

# Within-Host Evolution of *Pseudomonas aeruginosa* Reveals Adaptation toward Iron Acquisition from Hemoglobin

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**ABSTRACT** *Pseudomonas aeruginosa* airway infections are a major cause of mortality and morbidity of cystic fibrosis (CF) patients. In order to persist, *P. aeruginosa* depends on acquiring iron from its host, and multiple different iron acquisition systems may be active during infection. This includes the pyoverdine siderophore and the *Pseudomonas* heme utilization (*phu*) system. While the regulation and mechanisms of several iron-scavenging systems are well described, it is not clear whether such systems are targets for selection during adaptation of *P. aeruginosa* to the host environment. Here we investigated the within-host evolution of the transmissible *P. aeruginosa* DK2 lineage. We found positive selection for promoter mutations leading to increased expression of the *phu* system. By mimicking conditions of the CF airways *in vitro*, we experimentally demonstrate that increased expression of *phuR* confers a growth advantage in the presence of hemoglobin, thus suggesting that *P. aeruginosa* evolves toward iron acquisition from hemoglobin. To rule out that this adaptive trait is specific to the DK2 lineage, we inspected the genomes of additional *P. aeruginosa* lineages isolated from CF airways and found similar adaptive evolution in two distinct lineages (DK1 and PA clone C). Furthermore, in all three lineages, *phuR* promoter mutations coincided with the loss of pyoverdine production, suggesting that within-host adaptation toward heme utilization is triggered by the loss of pyoverdine production. Targeting heme utilization might therefore be a promising strategy for the treatment of *P. aeruginosa* infections in CF patients.

**IMPORTANCE** Most bacterial pathogens depend on scavenging iron within their hosts, which makes the battle for iron between pathogens and hosts a hallmark of infection. Accordingly, the ability of the opportunistic pathogen *Pseudomonas aeruginosa* to cause chronic infections in cystic fibrosis (CF) patients also depends on iron-scavenging systems. While the regulation and mechanisms of several such iron-scavenging systems have been well described, not much is known about how the within-host selection pressures act on the pathogens' ability to acquire iron. Here, we investigated the within-host evolution of *P. aeruginosa*, and we found evidence that *P. aeruginosa* during long-term infections evolves toward iron acquisition from hemoglobin. This adaptive strategy might be due to a selective loss of other iron-scavenging mechanisms and/or an increase in the availability of hemoglobin at the site of infection. This information is relevant to the design of novel CF therapeutics and the development of models of chronic CF infections.

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Iron is an essential component for virtually all forms of life. This includes bacterial pathogens that depend on acquiring iron from their hosts in order to replicate and cause disease (1). A general defensive mechanism of the host is therefore to withhold iron from invading bacteria to prevent their growth, but this defense is countered by bacterial pathogens since they possess specific systems to scavenge iron from their hosts. While the regulation and mechanisms of several of such iron-scavenging systems are well described (1), not much is known about how the within-host selection pressures act on the pathogens' ability to acquire iron. This is especially relevant in relation to long-term chronic infections in which invading bacteria acquire adaptive mutations in response to the selective pressures encountered in the host.

The opportunistic pathogen *Pseudomonas aeruginosa* is a common environmental inhabitant which is capable of causing long-

term chronic infections in the airways of patients with cystic fibrosis (CF), and *P. aeruginosa* infections are directly associated with the morbidity and mortality of CF patients. Chronic infections in CF patients provide an opportunity for long-term monitoring of the battle between the infecting bacteria and the host (2–6) and thus offer an opportunity for observing evolutionary adaptation of *P. aeruginosa* to the human host environment.

Most iron in the human body is bound in hemoglobin, which is an oxygen transport protein in red blood cells (1). If not bound by essential proteins, such as hemoglobin, iron is withheld and stored by binding to proteins like transferrin, lactoferrin, and ferritin. *P. aeruginosa* is known to scavenge iron from the human host by both siderophore-based systems and heme acquisition systems (7).

Siderophores are low-molecular-weight molecules secreted by

bacteria. The strong association of iron to siderophores enables them to remove iron from the human iron storage proteins, whereupon the siderophore-iron complex can be taken up by cognate receptors at the bacterial surface. The major siderophores secreted by *P. aeruginosa* are pyoverdine and pyochelin (7), and iron-loaded pyoverdine and pyochelin are taken up by the outer membrane receptors FpvA and FptA, respectively (8–10).

Alternatively, iron contained in the heme group of hemoglobin can be taken up by either of two heme uptake systems in *P. aeruginosa*. The two systems are the *Pseudomonas* heme utilization (*phu*) system and the heme assimilation system (*has*) (11). The two systems are different in the sense that the *phu* system is dependent on the direct uptake of heme by the outer membrane receptor PhuR, whereas the *has* system encodes a secreted hemophore, HasA, that returns heme to an outer membrane receptor, HasR.

Furthermore, *P. aeruginosa* can take up ferrous iron through the *feo* system (12) or ferric citrate through the *fec* system (13).

It is not clear in which way the different iron uptake systems in *P. aeruginosa* play a role for survival in the lungs of CF patients. Detection of pyoverdine in the sputa of some CF patients has led to the suggestion that pyoverdine plays a key role in the infection process (14, 15). On the other hand, measurements of the transcription levels of iron uptake systems in sputum samples have suggested that multiple systems are active and that siderophore-mediated uptake may not be the dominant iron acquisition mechanism in all patients (16, 17).

In an effort to understand the genetic adaptation of *P. aeruginosa* to the CF airways, we recently mapped all mutational changes in the *P. aeruginosa* DK2 lineage as it spread among 21 Danish CF patients by interpatient transmission (2). The study showed that the selective forces driving the evolution of *P. aeruginosa* in the CF airways could be inferred from convergent evolution of DK2 sublineages evolving in parallel in separate hosts. Here we further analyzed the genomic data, and we provide evidence that within-host evolution of *P. aeruginosa* is characterized by adaptation toward iron acquisition from hemoglobin.

## RESULTS AND DISCUSSION

**Parallel evolution of mutations in the promoter regions of the *phu* system.** It is known that *P. aeruginosa* undergoes genetic adaptation to CF patients during long-term chronic infections, and several studies have sequenced the genomes of *P. aeruginosa* isolates sampled longitudinally from the airways of CF patients to map the mutations that accumulate during infection (2–6). In one such study, we mapped all the mutations that had occurred in the *P. aeruginosa* DK2 lineage during 36 years of infection (2). Whole-genome analysis of 55 DK2 isolates enabled a fine-grained reconstruction of the evolutionary relationship of the DK2 lineage, and the study identified several genes to be targeted by mutation to optimize pathogen fitness within the host environment (pathoadaptation). Nonetheless, only intragenic mutations (i.e., mutations within genes) were examined to identify such pathoadaptive patterns of mutation. Here, we therefore reanalyzed the data with respect to intergenic regions, since selection might also act on such sequences due to their role in regulation and transcription of neighboring genes.

The 6,402,658-bp genome of the *P. aeruginosa* DK2 strain contains 4,883 intergenic regions with an average size of 146 bp, and the intergenic regions constitute a total of 714,368 bp. Marvig et al. (2) found 1,365 intergenic mutations, meaning that one would

expect an average-length intergenic region to be hit by 0.3 mutations (or 0.0019 mutation/bp). Searching for recurrent patterns of mutation of the same genetic loci makes it possible to identify positive selection for mutations affecting genes important for host adaptation (2, 18, 19). We therefore focused on the intergenic regions with the highest densities of mutations and interestingly found the 180-bp intergenic region containing the promoters of the *phu* system to be the most frequently mutated, with a total of 13 mutations (0.072 mutation/bp) (Fig. 1). This number of mutations is 38-fold higher than what would be expected by chance and represents a significant increase in mutation density [ $P(X \geq 13) \sim \text{pois}(X; 0.342) = 2.22e-16$ , where  $P(X \geq 13)$  is the probability of observing  $\geq 13$  mutations given a Poisson distribution with a mean of 0.342 mutations (0.0019 mutation/bp \* 180 bp)].

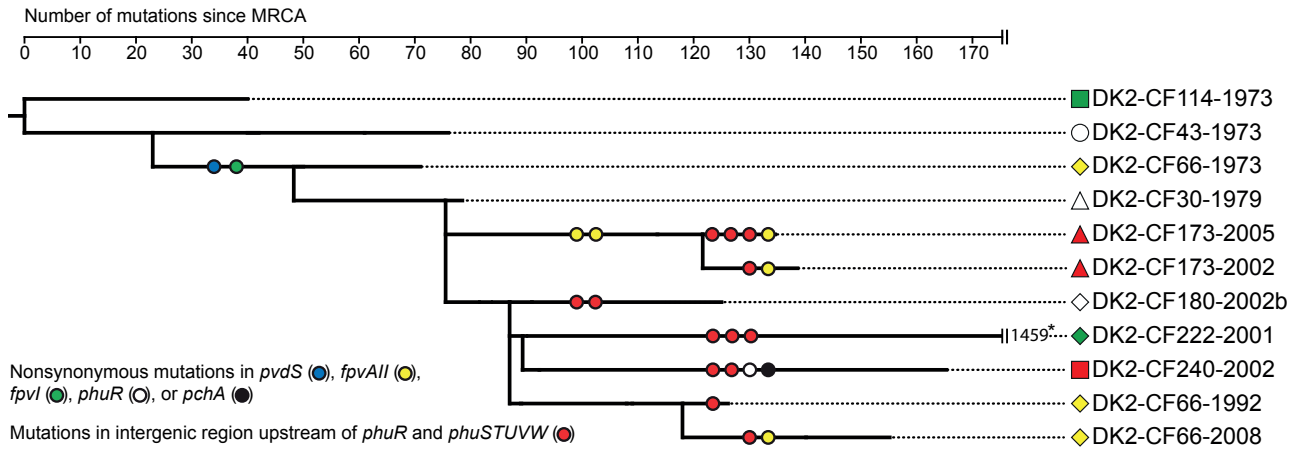
All of the 13 mutations are located within a narrow region from position -91 to -21 relative to the start codon of *phuR*, and eight of the mutations are within the annotated promoter regions of the *phu* system (Fig. 2). Furthermore, two positions (positions -35 and -57) were subject to convergent evolution, since they were independently mutated in parallel evolving DK2 sublineages.

**Correlation between promoter mutations and *phu* transcription in isolates DK2-CF173-2005 and DK2-CF66-2008.** Using Affymetrix GeneChips, we have previously measured the full transcriptomes of six of the 11 DK2 isolates listed in Fig. 1 (4), including four early DK2 isolates without *phu* promoter mutations and two isolates, DK2-CF173-2005 and DK2-CF66-2008, with *phu* promoter mutations. We hypothesized that the mutations, due to their location immediately upstream of *phuR* and *phuSTUVW*, could cause an effect on the transcription of the *phu* system. Accordingly, we found the transcription of the *phuRSTUVW* genes to be upregulated in both of the mutated isolates (DK2-CF173-2005 and DK2-CF66-2008) relative to that for their ancestors and a laboratory reference strain PAO1 (Fig. 3). Most highly upregulated was *phuR*, showing 116- and 25-fold upregulation, respectively, but also, the genes of the *phuSTUVW* operon were on average upregulated 8- and 4-fold, respectively.

The *phu* system is negatively regulated by the ferric uptake regulator (Fur) (11). As an alternative hypothesis, we therefore speculated that the increased transcription of the *phu* system in DK2-CF173-2005 and DK2-CF66-2008 might be due to a decreased level or activity of the Fur protein. Nonetheless, no mutations or changes in transcription of the *fur* gene were found (Table 1) (2).

Furthermore, in order to determine if iron acquisition systems in general were subject to evolutionary changes in transcription, we searched the transcriptomes for other iron acquisition systems to be differentially transcribed. This search revealed that the *feo* operon, encoding a ferrous iron uptake system (12), was upregulated in DK2-CF66-1973 and the four isolates sampled after 1973 (Table 1), indicating that several iron acquisition systems might play a role in adaptation of *P. aeruginosa* to the human host airways.

**Effect of intergenic mutations on activities of *phu* system promoters.** To further investigate the effect of the *phu* promoter mutations on the activity of the *phuR* promoter, we cloned the *phuR* promoter region from six of the mutated DK2 clones in front of a luciferase reporter (*luxCDABE*) and chromosomally integrated the transcriptional fusion into *P. aeruginosa* PAO1 at the *attB* site by use of the mini-CTX2-derived plasmid pHK-CTX-lux. The transcriptional fusions enabled us to compare *phuR::lux*



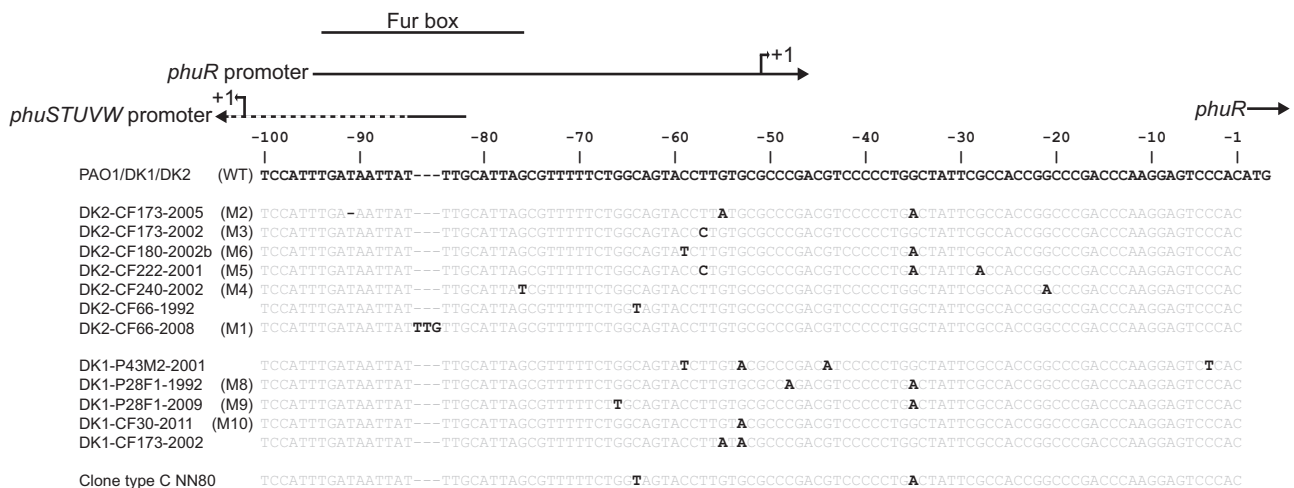
**FIG 1** Maximum-parsimony phylogenetic tree showing the genetic relationship of the 11 DK2 clones included in this study. The phylogenetic tree is a subset of a phylogenetic tree from the work of Marvig et al. (2), who recently reported the genome sequences of 55 DK2 isolates. The shown tree depicts the genetic relationship of the 11 DK2 isolates included in this study, and it represents a total of 1,827 mutations (1,486 SNPs and 311 insertion/deletions) identified from whole-genome sequencing. Lengths of branches are proportional to the numbers of mutations except in the case of the truncated branch leading to isolate DK2-CF222-2001. For this hypermutator isolate, the large number of mutations is indicated at the end of the truncated branch. We searched the genomes for nonsynonymous mutations within genes encoding components of the pyoverdine, pyochelin, *phu*, *has*, *feo*, and *fec* iron acquisition systems (7, 11–13), and circles on the evolutionary branches denote that the specified gene is mutated in the branch. Due to the large number of mutations in the branch leading to the hypermutable isolate DK2-CF222-2001, only *phuR* and *phuSTUVW* intergenic mutations are specified. \*, in addition to the three *phuR* and *phuSTUVW* intergenic mutations, this branch also contains nonsynonymous mutations in *pvdS*, *pvdI*, *fpvI*, the FpvAII gene, *fpvR*, *phuR*, *fptA*, *pchH*, *pchG*, *pchF*, *pchE*, and *pchD* (2).

expression from the mutated promoter regions (M1 to M6) (Fig. 2) relative to the expression from a construct with a wild type promoter region (WT) (Fig. 2). A construct without an inserted promoter region was used to correct for background expression from *lux* gene cassette integration.

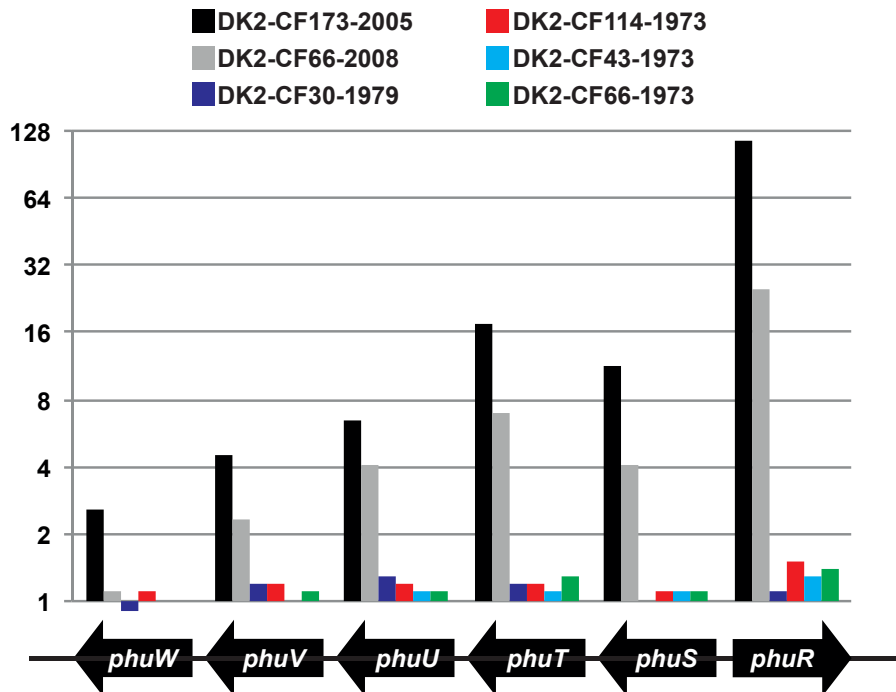
Measurements of *phuR::lux* expression at exponential growth (optical density at 600 nm [OD<sub>600</sub>] = 0.15) in Luria-Bertani (LB) medium revealed that all six mutant alleles (M1 to M6) caused a significant increase in promoter activity, with changes in expression from 5- to 112-fold (Table 2). The largest increases in expressions (93- and 112-fold) were observed for the alleles M1 and M2,

originating with clones DK2-CF66-2008 and DK2-CF173-2005, respectively. The M1 and M2 alleles contain a 3-bp insertion and a 1-bp deletion, respectively, in the repressor-binding site (Fur box) of the Fur regulator, known to control the expression of the *phuR* promoter (11). Since Fur mediates strong repression of *phuR* under iron-rich conditions (11), we find it likely that the indels in the M1- and M2-derived *phuR* promoters alleviate Fur repression (if there is any repression from Fur).

Using the same cloning strategy, we tested a *phuS::lux* reporter fusion to compare the expression from the mutated promoter region of DK2-CF173-2005 to the expression from a construct



**FIG 2** Overview of the intergenic region upstream of *phuR*. The alignment shows homologous sequences from different isolates with genetic variants highlighted in bold. Wild-type sequences of *P. aeruginosa* strains PAO1, DK1, DK2, and C are shown at the top of the alignment. Abbreviations of sequence alleles from different isolates are indicated in parentheses (WT and M1 to M10). Positions of promoters and a Fur box are indicated with black lines above the alignment (the *phuSTUVW* promoter is only partially shown). Positions are relative to the start codon of *phuR*.



**FIG 3** Relative transcriptional levels of genes encoding the *phu* system. The transcriptomes of six of the DK2 isolates included in this study have previously been measured at exponential growth phase in LB medium (4). The expression of the *phu* genes is shown for each of the six clinical isolates relative to that for laboratory reference strain PAO1. Values are averages for three replicates, and the values are normalized relative to the transcription of the respective gene in strain PAO1.

with a wild-type promoter region. Similar to the results for the *phuR* promoter, we observed that the mutations also resulted in a significant ( $P = 0.01$ ) increase in *phuS* promoter activity (Table 2), albeit the mutations had a larger effect on the activity of the *phuR* promoter.

***phuR* promoter mutations confer a growth advantage in the presence of hemoglobin.** The increased expression from the mutated *phu* promoters suggested that there has been positive selection in the CF airways toward iron acquisition from hemoglobin. To test this hypothesis, we replaced the wild-type *phu* promoters of isolate DK2-CF30-1979 with the mutated *phu* promoters of isolate DK2-CF173-2005 by allelic replacement and tested whether the constructed mutant strain, DK2-CF30-1979-M2, had a growth advantage relative to the isogenic wild-type strain, DK2-CF30-1979. We chose to test the consequence of the *phu* promoter mutations in the genetic background of isolate DK2-CF30-1979 because this isolate is an immediate ancestor of isolate DK2-CF173-2005 (4). For the growth experiment, we used FeCl<sub>3</sub>-free

ABTGC minimal medium (which contains glucose and Casamino Acids), supplemented with hemoglobin and apotransferrin.

Confirming our hypothesis, we found that the allelic replacement mutant DK2-CF30-1979-M2 grew significantly faster than its isogenic wild-type counterpart when hemoglobin was present as the sole iron source (Table 3), while no difference was observed for rich medium and medium supplemented with Fe<sup>3+</sup> as the sole iron source. We suggest that the growth advantage of the mutant is facilitated by an enhanced uptake of iron derived from hemoglobin.

**Adaptation toward heme utilization is a general adaptive mechanism.** Our results demonstrate parallel adaptation of the DK2 lineage toward hemoglobin utilization in five different CF patients. This indicates that similar selective conditions for heme utilization exist across different patients. Next, we speculated on whether the acquisition of *phu* promoter mutations is an adaptive mechanism specific to the DK2 lineage or if *phuR* promoter mutations constitute a general adaptive genetic mechanism of

**TABLE 1** Relative transcriptional levels of *fur* and genes encoding the *feo* iron acquisition pathway<sup>a</sup>

Gene	Relative transcription in strain:						
	PAO1	DK2-CF114-1973	DK2-CF43-1973	DK2-CF66-1973	DK2-CF30-1979	DK2-CF173-2005	DK2-CF66-2008
<i>feoA</i>	1	2.9	1.6	16.7	21.2	21.6	28.1
<i>feoB</i>	1	2	1.6	5.1	6	6.8	13.4
<i>feoC</i>	1	1.3	1.5	2.3	2.8	2.4	4.4
<i>fur</i>	1	1.1	1.5	1.4	0.9	1.1	1

<sup>a</sup> The transcriptomes of six DK2 isolates included in this study have previously been measured at exponential growth phase in LB medium (4). We searched the transcriptomes for genes encoding components of the pyoverdine, pyochelin, *phu*, *has*, *feo*, and *fec* iron acquisition systems (7, 11–13), and the table lists the transcription profiles of those systems in which at least one gene showed differential expression (>3-fold change) in the post-1973 isolates relative to that in the 1973 isolates or strain PAO1. Also, the transcription of the *fur* gene is shown. Values are averages for three replicates, and the values are normalized relative to the transcription of the respective gene in reference strain PAO1.

TABLE 2 Activities of the *phuR* and *phuS* promoters originating with different clinical isolates of *P. aeruginosa*<sup>a</sup>

Strain	Promoter	Origin of promoter	Allele	Mean luminescence (± SD)	Fold change	<i>P</i> value
PAO1	<i>phuR</i>	PAO1	WT	365 (±1,018)	1	
PAO1	<i>phuR</i>	DK2-CF66-2008	M1	34,111 (±3,379)	93	0.00021
PAO1	<i>phuR</i>	DK2-CF173-2005	M2	40,726 (±3,422)	112	0.00004
PAO1	<i>phuR</i>	DK2-CF173-2002	M3	1,879 (±3,422)	5	0.16
PAO1	<i>phuR</i>	DK2-CF240-2002	M4	7,584 (±496)	21	0.00038
PAO1	<i>phuR</i>	DK2-CF222-2001	M5	8,968 (±610)	25	0.00023
PAO1	<i>phuR</i>	DK2-CF180-2002	M6	6,723 (±701)	18	0.00088
PAO1	<i>phuR</i>	DK1-P28F1-1992	M8	13,329 (±1,482)	37	0.00024
PAO1	<i>phuR</i>	DK1-P28F1-2009	M9	12,205 (±603)	33	0.00007
PAO1	<i>phuR</i>	DK1-CF30-2011	M10	9,563 (±1,586)	26	0.0011
PAO1	<i>phuS</i>	PAO1	WT	7,444 (±1,777)	1	
PAO1	<i>phuS</i>	DK2-CF173-2005	M2	12,030 (±3,191)	1.6	0.01

<sup>a</sup> Luminescence production from laboratory reference strain PAO1 (37) with *phuR::lux* reporter fusions was measured at exponential growth ( $OD_{600} = 0.15$ ) in Luria-Bertani (LB) medium and normalized for differences in cell density. Mean luminescence production and standard deviations (SD) were calculated for three biological replicates. Statistical analysis concerning the difference between two means was done using a Student *t* test, and the *P* values denote the probability of the mutated alleles having expression equal to that of the wild type (WT).

*P. aeruginosa* toward heme utilization in the CF airways. To further investigate the generality, we compared our findings to other lineages of *P. aeruginosa* isolated from CF infections.

In addition to the DK2 lineage, our previous investigations have revealed another distinct clone type, known as the DK1 clone type, which has also spread among Danish CF patients (21). We sequenced and analyzed the *phuR* promoter region of five DK1 isolates sampled in the years 1992 to 2011 in addition to an ancestral DK1 isolate from 1973. Whereas the sequence of the *phuR* promoter of the ancestral 1973 isolate (DK1-P33F0-1973) was identical to the wild-type sequence of strains PAO1 and DK2, all five evolved DK1 isolates had accumulated 1 to 4 single nucleotide polymorphisms (SNPs) in the promoter region, and three of the DK1 SNPs were identical to SNPs found in the evolved DK2 isolates (Fig. 2). We tested the activities of three of the mutated promoters from the DK1 isolates (M8 to M10) and found that all three mutated promoters resulted in increased levels of transcription, similar to what has been observed for mutated DK2 alleles (Table 2). Our results provide strong evidence for convergent adaptive evolution of different lineages of *P. aeruginosa* toward iron acquisition from hemoglobin.

To rule out that the adaptive trait was specific for *P. aeruginosa* CF infections at the Copenhagen CF Center, we analyzed the available public data for the genomic evolution of the *P. aeruginosa* C lineage, which was isolated from a patient attending the CF clinic at Hannover Medical School, Germany (6). Interestingly, the C lineage, which has colonized this patient for a period of more than 20 years, also accumulated two SNPs in the *phuR* promoter region (Fig. 2). Remarkably, the two SNPs are identical to SNPs found in

the DK1 and DK2 lineages, and this observation suggests that these mutations were also positively selected for in the host environment.

The research team at Hannover Medical School also investigated the microevolution of a PA14 lineage as it infected a patient over 14 years. Nonetheless, the PA14 lineage did not accumulate SNPs in any iron acquisition systems. Likewise, a lineage investigated by Smith et al. (5) over an infection course of 90 months also did not reveal any mutations in iron acquisition systems, except for a nonsynonymous mutation in *pvdS* (which correlated with the loss of pyoverdine production) and an intergenic SNP upstream of *fptA* (5). We therefore conclude that despite an apparent selection for *phu* promoter mutations in three independent *P. aeruginosa* lineages, not all lineages accumulate *phu* promoter mutations during CF infections.

**Selection against pyoverdine secretion might lead to a shift in iron source.** The siderophore pyoverdine has previously been found in sputum of CF patients, and thus pyoverdine-mediated uptake of iron has been considered important for the survival of *P. aeruginosa* in the CF airways (14). Nonetheless, we observed that all three lineages (DK1, DK2, and C) had accumulated nonsynonymous mutations in the alternative sigma factor PvdS, which is required for pyoverdine synthesis (Fig. 1 and Fig. 4). Accordingly, the evolved C clone NN80 was observed to have lost its ability to produce pyoverdine, in contrast to its predecessors (C clones NN2 and NN11) (6).

This led us to examine the production of pyoverdine in the DK1 and DK2 isolates, and we observed a negative correlation between pyoverdine production and mutations in PvdS (Fig. 5).

TABLE 3 Growth rates of strains DK2-CF30-1979 and DK2-CF30-1979-M2 at exponential growth phase in different media<sup>a</sup>

Growth medium	Doubling time (h)		<i>P</i> value
	DK2-CF30-1979	DK2-CF30-1979-M2	
LB	1.27 ± 0.05	1.35 ± 0.07	0.16
ABTGC + 10 μM Fe <sup>3+</sup>	2.74 ± 0.02	2.69 ± 0.03	0.23
ABTGC + 10 μM Fe <sup>3+</sup> + 100 μg/ml apo-TF	3.08 ± 0.10	3.07 ± 0.04	0.91
ABTGC + 2.5 μM Hb + 100 μg/ml apo-TF	2.76 ± 0.24	2.13 ± 0.09	0.01

<sup>a</sup> The abbreviations Hb and apo-TF are used for hemoglobin and apotransferrin, respectively. Note that the ABTGC minimal medium standard recipe was modified so that no iron source other than the one stated in the table was added to the growth medium. Mean doubling times were calculated from three biological replicates. Statistical analysis concerning difference between two means was done using a Student *t* test, and the *P* values denote the probability of the two strains having equal means.



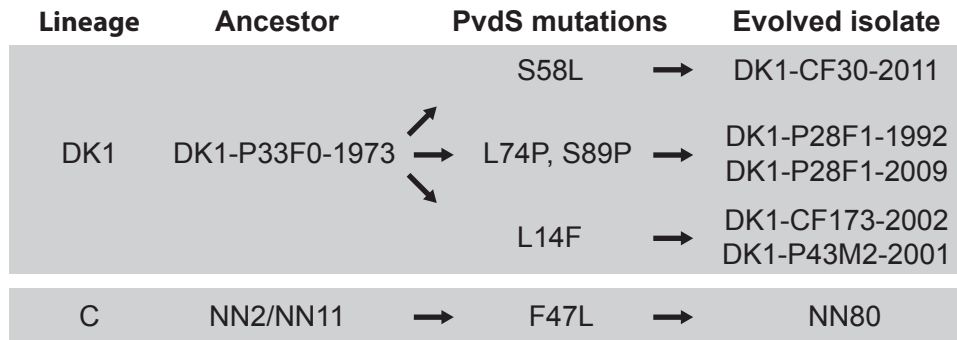


FIG 4 Overview of *pvdS* mutations in the DK1 and C lineages. Mutations that have accumulated in evolved isolates relative to sequences of their ancestor are shown. The *pvdS* mutation found in the DK2 lineage is shown in Fig. 1.

Accordingly, only the ancestral DK1 and DK2 isolates carrying wild-type alleles of *pvdS* were able to produce pyoverdine, whereas all isolates carrying mutated alleles of *pvdS* were unable to produce pyoverdine (DK1-CF173F-2002 was not tested).

Siderophores are generally regarded as highly immunogenic (22), and selection against pyoverdine production might have driven the accumulation of *pvdS* mutations, leading to a loss of pyoverdine production in the evolved isolates. At the same time, we observed a positive selection for *phuR* promoter mutations in the CF airways, leading to a bacterial growth advantage when acquiring iron from hemoglobin. We therefore propose a model in which the CF airways impose selective pressure on the invading bacteria, forcing them to adapt toward a shift to hemoglobin as an alternative iron source. This is of particular interest because in-

flammation may cause microbleeds, which lead to the presence of hemoglobin at the delicate CF lung epithelia in the presence of both host and bacterial proteases (23). Also, hemoglobin is reported to be expressed by alveolar epithelial cells (24).

**Other iron acquisition systems might be affected by mutations.** Several iron acquisition systems and mutations other than the ones that we have investigated in detail here might play a role in survival of *P. aeruginosa* in the lungs of CF patients. Accordingly, we also found nonsynonymous mutations in the FpvAII gene and the genes *fpvI*, *fpvR*, *phuR*, *pchA*, *pchDEFGH*, and *fptA* when searching for mutations in genes of the pyoverdine, pyochelin, *phu*, *has*, *feo*, and *fec* iron acquisition systems (Fig. 1). We anticipate that the identification of such mutations can facilitate further investigations of the adaptation of *P. aeruginosa* to human

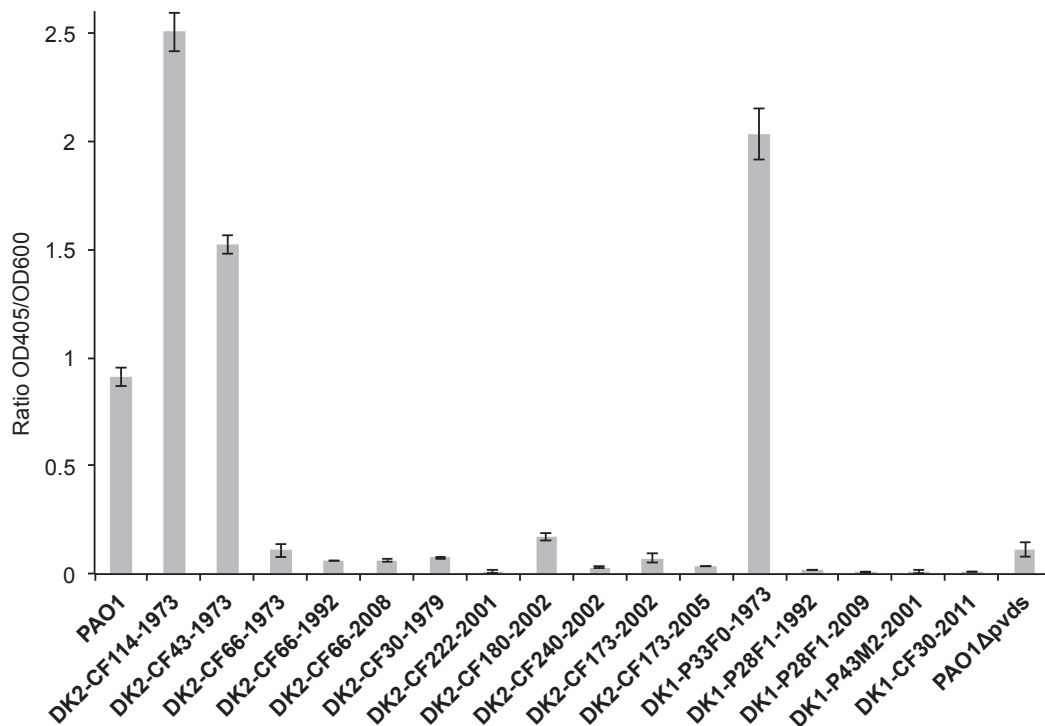


FIG 5 Pyoverdine production in isolates of *P. aeruginosa*. The presence of pyoverdine secreted into the supernatant of bacterial cultures grown in pyoverdine-inducing medium was quantified by measurement of the absorbance at OD<sub>405</sub> and normalized against the cell density (OD<sub>600</sub>). The means and standard deviations calculated from three biological replicates are shown in the bar plot.

host airways. For example, it remains to be elucidated whether the mutations in the *pch* and *fptA* genes affect the function of the pyochelin iron uptake system in the DK2 lineage and if isolates with mutations in the pyoverdine system are unable to cheat on other pyoverdine producers.

**Conclusions and implications.** Our results provide evidence that the selective conditions by which evolution is directed in the CF airways can result in acquisition of *phu* promoter mutations in *P. aeruginosa* during chronic CF infections and that such mutations provide a growth advantage in relation to acquisition of iron from hemoglobin. This adaptive trait may be directly selected for due to an abundance of heme-bound iron in the CF lung. Furthermore, we also observed that *phu* promoter mutations coincided with the loss of pyoverdine production, suggesting that selection for increased heme utilization may be secondary to the loss of the pyoverdine iron uptake system. Therefore, targeting heme utilization might be a promising strategy for the treatment of CF infections.

CF patients commonly experience iron deficiency, and *P. aeruginosa* possibly contributes to iron deficiency by depletion of the host iron storage and by causing inflammation (25, 26). In this regard, expanding our knowledge of adaptation of *P. aeruginosa* to the CF lung may help to lessen the impact of *P. aeruginosa* infection and improve the condition of patients.

## MATERIALS AND METHODS

**Bacterial strains and media.** Isolates of the *P. aeruginosa* DK1 and DK2 clone types were sampled from Danish CF patients attending the Copenhagen Cystic Fibrosis Clinic. Isolation and identification of *P. aeruginosa* from sputum were done as previously described (27). The isolates are named according to their clone type, the patient from whom they were isolated, and their isolation year (e.g., isolate DK2-CF30-1979). Luria-Bertani (LB) broth was used for routine preparations of bacterial cultures. ABTGC minimal medium was composed of 2 g/liter (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 6 g/liter Na<sub>2</sub>HPO<sub>4</sub>, 3 g/liter KH<sub>2</sub>PO<sub>4</sub>, 3 g/liter NaCl, 1 mM MgCl<sub>2</sub>, 0.1 mM CaCl<sub>2</sub>, 0.01 mM FeCl<sub>3</sub>, 2.5 mg/liter thiamine supplemented with 1% glucose, and 0.5% Casamino Acids. For the growth rate experiments (Table 3), no FeCl<sub>3</sub> was added to ABTGC minimal medium unless otherwise stated. Human hemoglobin (Sigma-Aldrich) and human apotransferrin (Sigma-Aldrich) were added to concentrations of 2.5 μM and 100 μg/ml, respectively. Pyoverdine-inducing medium was composed of ABTGC minimal medium with 50 μM iron chelator 2,2'-dipyridyl (DIPY). *Escherichia coli* strain CC118(λ*pir*) was used for maintenance of recombinant plasmids (28) in medium supplemented with 8 μg/ml of tetracycline. Allelic replacement constructs were transferred to *P. aeruginosa* by triparental mating using the helper strain *E. coli* HB101/pRK600 (29). For marker selection in *P. aeruginosa*, 50 μg/ml of tetracycline was used. Genetic techniques were performed using standard methods, and Sanger sequencing was used for verification of genetic construct and allelic replacement mutants.

**Sequencing of *phuR* promoter region and *pvdS* gene in DK1 isolates.** Sequencing of DK1 isolates was performed as described earlier (4). Accordingly, genomic DNA was purified from *P. aeruginosa* isolates using a Wizard Genomic DNA purification kit (Promega, Madison, WI) and sequenced on Illumina's GAIIx or HiSeq2000 platform. Reads were mapped against the reference genome sequence using the software program Novoalign (Novocraft Technologies, Selangor, Malaysia) (30), and pileups of read alignments were produced by the software program SAMtools, release 0.1.7 (31).

**Construction of reporter fusions and luminescence measurements.** The *lux* gene cassette (*luxCDABE*) was subcloned from the plasmid pUC18-mini-Tn7T-Gm-*lux* (32) fragment into mini-CTX2 (33) using the restriction sites XhoI and PstI to produce pHK-CTX2-*lux*, used for the

transcriptional fusion experiments. For *phuR::lux* reporter fusions, a 220-bp fragment containing the intergenic region upstream of *phuR* was amplified from genomic DNA using Phusion polymerase (Thermo Scientific) with the primers PhuR\_F-PstI (5' GAGACTGCAGAGGCTGGGAG TGCTGCTCAT 3') and PhuR\_R-XhoI (5' ACATCTCGAGAAGGGCGG GGAGAGCGGCAT 3') and ligated with T4 DNA ligase into pHK-CTX2-*lux* after double digestion of the PCR fragment and vector with the restriction enzymes XhoI and PstI. For *phuS::lux* reporter fusions, a 220-bp fragment containing the intergenic region upstream of *phuS* was amplified with the primers PhuS\_F-XhoI (5' ACATCTCGAGAGGCTG GGAGTGCTGCTCAT 3') and PhuS\_R-PstI (5' GAGACTGCAGAAGG GCGGGGAGAGCGGCAT 3') and ligated into pHK-CTX2-*lux* after double digestion of the PCR fragment and vector with the restriction enzymes XhoI and PstI. The resulting plasmids were introduced into *P. aeruginosa* strain PAO1 by transformation as previously described (32).

**Allelic replacement of *phuR* promoter region in DK2-CF30-1979.** A 1,296-bp fragment containing the intergenic region upstream of *phuR* was amplified from genomic DNA of DK2-CF173-2005 using Phusion polymerase (Thermo Scientific) with the primers PhuSi\_F-XbaI (5'-ACATT CTAGACGGACGTCGCTGGCCTCG-3') and PhuSi\_R-SacI (5'-GAGA GAGCTCTCTCGTGGCCCTGGCCGGTAG-3'). The PCR fragment was ligated into the vector pNJ1 (34) after digestion with the restriction enzymes XbaI and SacI. The allelic replacement construct was transferred into strain DK2-CF30-1979 by triparental mating, and merodiploid mutants were selected by plating the conjugation mixture on LB agar plates with tetracycline. Colonies were restreaked on selective plates before being streaked on 8% (wt/vol) sucrose-LB plates without NaCl. Sucrose-resistant and tetracycline-sensitive colonies were restreaked on sucrose-LB plates and screened for the presence of mutated alleles by PCR followed by restriction fragment length polymorphism (RFLP) analysis. Positive mutants were finally sequenced by Sanger sequencing at LGC genomics (Germany).

**Measurement of growth and luminescence in reporter fusion strains.** Overnight cultures of the reporter fusion strains were diluted 40 times in fresh LB, and aliquots of 100 μl were transferred to a black (clear-bottom) 96-well microtiter plate (Nunc). Three technical replicates were used for each strain, and measurements of growth (OD<sub>600</sub>) and luminescence were recorded in a Synergy Hybrid H1 reader (Bio-Tek) with 6-min intervals for 10 h and under shaking conditions (200 rpm) at 37°C. Data were analyzed using a custom-made script in the R software environment, version 2.15.2 (35). The experiment was repeated three times to obtain biological replicates.

**Growth rate measurements.** Growth rate experiments were carried out in 250 ml baffled shake flasks containing 50 ml of growth medium under shaking (200 rpm) at 37°C. Culture flasks were inoculated to a starting OD<sub>600</sub> of 0.005 in 50-ml minimal medium, and measurements of OD<sub>600</sub> were started 9 h after the inoculation and recorded every 30 min. In the experiment where the cells were cultivated in LB, the measurements were started after 2 h. The experiment was stopped when the cells reached stationary growth phase, typically after around 23 h of growth in minimal medium. Growth experiments were repeated three times for each strain under each condition to obtain biological replicates.

**Pyoverdine quantification assay.** Pyoverdine concentrations were quantified as previously described (36). All strains were grown in pyoverdine inducing medium for up to an OD<sub>600</sub> of >1.5. Cultures were moved into 2-ml microcentrifuge tubes and centrifuged at 16,000 × g for 2 min. The supernatants were diluted in 100 mM Tris-HCl buffer (pH 8), and pyoverdine concentrations were quantified by measurement of the absorbance at OD<sub>405</sub>. Finally, the values of absorbance at OD<sub>405</sub> were normalized against the cell densities (OD<sub>600</sub>) for each strain. The procedure was repeated for three independent biological replicates.

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