

Ferrous Iron Is a Significant Component of Bioavailable Iron in Cystic Fibrosis Airways

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ABSTRACT Chronic, biofilm-like infections by the opportunistic pathogen *Pseudomonas aeruginosa* are a major cause of mortality in cystic fibrosis (CF) patients. While much is known about *P. aeruginosa* from laboratory studies, far less is understood about what it experiences *in vivo*. Iron is an important environmental parameter thought to play a central role in the development and maintenance of *P. aeruginosa* infections, for both anabolic and signaling purposes. Previous studies have focused on ferric iron [Fe(III)] as a target for antimicrobial therapies; however, here we show that ferrous iron [Fe(II)] is abundant in the CF lung (~39 μ M on average for severely sick patients) and significantly correlates with disease severity ($\rho = -0.56$, $P = 0.004$), whereas ferric iron does not ($\rho = -0.28$, $P = 0.179$). Expression of the *P. aeruginosa* genes *bqsRS*, whose transcription is upregulated in response to Fe(II), was high in the majority of patients tested, suggesting that increased Fe(II) is bioavailable to the infectious bacterial population. Because limiting Fe(III) acquisition inhibits biofilm formation by *P. aeruginosa* in various oxic *in vitro* systems, we also tested whether interfering with Fe(II) acquisition would improve biofilm control under anoxic conditions; concurrent sequestration of both iron oxidation states resulted in a 58% reduction in biofilm accumulation and 28% increase in biofilm dissolution, a significant improvement over Fe(III) chelation treatment alone. This study demonstrates that the chemistry of infected host environments coevolves with the microbial community as infections progress, which should be considered in the design of effective treatment strategies at different stages of disease.

IMPORTANCE Iron is an important environmental parameter that helps pathogens thrive in sites of infection, including those of cystic fibrosis (CF) patients. Ferric iron chelation therapy has been proposed as a novel therapeutic strategy for CF lung infections, yet until now, the iron oxidation state has not been measured in the host. In studying mucus from the infected lungs of multiple CF patients from Europe and the United States, we found that ferric and ferrous iron change in concentration and relative proportion as infections progress; over time, ferrous iron comes to dominate the iron pool. This information is relevant to the design of novel CF therapeutics and, more broadly, to developing accurate models of chronic CF infections.

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Both culture-dependent and molecular identification methods suggest that *Pseudomonas aeruginosa* is a dominant bacterial pathogen of patients with cystic fibrosis (CF) (1). As *P. aeruginosa* adapts to the host environment, it adopts a biofilm-like lifestyle associated with enhanced antibiotic resistance, persistent infections, and poor pulmonary prognosis (2). Numerous studies have documented how *P. aeruginosa* responds to specific environmental cues, including hypoxia, that are thought to be relevant for biofilm formation and chronic colonization *in vivo* (3, 4); however, only a few studies have directly measured chemical parameters within the host, and usually only at a single time point (5, 6). This knowledge gap is an important one to fill because pathogens are known to coevolve with host environmental chemistry with respect to their metabolic programs and growth phenotypes, in-

cluding biofilm formation (2, 7). Toward this end, we sought to characterize the oxidation state of iron within CF sputum and the biological response of *P. aeruginosa* at different disease states for a cross section of CF patients.

The competition for iron between pathogens and the human host has been extensively studied due to its critical importance in pathogenesis (8, 9). In particular, iron has been identified as an important parameter that plays a central role in the development and maintenance of *P. aeruginosa* biofilm infections within the lung (10–12). On this basis, iron uptake and acquisition pathways have been identified as potential antimicrobial targets (10–15). While microbial ferrous iron [Fe(II)] acquisition pathways are known (16), therapeutic strategies designed to iron-limit pathogens have focused on blocking ferric iron [Fe(III)] acquisition

because it is commonly assumed to be the dominant physiologically relevant form. For example, Fe(III) chelation by the host immune protein lactoferrin and its analog, conalbumin, has been shown to dramatically reduce biofilm formation under oxic conditions (11–13). EDTA and FDA-approved iron chelation compounds are similarly effective at mitigating biofilm growth while concomitantly increasing the efficacy of conventional antibiotics (13, 15). In addition, the transition metal gallium (also FDA approved) has been shown to disrupt biofilm formation by virtue of its chemical similarity to iron, disrupting Fe(III) uptake and interfering with Fe signaling (17, 18).

Despite the success of these Fe sequestration strategies in laboratory models, for many of them to be effective *in vivo*, iron would need to exist in the ferric form. However, in late-stage disease, oxygen tension is reduced in the lung (5) and neutrophil-mediated superoxides are generated that can reduce organically complexed Fe(III) to Fe(II) (19, 20). In addition, dense, biofilm-like microcolonies form within sputum that can create hypoxic microenvironments that can maintain iron in its reduced state (21). Furthermore, *P. aeruginosa* produces a variety of redox-active metabolites in CF sputum (e.g., phenazines) (22) that can reduce Fe(III) to Fe(II), even when iron is bound to host chelation proteins (23). While the CF lung environment is likely to be chemically heterogeneous, with oxic, hypoxic, and anoxic zones, the potential for locally anoxic and reducing conditions caused us to suspect that Fe(II) might be abundant in the airways. This report describes how we tested this hypothesis and explored its consequences for the development of iron-specific therapeutic strategies.

RESULTS

Total and ferrous iron concentrations increase within the lung environment as infections progress. Although the total concentration of iron has been measured in the airways (24, 25) and is known to accumulate in the lavage and explanted lungs of CF patients (26), its oxidation state has not been defined. We therefore set out to measure Fe(II) abundance in CF sputum at different stages of disease progression. Accurately measuring the iron oxidation state is complicated by the rapid oxidation of Fe(II) to Fe(III) once expectorated sputum is exposed to ambient oxygen. With this in mind, we designed a sputum collection and processing approach to better preserve and measure iron in its *in vivo* oxidation state. Twenty-four pediatric patients from across the spectrum of disease severity provided 115 sputum samples that were rapidly flash frozen upon expectoration. Samples were then moved to an anaerobic chamber to impede oxidation and mechanically homogenized by syringe, and ratios of free Fe(II)/Fe(III) concentrations were then determined using the ferrozine assay. As controls, total iron levels were also assayed using inductively coupled plasma mass spectrometry (ICP-MS) and untreated samples stored under argon were compared to flash-frozen samples to test whether the iron oxidation state was faithfully preserved during cryostorage (see Fig. S1 in the supplemental material).

Due to the temporal variability of sputum iron concentrations and differences in the number of samples that we were able to collect for a given patient (see Table S1 in the supplemental material), we grouped data over the entire period of the study and treated each patient's iron measurements as an average, rather than as independent observations, in order to test for correlations

TABLE 1 Summary of average Fe concentrations grouped by disease severity^a

Disease severity	FEV1%	<i>n</i>	Total Fe (μ M)	Fe(II) (μ M)	Fe(II) %
Normal to mild	>70	7	18 \pm 14	7 \pm 8	41 \pm 28
Moderate	40–69	12	48 \pm 38	28 \pm 27	52 \pm 10
Severe	<40	5	62 \pm 20	39 \pm 22	56 \pm 15

^a Reported values are mean concentrations \pm 1 standard deviation of iron detected in sputum samples collected over the study period. Values are conservative estimates based on ferrozine and ICP-MS measurements (see Fig. S1 in the supplemental material).

with patient disease state (Table 1). Using this data set, clustered by patient ($n = 24$), Spearman rank analysis revealed a significant negative correlation ($\rho = -0.48$, $P = 0.018$) between total iron and declining lung function (measured by forced expiratory volume [FEV1%]) (Fig. 1A; Table 2). Elevated iron levels (62 \pm 20 μ M for severely infected patients) (Table 1) were consistent with previous studies that quantified total iron levels and iron-related proteins in the CF airways (24–26). Consistent with our hypothesis, a considerable amount of this iron was found in its ferrous form, as sputum from severely infected patients had 39 \pm 22 μ M Fe(II). Here, we found a highly significant negative correlation between absolute Fe(II) concentrations and disease status ($\rho = -0.56$, $P = 0.004$) (Fig. 1B), though a similar relationship was not found for Fe(III) ($\rho = -0.28$, $P = 0.179$) (Fig. 1C). The percentage of the total iron pool that was present as Fe(II) was also higher (though not significantly: $\rho = -0.36$, $P = 0.083$) in patients with advanced disease states; in patients with severe lung obstruction (FEV1%, <40), Fe(II) constituted 56% \pm 15% of the total iron pool (Fig. 1D). These data reveal that the chemical environment of the lung is dynamic and evolves with respect to its iron redox chemistry as CF disease progresses.

Increased Fe(II) correlates with elevated phenazine concentrations. The alteration of total iron concentrations and the rise in Fe(II) over time likely result from multiple inputs by both host and pathogen (24, 26). For example, iron levels are known to increase due to inflammation (27); loss of intracellular iron by Δ F508 epithelial cells (28); altered production of the iron-related proteins heme, ferritin, and transferrin (26); and their proteolysis (29). In addition, redox-active phenazine metabolites produced by *P. aeruginosa* are abundant in CF sputum (22), some of which can readily reduce Fe(III) to Fe(II) (30). Iron reduction by phenazines has been demonstrated to circumvent iron chelation *in vitro*, promoting the formation of biofilms (31). Based on our recent demonstration of a strong correlation between sputum phenazine levels and pulmonary decline (22), we used high-pressure liquid chromatography (HPLC) to assess whether elevated levels of two phenazines, pyocyanin (PYO) and phenazine-1-carboxylic acid (PCA), also correlated with high Fe(II) concentrations. Consistent with our previous findings from an independent adult patient cohort, the majority of sputum samples tested had detectable phenazine concentrations (76 of 97 samples tested contained >10 μ M total phenazine; see Table S1 in the supplemental material). In sputum samples with low concentrations of phenazines, the percentage of the total iron pool that was Fe(II) ranged anywhere from 0 to 100%, revealing that phenazines are not required for the presence of ferrous iron (Fig. 2A). Yet, phenazines may facilitate Fe(III) reduction *in vivo*, as evidenced by the generally high percentage of Fe(II) once phenazine levels

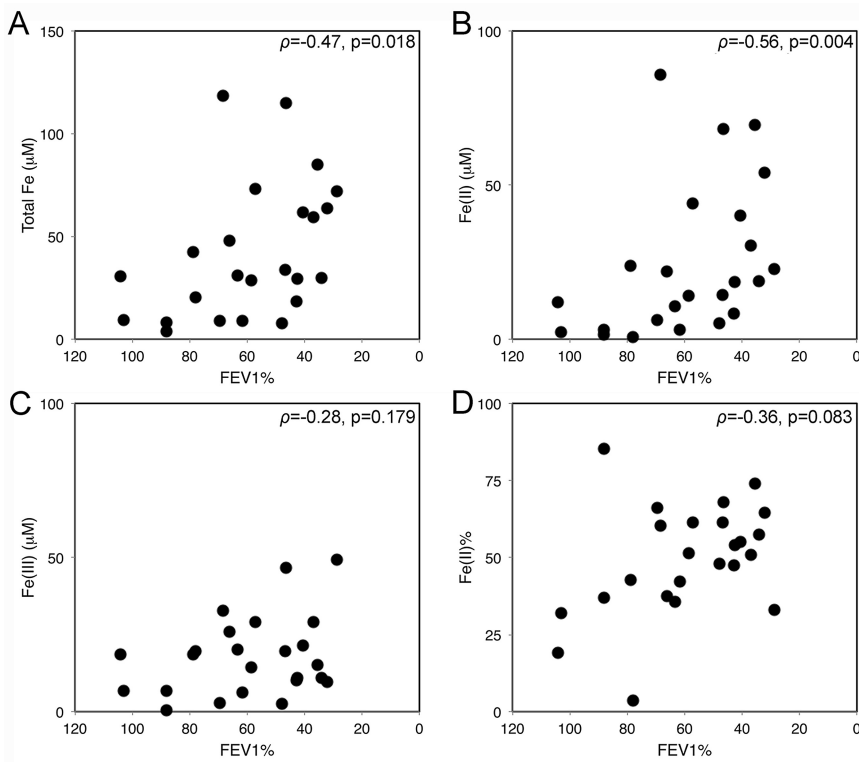


FIG 1 Direct detection of iron abundance and oxidation state within CF sputum. Total iron [Fe(III) plus Fe(II)] (A), Fe(II) (B), and Fe(II) % (D) all increase as pulmonary function (FEV1%) declines. There is no significant increase in Fe(III) (C). Each point represents the average of measurements on multiple sputum samples from a single CF patient.

rise above $\sim 50 \mu\text{M}$ in expectorated sputum. Treating each sputum sample independently, we found a strong trend between PCA abundance and Fe(II) % ($\rho = 0.185$, $P = 0.069$), yet no correlation between PYO abundance and Fe(II) % ($\rho = 0.042$, $P = 0.67$) (Fig. 2B and C). This may reflect that PCA can reduce Fe(III) much faster than can PYO under anoxic conditions (30). It remains to be determined whether the PCA/Fe(II) % trend would pass our test of statistical significance ($P < 0.05$) with additional sampling. We treated samples independently in this analysis in order to compare phenazine concentrations and Fe(II) % within a particular environment; given the variability of sputum chemistry over time (and likely also space) for individual patients (see Fig. S2 and Table S1), averaging and comparing these values per patient would not have been meaningful.

Fe(II)-responsive genes and multiple iron uptake pathways are expressed by *P. aeruginosa* within CF sputum. Given the abundance of Fe(II) *in vitro*, we predicted that extracellular Fe(II) would be bioavailable to *P. aeruginosa* within the airways. To test

TABLE 2 Summary of statistical relationships between iron concentrations and disease severity (FEV1%)

	<i>n</i>	Spearman rank coefficient	Sig. (two-tailed)
Total iron	24	-0.48	0.018
Fe(III)	24	-0.21	0.316
Fe(II)	24	-0.56	0.004
Fe(II) %	24	-0.36	0.083

this, we used a quantitative real-time PCR (qRT-PCR) approach to measure the expression patterns of two Fe(II)-responsive genes within expectorated sputum relative to their expression patterns under controlled conditions. The *P. aeruginosa* genes *bqsR* and *bqsS* encode a putative response regulator and sensor kinase, respectively, of a two-component system that was previously shown to be specifically upregulated in response to extracellular Fe(II) (32). We mined the NCBI Gene Expression Omnibus (GEO) database (<http://www.ncbi.nlm.nih.gov/geo/>) for microarray data generated for *P. aeruginosa* grown under conditions relevant to the CF lung environment. These data sets revealed no differential expression of *bqsRS* in response to multiple environmental stimuli, including low oxygen, pH, phosphate starvation, oxidative stress, biofilm formation, and various antibiotic treatments (see Table S2 in the supplemental material). Thus, *bqsRS* expression levels (relative to the constitutively expressed gene *oprI*) serve as a reliable proxy for the bioavailability of Fe(II) in the lung.

As previously observed, anaerobically grown laboratory cultures of *P. aeruginosa* upregulated *bqsS* (>90 -fold) in response to $50 \mu\text{M}$ Fe(II) relative to no treatment or treatment with $50 \mu\text{M}$ Fe(III) (Fig. 3A). Likewise, *bqsR* was highly expressed (>70 -fold) in response to Fe(II) relative to other treatments. We then utilized these gene expression patterns in controlled cultures to gain insight into the iron oxidation state perceived by *P. aeruginosa* in sputum. Consistent with our direct iron analyses, when *bqsR* and *bqsS* transcripts from sputum samples were quantified, expression was detected within the majority of patients (Fig. 3A). Transcriptional activity varied between sputum samples; however, 8 of 16 patients harbored relative *bqsS* expression patterns comparable to those for Fe(II)-treated laboratory cultures. *bqsR* transcripts were also detected in the majority of sputum samples that we tested, and many had relative expression levels comparable to those for Fe(II)-treated planktonic cultures. Unfortunately, technical limitations prevented us from measuring gene expression and iron content in the same sputum sample (see Materials and Methods). Despite potential differences in degradation rates for each transcript (see Fig. S3 in the supplemental material), given the high direct measurements of Fe(II) in sputum, previous microarray data, and our control experiments showing specific upregulation of *bqsRS* in response to Fe(II), we favor the interpretation that the iron pool within the CF airways is of a mixed oxidation state and that the infected lung environment frequently includes a ferrous portion that is sensed by *P. aeruginosa*.

In a majority (75%) of patients, the prevalences of *bqsR* and *bqsS* gene expression were comparable within patients (see Fig. S4 in the supplemental material); however, there was not a significant correlation between *bqsRS* transcriptional levels and disease sever-

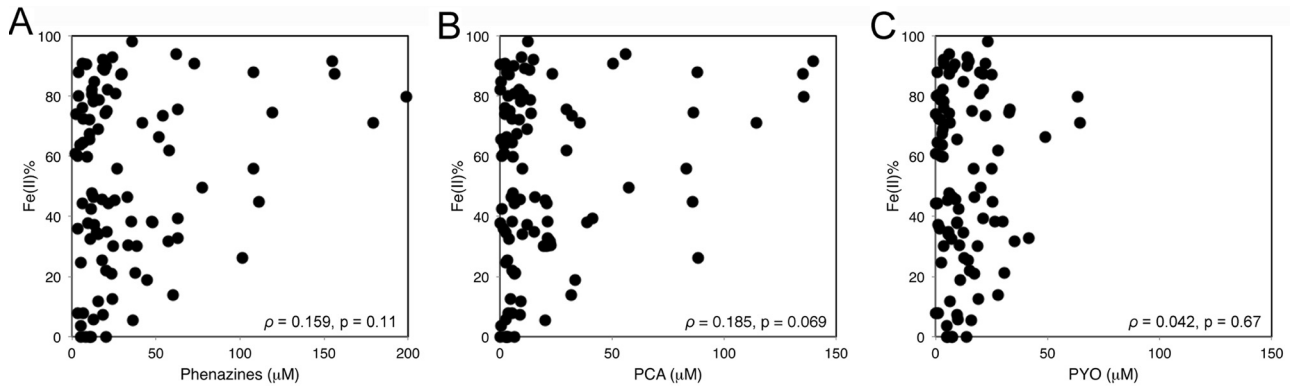


FIG 2 Fe(II) percentage of the total iron pool relative to sputum phenazine content. Fe(II) dominates the iron pool at high concentrations of total phenazines (PYO plus PCA) (A) and phenazine-1-carboxylic acid (PCA) (B) but not pyocyanin (PYO) (C). These data likely reflect the higher reactivity of PCA with Fe(III) under anoxic conditions (30).

ity (*bqsR*, $\rho = 0.13$; *bqsS*, $\rho = 0.01$) (see Fig. S5). This is not surprising because the relative expression of each gene was previously shown to be upregulated in response to Fe(II) levels greater than 10 μM . In our patient cohort, on average, Fe(II) was frequently detected at levels above 10 μM , even in patients in the early stages of disease. Thus, one would expect expression of these Fe(II)-sensitive genes across the spectrum of disease severity in response to the mixed-oxidation state of the iron pool.

A mixed-oxidation state of lung iron is further supported by our detection of *P. aeruginosa* gene transcripts encoding diverse Fe(II)- and Fe(III)-specific uptake proteins (Fig. 3B). We targeted *feoA/B* (encoding a ferrous iron transporter), *fptA* (ferrityochelin receptor), *pvdA* (pyoverdine biosynthetic protein), and *hasAp* (heme uptake protein) and quantified their relative expression levels in each sputum sample relative to *oprI*. Transcripts for each gene were detected in the majority of sputum samples analyzed (*feoA*, 15/16 patients; *feoB*, 11/16; *fptA*, 13/16; *pvdA*, 11/16; *hasAp*,

14/16), though relative expression levels of each gene varied over 5 orders of magnitude between patients. Uptake systems for the two iron oxidation states were simultaneously expressed within several individual patients (see Fig. S4 in the supplemental material), consistent with a recent study that investigated the expression of these genes in an independent patient cohort (33). Yet, the expression of Fe(II) uptake pathways did not correlate with the suppression of uptake pathways specific for Fe(III), or vice versa. Furthermore, because the regulation of these iron uptake pathways is complex (34, 35) and some (*pvdA*, *fptA*, and *hasAp*) appear to be independent from the oxidation state under anoxic conditions (Fig. 3B), these expression patterns alone are not predictive of the iron oxidation state *in vivo*. Rather, the expression of multiple iron uptake systems is supportive of our interpretation that *P. aeruginosa* utilizes a mixed-oxidation pool of iron within the CF sputum environment.

Interfering with bioavailable Fe(II) limits biofilm formation

under anoxic conditions. Given that the CF sputum environment contains a mixture of Fe(III) and Fe(II), what implications does this have for treating biofilm infections? Might abundant Fe(II) levels in infected environments compromise the success of Fe(III)-specific chelation therapies targeting *P. aeruginosa*? This was first suggested in a recent study that tested the efficacy of several iron-binding compounds in the disruption of *P. aeruginosa* biofilm growth under both oxic and hypoxic conditions (14). While biofilm formation was prevented under most conditions tested, the specific oxidation state of iron was unknown. Motivated by these experiments, we utilized a high-throughput biofilm assay to measure biofilm formation in the presence of Fe(III) and Fe(II) with or without oxidation-state-specific iron chelators. First, we tested whether ferrozine, a Fe(II)-specific chelator, could act synergistically with conalbumin, a Fe(III)-specific chelator, to prevent biofilm de-

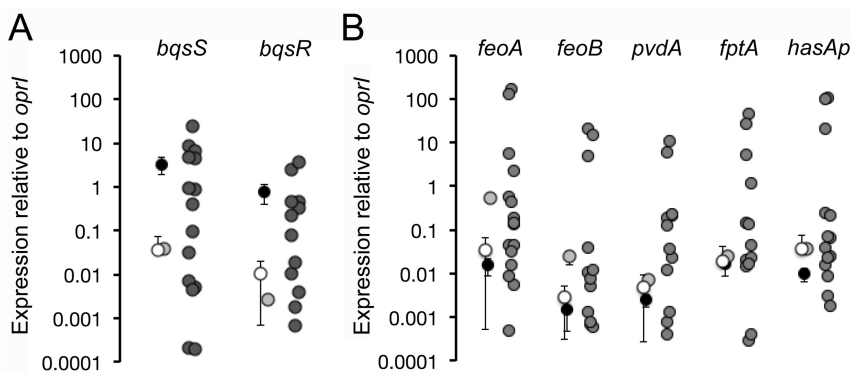


FIG 3 (A) Fe(II)-relevant gene expression in CF sputum. *bqsS* is upregulated in planktonic cultures of *P. aeruginosa* in response to 50 μM Fe(II) (black) relative to 50 μM Fe(III) (white) or no treatment (light gray). A similar result is seen with *bqsR*. Points represent average C_T values from three independent experiments; bars represent the standard deviations. By comparison, expression levels of these Fe(II)-sensitive genes in CF sputum (dark gray) vary over 5 orders of magnitude. Points represent relative gene expression calculated from C_T values from triplicate measurements of an individual sputum sample. Transcriptional activity is shown relative to the endogenous housekeeping gene *oprI*. (B) Expression of diverse iron uptake pathways within CF sputum. *feoA* and *feoB* encode proteins that transport Fe(II), while *fptA*, *pvdA*, and *hasAp* encode proteins that are involved in Fe(III) acquisition. Expression levels are shown compared to those in laboratory cultures treated with Fe(II), Fe(III), and no iron as described above.

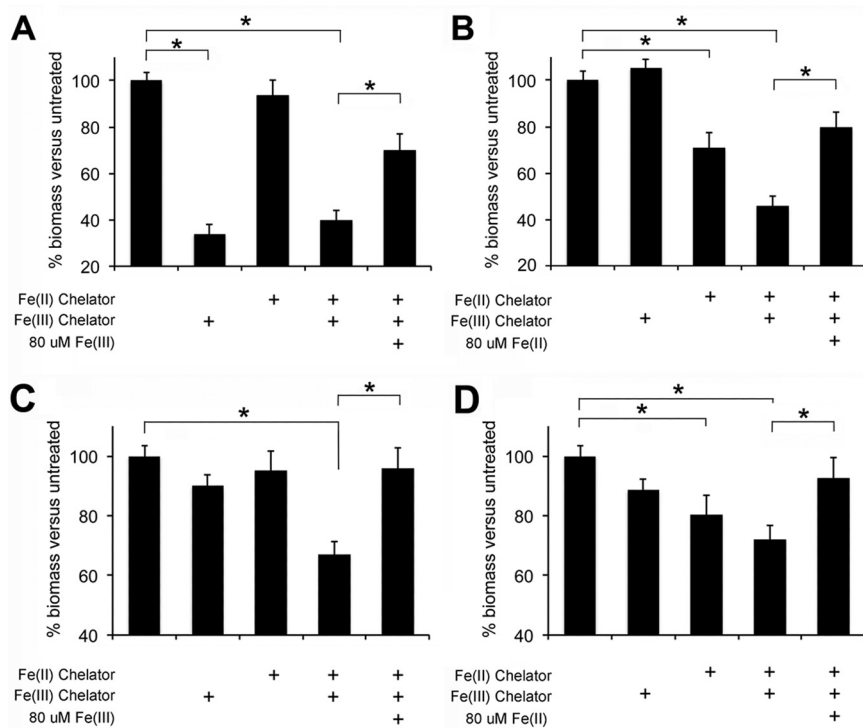


FIG 4 (A and B) Biofilm growth prevention under aerobic conditions [$\sim 98\%$ Fe(III)] (A) and anaerobic conditions [$\sim 10\ \mu\text{M}$ Fe(II) and $10\ \mu\text{M}$ Fe(III)] (B) by conalbumin [a Fe(III) chelator] and ferrozine [a Fe(II) chelator]. (C and D) Biofilm dissolution under aerobic (C) and anaerobic (D) conditions by conalbumin and ferrozine. In all cases, chelator effects are mitigated by the addition of Fe in excess of the chelation capacity [$80\ \mu\text{M}$ Fe(III) under oxic conditions; Fe(II) under anoxia]. Asterisks represent significance versus untreated controls. Error bars represent standard errors of the means ($n = 12$).

velopment. Consistent with previous studies (10, 14), $100\ \mu\text{M}$ conalbumin prevented biofilm formation by 66% ($P < 0.001$) under aerobic conditions where all iron ($20\ \mu\text{M}$) was Fe(III) (Fig. 4A). In contrast, $200\ \mu\text{M}$ ferrozine [Fe(II) specific] had no significant effect, nor did the combination of ferrozine and conalbumin treatments relative to conalbumin alone. Conversely, under hypoxic conditions designed to mimic airway microenvironments during late-stage infection, conalbumin was ineffective in preventing biofilm accumulation when $\sim 10\ \mu\text{M}$ Fe(II) and $10\ \mu\text{M}$ Fe(III) were present (Fig. 4B). Here, $200\ \mu\text{M}$ ferrozine significantly reduced biofilm accumulation by 29% ($P = 0.012$), and more notably, the combination of $100\ \mu\text{M}$ conalbumin and $200\ \mu\text{M}$ ferrozine reduced biofilm accumulation by 54% ($P < 0.001$), suggesting that targeting both oxidation states of iron *in vivo* might be more effective than targeting Fe(III) alone in the prevention of biofilm growth. Under both oxic and anoxic conditions, the addition of $80\ \mu\text{M}$ iron (resulting in $100\ \mu\text{M}$ total) in excess of the chelation capacity (conalbumin binds iron in a 2:1 ratio; ferrozine binds in a 3:1 ratio) restored biofilm accumulation, demonstrating that the chelator effect is likely due to iron sequestration rather than nonspecific interactions.

Combined Fe(III)/Fe(II) chelation promotes biofilm dissolution under anoxic conditions. In addition to signaling biofilm formation, iron is essential for maintenance of established biofilm communities (12). We therefore performed similar mixed Fe(II)/Fe(III) chelation experiments targeting mature biofilms to test the ability of conalbumin and ferrozine to dissolve bacterial biofilms

that have already formed. Under aerobic conditions (Fig. 4C), the application of either conalbumin ($100\ \mu\text{M}$) or ferrozine ($200\ \mu\text{M}$) in molar excess of iron in the growth medium showed minimal effect on biofilm dissolution. We hypothesized that this is due to the presence of both Fe(III) and Fe(II) in the hypoxic interior of aerobically grown biofilms. Consistent with this prediction, the combined application of both Fe(III) and Fe(II) chelators revealed a synergistic dissolution effect, resulting in a 33% reduction ($P = 0.01$) of biomass in the presence of oxygen. The addition of excess iron restored the untreated phenotype, corroborating an iron-specific mechanism of chelator-induced dispersal. Similarly, $100\ \mu\text{M}$ conalbumin did not significantly reduce established biofilm growth under anoxic conditions (Fig. 4D). However, significant biofilm dissolution (20%; $P < 0.001$) was observed in the presence of $200\ \mu\text{M}$ ferrozine, indicating that *P. aeruginosa* biofilms can reduce Fe(III) present in the growth medium. More notably, when applied together with conalbumin, ferrozine promoted further dissolution of established biofilms at levels comparable to those under oxic conditions (28%; $P < 0.001$), supporting the case for targeting both Fe(III) and Fe(II) to disrupt *P. aeruginosa* biofilm growth in the CF

airways. In contrast to previous experiments performed under oxic conditions (15), dual exposure to iron chelators and tobramycin did not exhibit a synergistic effect under anoxic conditions (see Fig. S6 in the supplemental material).

DISCUSSION

The dependence of bacteria on iron acquisition for biofilm formation has led to its identification as a novel therapeutic to eliminate *P. aeruginosa* infections within the host, particularly for CF patients. However, as recently pointed out (14), there is a gap in our understanding of the *in vivo* chemical environments under which these treatments might be administered, which might significantly impact their efficacy. Therefore, the goal of this study was to gain a better understanding of iron chemistry within the lungs of CF patients and determine how the *in vivo* environment might impact the success of iron-specific therapies. Using a unique sputum sampling and processing approach, we determined that Fe(II) comprises a significant component of the airway iron pool. We then used quantitative PCR analysis to confirm that elevated Fe(II) levels were freely available for the infecting bacterial population. Finally, we found that interfering with a mixed-oxidation state iron pool can limit biofilm formation and promote biofilm dissolution.

On average, each patient had $42\ \mu\text{M}$ total iron (range, 3.7 to $118\ \mu\text{M}$) present in his or her sputum, which was highly dependent on the stage of disease (Table 1). These values are consistent with a range of studies reporting elevated iron levels in CF sputum

and bronchoalveolar lavage. For example, Reid et al. (24) and Stites et al. (25) both determined that free iron and ferritin negatively correlate with pulmonary function in clinically stable patients. Similarly, Gifford et al. (36) reported elevated sputum iron levels in CF patients and yet found no association between Fe concentrations and lung function in a mixed cohort of stable and acutely ill subjects. Although we found a correlation between total iron and FEV1%, this derives from Fe(II), not Fe(III).

Despite the wealth of data on total iron abundance in the CF airways, to our knowledge, direct measurements of the iron oxidation state have not been made previously. It has been suggested that the abnormal acidification of the CF airways (37) and the development of anoxic microenvironments in sputum (5) favor the maintenance of a bioavailable Fe(II) pool; however, the rapid oxidation of Fe(II) upon exposure to ambient oxygen has made this hypothesis difficult to test. Our novel sputum sampling and processing approach was able to circumvent this problem, and it is clear that the percentage of the total iron pool that is Fe(II) is substantial, particularly in severely ill patients ($56\% \pm 15\%$). While our data support the aforementioned low-pH/anoxic hypothesis for iron reduction, it also seems likely that the accumulation of redox-active phenazines over time contributes to a highly reducing airway environment that can foster Fe(III) reduction to Fe(II) (Fig. 2). Based on our laboratory observations that PCA-mediated iron reduction facilitates biofilm formation (31) and a correlation between concentrations of these metabolites and pulmonary decline (22), we suggest that PCA-mediated iron reduction facilitates disease progression in CF patients.

We recognized a need to distinguish between detectable Fe(II) levels (by ferrozine) and those which are bioavailable for *P. aeruginosa in vivo*. For example, the total soluble iron measurements determined by our analytical approach likely include a portion that is bound to complexing ions or ligands that keep it in solution, though it might not be bioavailable. Moreover, ferrozine does not react with heme-associated iron (38), which may represent an important iron source within the lung (4, 9). However, because we could detect a high level of Fe(II)-specific *bqsRS* expression (relative to *oprI*) in a majority of sputum samples (compared to tightly controlled laboratory cultures), we can conclude that some portion of the Fe(II) pool is sensed by *P. aeruginosa in vivo*. Consistent with previous *in vitro* studies demonstrating that *P. aeruginosa* grown in the presence of a sputum-derived medium expresses diverse iron-acquisition-related genes (4), we found genes involved in pyoverdine, pyochelin, and heme uptake to be expressed in sputum, similar to another recent study (33). In addition, direct measurements have also confirmed the presence of the siderophore pyoverdine in a high percentage of CF patients, but not all, indicating that *P. aeruginosa* uses multiple mechanisms for iron acquisition within the host (52). Intriguingly, our data indicate that multiple iron uptake pathways are expressed simultaneously in several patients and that several (e.g., *pvdA*, *fptA*, and *hasAp*) do not appear to be iron responsive under anoxic conditions (Fig. 3B). This apparent loss of Fur regulation may also reflect mutations that accrue as infections progress, as has been documented elsewhere (39). A more thorough understanding of iron-relevant gene regulation *in vivo* will be necessary to determine how expression patterns of iron uptake machinery may inform us about the chemical environment of the airways.

Because bioavailable iron serves as a signal for biofilm formation (12) and as an integral cation for biofilm stability (40), it is

thought to be required for both the establishment of *P. aeruginosa* biofilms and their chronicity in CF patients (14, 24). It has also been established that an optimal concentration of iron is required for the formation of *P. aeruginosa* biofilms (10, 41). In a biofilm mode of growth, it is generally accepted that bacterial cells are inherently more resistant to antibiotics and components of the host immune system, and only once they revert to their planktonic state are they readily cleared (42). In our studies, the mixed-oxidation approach (conalbumin/ferrozine) to iron chelation appears to be promising in preventing biofilm formation, and we predict that under oxic conditions, it will further sensitize biofilm cells to conventional antimicrobial treatments, as has previously been shown (13, 15). However, mixed Fe(III)/Fe(II) chelation may be most significant under anoxic conditions (thought to be prevalent throughout the CF airways [5]), by preventing or disrupting biofilms. While cells reverting to a planktonic lifestyle would likely remain tolerant to conventional antibiotics due to slow anaerobic growth and physiological changes (43), they would no longer be as protected from the host immune response, and possibly more readily cleared from the host environment. Testing this hypothesis in infection models is a logical next step. A challenge will be to ensure that these infection models accurately mimic the environment of the human host for chronic infections.

Collectively, these studies underscore the importance of a dialectic between laboratory and environmental studies of pathogens such as *P. aeruginosa*. To complement mechanistic studies at the bench, characterization of the microbial community *in vivo* must also include an analysis of the chemical conditions under which it lives. Such combined efforts will provide insight into how infected environments coevolve with the composition and activities of the constituent microbiome at different stages in disease progression. As suggested here, this integrated approach has the potential to inform effective design and application of novel therapeutic strategies for *P. aeruginosa* biofilm control.

MATERIALS AND METHODS

Study design and sample collection. Twenty-five participants (aged 7 to 20 years) and eight participants (aged 16 to 38 years) were recruited from Children's Hospital Los Angeles (CHLA) and the Academic Hospital UZ Brussel, respectively. Inclusion criteria were a positive diagnosis of CF, ability to expectorate sputum, and informed consent. Sputum was flash frozen in liquid nitrogen shortly after expectoration to minimize oxidation and/or mRNA degradation and stored at -80°C until processing. Disease severity was determined by FEV1% scores, and patients were clustered using published guidelines (44, 45). This study was approved by the ethical commissions of the California Institute of Technology, Children's Hospital Los Angeles, and the Academic Hospital UZ Brussel.

Sputum processing. Frozen samples were thawed in an anaerobic chamber. Sputum was disrupted using a 16-gauge needle and was homogenized by vortexing in an equal volume of anoxic 50 mM HEPES buffer. Sputum was centrifuged at $8,000 \times g$ for 10 min, and supernatants were filtered through 0.22- μm -pore-size columns for 20 min at $10,000 \times g$. Filtrates were analyzed for iron content. When sufficient sputum material was obtained, 200 μl of filtrate was stored at -80°C for inductively coupled plasma mass spectrometry (ICP-MS) analysis.

Iron quantification. Iron levels were quantified using the ferrozine assay (46). Briefly, 50 μl of sputum filtrate was carefully added (to avoid introducing bubbles) to 50 μl of 1 M HCl to quantify Fe(II). For total iron, 50 μl was treated with 50 μl of 10% hydroxylamine hydrochloride in 1 M HCl to reduce Fe(III) to Fe(II). Samples were added to 100 μl of ferrozine (0.1% [wt/vol] in 50% ammonium acetate) and incubated for 15 min, and absorbance was measured at 562 nm. Ferrous ammonium sulfate was

used as the iron standard. Samples were also analyzed by inductively coupled plasma mass spectrometry (ICP-MS). Briefly, 50 μ l of filtrate was digested in 100 μ l 8 N nitric acid and brought to a total of 1.5 ml in 5% nitric acid-indium standard. Samples were analyzed on an Agilent 7500cx analyzer equipped with a reaction cell, using He (2 ml/min) and H₂ (2.5 ml/min) as reaction gases. Fe concentrations were calculated using ⁵⁶Fe and ⁵⁷Fe signal intensities.

Sputum mRNA extraction and quantitative real-time PCR. Sputum samples were collected, frozen, and homogenized as described above. Under anoxic conditions, homogenate was added to 1 vol of 0.1-mm zirconia beads and 3 volumes of Trizol LS, and mRNA was extracted as described by Lim et al. (47). Purity and degradation were assessed using NanoDrop spectrophotometry, agarose gel electrophoresis, and an Agilent 2100 Bioanalyzer. cDNA was reverse transcribed from 1 μ g of total RNA with the first-strand cDNA synthesis kit (Amersham Biosciences) or iScript (Bio-Rad) according to the manufacturer's protocols. cDNA was then used as a template for quantitative PCR (RealTime 7500 PCR machine; Applied Biosystems) using SYBR green with the ROX detection system (Bio-Rad). Triplicate measurements were made on each sputum sample. As controls, anaerobically grown *P. aeruginosa* PA14 treated with 50 μ M Fe(II), 50 μ M Fe(III), or water (no iron) was assayed as previously described (32). For all primer sets (see Table S3 in the supplemental material), the following cycling parameters were used: 94°C for 3 min followed by 40 cycles of 94°C for 60 s, 55°C for 45 s, and 72°C for 60 s, followed by 72°C for 7 min. *oprI* and *clpX* were used to normalize levels of gene expression (48, 49) (see Fig. S3). Primer efficiencies were determined using iQ5 optical system software (Bio-Rad), and standard curves were constructed based on four different known quantities of genomic DNA of *P. aeruginosa* PAO1 (100 ng, 50 ng, 10 ng, and 5 ng) (see Table S3). The threshold cycle (C_T) values of each gene were used to calculate relative gene expression using the $2^{-\Delta\Delta CT}$ method (50). The mRNA extraction protocol precluded a concurrent Fe(II) measurement because the coloration of the Trizol reagent interferes with the ferrozine assay.

HPLC quantification of phenazines. Phenazine extraction and quantification were performed anaerobically as previously described (22). Ninety-seven out of 115 samples contained sufficient sputum material for phenazine analysis (3).

MBEC assay for biofilm prevention and dissolution. We used a high-throughput biofilm assay (MBEC physiology and genetics assay) consisting of a 96-well plate and 96-peg lid. Inoculum was prepared by diluting (30-fold) a 10⁷-cell/ml suspension of *P. aeruginosa* PA14 in Trypticase soy broth (TSB). One hundred fifty microliters was dispensed into each of the 60 inner wells, while 200 μ l of sterile TSB was placed in each perimeter well. For dissolution experiments, plates were incubated at 37°C for 24 h, and lids were transferred to a fresh 96-well TSB plate for 24 h at 37°C or to an anaerobic chamber for 24 h at 37°C in anaerobic TSB containing 50 mM KNO₃. Biofilms were exposed to 100 μ M conalbumin and/or 200 μ M ferrozine for 24 h (concentrations were selected such that they were in molar excess of medium iron concentrations). The dual chelator treatment was also complemented with 8 μ g/ml tobramycin or 80 μ M ferrous ammonium sulfate where indicated. After treatment, lids were rinsed once in 50 mM HEPES, air dried for 10 min, and quantified by crystal violet staining (51). For biofilm growth prevention assays, both aerobic and anaerobic inocula were amended with 100 μ M conalbumin and/or 200 μ M ferrozine. For aerobic experiments, biofilms developed for 24 h. For anaerobic growth, the medium was replaced every 24 h by transferring the lid to a sterile plate containing TSB with or without treatments, and biofilms were developed for 168 h.

Statistical analysis. Spearman rank analysis (ρ) was performed on iron and phenazine concentrations versus lung function (Fig. 1 and 2). Two-tailed Student *t* tests were used for pairwise comparisons between chelator treatments and controls (Fig. 4). In all cases, $P < 0.05$ was considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00557-13/-DCSupplemental>.

Text S1, DOCX file, 0 MB.
Figure S1, JPG file, 0.3 MB.
Figure S2, JPG file, 0.3 MB.
Figure S3, JPG file, 0.5 MB.
Figure S4, JPG file, 1.2 MB.
Figure S5, JPG file, 0.1 MB.
Figure S6, JPG file, 0.1 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.
Table S3, DOCX file, 0.1 MB.

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