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Omics-based tracking of *Pseudomonas aeruginosa* persistence in 'eradicated' CF patients

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Take home message: For 80 cystic fibrosis patients, we used omics and positive culture history of Pseudomonas aeruginosa infections to show that strains routinely persist over lengthy Pseudomonas-free periods, and we recommend using genomic data in 'eradication' metrics.

Key words: *Pseudomonas aeruginosa*, whole genome sequencing, persistent clone types, cystic fibrosis, early eradication therapy

Abstract

Whenever *Pseudomonas aeruginosa* (PA) is cultured from cystic fibrosis (CF) patient airways, the primary goal is eradication by antibiotic therapy. Success is defined by at least six months of negative bacterial airway cultures. However, we suspect that PA persists in airways without clinical detection for long periods.

Of 298 PA-infected Copenhagen CF patients, we identified 80 with complete PA monitoring records and measured their maximum PA-free eradication periods (MEP). Isolates from 72 patients were whole genome sequenced (n=567) and clone typed. Select isolate relatedness was examined through phylogenetic analysis and phenotypic multivariate modelling.

Sixty-nine patients (86%) exhibited eradication in the monitoring period (2002-2018). Sequenced isolates bridged the MEP of 42 patients, and the same clone type persisted over the MEP in 18 (43%). Patients with failed eradication were on average treated more intensively with antibiotics, but this may be linked to their more severe pre-MEP infection trajectories. Of the 42 patients, 26 also had sinus surgery; the majority (15) show MEPs adjacent to surgery, and only 5 had persisting clone types. Importantly, combined phylogenetic-phenomic evaluation suggests that persisting clone types are a result of re-emergence of the same strain rather than re-infection from the environment, and similar relatedness is exhibited by paired lower and upper airway samples and in transmission cases.

In conclusion, nearly half of CF patients with supposed eradication may not truly be cleared of their original bacteria according to omics-based monitoring. This distinct cohort that is persistently infected would likely benefit from tailored antibiotic therapy.

Introduction

The positive experience from early and aggressive antibiotic treatment, which has prolonged the lives of CF patients, has promoted the clinical conclusion that populations of colonizing bacteria early in the treatment timeline can be eradicated from CF airways, and thus delay the onset of chronic infection.[1–3] The progression of *Pseudomonas aeruginosa* (PA) lung infection in CF patients is currently divided into two components: 1) intermittent colonization, where a bacterial species cannot be cultured from the next sputum sample after antibiotic therapy, and 2) chronic infection, denoted by continuous culturing of PA (the 'Leeds criteria'[4]), and/or an increased antibody response or mucoidity of cultures (the 'Copenhagen criteria').[5,6] These guidelines have been structured around avoiding chronic infections of PA, the historically dominant pathogen in CF patients' lungs[7]. At the Copenhagen CF Center, the frontline approach to avoiding chronic infections for more than 2 decades has been routine use of early eradication therapy (EET), a standardized treatment period predominantly consisting of inhaled colistin and per-oral ciprofloxacin, for every new positive culture of PA after a period of at least 6 months of PA-negative culture[1]. The EET approach has been recommended in best practice guidelines by the European Cystic Fibrosis Society, though no specific antibiotic regimen has been recommended due to lack of evidence[8,9]. While several publications have stated that antibiotic therapy applied to 'early PA infection' has in fact achieved eradication in 66-80% of patients[2,10,11] and postponed chronic infection in up to 80%[1], few of these incorporated strain genomics in their assessment of eradication. However, integration of pathogen genotyping and genomics in CF clinics is growing, offering increased resolution into strain dynamics within patient cohorts such as genotype prevalence within and between patients[10,12–14], strain origin[15] and diversification[16–18] and transmission[7,10]. The adoption of bacterial whole genome sequencing (WGS) in particular provides a reliable, quantitative method of tracking the persistence and adaptation of specific strains.

Given PA's extensive toolset for evading treatment, we expect that strain persistence in CF patients is common; however, the incidence and underlying mechanisms of this persistence are poorly tracked in current clinical practice. The purpose of this study was to contextualize clinical bacteriology records with genomic and phenomics screening data to 1) further our understanding of infection dynamics and strain persistence and 2) demonstrate the utility of WGS-based tools in routine care and in defining type of infection. We focus on the longest Pseudomonas-free interval (PFI) in each patient's culture records (referred to as the maximum eradication period, or MEP, when longer than .5 years), as this represents

the period most responsive to therapy and most likely to result in true strain eradication. We then compare strains via available genomic and phenomic data to assess strain presence, persistence over MEPs, response to treatment, and adaptation during these periods and between different airway environments. This work provides an important new assessment of true strain persistence and therefore treatment effectiveness in a large patient cohort using WGS-based tools, which illustrates the potential for treatment redesign from an omics-based perspective.

Methods

Patient cohort and sampling. Since 1971, all Danish CF patients have been followed ~monthly in the outpatient clinic at the Copenhagen CF Center at Rigshospitalet for clinical status, pulmonary function tests and bacteriological investigations of lower respiratory tract secretions.[5,19,20] Since late 2004, we have collected all first and most subsequent PA lung isolates from CF children and young adults in a frozen biobank. Since 2007, all PA isolates cultured from sinus surgery samples have also been stored.[21] In total, airway cultures of PA assessed in this study were derived from ~monthly samples that included expectorate, endolaryngeal suction, bronchial, and/or nasopharyngeal secretions, sinus surgery tissue, pus and fluid samples, bronchiolar lavage or saliva. Further details on standard culture methods and patient classification at the CF Center at Rigshospitalet are described in **Supplemental Methods**. Through a database maintained by the Department of Clinical Microbiology at Rigshospitalet with systematic entries of all airway bacteriological samples since 2002, we had access to records for 399 patients with CF treated at the Copenhagen CF Center between January 2002 and December 2018. During the study period, 298 out of 399 patients (75%) had one or more PA positive cultures from their airways, and we focus on 80 of these 298 patients (27%) which were divided into the 4 cohorts described below. Further details on this selection based on factors such as sample frequency and exclusion parameters are discussed in detail in **Supplemental Methods (Figures S1** and **S2)**.

We hereafter refer to four nested patient groups in our analysis (**Table 1**):

1) Cohort 1 (C1): This group of 80 patients has a complete and continous record of PA cultures with their first PA culture collected within the study period, i.e. after January 2002 and before July 2018. These patients were still under continuous care at the CF Center at Rigshospitalet at the end of 2018, and we thus had their complete PA infection history covered in the bacteriological database. For each patient, we calculated the length of every period where the clinic did not detect any PA in airway culture, termed a *Pseudomonas*-free interval (PFI).

2) Cohort 2 (C2): This group of 72 patients from C1 has had at least one isolate whole genome sequenced. Sequencing was performed either for genotypic monitoring in the clinic or, for 23 patients, during a 2015 study of 474 longitudinal whole genome sequenced (WGS) PA isolates[17].

3) Cohort 3 (C3): This group of 57 patients from C2 has at least one instance of a PFI which lasts longer than 6 months, qualifying according to the Copenhagen CF Center as a true 'eradication period' (EP).

4) Cohort 4 (C4): This group of 42 patients from C3 has a sufficient dataset of sequenced isolates (see **Supplemental Methods**) to evaluate whether an early strain persists over the longest EP, or 'maximum eradication period' (MEP).

Definition of infection status. Since 1974, CF patients have been defined as intermittently colonized from the first positive culture of PA until chronic diagnosis according to either the Copenhagen definition or the Leeds criteria.[4–6] In this study, patients are defined as chronically infected when PA is cultured in six consecutive monthly sputum samples and/or there are elevated or increasing precipitating antibodies against PA and/or if the bacteria is observed to have a mucoid phenotype.[5,6] We define eradication periods as *Pseudomonas*-culture free intervals of at least 6 months in length, and the maximum eradication period as the longest lasting eradication period for all patients who have shown at least one eradication period.

Patient interventions. The Copenhagen CF Center follows a positive culture-based model of treatment supported by the ~monthly patient clinic visits. CF patients treated at the Copenhagen CF Center undergo early eradication therapy (EET) of three weeks (before 2008: three months) of predominantly peoral ciprofloxacin and nebulized colistin for treatment of first PA isolation (a few patients are treated with ciprofloxacin and tobramycin based on patient-specific sensitivities)[1,22]. If PA is subsequently cultured within the following six months, an i.v. course of two weeks (ß-lactam + aminoglycoside based on antibiogram) is initiated followed by treatment with inhaled antibiotics, preferably colistin in combination with oral ciprofloxacin for 3 months. If PA is not detected within the six months after the early eradication, the next positive culture is treated as a new infection using EET with ciprofloxacin and colistin unless antibiotic susceptibility tests support use of alternative treatment. Sinus surgery is considered when patients have non-continuous positive PA culture, they have declining lung function, specific antibody levels are increasing, lung transplantation occurred within the prior year, or symptoms of severe rhinosinusitis are exhibited.[21]

Sequencing and genotyping. Both in-house and external sequencing was performed during the study period. For in-house sequencing, a DNEasy Blood and Tissue Kit (Qiagen) was used to purify DNA from overnight (in liquid or on blood agar plates) cultures of single colonies. Sequencing of libraries constructed using Nextera XT was performed on an Illumina MiSeq using a v2 250 x 2 kit. External sequencing was performed by BGI Genomics, Europe (2200 Copenhagen N, Denmark) on their DNBseq platform. *De novo* assemblies were aligned and clone types were demarcated on the basis of >10,000 differential SNPs post assembly alignment as described in Marvig *et al.* 2015.[17]

Statistics. Statistical comparisons between groups were performed using unpaired Wilcoxon-Mann-Whitney tests to compare group means in pairwise fashion. All data analysis and modelling was conducted in R (v. 3.4.0).

Phylogenetic reconstruction. A maximum likelihood phylogeny was produced with MEGA (v. 7.0.26),[23] based on concatenated SNPs from Marvig *et al.* 2015[17] using 1000 bootstraps. This was performed for multi-patient clone types as shown in Figure S4.

Archetype analysis. Isolates related to eradication periods in 19 patients of C4 were analyzed with respect to their similarity to phenotypic 'archetypes' defined by the archetypal model constructed in Bartell *et al.* 2019[24] using 443 phenotyped PA isolates from our young Copenhagen CF cohort. In-depth analysis was performed for prominent and well-characterized clone types DK19 and DK06[17]. Archetype analysis is a multidimensional statistical modeling approach where the extremal points of a multi-feature dataset are defined as 'archetypes' and all samples are defined with respect to their similarity to each archetype. In this analysis, we evaluate 5 phenotypic traits (features) that are known to adapt during CF infections and can be assessed by continuous values (growth rate, susceptibility to antibiotics ciprofloxacin and aztreonam, aggregation, and adhesion). We refer to these measures as a phenomics dataset because they were collected at strain library scale for integration with genomics data via modeling. Archetype-based assessment combines and translates this multi-dimensional data into novel insights into systems-level pathogen evolution. This method has proven useful because the predicted archetypes match naïve and adapted evolutionary states. The projected simplex visualizations (**Figure 1**) allow simultaneous assessment of multiple traits for each sample based on their localization relative to the archetypes and illustrate the multi-trait adaptive trajectory of an evolving clone type between archetypes.

Results

Eradication assessed by culture. Eighty-six percent of patients (n=69/80, C1) exhibited an eradication period (0.5 years or greater) at least once during the course of their care between 2002 and 2018, including 8 patients with a single PA positive culture. For each patient, we evaluated the longest period between PA cultures in the course of their monitoring period (**Figure 2A, Figure S3)**, and define this as the 'maximum eradication period' (MEP) if longer than 0.5 years in length. In those patients with at least two positive PA cultures, we observed a median MEP of 2.4 years (SD: 2.8 years) with the longest period lasting 14.8 years. The length of colonization time before an MEP ranges from 0 to 12.2 years (median time to MEP: 1.6 y, SD: 3.0 y, **Figure 2B**), indicating that there is still a substantial therapeutic window for mitigation of PA for many patients who have not been successfully eradicated after the first PA culture.

Eradication assessed by clonal persistence. An eradication period theoretically represents the total clearance of an infecting strain, but despite lengthy periods without positive cultures, the strain may still be present in a patient's airways. In an effort to track strain presence, 72 patients had at least one isolate sequenced (C2), resulting in the identification of 96 distinct strain genotypes (or 'clone types'). Twenty of these clone types occurred in multiple patients, with the most abundant being DK06, DK19, DK26, DK36, and DK54 (range: 6-9 patients). Figure S4 shows close genetic relationships between sequenced isolates from the same patient at the start and end point of eradication periods for clone types infecting multiple patients, supporting that recurrence of the same clone type is in fact undetected persistence of this strain specific to that patient's lungs. We thereafter identified 57 patients with at least one eradication period (C3) and then selected a group of patients with sufficient data (both sequencing frequency and the colonization timespan over which isolates were sequenced) for further analysis (**Table 2, Supplemental Methods Figures S1-S2)**. The 42 patients in C4 had frequent isolate sequencing that established the genotypic profile of early colonization strains and spanned their MEP.

To evaluate how often patients retain a clone type over the MEP, we assessed the post-MEP recurrence of any clone types appearing before the MEP in the 42 patients of C4. Ultimately, at least 43% of patients (N=18) showed the same clone type persisting over their MEP (ranging from 0.5 to 3.6 years, median: 1.1 years). In the 22 patients where the clone type switched after the MEP, indicating truly successful eradication, the period ranged from 1.2 to 11.7 years (median: 2.8 years) (**Figure S3**). Length of colonization before the MEP occured did not differ significantly between patient groups, but there

was a significant difference between mean length of MEP of patients whose clone types persisted or switched (1.4 versus 4.1 years, respectively, p= 2.2e-05, adj. Wilcoxon) (**Figure 3A**).

Persistence versus patient outcomes. During our monitoring period, 7 patients of C4 were diagnosed as chronically infected. Four of these patients had clone types persisting over their MEP, and the most extreme case was initially colonized by clone type DK41 for 6.98 years (including 2.41 years of 'eradication') prior to diagnosis of chronic infection. Interestingly, in our C4 analysis, we observe a potential link between the MEP and functional endoscopic sinus surgery (FESS) combined with intravenous antibiotics and a colistin rinse, a common intervention at the Copenhagen CF Center for patients with intermittent but progressing infection[25]. The MEP was adjacent to an FESS in the majority (15 out of 26 patients) who received this intervention, which aligns with past studies indicating FESS reduces positive culture[26]. We extend these findings by determining that only five of these 15 patients had a clone type which persisted over both the FESS and MEP.

Phenotypes for persistent versus switched strains. Leveraging our genetic and phenotypic evolution study in a subset of the patients[24], we compared patients with switched versus persisting clone types within the 'eradication' cohort to assess other potential differences between these groups. **Figure 3B** illustrates an extreme case of clone type switching, where 4 different clone types sequentially colonize patient P2204 and then are eradicated over a period of 7.5 years. All isolates show naïve phenotypic profiles (normal growth rate, low antibiotic resistance, and low biofilm-linked traits) based on their localization near the naïve archetypes (A3 and A5). In contrast to this 'Switch' patient, the 'Persist' patient of **Figure 3C** shows both genetic and phylogenetic adaptation of the same clone type over 7 years in patient P1404. Trait profiles of isolates shift from the 'naïve' archetype A3 towards prototypical 'adapted' traits, adapting towards slow growth rate and increased ciprofloxacin resistance at A1 and then slow growth and increased adherence at A2/A6. The last set of traits are maintained in isolates spanning an FESS and consequent MEP of 1.7 years. The isolates spanning this MEP are also more similar to each other than to the deviating early isolate (denoted with #) similar in trait profile to A4 as well as other DK12 isolates colonizing a different patient (P2605). This also holds true with respect to genetic adaptation in the included phylogenetic tree (**Figure 3C**, right). In examining the other 'Persist' patient for which we have trait data for isolates at the start and end of their MEP, we saw the same similarity in genetic and phenotypic traits between the isolates bridging the MEP. As a final summary, 17 additional patients had phenotypic data available for isolates sampled at the start or end of an eradication period, which we highlight in the archetype model in **Figure S5** (showing either isolates linked to a patient's MEP

or longest eradication period for which we have trait data). This figure emphasizes the diversity of adaptive trajectories of strains colonizing different patients in alignment with our previous studies of adaptive trajectories[24] – different adaptive paths can lead to equal strain persistence. While 'starting' isolates for 'switch' patients tend to have more naïve traits than the more diverse traits observed in 'persist' patients (as illustrated in the guide in **Figure 1**), 'starting' isolates do not cluster dramatically with a specific, shared archetypal profile.

Persistence versus strain origin. Strain persistence and adaptation is theorized to be influenced by patient-specific environmental effects that include inherent differences such as immune function, external differences such as individualized antibiotic therapy, and spatial differences as strains may localize in different regions of the airway. For example, it has been argued that the sinuses may serve as an infectious focus, and consequently more adapted PA may repeatedly spread from the upper to the lower airways[3,21,27]. This assertion has been investigated primarily in older chronically infected patients[16,28]. In this young cohort, we do not see any evidence of the UAW and LAW being segregated (**Figure 4A*)***, supportive of the hypothesis of united airways. For each of the clone types that were cultured in both the UAW and the LAW (15 clone types in 18 patients, 3 of which were shared clone types DK03, DK19 and DK36), we again looked for clustering of isolates both through AA and phylogenetic analyses. However, we were not able to identify specific clades or clustering of UAW isolates as compared to LAW isolates (**Figure 3C**, **Figure 4A,** and **Figure S4**).

A more extreme example of environmental differences is presented by cases of patient-to-patient transmission of PA clone types, mainly a concern before introduction of appropriate infection control.[10,29,30] We evaluated three probable direct transmission cases (0-29 SNPs different, mean of 10.2) and 10 clone types present in multiple patients by likely indirect means (54-504 SNPs different, mean of 211.5). We find remarkable similarities in traits between transmitted strains showing close genetic relatedness, as shown for DK06 in **Figure 4B**. In patients without obvious evidence of a direct transmission, strains from the same clone type are often less phenotypically similar (environment) (**Figure 3C**, **Figure 4**).

Persistence versus patient treatment. The lung environment of CF patients is likely dramatically affected by the intense regimen of individualized antibiotic therapy that each patient receives in an effort to eradicate bacteria. Using clinical treatment records, we examined the effect of treatment on patients with persisting versus switching clone types over their MEPs (N=42, Cohort 4). We evaluated the months in which twelve antibiotics which target *P. aeruginosa* (amikacin, azithromycin, aztreonam,

ciprofloxacin, ceftazidime, clarithromycin, colistin, imipenem, meropenem, ofloxacin, piperacillin/tazobactam, and tobramycin) were in use among the 42 patients within 6 months of the start of their MEP; the antibiotics were grouped by antibiotic class (**Figure 5A**). In some patients, a given antibiotic was in use every month of the 12 month period (6 months before and after the start of the MEP to account for the influence of prolonged treatment on the continuation of the eradication period). The 4 classes most often in use were aminoglycosides, fluoroquinolones, macrolides, and polymyxins (relative number of patient cohort treated with a given antibiotic class is indicated by boxplot width in **Fig. 5A**), and **Figure S6** shows that most patients received combination therapy using at least 3 of these classes within their MEP timeframe. Interestingly, **Figure 5** shows that the distribution of each antibiotic class's usage in the 'persist' patients is generally similar or elevated compared to the 'switch' patients, with significantly different means between months in use for aminoglycosides, macrolides, and polymyxins (by Wilcox test). To add context to this finding, we estimated age of each patient's PA infection (**Figure 5B**) and estimated severity of infection by counting the positive PA cultures from their ~monthly clinic visits in the year prior their MEP (**Figure 5C**). Patients in the 'persist' cohort tend to have MEPs which occur later in their infection period, though the means of each cohort are not significantly different. However, there is a significant difference in the average positive PA cultures which occur the year prior to the MEP for 'persist' versus 'switch' patients, supporting that 'persist' patients have more serious infections than 'switch' patients.

Discussion

We leverage the implementation of bacterial WGS in the clinical routine at the Copenhagen CF Center to demonstrate that nearly half of patients in a longitudinal infection cohort are infected with the same clone type before and after their maximum eradication period, which should represent the most likely instance of true eradication. Through omics methods and statistical modeling, we observe remarkable phylogenetic and phenotypic similarity in isolates separated by long eradication periods, infection niche, and during transmission between patients. We see a significant difference in months of treatment with 3 classes of drugs in 'persist' patients versus 'switch' patients; the elevated treatment in 'persist' patients accompanied by a higher number of PA-positive cultures in the 'persist' patients before their MEP suggests a worse infection that requires more antibiotics to achieve the MEP, yet still does not result in true eradication. Our experience with WGS implementation in the clinic has major implications for effective monitoring and eradication of persisting infections. We support the pairing of positive-PA culture records with omics data for an improved understanding of patient infection trajectory, and

recommend the reassessment of studies of treatment efficacy which might benefit from improved, omics-based eradication metrics.

First, our observations of continuous persistence independent of a chronic diagnosis highlight a shortcoming of the current view of PA airway infections in CF.[4,31] Treatment regiments are generally based on the infection status as either 'intermittently colonized' or 'chronically infected'.[4,10,31] In reality, the infecting clone type in many 'intermittently colonized' patients is not being eradicated. This has been suspected at other CF centers which track the genotype of infecting bacterial isolates via multilocus sequence typing,[14] but WGS offers new resolution in identifying re-emergent strains via genetic relatedness to other adapted isolates previously found in the patient (**Figure S4**). We estimate that 43% of patients (N=18) in our initially non-chronic, young cohort (C4) experience this clonal persistence over at times quite lengthy 'eradication' periods, and improved diagnostic approaches should highlight these patients that could benefit from customized therapy in the clinic. Our work suggests, for example, that sinus surgery may be an effective way of initiating a lengthy eradication period and reducing clone type recurrence rates, and an omics-based assessment of eradication versus treatment could highlight particularly beneficial antibiotic regimens which evaded prior positive-culture-only studies showing no difference between regimens.

Currently, we observe that many patients with a persistent clone type have been treated with more antibiotics in the timeframe of their MEP (**Figure 5A**). This unexpected trend could be interpreted to mean that intensive antibiotic therapy is actually counterproductive, but alternative explanations are available. We speculate that this treatment difference can be partly explained by the timing of MEPs with persisting versus switching clone types. The mean time between first ever PA and MEP is about a year shorter for 'switch' patients versus 'persist' patients (2.07 versus 2.92 years, C4), though the difference is not statistically significant (**Figure 5B**). These 'persist' patients with longer infection histories may be undergoing heavier treatment as their strains have adapted and persisted, while a true eradication is likely easier in the 'switch' patients with less adapted strains. There is also a significant difference in the average number of positive PA cultures between 'persist' and 'switch' patients in the year prior to their MEP, with 'persist' patients showing more frequent positive cultures, which lead to additional antibiotic courses per CF Center treatment guidelines (**Figure 5C**). Ultimately, our findings support the idea of tracking age of infection by both PA culture incidence and clone type recurrence as a predictor of strain persistence and mode of assessing treatment. Effectiveness will be dependent on the frequency of clinical sampling and ability to perform comprehensive sequencing of patient bacterial

isolates to identify clonal incidence at the earliest possible date. We acknowledge that while this is becoming feasible to implement in Danish clinics, it may require advances in at-home sampling and analytics in other treatment environments. Larger studies of the outcome of eradication periods of similar duration and timing paired with our omics-centered approach will hopefully provide answers as to 1) the utility of this approach in customizing treatment strategies supporting eradication and 2) the scope of data necessary to implement this approach to infection monitoring.

Our analysis of genetic and phenotypic similarity of isolates spanning eradication periods and spatial separation is notable as we also have observed clear adaptive trajectories in other persisting infections. Here, we show that the UAW and LAW are not separate entities with different sub-populations. We instead highlight the shared persistence in UAW and LAW by similar populations of bacteria in similar timeframes, which may ultimately render a diagnosed infection focus irrelevant for most patients. However, infecting strains are able to persist and adapt within patients' airways to a significant degree in these early stages of colonization, and the likelihood of persistence of a specific clone type from its first appearance in lab culture should be incorporated in treatment decisions. We previously found that bacterial growth rate and resistance to ciprofloxacin adapt rapidly in an initial 2-3 year period after first colonization.[24] The re-emergence of phylogenetically and phenotypically similar isolates after long eradication periods in this study adds a new perspective to our prior work, as the clone type persists but may undergo limited adaptation while surviving in an inactive 'persister' state[32]. However, acknowledging that the same adapted bacterial strain can re-emerge after 'eradication' is important for accurately estimating its exposure to antibiotics. This study highlights the likely underestimation of risk of treatment tolerance, resistance and persistence for these patients.

It is important to note that the findings of this study are affected by several factors. First, the young patients in our cohort cannot expectorate easily so we likely underestimate the true presence of PA via positive culture. We also do not address the potential impact of other CF pathogens on PA clearance. It can also be possible that a post-eradication isolate genetically related to prior adapted isolates from a patient has been re-acquired from the patient's living environment after 6+ months of true eradication, but this argument against clone type persistence seems very unlikely outside of young co-habiting siblings infected with the same strain. Furthermore, the ability to monitor the reappearance of a previously adapted strain by omics-based approaches is still relevant for these patients and their treatment design. With respect to our isolate similarity studies via archetypal analysis, we constructed these models using *in vitro* screening of five phenotypes (growth rate, susceptibility to antibiotics

ciprofloxacin and aztreonam, aggregation, and adhesion), but further phenotypes may also be undergoing adaptation in the lungs. We currently offer detailed examples of the potential of this approach (Figures 3-4) rather than an expansive analysis of the spectrum of archetypes associated with MEPs – preliminary evidence suggests they are likely quite diverse (Figure S5). We view the present work as a foundational surveillance study from which we can expand our understanding of bacterial adaptation in the complex environment of the CF lungs as well as improve our means of precision medicine in the CF clinic via integrated WGS and omics assays. We envision a treatment approach where persistence and adaptation of a colonizing strain is tracked both by functional genetic and phenotypic markers individualized to each patient, and a strain of the same clone type re-emerging after an 'eradication' period will be quickly compared to previous isolates to check degree of adaptation and any novel traits. This data can then inform a new antibiotic regimen design rather than repeating a prior regimen that has not prevented the adapted strain's persistence. We ultimately aim to leverage omics data that is feasible for a clinic to obtain and support a more structured and evidence-based approach to treatment for specific infection cases such as intermittently-cultured persistent strains.

Our findings support the centering of bacterial genomics data in evaluating infection and achieving strain eradication in CF airways. Clinicians presented with a positive PA culture following a substantial period of PA-negative samples (including multiple years in length) should consider it equally likely that a previously treated, possibly adapted strain has re-emerged versus assuming that a new, susceptible strain has colonized the airways. Our findings are important for updating definitions of PA airway infections, diagnosis and treatment protocols in CF patients.

Ethics

The local ethics committee at the Capital Region of Denmark (Region Hovedstaden) approved the use of the stored PA isolates (registration numbers H-4-2015-FSP and H-19029688) and the sinus surgery procedure (registration number H-A-2008-141). Access to the microbiological and treatment records was approved by both the management of the Department of Clinical Microbiology at Rigshospitalet and the Danish Agency for Patient Safety (registration number 31-1521-428), and all data analysis was performed on pseudonymized data.

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CONTRIBUTIONS

HKJ collected all the bacterial isolates and contributed to the conception and study design together with JAB, LMS and SM. MS and TP provided the clinical data. JAB, LMS, RLM, SM and HKJ contributed to the analysis and interpretation of data. JAB, LMS and HKJ drafted the manuscript and all authors commented on the manuscript prior to submission and approved the final version.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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TABLES

Table 1. Cohort overview. Counts of males and females, median age in years at first *Pseudomonas aeruginosa* sample, the time covered by *P. aeruginosa* positive cultures (timespan) in years and the total number of positive *P. aeruginosa* samples.

		Patients			Patient age*		Sample Timespan§		
Cohort	**CF Patient Filtering**	*Total*	*M*	*F*	*Median*	*Range*	*Median*	*Range*	**Total samples**
C1	First *P. aeruginosa* positive culture after 2002	**80**	36	44	4.3	0.3-17.2	7.0	0.0-16.7	2093
C2	1 or more sequenced isolates	**72**	30	42	5.1	0.3-24.5	5.1	0.0-15.3	567
C3	Eradication period greater than 0.5 years	**57**	24	33	5.8	0.8-24.5	7.5	0.0-15.3	525
C4	Sufficient data to assess persisting/switched strains	**42**	20	22	4.6	0.8-17.2	7.5	0.7-15.3	438

Culture

Sequence Data

**C1: Age at first P. aeruginosa positive culture; C2-4: Age at first sequenced P. aeruginosa isolate.*
§C1: timespan from first P. aeruginosa positive culture to last; C2-4: timespan of sequenced isolates.

Table 2. Number of isolates sequenced per patient by cohort.

	Sequenced Samples Per Patient				
Cohort	Min	1st Qu.	Median	3rd Qu.	Max
C2	1	2	3	12.3	34
C3	1	2	6	15	34
C4	2	3	8	16.8	34

FIGURE LEGENDS

Figure 1. *Archetype Analysis simplex projection.* In center: a simplex visualisation with individual isolates projected as transparent grey dots. Archetypes are denoted on the edge of the simplex (A1 to A6) ordered with the archetypes showing the most naïve phenotypic characteristics on the left (A3 and A5) and the archetypes with the more adapted phenotypic profiles on the right (A1, A2 and A6). Generally, naïve isolates grow quickly and are antibiotic susceptible, while adapted isolates grow slowly and are not antibiotic susceptible. The degree of adaptation within a specific phenotype is indicated by size of the points and the phenotype evaluated stated beneath (cip: ciprofloxacin MIC, azt: aztreonam MIC, adh: adhesion, agg: aggregation, grASM: growth rate in ASM). Absence of points indicate the archetypes with the most naïve version of the phenotype (e.g. A5 and cip or adh) and the largest circles indicate archetypes with the most evolved version of the phenotype (e.g. A1 and cip).

Figure 2. *Pseudomonas-free interval data by patient count.* All Pseudomonas-free intervals (PFIs) greater than 6 months qualify as eradication periods (and are marked in red). Histogram bins are 3 months. **(A)** Maximum PFI for each patient over their complete monitoring period. **(B)** Time between first *Pseudomonas aeruginosa* (PA) isolation in a patient and the occurrence of their max PFI. Many patients experience their max PFI directly after their first PA isolate (t=0).

Figure 3. *Eradication of Pseudomonas aeruginosa.* (A) The maximum eradication period (MEP) is significantly different (Wilcoxon, p=7.2E-6) in patients with clone types that 'Persist' versus 'Switch' over the MEP in C4 (N=42). (B) Example of phenotypic clustering of 4 clone types (DK21-DK24) sequentially colonizing and then being eradicated in a 'Switch' patient (illustrated by an archetypal analysis simplex plot[24]). The last eradication was supported by a functional endoscopic sinus surgery (FESS) (marked by *). Years since first isolation are indicated by symbol opacity, and clone type is indicated by hue. (C) Phenotypic and phylogenetic clustering of clone type DK12 in 'Persist' patient P1404, where genetic and trait similarity is maintained over a 1.7 year eradication period, supported by a FESS, marked by *, and between the upper and lower airway (UAW versus LAW). Years since first isolation are indicated by symbol opacity and patient of origin is indicated by color. Phylogenetic relationships are indicated by the cladogram (left) with accompanying phylogenetic tree (center, showing the distance between isolates to scale) of concatenated SNPs accumulated within the clone type[17] with black squares denoting bootstrap values >= 50. Isolates from P1404 with trait data (filled symbols) are shown in the archetypal analysis simplex plot on the right. For context, samples from patient P2605 infected with the same clone type are included to show the phenotypic and phylogenetic difference between patients that are likely infected from different environmental sources. A single isolate from P1404 (marked by #) suggests a

heterogeneous initial infection population, as it shows higher similarity to isolates from P2605 than others from P1404. Note the true scale of genotypic differences as indicated by the small phylogeny in the middle.

Figure 4. *Maximum likelihood phylogenetic analysis and Archetypal analysis (multi-dimensional clustering) of two clone types (A) DK19 and (B) DK06.* Isolates are indicated by circles (lower airway, LAW) or triangles (upper airway, UAW) and the colour indicates patient origin as indicated by the phylogenies in the left panels of A and B, with the hue indicating years since the clone type was first isolated from any patient. Isolates with a white center are not included in the cluster analysis to the right. To the left, cladograms with accompanying phylogenetic trees (smaller tree to the right showing the distance between isolates to scale) of concatenated SNPs accumulated within the clone type[17] with black squares denoting bootstrap values >= 50. Likelihoods: -1136.76 (DK19) and -1466.05 (DK06). To the right, cluster analysis (archetype analysis) of five phenotypes (see main text and prior study[24] for further explanation). Isolates marked by (*) indicate the phylogenetically closest isolates of three different patients all infected by DK06, and this similarity is reflected on the right where the same isolates are also identified in the (*) box.

Figure 5. *Use of* Pseudomonas*-targeting drugs in treatment period surrounding MEP.* (A) In C4 patients, 8 drug classes (covering 13 drugs that target *P. aeruginosa*) were in use in at least one month in one patient within 6 months of their maximum eradication period (MEP) (both before and after MEP start). The months an antibiotic class was in use by each patient treated with that antibiotic class are shown by boxplots, where the width of each box (in the y-axis direction) correlates with the number of patients treated with that drug (patients with zero months of treatment with a given drug are not included in the analysis). Patients with clone types that persisted versus switched over their MEP are separated for comparison, and significant differences between these groups are shown by bolded p values (Wilcoxon test of means). (B) We show the years from first ever positive culture of *P. aeruginosa* (PA) to start of the MEP for ‘persist’ and ‘switch’ cohorts (difference between cohorts is nonsignificant, Wilcoxon test of means). (C) We show the number of positive PA cultures in the year prior to the start of the MEP for ‘persist’ and ‘switch’ cohorts (difference between cohorts is significant, Wilcoxon test of means).

SUPPLEMENTAL METHODS

Cystic fibrosis (CF) diagnosis. Patients are defined as having CF if they are homo- or heterozygous with one or more of the CF causing mutations of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene. *Pseudomonas aeruginosa* infected and non-infected patients are segregated into separate wards and seen on different days in the outpatient clinic according to bacteriology in the lower respiratory tract.[33]

Culture and identification of PA*.* PA is isolated from the airways using standard microbiological methods.[34] Briefly, samples are plated on a Sabouraud plate, a 7% NaCl plate, a *Burkholderia cepacia* plate containing colistin and gentamicin, a 'blue plate' (modified Conradi Drigalski's medium) selective for Gram-negative rods, a 5% Danish blood agar plate, and a chocolate agar plate (SSI Diagnostica®, Hillerød, Denmark). Isolated bacteria are identified as described previously.[34] Before 2011, biochemical profiling of PA was based on API 20NE (bioMérieux), and from 2011 on MALDI-TOF mass spectrometry (Bruker, Germany).

Cohort evaluation. Our base cohort of 80 CF patients was determined by excluding patients with their first PA positive culture before January 2002 or after July 2018, and those that between January 2002 to December 2018 moved treatment centers, were given a lung transplant, or were deceased. We then examined continuity of treatment by evaluating the dates of all patient microbiology samples by both visualization of longitudinal sampling and calculating various metrics of sample gaps (periods of time where no sample results, pathogen positive or normal, were reported in our database). We further excluded patients with any sample gaps greater than one year (n=5, retained one patient with a year long sample gap which ended 4 years before first positive PA culture). Patients with sample gaps between 0.5 and 1 year in length or at least 3 instances of a sample gap greater than 0.25 years were manually evaluated to ensure that sample gaps did not overlap with first or maximum eradication periods or otherwise indicate routinely poor clinic attendance by the patient. Patients with 4 or more sample gaps greater than 0.25 years were predominantly diagnosed as chronic during the study period and therefore none were excluded from the study; these patients often attend the clinic less regularly as monitoring for new PA infection is no longer a primary goal in their care (n=4 of 5).

In the resulting complete-record cohort, a median of 6 PA samples (SD: 32.7, range: 1-238) per patient were obtained over a median of 7 years (SD: 5.4, range: 0-16.7 years). A comparison of samples versus sequenced samples over time is shown in **Figure S2A**, highlighting a higher ratio of sequence to sample

in early colonization, including both the 72 patients with sequenced samples and the remaining 8 patients with no sequenced isolates. Because we do not have all PA isolates sequenced, it is important to be aware of how sequenced isolates span both colonization time and number of samples (**Figure S2B)**. Patients with many sequenced isolates spanning a large proportion of the colonization of the given patient are large, green circles, while patients with few or no sequenced samples, that thus covers limited or no time of the entire colonization, are small yellow circles. Examples of the latter group would be patients who have been diagnosed with chronic PA infection and shown frequent or continuous positive culture since that chronic diagnosis date but with few or no samples sequenced after their diagnosis as chronically infected under the assumption that the same clone type is present. In summary, while we have sequence coverage variation across patients, our sequenced patients cover almost the entire spectrum of colonization lengths (that is, the green medium-large circles are present along the entire x-axis).

SUPPLEMENTAL FIGURE LEGENDS

Figure S1. *Summary of sampled data for all patients with more than 1 positive Pseudomonas aeruginosa culture.* Data from C2 to C4 is based on sequenced samples only. Both number of samples and sample range increase from C2 to C4 as completeness of patient records is improved through filtering (as described in Figure S2). A. Number of samples per cohort. B. Range of sampling periods per cohort in years. Boxplots show median and quartiles.

Figure S2. *Bacterial sampling from 80 young CF patients.* **(A)** Samples of *Pseudomonas aeruginosa* cultured over the monitoring period from patients with complete *P. aeruginosa* culture records (C1). Proportion of samples sequenced is indicated in red. **(B)** Sequence coverage of individual patients (circles) contextualized by total length of colonization (x axis), cohort membership (circle outline color), fraction of samples sequenced (size), and temporal range of sequenced samples (circle fill color).

Figure S3. Maximum eradication period (MEP) length per patient versus length of colonization (max time from 1st *Pseudomonas aer*uginosa culture) for C3, highlighting patients with clone types that persist versus switch over the MEP (C4).

Figure S4. *Maximum likelihood cladograms and phylogenies of clone types found in multiple patients.* Bootstrap values >= 40 are shown with large text size in the cladogram where patients are also indicated with lines and patient IDs. In the phylogeny to the right the isolate IDs are indicated, which can be traced back to genotypes in Marvig *et al*[17] and phenotypes in Bartell *et al*[24]. The log likelihoods of trees are: (A)

DK03: -2665.62, (B) DK08: -853.02, (C) DK12: -2567.09, (D) DK15: -2931.71, (E) DK26: -800.92, (F) DK36: -6641.36. The likelihood of mutation for the maximum likelihood phylogenies to the right are indicated with a bar and likelihood in the center of the phylogenies. In the phylogenies to the right, instances of eradication periods (either the MEP or the longest PFI as noted) are highlighted such that isolates at period start are yellow and isolates at period end are green. Length of eradication period and relevant context is included in text. The closest phylogenetic linkage between start and end isolates is also in bold. In the cladograms on the left, isolates derived from the upper airway (UAW) are highlighted with (*).

Figure S5. *Archetypal analysis of isolates associated with MEP or longest PFI.* In addition to two patients for which we had data to model phenotypic adaptation over their MEP, seventeen patients had phenotypic data available to model isolates (points) at the start (yellow) or end (green) of an eradication period (MEP – circles, longest PFI – triangles). Here, we show all 19 patients, separated into clone types that persisted (panel A, 8 strains) versus switched over the eradication period (panel B, 11 strains).

Figure S6. *Archetypal analysis of isolates associated with MEP or longest PFI.* The 4 drug classes prescribed to the most patients in Panel A (aminoglycosides – amg, fluoroquinolones – flq, macrolides – mcl, and polymyxins – pmx, prevalence indicated by y-axis boxplot width) were evaluated with respect to usage combinations versus number of ‘persist’ or ‘switch’ patients.

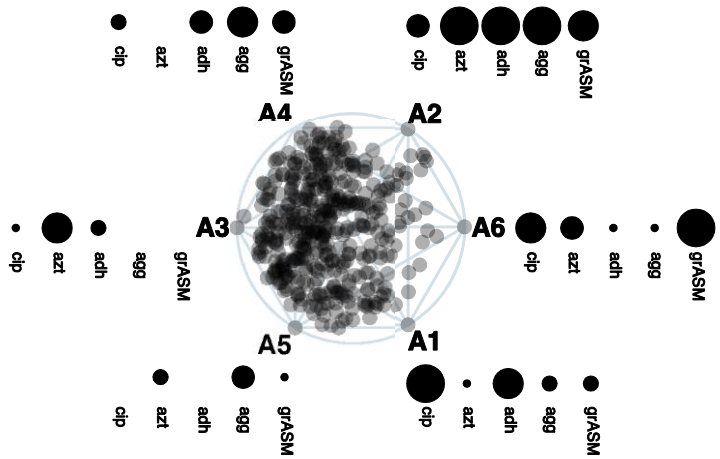

A4
cip
azt
adh
agg
grASM
A2
cip
azt
adh
agg
grASM
A3
cip
azt
adh
agg
grASM
A6
cip
azt
adh
agg
grASM
A5
cip
azt
adh
agg
grASM
A1
cip
azt
adh
agg
grASM

FIGURE 1

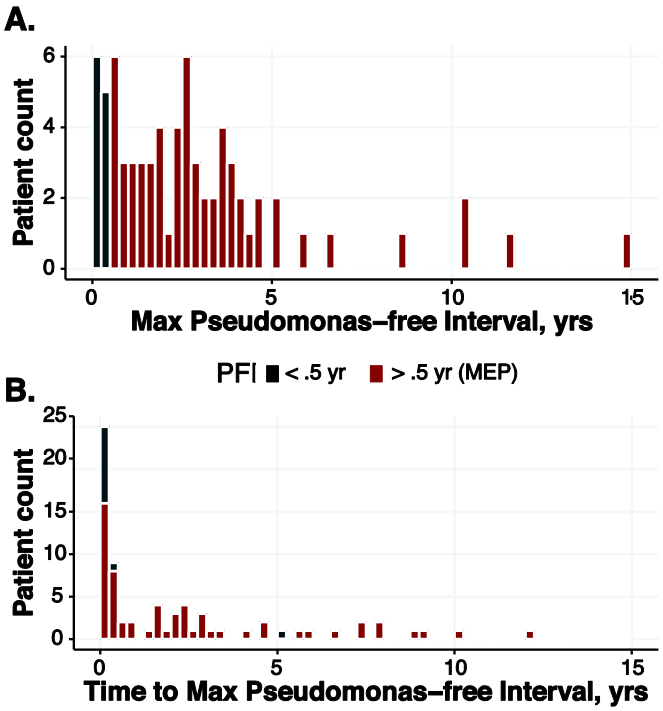

A.
Patient count
6
4
2
0
0
5
10
15
Max Pseudomonas–free Interval, yrs
PFI < .5 yr > .5 yr (MEP)
B.
Patient count
25
20
15
10
5
0
0
5
10
15
Time to Max Pseudomonas–free Interval, yrs

FIGURE 2

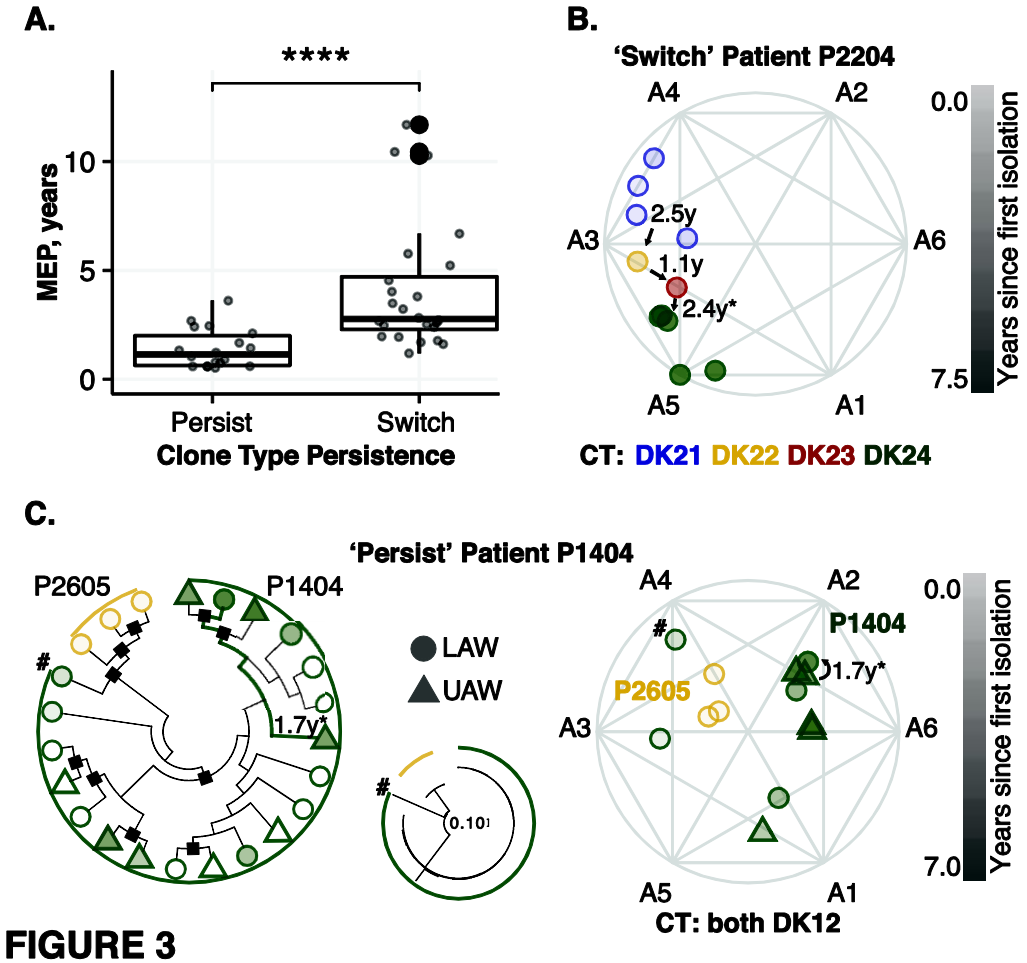

A.
****
MEP, years
10
5
0
Persist
Switch
Clone Type Persistence
B.
'Switch' Patient P2204
A4
A2
A3
A6
A5
A1
2.5y
1.1y
2.4y*
0.0
7.5
Years since first isolation
CT: DK21 DK22 DK23 DK24
C.
'Persist' Patient P1404
P2605
P1404
#
1.7y*
LAW
UAW
#
0.10
A4
A2
P1404
#
P2605
1.7y*
A3
A6
A5
A1
0.0
7.0
Years since first isolation
CT: both DK12

FIGURE 3

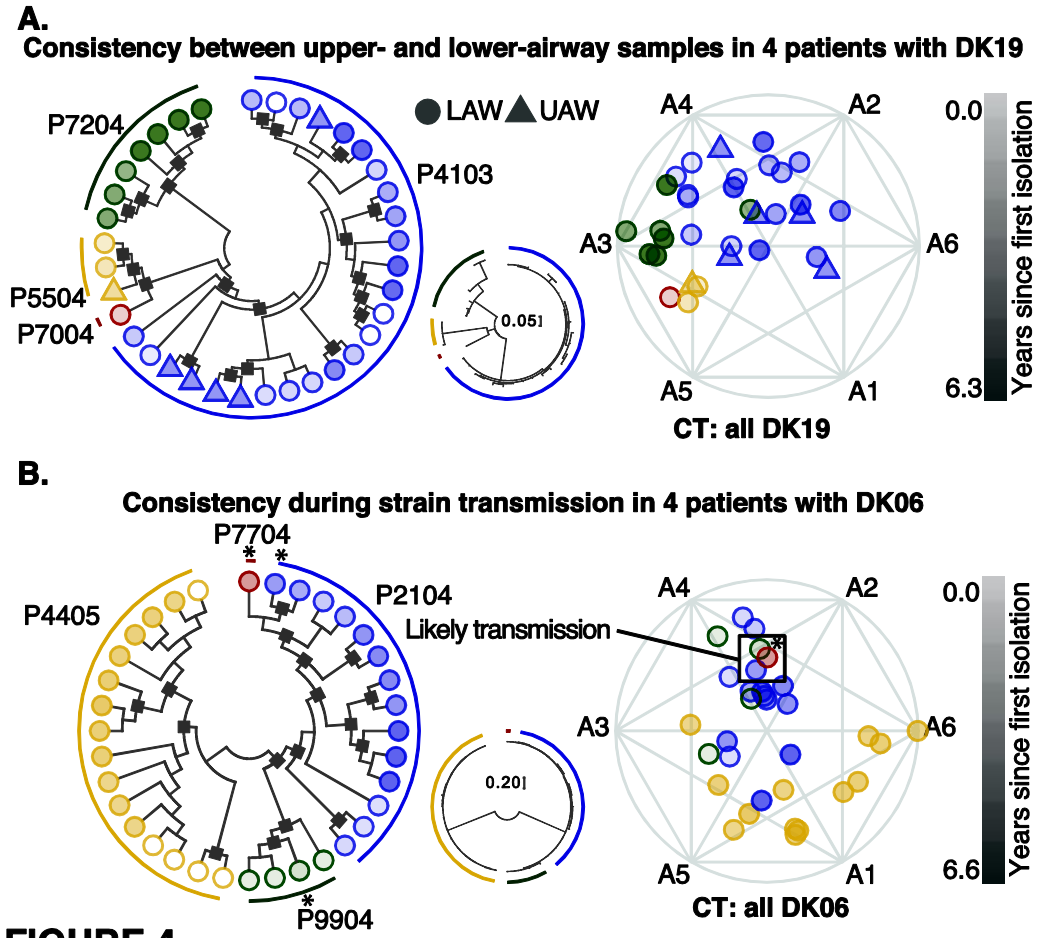
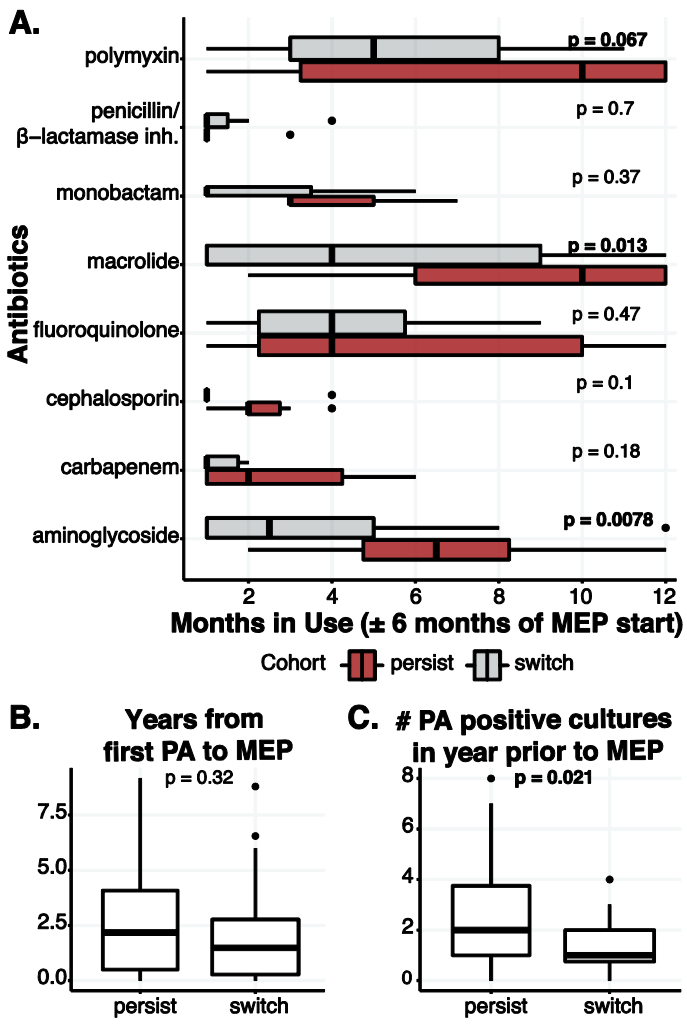

A.
Consistency between upper- and lower-airway samples in 4 patients with DK19
P7204
P5504
P7004
P4103
LAW
UAW
0.05
A4
A2
A3
A6
A5
A1
CT: all DK19
0.0
6.3
Years since first isolation
B.
Consistency during strain transmission in 4 patients with DK06
P7704
P4405
P2104
Likely transmission
P9904
0.20
A4
A2
A3
A6
A5
A1
CT: all DK06
0.0
6.6
Years since first isolation

FIGURE 4


A.
polymyxin
penicillin/
β–lactamase inh.
monobactam
macrolide
fluoroquinolone
cephalosporin
carbapenem
aminoglycoside
Antibiotics
p = 0.067
p = 0.7
p = 0.37
p = 0.013
p = 0.47
p = 0.1
p = 0.18
p = 0.0078
2
4
6
8
10
12
Months in Use (± 6 months of MEP start)
Cohort
persist
switch
B.
Years from
first PA to MEP
p = 0.32
7.5
5.0
2.5
0.0
persist
switch
C.
PA positive cultures
in year prior to MEP
p = 0.021
8
6
4
2
0
persist
switch

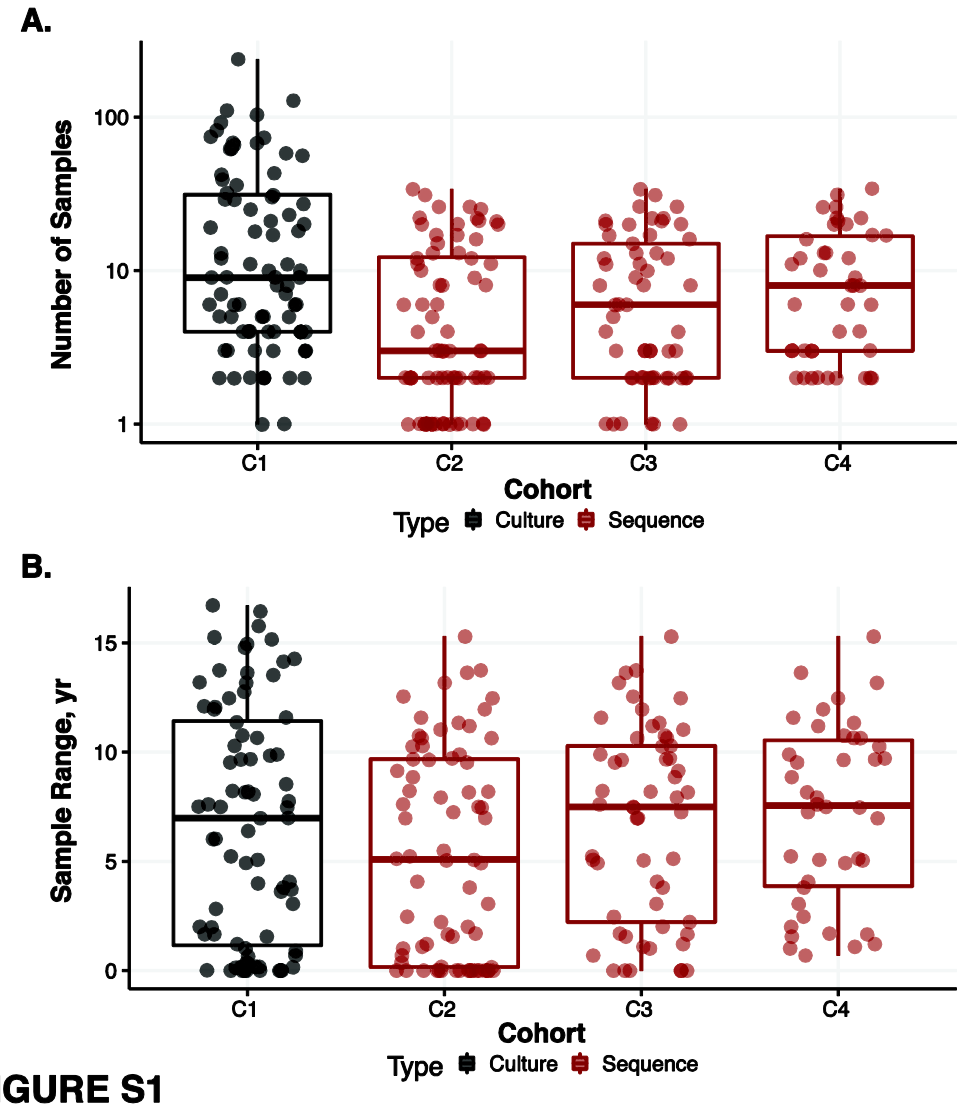

A.
Number of Samples
100
10
1
C1
C2
C3
C4
Cohort
Type
Culture
Sequence
B.
Sample Range, yr
15
10
5
0
C1
C2
C3
C4
Cohort
Type
Culture
Sequence

FIGURE S1

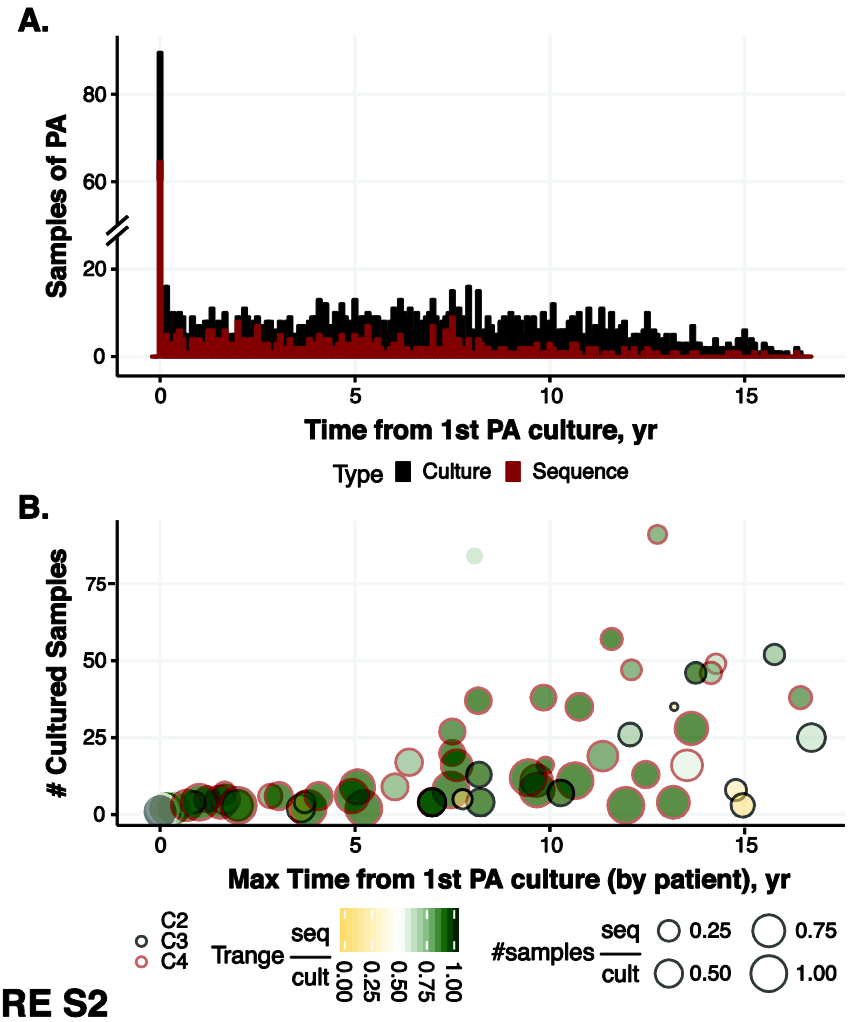

A.
Samples of PA
80
60
20
0
0
5
10
15
Time from 1st PA culture, yr
Type
Culture
Sequence
B.
Cultured Samples
75
50
25
0
0
5
10
15
Max Time from 1st PA culture (by patient), yr
C2
C3
C4
Trange
seq
cult
0.00
0.25
0.50
0.75
1.00
#samples
seq
cult
0.25
0.50
0.75
1.00

FIGURE S2

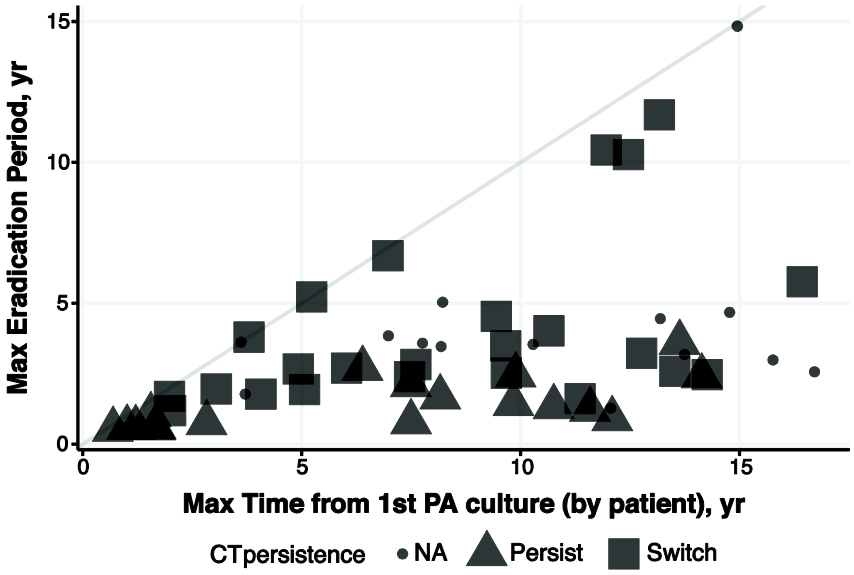
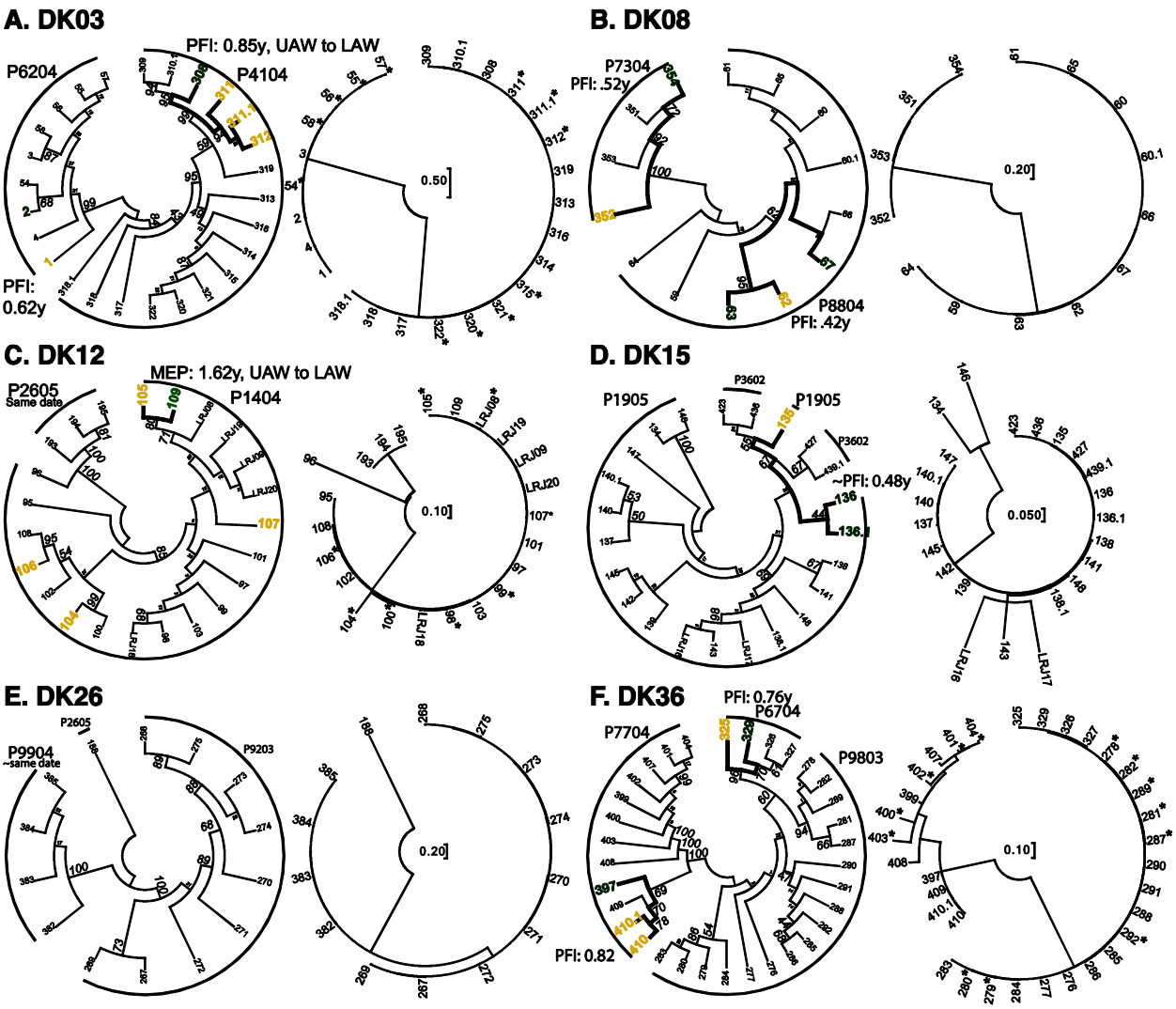
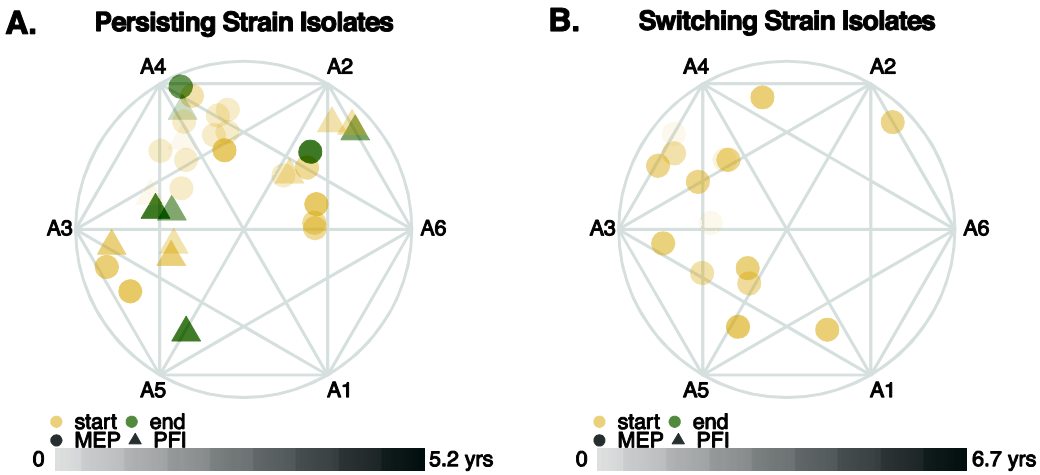

Max Eradication Period, yr
15
10
5
0
0
5
10
15
Max Time from 1st PA culture (by patient), yr
CTpersistence
NA
Persist
Switch

FIGURE S3



Isolates bridging longest MEP or PFI within CT phylogeny denoted as yellow (start) and green (end), * = UAW

FIGURE S4


A.
Persisting Strain Isolates
A4
A2
A3
A6
A5
A1
start
end
MEP
PFI
0
5.2 yrs
B.
Switching Strain Isolates
A4
A2
A3
A6
A5
A1
start
end
MEP
PFI
0
6.7 yrs

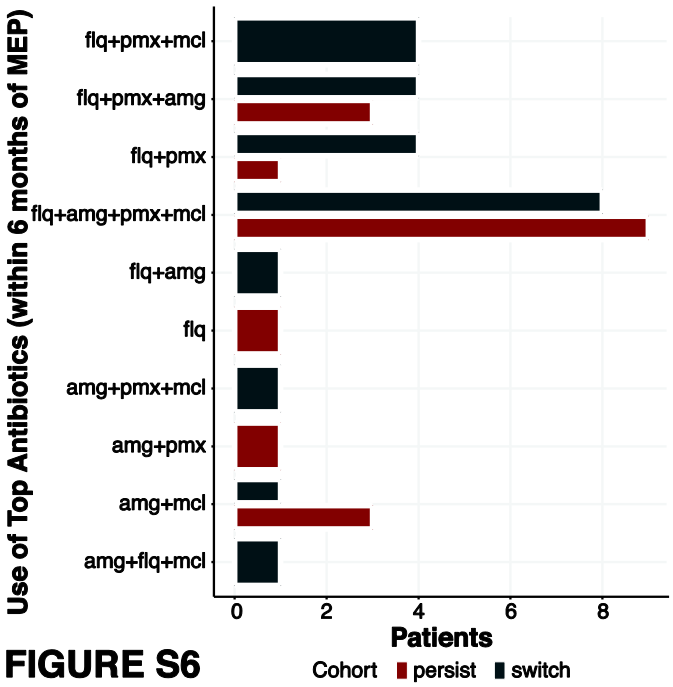

Use of Top Antibiotics (within 6 months of MEP)
flq+pmx+mcl
flq+pmx+amg
flq+pmx
flq+amg+pmx+mcl
flq+amg
flq
amg+pmx+mcl
amg+pmx
amg+mcl
amg+flq+mcl
0
2
4
6
8
Patients
Cohort
persist
switch

FIGURE S6