosis. J Cyst Fibros 20:8–16. https://doi.org/10.1016/j.jcf.2020.10.008
4. Cafora M, Deflorian G, Forti F, Ferrari L, Binelli G, Briani F, Ghisotti D, Pistocchi A. 2019. Phage therapy against *Pseudomonas aeruginosa* infections in a cystic fibrosis zebrafish model. Sci Rep 9:1527. https://doi.org/10.1038/s41598-018-37636-x
5. Hahn A, Sami I, Chaney H, Koumbourlis AC, Del Valle Mojica C, Cochrane C, Chan BK, Koff JL. 2023. Bacteriophage therapy for pan-drug-resistant *Pseudomonas aeruginosa* in two persons with cystic fibrosis. J Investig Med High Impact Case Rep 11:23247096231188243. https://doi.org/10.1177/23247096231188243
6. Singh J, Yeoh E, Fitzgerald DA, Selvadurai H. 2023. A systematic review on the use of bacteriophage in treating *Staphylococcus aureus* and *Pseudomonas aeruginosa* infections in cystic fibrosis. Paediatr Respir Rev 48:3–9. https://doi.org/10.1016/j.prrv.2023.08.001
7. Bernabéu-Gimeno M, Pardo-Freire M, Chan BK, Turner PE, Gil-Brusola A, Pérez-Tarazona S, Carrasco-Hernández L, Quintana-Gallego E, Domingo-Calap P. 2024. Neutralizing antibodies after nebulized phage therapy in cystic fibrosis patients. Med 5:1096–1111. https://doi.org/10.1016/j.medj.2024.05.017
8. Pirnay J-P, Djebara S, Steurs G, Griselain J, Cochez C, De Soir S, Glonti T, Spiessens A, Vanden Berghe E, Green S, et al. 2024. Personalized bacteriophage therapy outcomes for 100 consecutive cases: a multicentre, multinational, retrospective observational study. Nat Microbiol 9:1434–1453. https://doi.org/10.1038/s41564-024-01705-x
9. Costa AR, van den Berg DF, Esser JQ, Muralidharan A, van den Bossche H, Bonilla BE, van der Steen BA, Haagsma AC, Fluit AC, Nobrega FL, Haas P-J, Brouns SJJ. 2024. Accumulation of defense systems in phage-resistant strains of *Pseudomonas aeruginosa*. Sci Adv 10:eadj0341. https://doi.org/10.1126/sciadv.adj0341
10. Winsor GL, Lam DKW, Fleming L, Lo R, Whiteside MD, Yu NY, Hancock REW, Brinkman FSL. 2011. *Pseudomonas* Genome Database: improved comparative analysis and population genomics capability for *Pseudomonas* genomes. Nucleic Acids Res 39:D596–D600. https://doi.org/10.1093/nar/gkq869
11. Deep A, Gu Y, Gao YQ, Ego KM, Herzik MA, Zhou H, Corbett KD. 2022. The SMC-family Wadjet complex protects bacteria from plasmid transformation by recognition and cleavage of closed-circular DNA. Mol Cell 82:4145–4159. https://doi.org/10.1016/j.molcel.2022.09.008
12. Hu H, Popp PF, Hughes TCD, Roa-Eguiara A, Rutbeek NR, Martin FJO, Hendriks IA, Payne LJ, Yan Y, Humolli D, Klein-Sousa V, Songailiene I, Wang Y, Nielsen ML, Berry RM, Harms A, Erhardt M, Jackson SA, Taylor NMI. 2024. Structure and mechanism of the Zorya anti-phage defense system. Nature. https://doi.org/10.1038/s41586-024-08493-8
13. Doron S, Melamed S, Ofir G, Leavitt A, Lopatina A, Keren M, Amitai G, Sorek R. 2018. Systematic discovery of antiphage defense systems in the microbial pangenome. Science 359:eaar4120. https://doi.org/10.1126/science.aar4120
14. Pinilla-Redondo R, Mayo-Muñoz D, Russel J, Garrett RA, Randau L, Sørensen SJ, Shah SA. 2020. Type IV CRISPR-Cas systems are highly diverse and involved in competition between plasmids. Nucleic Acids Res 48:2000–2012. https://doi.org/10.1093/nar/gkz1197
15. Taylor SL, Leong LEX, Sims SK, Keating RL, Papanicolas LE, Richard A, Mobegi FM, Wesselingh S, Burr LD, Rogers GB. 2021. The cystic fibrosis gut as a potential source of multidrug resistant pathogens. J Cyst Fibros 20:413–420. https://doi.org/10.1016/j.jcf.2020.11.009
16. Castañeda-Barba S, Top EM, Stalder T. 2024. Plasmids, a molecular cornerstone of antimicrobial resistance in the One Health era. Nat Rev Microbiol 22:18–32. https://doi.org/10.1038/s41579-023-00926-x
17. Ofir G, Melamed S, Sberro H, Mukamel Z, Silverman S, Yaakov G, Doron S, Sorek R. 2018. DISARM is a widespread bacterial defence system with broad anti-phage activities. Nat Microbiol 3:90–98. https://doi.org/10.1038/s41564-017-0051-0
18. Maestri A, Pons BJ, Pursey E, Chong CE, Gandon S, Custodio R, Olina A, Agapov A, Chisnall MAW, Grasso A, Paterson S, Szczelkun MD, Baker KS, van Houte S, Chevallereau A, Westra ER. 2024. The bacterial defense system MADS interacts with CRISPR-Cas to limit phage infection and escape. Cell Host Microbe 32:1412–1426. https://doi.org/10.1016/j.chom.2024.07.005
19. Ling KM, Stick SM, Kicic A. 2023. Pulmonary bacteriophage and cystic fibrosis airway mucus: friends or foes? Front Med (Lausanne) 10:1088494. https://doi.org/10.3389/fmed.2023.1088494
20. Bentkowski P, Van Oosterhout C, Mock T. 2015. A model of genome size evolution for prokaryotes in stable and fluctuating environments. Genome Biol Evol 7:2344–2351. https://doi.org/10.1093/gbe/evv148
21. Merhej V, Royer-Carenzi M, Pontarotti P, Raoult D. 2009. Massive comparative genomic analysis reveals convergent evolution of specialized bacteria. Biol Direct 4:1–25. https://doi.org/10.1186/1745-6150-4-13
22. Rossi E, La Rosa R, Bartell JA, Marvig RL, Haagensen JAJ, Sommer LM, Molin S, Johansen HK. 2021. *Pseudomonas aeruginosa* adaptation and evolution in patients with cystic fibrosis. Nat Rev Microbiol 19:331–342. https://doi.org/10.1038/s41579-020-00477-5
23. Tesson F, Planel R, Egorov AA, Georjon H, Vaysset H, Brancotte B, Néron B, Mordret E, Atkinson GC, Bernheim A, Cury J. 2024. A comprehensive resource for exploring antiphage defense: DefenseFinder Webservice, Wiki and Databases. Peer Community J 4. https://doi.org/10.24072/pcjournal.470
24. Akhter S, Aziz RK, Edwards RA. 2012. PhiSpy: a novel algorithm for finding prophages in bacterial genomes that combines similarity- and composition-based strategies. Nucleic Acids Res 40:e126. https://doi.org/10.1093/nar/gks406
25. Ebbert D. 2019. A post hoc analysis for Pearson's Chi-squared test for count data. Available from: http://chisq-posthoc-test.ebbert.nrw
26. Blighe K, Rana S, Lewis M. 2018. EnhancedVolcano: publication-ready volcano plots with enhanced colouring and labeling. Available from: https://github.com/kevinblighe/EnhancedVolcano