ORIGINAL ARTICLE

# *Pseudomonas aeruginosa In Vitro* Phenotypes Distinguish Cystic Fibrosis Infection Stages and Outcomes

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## Abstract

**Rationale:** *Pseudomonas aeruginosa* undergoes phenotypic changes during cystic fibrosis (CF) lung infection. Although mucoidy is traditionally associated with transition to chronic infection, we hypothesized that additional *in vitro* phenotypes correlate with this transition and contribute to disease.

**Objectives:** To characterize the relationships between *in vitro* *P. aeruginosa* phenotypes, infection stage, and clinical outcomes.

**Methods:** A total of 649 children with CF and newly identified *P. aeruginosa* were followed for a median 5.4 years during which a total of 2,594 *P. aeruginosa* isolates were collected. Twenty-six *in vitro* bacterial phenotypes were assessed among the isolates, including measures of motility, exoproduct production, colony morphology, growth, and metabolism.

**Measurements and Main Results:** *P. aeruginosa* phenotypes present at the time of culture were associated with both stage of infection (new onset, intermittent, or chronic) and the primary clinical outcome, occurrence of a pulmonary exacerbation (PE) in the subsequent 2 years. Two *in vitro P. aeruginosa* phenotypes best distinguished infection stages: pyoverdine production (31% of new-onset cultures, 48% of intermittent, 69% of chronic) and reduced protease production (31%, 39%, and 65%, respectively). The best *P. aeruginosa* phenotypic predictors of subsequent occurrence of a PE were mucoidy (odds ratio, 1.75; 95% confidence interval, 1.19–2.57) and reduced twitching motility (odds ratio, 1.43; 95% confidence interval, 1.11–1.84).

**Conclusions:** In this large epidemiologic study of CF *P. aeruginosa* adaptation, *P. aeruginosa* isolates exhibited two *in vitro* phenotypes that best distinguished early and later infection stages. Among the many phenotypes tested, mucoidy and reduced twitching best predicted subsequent PE. These phenotypes indicate potentially useful prognostic markers of transition to chronic infection and advancing lung disease.

**Keywords:** epidemiology; risk factors; exacerbation; pulmonary function; mucoid *Pseudomonas aeruginosa*

Airway infection and inflammation are key contributors to lung disease in people with cystic fibrosis (CF), and the opportunistic gram-negative bacterium *Pseudomonas aeruginosa* is among the most common and important CF respiratory pathogens (1). The association between *P. aeruginosa* infection and measures of CF disease severity is clearly established, including increased rate of lung function decline, greater need for antibiotics and hospitalization, and decreased survival (2–10). Although there is a parallel

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## At a Glance Commentary

**Scientific Knowledge on the Subject:** *Pseudomonas aeruginosa* undergoes phenotypic changes during cystic fibrosis (CF) lung infection, and the mucoid phenotype is perhaps the best-characterized change known to be associated with poor clinical outcome. Although numerous other adaptive changes have been identified, it is unclear from prior relatively small studies how these changes are associated with the transition from early to chronic *P. aeruginosa* infection, the relative prevalence of these changes throughout the course of infection, and whether any are changes associated with markers of disease pathogenesis.

**What This Study Adds to the Field:** This study characterizes the multiple *in vitro* phenotypic changes that *P. aeruginosa* can undergo during the transition from early to chronic *P. aeruginosa* infection within the setting of a large, prospective observational study. Beyond describing the phenotypic changes that occurred most often throughout CF infection, this study characterizes their relative clinical importance through their association with outcomes reflective of CF disease pathogenesis.

between declining health and the progression from early to chronic *P. aeruginosa*, gaps remain in understanding the changes that *P. aeruginosa* undergoes during this critical transition period (11). *P. aeruginosa* isolates have been observed to exhibit a series of adaptive phenotypic and genotypic changes during this progression, but the relative roles of these changes in pathogenesis and persistence are poorly understood. These adaptations likely occur in response to the host airway environment, which includes specific nutrients; innate and adaptive immune system components; and medications, such as antibiotics (12). An improved understanding of these adaptations and their clinical consequences is critical for defining how *P. aeruginosa* persists and causes disease in the CF lung, and for identifying novel therapeutic approaches.

In perhaps the most extensive longitudinal study of *P. aeruginosa* phenotypic change, Li and coworkers (10) followed 56 children with CF from birth to 16 years, describing the association between the emergence of the best-characterized *in vitro P. aeruginosa* phenotype, mucoidy, and deterioration in chest radiograph scores and pulmonary function. Prior studies have identified numerous other *P. aeruginosa in vitro* phenotypes that change throughout infection (13), including colony morphology (14); motility (15); cell signaling (16); and production of pigments (17), proteases (18, 19), and other exoproducts (19, 20). However, these small studies, which each generally focused on one *P. aeruginosa* phenotype, were limited in their ability to describe the interrelatedness of multiple adaptive changes across the course of *P. aeruginosa* infection and their associations with clinical outcomes. For example, although mucoidy has been associated with failure of eradication by antibiotic treatment in small, focused studies (21), our subsequent comparative analysis of many *P. aeruginosa* phenotypes in a larger clinical population found other, more common *P. aeruginosa* phenotypes to be much more highly associated with this outcome (22). To rigorously define the natural history of *P. aeruginosa* phenotypic changes, and the relationship between these changes and disease progression, large longitudinal studies comparing multiple *P. aeruginosa* phenotypes are required (13).

Using longitudinally collected *P. aeruginosa* isolates and clinical data from a large, multicenter, prospective observational study of *P. aeruginosa* infection among a cohort followed across the transition from early to chronic *P. aeruginosa* infection (23), we defined the prevalence of multiple *P. aeruginosa* phenotypes and their association with disease progression. This study represents the largest comparative study of *P. aeruginosa* phenotypes to date. We hypothesized that specific *P. aeruginosa in vitro* phenotypes would increase in prevalence during the transition from early to chronic infection and correlate with measures of lung disease severity, including frequency of pulmonary exacerbations, emergence of mucoid *P. aeruginosa*, and reduced lung function. We chose phenotypic assays that could be reproducibly performed by clinical laboratories using commonly available resources. By simultaneously comparing multiple *in vitro* *P. aeruginosa* phenotypes with subsequent clinical outcomes, we aimed to identify useful candidate markers for the establishment of chronic *P. aeruginosa* infection and CF lung disease pathogenesis. Some of the results of these studies have been previously reported in the form of an abstract (24).

## Methods

### Study Design

The EPIC Observational Study is a prospective, multicenter study investigating risk factors for and outcomes of *P. aeruginosa* acquisition in CF (23). Participants in the current study had newly identified *P. aeruginosa* within 6 months of enrollment in the EPIC study or during the follow-up period at the time this study was initiated, termed "new-onset *P. aeruginosa*" and defined as the first lifetime *P. aeruginosa*–positive culture or the first positive after greater than or equal to 2 years absence of *P. aeruginosa* (with $\geq$1 negative culture per year) (23). Respiratory cultures over the follow-up period were performed at site clinical microbiology laboratories as part of standard clinical care, with results and clinical data recorded in the CF Foundation National Patient Registry (CFFNPR). *P. aeruginosa* isolates were shipped from site microbiology laboratories to a core microbiology laboratory (23). This study was approved by the Seattle Children's Hospital institutional review board.

### *Pseudomonas aeruginosa In Vitro* Phenotypic Characterization

Because of the large number of isolates collected, and to maximize future use for diagnostic microbiology laboratories, phenotypic assays were selected for simplicity and reproducibility in a high-throughput format (*see* Figures E1 and E2 in the online supplement). All assays were developed using *P. aeruginosa* strains and isolates known to be competent and defective for those specific phenotypes; for quantitative assays, these strains were included as controls. Phenotypes assessed included motility (twitching, swimming), exoproduct production (β-lactamase, protease, and colorimetric assays for pyoverdine and pyocyanin), colony morphology (size, color, mucoidy, surface wrinkliness, edge regularity, sectoring, autolysis, sheen, Congo red binding), and growth and metabolism (auxotrophy, relative growth in added nitrate).

### Endpoints

Phenotype prevalence at time of culture, defined as present if exhibited by any isolate from the same culture, was stratified by stage of infection: (*1*) new-onset *P. aeruginosa*; (*2*) chronic *P. aeruginosa*, defined as the third positive *P. aeruginosa* culture within a 2-year period (modified Leeds criteria, requiring at least two *P. aeruginosa* culture-positive quarters within a year to meet the definition [25, 26]); and (*3*) intermittent *P. aeruginosa*, defined as neither new onset or chronic. Although isolates were not available from every *P. aeruginosa*–positive culture identified over the follow-up period, infection stage at the time of each culture for which an isolate was available was defined in reference to all observed *P. aeruginosa*–positive cultures during the study as recorded in the CFFNPR, not only those for which isolates were available. The primary clinical outcome was occurrence of a physician-defined pulmonary exacerbation requiring intravenous (IV) antibiotics and/or hospitalization during the 2 years after each culture, chosen to reflect a clinically relevant time period and to reflect the available follow-up time after the collection of isolates obtained later in the study. Secondary endpoints included the emergence or reappearance of mucoid *P. aeruginosa* as recorded in the CFFNPR, and change in $FEV_1$ % predicted.

### Statistical Analyses

Repeated measures logistic regression models were used to identify phenotypes that best distinguished between one infection stage and another (27), and phenotypes that distinguished between infection stages at a 0.05 level two-sided error for all three infection stage comparisons were identified (equivalent to a type 1 error $<0.05^3 = 0.0001$). Multivariable repeated measures logistic or linear regression models were used to identify phenotypes significantly associated with outcomes of interest.

## Results

### Cohort Description

A total of 649 EPIC Observational Study participants had at least one *P. aeruginosa* isolate available at the core microbiology laboratory and comprised the current cohort. Characteristics at the time of new-onset *P. aeruginosa*, which occurred between 2004 and 2011, are described in Table 1. The median follow-up time after new-onset *P. aeruginosa* among the study cohort was 5.4 years (maximum follow-up, 7.0 years), during which 2,594 *P. aeruginosa* isolates were obtained and phenotyped from 1,707 *P. aeruginosa*–positive respiratory cultures (representing ~54% of all *P. aeruginosa*–positive cultures during the follow-up period). A total of 51 of those 2,594 isolates grew too poorly *in vitro* to be phenotyped in any assay and were considered "absent" for this analysis. The average number of cultures per participant from which isolates were collected was 2.63 (median, 2; range, 1–16). There were 602 participants in the EPIC Observational Study with new-onset *P. aeruginosa* for whom no *P. aeruginosa* isolates were available and who were therefore not included in the current cohort; these participants were on average slightly older, but comparable with the current study cohort with respect to other characteristics (Table E1). Antipseudomonal therapy at the time of new-onset *P. aeruginosa* in the EPIC Observational Study cohort has been previously described, with approximately 91% receiving antipseudomonal antibiotics within the first 6 months after initial *P. aeruginosa* isolation (26).

### Relationship between *Pseudomonas aeruginosa* Phenotypes and Infection Stage

Figure 1A displays the availability of *P. aeruginosa* cultures from which isolates were sampled by age and infection stage. A total of 580 cultures representing new-onset *P. aeruginosa* from 580 of 649 (89%) participants were sampled, 459 cultures categorized as reflecting intermittent *P. aeruginosa* infection from 307 (47%) participants, and 668 cultures categorized as chronic from 211 (33%) participants. Overall, 57% of the 1,707 cultures were oropharyngeal (OP), 20% from sputum, 1% from bronchoalveolar lavage, and 22% had missing culture source; OP cultures were more commonly used at new-onset infection as compared with persistent (Table E2). The average age at new-onset *P. aeruginosa* culture was 6.3 years, 8.1 years at the time of an intermittent culture, and 10.8 years at the time of a chronic culture. Figure 1B displays the prevalence of each phenotype by infection stage (*see* Figure E3 for prevalence by age group). The most prevalent phenotypes among the new-onset cultures were tan colony color (67%), pyocyanin production (49%), green broth color (37%), and abnormal colony size (36%). Across infection stage, mucoidy was present among 8% of new-onset cultures, 14% of intermittent cultures, and 29% of chronic cultures, and further

**Table 1.** Description of the Study Cohort at New-Onset *Pseudomonas aeruginosa* (n = 649)

| | N (%) or Mean (SD) |
|---|---|
| Sex, male | 325 (50%) |
| Age at new-onset *P. aeruginosa*, yr | 6.3 (3.86) |
| Age distribution at new-onset *P. aeruginosa* | |
| >0–3 yr | 166 (26%) |
| >3–6 yr | 147 (23%) |
| >6–12 yr | 280 (43%) |
| >12 yr | 56 (9%) |
| Genotype | |
| F508 del heterozygous | 233 (36%) |
| F508 del homozygous | 345 (53%) |
| Other/unknown | 71 (11%) |
| Race | |
| White/non-Hispanic | 595 (92%) |
| Hispanic | 24 (4%) |
| Black/African American | 17 (3%) |
| Other | 13 (2%) |
| First lifetime *P. aeruginosa* + culture at new onset | 389 (60%) |
| $FEV_1$ % predicted* | 94% (18) |

*A total of 266 participants had $FEV_1$ % predicted measurement at time of new-onset *P. aeruginosa*.

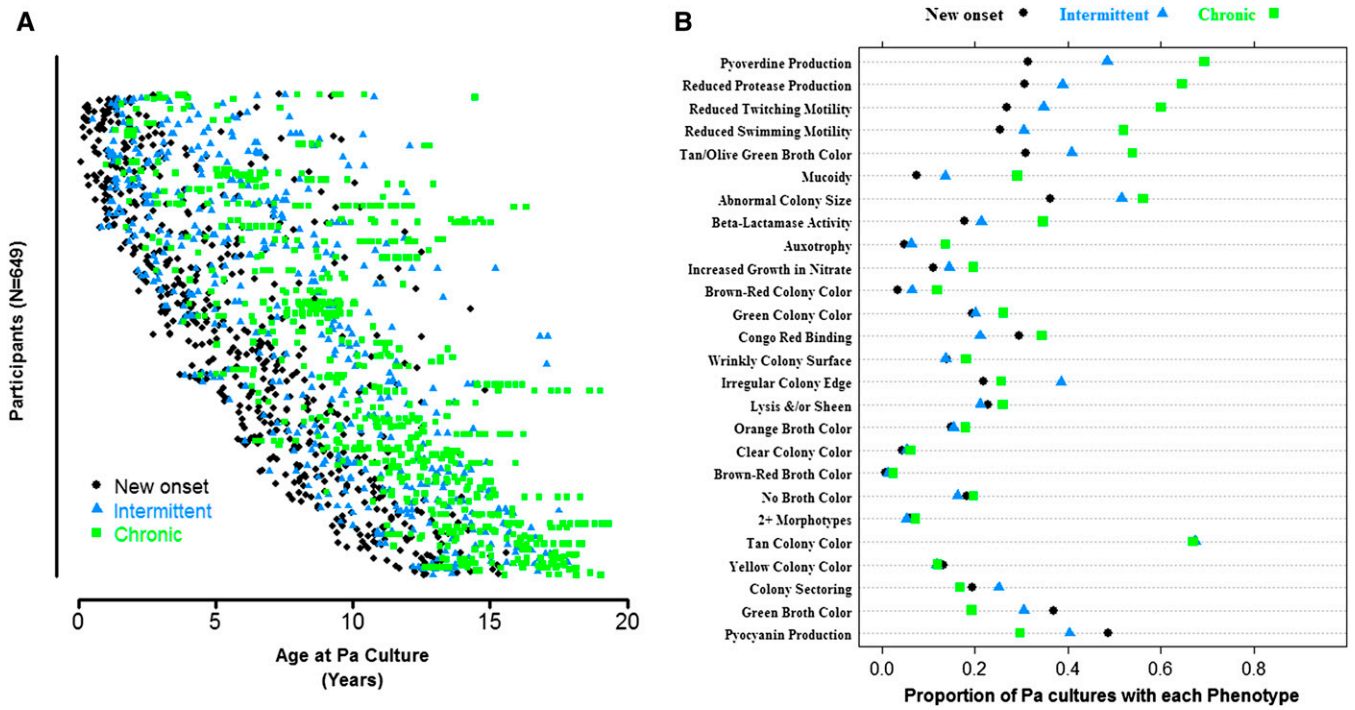


**Figure 1.** (*A*) *Pseudomonas aeruginosa* cultures from which isolates were sampled from by age and infection stage. Each *horizontal plane* represents a single participant in the study cohort. (*B*) Prevalence of each phenotype by infection stage (corresponding percentages provided in Table E3).

analyses demonstrated these prevalence estimates did not differ significantly by lifetime *P. aeruginosa* history (i.e., between participants with no prior *P. aeruginosa* vs. those with *P. aeruginosa* detected ⩾2 years before enrollment). By comparison, several other *in vitro* phenotypes exhibited more significant differences in prevalence across infection stages (e.g., were more prevalent in cultures categorized as chronic as compared with intermittent, and more prevalent in intermittent cultures as compared with new onset). Phenotypes demonstrating greater prevalence and differentiation among infection stages in Figure 1B included pyoverdine production, reduced protease production, reduced twitching motility, and reduced swimming motility.

Figure 2 is a schematic of the phenotypes with significantly higher odds of occurring during one infection stage than another based on multivariable modeling. Two phenotypes, pyoverdine production and reduced protease production, were significantly different across all three infection stage comparisons. As shown in Figure 2, reduced protease production and intact pyoverdine production were associated with significantly higher odds of occurring during chronic infection as compared with new onset (odds ratio [OR], 3.24; 95% confidence interval [CI], 2.31–4.56 and OR, 4.04; 95% CI, 2.99–5.47, respectively), with similar findings in the other two infection stage comparisons. Further modeling demonstrated a "dose-response" across infection stages with respect to reduced protease; in other words, there were significantly higher odds of a culture exhibiting reduced protease among at least one isolate during chronic and intermittent infection as compared with new-onset infection (OR comparing chronic vs. new-onset infection, 4.1; 95% CI, 3.1–5.5; OR comparing intermittent vs. new onset, 1.4; 95% CI, 1.1–1.9). Similarly, there were significantly higher odds of a culture exhibiting pyoverdine production during chronic and intermittent infection as compared with new-onset infection (OR comparing chronic vs. new onset, 5.0; 95% CI, 3.8–6.5; OR comparing intermittent vs. new onset, 2.1; 95% CI 1.6–2.7).

### Association between *Pseudomonas aeruginosa* Phenotypes and Subsequent Pulmonary Exacerbation Occurrence

Although there was clear evidence for *in vitro* phenotypic changes distinguishing stage of infection, another aim of our study was to assess whether these phenotypes were associated with clinical outcomes reflecting morbidity. Table 2 presents results from the multivariable model used to identify phenotypes significantly associated with the occurrence of a pulmonary exacerbation requiring hospitalization and/or IV antibiotics in the following 2 years. The model was adjusted for time from new-onset infection to identify phenotypes that were associated with poor clinical outcome not solely caused by the fact that they were more prevalent during later infection. Mucoidy had the strongest association with the subsequent occurrence of a pulmonary exacerbation (OR, 1.75; 95% CI, 1.19–2.57), followed closely by defective twitching motility (OR, 1.43; 95% CI, 1.11–1.84). Univariate regression analyses indicated that defective swimming motility was also significantly associated with pulmonary exacerbations but was highly correlated with defective twitching, the latter of which was the better predictor in multivariable regression modeling. Although other phenotypes, particularly those more prevalent later in infection, such as reduced protease production, demonstrated significant univariate associations with the occurrence of a pulmonary exacerbation

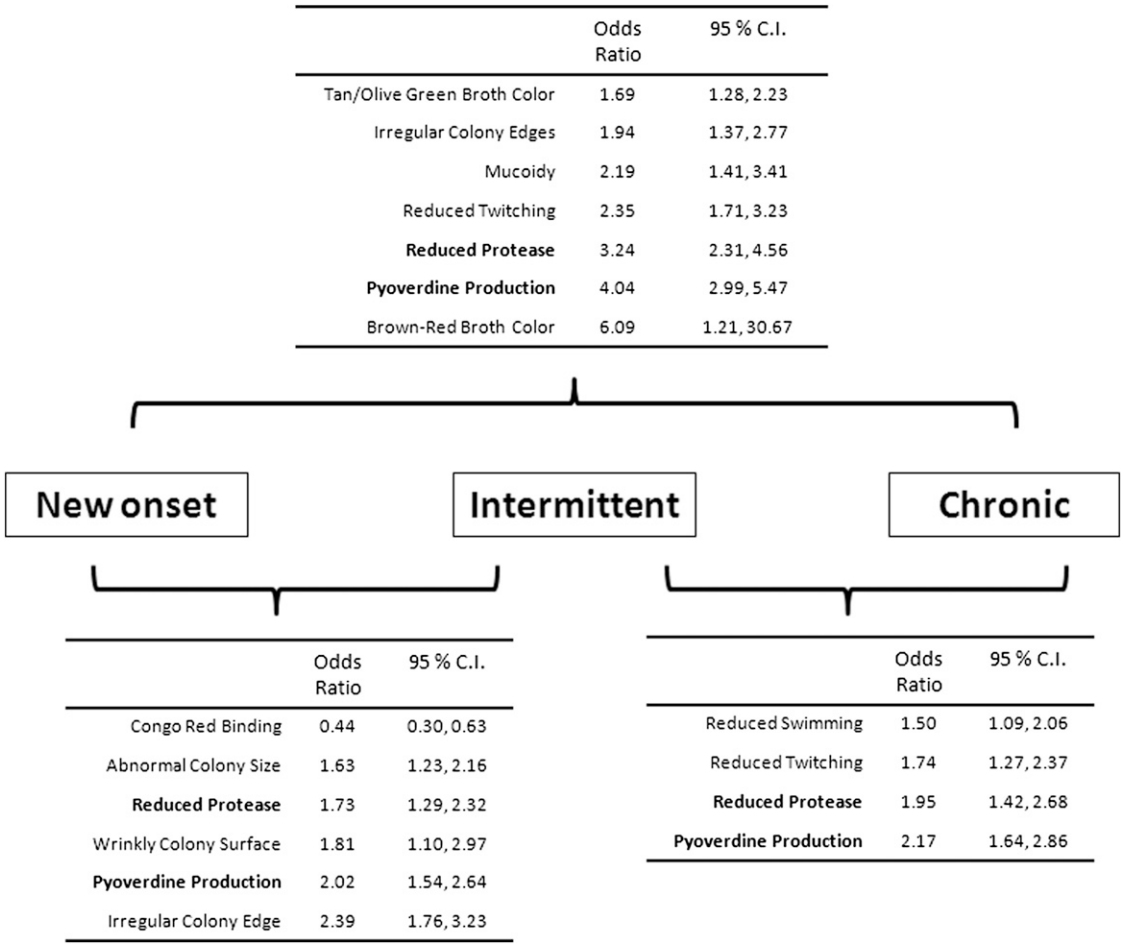


**Figure 2.** Results from the three multivariable models used to identify the set of phenotypes best distinguishing one infection stage from another. The set of phenotypes significantly associated with the more "advanced" infection stage as compared with the less advanced infection stage as identified using multivariable, repeated measures logistic regression. For example, reduced protease had significantly higher odds of being present during chronic infection as compared with new-onset infection (odds ratio [OR], 3.24), in addition to significantly higher odds of being present in chronic infection as compared with intermittent infection (OR, 1.95) and in intermittent infection as compared with new-onset infection (OR, 1.73). *Bold* phenotypes (reduced protease and pyoverdine production) showed significance across all three comparisons. C.I. = confidence interval.

(Figure E4), no other phenotypes besides mucoidy and reduced twitching motility were significantly associated with pulmonary exacerbations in the multivariable model after adjusting for time from infection. Additional sensitivity analyses revealed that mucoidy and reduced twitching motility remained significantly associated with the occurrence of an exacerbation over both a 6-month and 1-year follow-up period.

### Association between Phenotypes and Emergence or Reappearance of Mucoidy

The significant association between mucoidy and subsequent pulmonary exacerbation observed here underscored the clinical importance of this phenotype. Because mucoidy commonly emerges relatively late during chronic infection, phenotypic predictors of the subsequent emergence of mucoidy may be clinically useful. Table 3 presents multivariable model results for the association between phenotypes and the reappearance or new

**Table 2.** Results from a Multivariable Model for the Association between Phenotypes and the Occurrence of a Pulmonary Exacerbation Requiring Intravenous Antibiotics and/or Hospitalization over the Following Two Years

| | Odds Ratio | 95% CI | *P* Value |
|---|---|---|---|
| Mucoidy | | | |
| Nonmucoid | — | — | — |
| Mucoid | 1.75 | 1.19–2.57 | 0.004 |
| Twitching | | | |
| Normal | — | — | — |
| Reduced/none | 1.43 | 1.11–1.84 | 0.006 |
| Time from new-onset infection, yr | 1.03 | 0.96–1.10 | 0.427 |

*Definition of abbreviation*: CI = confidence interval.

**Table 3.** Results from the Multivariable Model for the Association between Phenotypes and the Emergence or Reappearance of Mucoidy over the Following Two Years

| | Odds Ratio | 95% CI | *P* Value |
|---|---|---|---|
| Mucoidy | | | |
| Nonmucoid | — | — | — |
| Mucoid | 6.04 | 4.14–8.81 | <0.001 |
| Increased growth in nitrate | | | |
| Absent | — | — | — |
| Present | 2.37 | 1.65–3.40 | <0.001 |
| β-Lactamase activity | | | |
| Absent | — | — | — |
| Present | 1.68 | 1.20–2.34 | <0.001 |
| Time from new-onset infection, yr | 1.31 | 1.21–1.41 | <0.001 |

*Definition of abbreviation*: CI = confidence interval.

emergence of mucoidy over the following 2 years. Included in the model was the presence of mucoidy at any time point, which was highly associated with its reappearance over the following 2-year period (OR, 6.04; 95% CI, 4.14–8.81). Two additional phenotypes were associated with the emergence of mucoidy even after adjusting for time from infection, which itself was significantly associated with increased odds of developing mucoidy (OR, 1.31; 95% CI, 1.21–1.41). The presence of increased growth in nitrate and β-lactamase activity were independently associated with increased odds of future mucoidy (OR, 2.37; 95% CI, 1.65–3.40 and OR, 1.68; 95% CI, 1.20–2.34, respectively). After adjusting for time from new-onset infection, several phenotypes that were associated with the emergence or reappearance of mucoidy in unadjusted univariate analyses (Figure E5) were no longer significant, suggesting a strong relationship between these phenotypes and duration of infection.

### Association between *Pseudomonas aeruginosa* Phenotypes and Subsequent Change in Lung Function

No phenotypes were significantly associated with the subsequent 2-year change in $FEV_1$ % predicted, although mucoidy exhibited a nonsignificant trend toward association with more rapid decline in lung function during chronic infection as compared with new-onset or intermittent infection (Tables E5 and E6). The 2-year change in $FEV_1$ % predicted associated with mucoidy observed at new-onset infection was 2.11% (95% CI, −3.49 to 7.71), as compared with a 1.57% decline during intermittent infection (95% CI, −6.43% to 3.30%) and a 3.01% decline during chronic infection (95% CI, −7.02% to 1.01%). Of note, infection stage was not independently associated with significant 2-year changes in $FEV_1$ % predicted in this model.

## Discussion

Among the largest cohort of individuals with CF for whom *P. aeruginosa in vitro* phenotypes have been characterized, this study identified the phenotypic changes most associated with stage of *P. aeruginosa* infection and defined the associations between these phenotypes and clinical outcomes. Surprisingly, we found that even at the earliest stages of *P. aeruginosa* detection in children with CF, many *in vitro* phenotypes occurred frequently that were previously considered to represent adaptation during chronic infection. Despite the highly diverse and dynamic *in vitro* phenotypes exhibited by *P. aeruginosa* isolates, only a limited number of phenotypes were associated with measures of the progression of infection and lung disease. Although the prevalence of mucoidy increased during the course of infection, other *in vitro* phenotypic changes performed better at distinguishing new-onset, intermittent, and chronic *P. aeruginosa* infection periods. However, the most important finding of our study was the identification of *P. aeruginosa* phenotypes most associated with key clinical outcomes in CF that, surprisingly, were not always those that occurred later in infection.

The two *P. aeruginosa* phenotypes that best distinguished infection stages, pyoverdine production and reduced protease activity, have been studied before. We previously reported production of both pyoverdine and protease (among other phenotypes) to be decreased in late versus early infection isolates in a clonal lineage from one patient with CF (28), and both phenotypes were observed to vary among seven CF chronic infection isolates in one lineage (29). In the current large, comparative phenotypic study, these two phenotypes significantly differed in prevalence across initial, intermittent, and chronic infection stages. Both *in vitro* phenotypes, as for all characteristics tested in this study, can be easily and reproducibly measured by clinical laboratories, underscoring their potential clinical use as markers of the transition from early to chronic infection (a role traditionally played by mucoidy).

Despite the striking variety of *in vitro* phenotypic changes undergone by *P. aeruginosa*, our results found very few of these phenotypes to be associated with symptomatic lung disease. Although it must be noted that bacterial characteristics measured *in vitro* are not necessarily representative of behavior *in vivo*, only the characteristics that can be robustly measured *ex vivo* represent candidate disease markers. The primary outcome in this study, occurrence of a pulmonary exacerbation requiring IV antibiotics and/or hospitalization within 2 years, was chosen for its demonstrated indication of disease severity and impact on quality of life (30, 31), and the ability to assess its occurrence across all ages after new-onset *P. aeruginosa* infection for all subjects (unlike pulmonary function, which was only available for 41% of our cohort at the time of new-onset *P. aeruginosa*). The 2-year observation period was chosen based on availability of clinical data after assessment of the last isolates phenotyped in this study, representing a proximal window of clinical relevance. Additionally, although pulmonary exacerbations have been associated with both short- and long-term morbidity and mortality (9, 32, 33), less is understood about the associations between rate of decline in lung function and long-term outcomes, such as mortality, in part because of complexities in model development (34).

Our study found mucoidy to be the *in vitro* phenotype most significantly associated with risk of a subsequent pulmonary exacerbation regardless of infection stage. However, reduced twitching motility, which was more common and also better distinguished infection stage than mucoidy in this study, was also significantly associated with risk of exacerbation. Although mucoidy is often simple to identify, the expression of this phenotype can vary with culture conditions and can revert over time (35). Additional analyses from our cohort demonstrated that during persistent infection, 58% of participants with mucoid isolates maintained this phenotype in a second culture sampled within the year; in contrast, 65 and 75% of participants with persistent infection who had isolates with reduced twitching and swimming present, respectively, maintained these phenotypes in a second culture within the year, highlighting the relative stability of the latter motility phenotypes. Because motility measures can be reproducibly and quantitatively measured, twitching motility represents a promising candidate biomarker. Defective motility (both twitching and swimming) was observed previously in isolates from late versus early CF infections, a change that favors aggregation and may confer protection against antibiotics and host immunity (15, 36). Importantly, a recent analysis (26) found a significant association between *P. aeruginosa* acquisition and risk of exacerbation, but not decline in lung function in the EPIC Observational Study cohort, supporting a relatively strong relationship between *P. aeruginosa* and exacerbations.

Given this finding, it is perhaps less surprising that those phenotypes most associated with risk of exacerbation were not significantly associated with lung function outcomes. Notably, our findings differed from those of Li and coworkers (10), who reported an association between the transition from nonmucoid to mucoid *P. aeruginosa* and faster rate of lung function decline among children with CF from the Wisconsin Newborn Screening Study. This single-center study followed 56 children born between 1985 and 2004 from birth to 16 years of age. There are several potential explanations for this discrepancy. First, the current 2-year follow-up period may have been too short to clearly assess lung function decline, because the availability of spirometric data subsequent to the emergence of mucoidy was limited. By contrast, the average follow-up time was 16 years for a recent, expanded Wisconsin study that found mucoidy to be a significant predictor of worsening lung function (37). Second, our cohort represents a more diverse and contemporary cohort than that studied by Li and colleagues, and CF antimicrobial strategies have evolved in the interim. Not only was tobramycin inhalation therapy approved in 1998 (38), but aggressive early *P. aeruginosa* eradication approaches have become standard of care (39).

In contrast with lung function, we identified a significant relationship between mucoidy and exacerbations that was consistent across infection stage. This finding was not reported in prior longitudinal observational studies (10, 37). Because of the clinical significance of mucoidy, we sought other phenotypes that correlated with the subsequent development of mucoidy. Two phenotypes, increased growth in added nitrate and β-lactamase production, were significantly associated with subsequent mucoidy regardless of infection stage. Altered nitrate use was shown previously to be conferred by at least two other adaptive changes in chronic CF *P. aeruginosa* isolates: *lasR* mutation (40) and mucoidy (41). Increased β-lactamase production has been observed in late versus early CF isolates both caused by *lasR* mutation (40) and β-lactam treatment (42). Thus, these phenotypic changes may represent earlier prognostic markers of important CF outcomes and be relevant targets for novel, early interventional strategies.

Our study had several strengths, including a large, diverse cohort and the relatively large numbers of both *P. aeruginosa* isolates and *in vitro* phenotypes characterized longitudinally over the course of infection. In addition, most phenotypic assays used were quantitative rather than qualitative, limiting bias. However, there were several notable limitations. First, the epidemiologic nature of this study prevents us from concluding that the associations we found between phenotypes and outcomes are causal. We can conclude that these associations were not simply driven by higher prevalences of phenotypes during later stages of infection because our models adjusted for time from new-onset *P. aeruginosa*, a surrogate for several measures of disease severity that could represent potential confounders that may be in the causal pathway.

Second, our analyses used data from the CFFNPR, restricting the pulmonary exacerbation events that were recorded to those requiring IV antibiotics and/or hospitalization. It was possible that physicians were more likely to prescribe IV antibiotics for specific symptoms if a patient was known to have mucoidy; however, in a survey of centers participating in the EPIC Observational Study, most physicians did not indicate their treatment preferences to be influenced by the presence of mucoidy (M. Rosenfeld, personal communication). Furthermore, the practicality of assessing more than 2,500 *P. aeruginosa* isolates limited the number and types of phenotypes assessed. Several other *P. aeruginosa* characteristics have previously been found to change throughout infection that we did not assay, because of the difficulty of adapting those assays for high-throughput testing, expense, or poor reproducibility. Among these characteristics are type III secretion and cytotoxicity, metabolic changes, antibiotic susceptibilities, growth in sputum or anaerobically, hypermutability, biofilm formation, membrane structural changes, and serum sensitivity (12, 40, 43). These characteristics represent potential possibilities for future studies. Also, recent analyses showed that traditional culture methods (i.e., isolating a small number of morphologically different bacterial colonies from each culture) frequently underrepresent the *P. aeruginosa* diversity in individual samples of CF respiratory secretions during chronic infection with respect to several phenotypes (44–47); therefore, even the large isolate collection in this study likely undersampled the phenotypic diversity of *P. aeruginosa* in each subject. Similarly, it is possible that sputum and OP specimens are not sufficiently sensitive to reflect the earliest stages of infection, whether of the lower or upper airways (48, 49), potentially explaining the observed high frequency in our "early" isolates of phenotypes commonly associated with adaptation.

Lastly, because of the EPIC Study design, we were unable to capture isolates from 100% of all *P. aeruginosa*–positive cultures during the study, limiting our ability to determine the exact timing of the emergence of the phenotypes. Thus, an unanswered question is whether specific

clinical characteristics predict the emergence of the key phenotypes identified in our study, and in particular whether antibiotic intensity or other factors over the duration of infection favor *P. aeruginosa* phenotypic adaptation. Unfortunately, lack of detailed data regarding the timing and frequency of antibiotic courses in this observational study prohibited such an analysis. Further research is therefore needed to identify potential confounders in the causal pathway that may help to explain the associations between *P. aeruginosa* phenotypes and clinical outcomes.

In summary, *P. aeruginosa* undergoes multiple phenotypic changes that are associated with the transition from early to chronic CF infection. Although mucoidy remained one of two clinically useful markers of risk of a pulmonary exacerbation, two adaptive phenotypes other than mucoidy emerged as candidate markers of the transition to chronic infection. The association between these adaptive changes and progression of infection and lung disease may indicate important mechanisms of persistence and pathogenesis. Thus, more research is needed to identify novel therapeutic approaches to prevent and eradicate *P. aeruginosa* exhibiting mucoidy and other adaptive changes. Phenotypic changes, such as those identified in this study, have the potential to provide valuable mechanistic insight, and they may serve as clinically useful markers of advanced infection or risk of a pulmonary exacerbation, potentially identifying patients who would benefit from more aggressive treatment. ■

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