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Adaptations of *Pseudomonas aeruginosa* to the Cystic Fibrosis Lung Environment Can Include Deregulation of *zwf*, Encoding Glucose-6-Phosphate Dehydrogenase

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Cystic fibrosis (CF) patients are highly susceptible to chronic pulmonary disease caused by mucoid *Pseudomonas aeruginosa* strains that overproduce the exopolysaccharide alginate. We showed here that a mutation in *zwf*, encoding glucose-6-phosphate dehydrogenase (G6PDH), leads to a ~90% reduction in alginate production in the mucoid, CF isolate, *P. aeruginosa* FRD1. The main regulator of alginate, sigma-22 encoded by *algT* (*algU*), plays a small but demonstrable role in the induction of *zwf* expression in *P. aeruginosa*. However, G6PDH activity and *zwf* expression were higher in FRD1 strains than in PAO1 strains. In PAO1, *zwf* expression and G6PDH activity are known to be subject to catabolite repression by succinate. In contrast, FRD1 *zwf* expression and G6PDH activity were shown to be refractory to such catabolite repression. This was apparently not due to a defect in the catabolite repression control (Crc) protein. Such relaxed control of *zwf* was found to be common among several examined CF isolates but was not seen in other strains of clinical and environmental origin. Two sets of clonal isolates from individual CF patient indicated that the resident *P. aeruginosa* strain underwent an adaptive change that deregulated *zwf* expression. We hypothesized that high-level, unregulated G6PDH activity provided a survival advantage to *P. aeruginosa* within the lung environment. Interestingly, *zwf* expression in *P. aeruginosa* was shown to be required for its resistance to human sputum. This study illustrates that adaptation to the CF pulmonary environment by *P. aeruginosa* can include altered regulation of basic metabolic activities, including carbon catabolism.

Pseudomonas aeruginosa is an important opportunistic and nosocomial bacterial pathogen that contributes to a high rate of fatality among cystic fibrosis (CF) patients. During pathogenesis, the CF lung environment promotes and selects for multiple phenotypic and genotypic alterations in *P. aeruginosa* (29). The most obvious and predominant alteration selected within the CF lung is overproduction of the exopolysaccharide alginate (7). The appearance of these alginate-overproducing strains, also known as mucoid variants, correlates with the establishment of a chronic lung infection and a poor prognosis for the CF patient (8). The roles for alginate in virulence are varied and include neutralization of oxygen radicals (38), inhibition of phagocytosis (30), inhibition of antibiotic penetration (10), and inhibition of complement activation (32). Although a significant number of *P. aeruginosa* isolates from adult CF patients are mucoid (7), presentations of the mucoid phenotype in each isolate differ with respect to amount of alginate produced, stability of the phenotype, and growth conditions that promote alginate production. These variations imply that there are multiple factors that affect alginate production, which may be influenced by the CF lung environment. Given the correlation of alginate overproduction with pulmonary deterioration in CF patients, a better understanding of alginate regulation and production may suggest strategies for down-regulating alginate within the CF lung environment (15).

Production of alginate is an energy-costly process that diverts carbon sources from being utilized for energy and growth towards alginate production. The fact that the majority of *P. aeruginosa* CF isolates produce copious amount of alginate suggests that these isolates require alginate production in vivo. Carbon metabolism and alginate production are intimately related such that defects in carbon catabolism have dramatic effects on alginate production (27). Much of our current knowledge regarding carbon catabolism in *P. aeruginosa* is derived from studies with nonmucoid strain PAO1, a wound isolate, whereas much of our current knowledge of alginate production is derived from CF isolates like FRD1. In PAO1, the genes that encode enzymes for the major carbon catabolic pathway are organized into several operons that comprise the hexose regulon (hex-regulon). The hex-regulon is induced by growth on carbon sources such as glucose, gluconate, and glycerol but not by succinate and other intermediates of the tricarboxylic acid (TCA) cycle (12, 14). To date, only two regulatory proteins that control the hex-regulon in PAO1 have been identified, and both are repressors: Crc (*c*atabolite *r*epression *c*ontrol) and HexR (2, 42). However, a molecular mechanism by which Crc mediates catabolite repression in *P. aeruginosa* has not yet been elucidated, and the physiological role of HexR in carbon catabolism is unknown. Moreover, neither of these regulators, or even carbon metabolism in gen-

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TABLE 1. Bacterial strains and plasmids[a]

Strain(s) or plasmid	Genotype, relevant characteristics	Source
Strains		
FRD1	CF isolate, mucoid, rough phenotype	28
PAO1	Wound isolate, nonmucoid	11
PDO300	PAO1*mucA22*, mucoid	24
PAO1*zwf* (LS637)	PAO1*zwf101*::*aacCI*	This study
PAO1*crc* (LS1441)	PAO1Δ*crc101*	This study
FRD1*crc* (LS1439)	FRD1Δ*crc101*	This study
FRD1*zwf* (LS636)	FRD1*zwf101*::*aacCI*	This study
FRD1*algD* (FRD1131)	FRD1*algD*::Tn*501*, nonmucoid	1
FRD1*algT* (LS586)	FRD1 *algT101*::*aacCI*, polar, nonmucoid	37
FRD1*zwf*+ (LS974)	FRD1*zwf* complemented for *zwf* in *trans*	This study
PAO1*zwf*+ (LS637)	PAO1*zwf* complemented for *zwf* in *trans*	This study
FRD1*zwf*+ (LS1518)	FRD1*zwf* complemented for *zwf* in *cis*	This study
PAO1*zwf*+ (LS1520)	PAO1*zwf* complemented for *zwf* in *cis*	This study
FRD1*crc*+ (LS1447)	FRD1*crc* complemented for *crc*	This study
PAO1*crc*+ (LS1448)	PAO1*crc* complemented for *crc*	This study
DO5 and DO249	CF isolates, mucoid	This study
DO60	CF isolate, nonmucoid	37
DO62	CF isolate, nonmucoid	This study
DO133 and DO326	CF isolate, mucoid	37
P1 and P2	Blood isolates, nonmucoid	This study
P15 and P16	Peritoneal fluid isolates, nonmucoid	This study
PA14	Wound isolate, nonmucoid	33
3064	CF isolate, mucoid	47
PAM57-15 and PA2192	CF isolate, mucoid	44
ENV2, ENV8, and ENV10	Garden vegetable isolates, nonmucoid	22
ENV46, ENV48, and ENV54	River isolates, nonmucoid	22
CF09-CF40	CF isolates, patients 12, 13 and 17	21, this study
PA2192*zwf*	PA2192 *zwf101*::*aacCI*	This study
Plasmids		
pEX100T	Cloning vector with *sacB*	36
pLS214	pUC19 with *moriT* at HindIII	40
pLS217	pUC18 with *moriT* at EcoRI	40
pLS594	*zwf-lacZ* transcriptional fusion in pSS223	This study
pLS1436	Δ*crc101* in pEX100T	This study
pLS1446	*crc* complementing plasmid	This study
pLS1051	*crc-lacZ* translational fusion in pSS361	This study
pSS223	*lacZ* operon fusion vector	47
pSS366	*zwf* complementing plasmid, *trans*	This study
pLS1517	*zwf* complementing plasmid, *cis*	This study
pUCGM1	ColE1 Amp^r Gm^r	35
pLS1155	Regulatable promoter/repressor system	This study
pLS1393	Regulatable *crc* upon plasmid integration	This study

[a] Abbreviations used for genetic markers are described by Holloway et al. (11). Ap^r, ampicillin resistance. Alternate strain names are shown in parentheses.

eral, have been extensively investigated in CF isolates of *P. aeruginosa*, which are reported to differ extensively from non-CF isolates in a number of characteristics (4, 6, 9, 16, 17, 22, 43, 48). In this study, a key enzyme of carbon catabolism and the Entner-Doudoroff pathway, glucose-6-phosphate dehydrogenase (G6PDH, or Zwf) was examined in a mucoid CF isolate because of its potential role in alginate overproduction.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this study are listed in Table 1. Unless otherwise indicated, bacteria were grown in L broth, L broth without NaCl, or L broth supplemented with appropriate antibiotics at 37°C with aeration. No carbon-E minimal medium (NCE) (5) was supplemented with 0.1% Casamino Acids and with glycerol (40 mM), succinate (40 mM), or glycerol and succinate as the carbon source(s). Basal salts medium was supplemented with succinate (40 mM) and lactamide (20 mM). L agar without NaCl was supplemented with 8% sucrose for use in negative selection of *sacB*. *Pseudomonas* isolation agar supplemented with appropriate antibiotics was used to select for *P. aeruginosa* transconjugants and to counterselect *Escherichia coli*. The following amounts of antibiotics were used in this study (per milliliter): 100 μg ampicillin for *E. coli*, 125 μg carbenicillin for *P. aeruginosa*, and 20 and 180 μg gentamicin (Gm) for *E. coli* and *P. aeruginosa*, respectively.

DNA manipulations, transformations, and conjugations. *E. coli* strain DH10B was routinely used as a host strain for cloning. DNA was introduced into *E. coli* by electroporation and into *P. aeruginosa* by conjugation as previously described (41). Plasmids were purified with QIAprep spin miniprep columns made by QIAGEN (Valencia, CA). DNA fragments were excised from agarose gels and purified using the Qiaex II DNA gel extraction system (QIAGEN) according to the manufacturer's instructions. Restriction enzymes and DNA modification enzymes were purchased from New England Biolabs (Beverly, MA). Either *Pfu* from Stratagene (La Jolla, CA) or *Taq* from New England Biolabs was used for PCR amplification of DNA. Oligonucleotides were purchased from Operon, Inc. (Alameda, CA), or Integrated DNA Technologies, Inc. (Coralville, IA).

Construction of *zwf and crc* mutants. Derivatives of FRD1 and PAO1 with a mutation in *zwf* were constructed as follows: a 1.55-kb fragment containing *zwf* was PCR amplified from FRD1 with *Pfu*, digested with EcoRI and XbaI and then cloned into pBluescript K(+) between the EcoRI-XbaI sites. A gentamicin resistance cassette isolated from pUCGM1 (35) as a BamHI fragment was then cloned into the internal BamHI site within *zwf* to disrupt the open reading frame (ORF). Next, an origin of transfer (*oriT*) from pLS217 was added as an EcoRI

fragment. The final construct, pLS635, was conjugated into FRD1 and PAO1, and potential *zwf* mutants were isolated as gentamicin-resistant (Gm^r) and carbenicillin-sensitive colonies. The presence of the mutant allele and the absence of the wild-type allele were verified by PCR analysis. To complement the *zwf* mutation in *trans*, *zwf* was PCR amplified with *Pfu* and cloned into the *P. aeruginosa-E. coli* shuttle vector, pUCP19, as a 1,850-bp EcoRI-HindIII fragment. The resulting plasmid, pSS354, was converted into a mobilizable plasmid, pSS366, by the addition of a m*oriT* (40) of plasmid RP4. To complement the *zwf* mutation in *cis*, *zwf* was PCR amplified with *Pfu* and cloned into pBK^+ as a 2,380-bp EcoRI-SmaI fragment. The resulting plasmid, pLS1515, was converted to a mobilizable plasmid, pLS1517, by the addition of a mini-*oriT* (m*oriT*) to the HindIII site. pSS366 and pLS1517 were introduced into *P. aeruginosa* via triparental mating. To construct a *crc* mutant in *P. aeruginosa*, an internal 343-bp fragment was deleted from the *crc* coding region by using the splicing by overlap extension technique (SOEing) (45). First, an 820-bp fragment, 5′ to and containing the first 180 bp of the *crc* ORF, was PCR amplified from FRD1 with *Pfu*. Concurrently, a 999-bp fragment, 3′ to and containing the last 255 bp of the *crc* ORF was PCR amplified with *Pfu*. The two fragments shared approximately 20 bp of overlap so that they could be joined by SOE PCR. In the second round of PCR amplification, the fragments described above served as DNA templates by use of the 5′ primer of the first fragment and the 3′ primer of the second fragment to generate a 1,819-bp product. This product, with 343 bp deleted from the *crc* coding sequence, was cloned into pEX100T, which carries a carbenicillin resistance gene and a counterselectable marker, *sacB* (36), to generate pLS1436. Following the conjugation of pLS1436 into *P. aeruginosa*, merodiploid colonies with the plasmid integrated into the *P. aeruginosa* chromosome via homologous recombination were selected for their resistance to carbenicillin. The merodiploids were subsequently resolved by selecting for growth on medium containing sucrose to promote the loss of plasmid DNA sequences carrying *sacB*. This resulted in allelic exchange between the wild-type *crc* and Δ*crc* alleles. The presence of the mutant allele and the loss of plasmid and the wild-type copy of *crc* in the putative *crc* mutants were verified by PCR. To complement the *crc* mutation, the *crc* coding sequence, along with approximately 800 bp of upstream sequence and 80 bp of downstream sequence, was PCR amplified from FRD1 by using *Pfu* and cloned into pUCP19 as a BamHI-EcoRI fragment. A m*oriT* isolated from pLS214 was then cloned as a HindIII fragment to generate pLS1446 and the plasmid was introduced into *P. aeruginosa crc* mutants via conjugation. The complemented FRD1*crc* and PAO1*crc* mutants were designated FRD1*crc*+ (LS1447) and PAO1*crc*+ (LS1448), respectively.

Construction of transcriptional and translational fusions. To construct the *zwf*::*lacZ* and *crc*::*lacZ* fusions, DNA fragments containing the promoter for the genes and a portion of the 5′ coding sequence were PCR amplified from FRD1 genome using *Pfu* DNA polymerase, digested with the appropriate enzymes and cloned into either pSS223 for a transcriptional fusion (*zwf*::*lacZ*) or pSS361 for a translational fusion (*crc*::*lacZ*). pSS361 is a mobilizable *lacZ* translational fusion vector that can replicate in *P. aeruginosa* (40). All plasmid constructs were verified by PCR analysis and restriction digestion before they were conjugated into *P. aeruginosa*.

Biochemical assays. Alginate was isolated from *P. aeruginosa* culture supernatants that were dialyzed against distilled water as previously described (41), and alginate (i.e., uronic acid) level was quantified by the carbazole method (13) using *Macrocystis pyrifera* alginate (Sigma) as a standard. G6PDH activity was determined as described previously (18), and β-galactosidase assays were preformed as described by Miller (25). Pyocyanin was quantified from 20-h cultures as previously described (3). Amidase activity was determined as previously described (20).

RAPD analysis of CF isolates. CF isolates 132 to 137 were previously typed by Mahenthiralingam et al. (21). To type CF 139 and 140, random amplified polymorphic DNA (RAPD) analysis of these isolates by using primer 208 (ACG GCCGACC) was conducted as previously described, and the results were compared to RAPD analysis of CF isolates 132 to 137 with the same primer.

Sputum inhibition assay. NCE agar plates supplemented with 20 mM glycerol were seeded with 5 μl of an overnight culture of *P. aeruginosa* diluted into 100 μl of saline. A 5-μl drop of sputum (1.3 μg/ml protein) was placed in the center of the plate and the plates were incubated overnight at 32°C.

Oxidative stress assays. Overnight bacterial cultures were adjusted to an optical density at 600 (OD_{600}) of ~2.0; 5 μl was inoculated into 1 ml fresh L broth (minus NaCl for paraquat assays) containing increasing concentrations of paraquat or hydrogen peroxide as described by Ma et al. (18). The samples were incubated for 20 h at 37°C with aeration after which the final OD_{600} was recorded.

DNA sequencing and statistical analysis. The PCR amplified *crc* gene from FRD1 (carried on pLS1334) was sequenced by The Auburn University Research and Instrumentation Facility (Auburn University, AL.). Statistical analysis was performed using In Stat (GraphPad software, San Diego, CA).

RESULTS

G6PDH promotes alginate production. Glucose-6-phosphate dehydrogenase is encoded by *zwf* and converts glucose-6-phosphate to 6-phosphogluconate; it is the first enzyme in the Entner-Doudoroff pathway, which is central to carbon metabolism in *Pseudomonas* sp. We constructed a *zwf*::*aacCI* (Gm^r) insertion mutant in the CF mucoid isolate, *P. aeruginosa* FRD1, as a starting point to elucidate the relationship between carbon catabolism and alginate production in CF strains. Cell extracts of FRD1 in L broth logarithmic culture contained ~105 mIU of G6PDH, whereas, the FRD1*zwf* mutant contained no detectable G6PDH activity. FRD1 produced ~675 ± 42 μg/ml of alginate, whereas FRD1*zwf* produced 75 ± 8.25 μg/ml of alginate, or 11% compared to the wild-type mucoid strain. This defect in alginate production levels was fully complemented (780 ± 89.7 μg/ml) by wild-type zwf^+ on plasmid pSS366. Thus, the flux of carbon via G6PDH is important for high-level alginate production in mucoid *P. aeruginosa* FRD1.

Effect of alginate production on G6PDH activity. Since G6PDH is required for high-level alginate production in FRD1, we hypothesized that mucoid strains might possess higher levels of G6PDH activity than nonmucoid strains. Mucoid conversion usually occurs as a result of a mutation in an anti-sigma factor encoded by *mucA* (23), which leads to the activation (i.e., deregulation) of sigma-22, which is also known as AlgT or AlgU. This alternative extracytoplasmic-function sigma factor is at the top of a hierarchy of regulators for alginate biosynthesis (49). However, mucoid FRD1 (with a *mucA22* allele) contained G6PDH levels that were only about 30% higher than those of the nonmucoid FRD1*algT* mutant when grown in L broth ($P < 0.001$) (Fig. 1). To test whether disruption of the alginate biosynthesis pathway affected G6PDH activity, an FRD1*algD* nonmucoid mutant was also tested. However, a mutation in *algD*, the first gene in the alginate biosynthetic operon (1), did not significantly affect G6PDH activity ($P > 0.05$) in FRD1. As described above, the FRD1*zwf* mutant contained no G6PDH activity. Thus, there was only a minor correlation between alginate overproduction and G6PDH levels by comparing these FRD1 derivatives. The G6PDH activities of the nonmucoid non-CF isolate, PAO1, and its mucoid *mucA22* mutant derivative, PDO300, were also compared. PDO300 displayed a slightly higher level of G6PDH activity than its nonmucoid parent, PAO1 ($P < 0.01$) (Fig. 1). However, it was striking that the FRD1 derivatives contained about 10-fold more G6PDH activity than the PAO1 derivatives when grown in L broth (Fig. 1). This suggested that FRD1, which had undergone adaptation to the CF pulmonary environment, may have undergone a genetic change that up-regulated G6PDH activity.

Catabolite repression of *zwf* transcription. To study the transcriptional control of *zwf*, a *zwf-lacZ* transcriptional fusion was constructed and designated pLS594. The results showed that L broth-grown *P. aeruginosa* strains expressed *zwf-lacZ* in a manner that closely mimicked the G6PDH activity patterns described above. Mucoid FRD1 contained approximately 40%

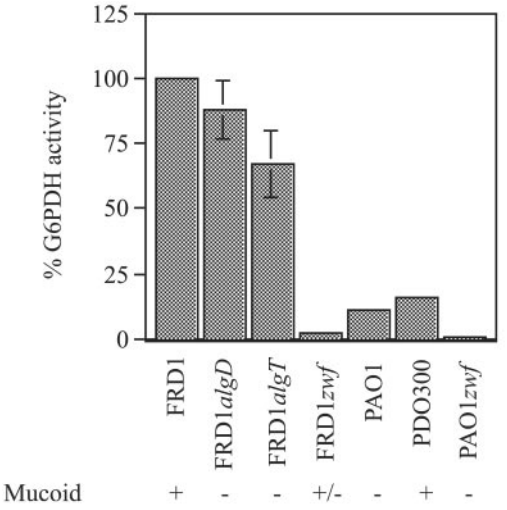

FIG. 1. G6PDH activity in FRD1, PAO1 and their derivatives. G6PDH activity was measured from cells grown to an OD_{600} of approximately 1.0 in L broth. G6PDH activity was normalized to protein concentrations, calculated for mIU, and one hundred percent assigned to the activity observed for FRD1 (~105 mIU). The data represent the averages (±standard deviations) of two independent experiments conducted in duplicate. Alginate production (+/–) is shown below the graph.

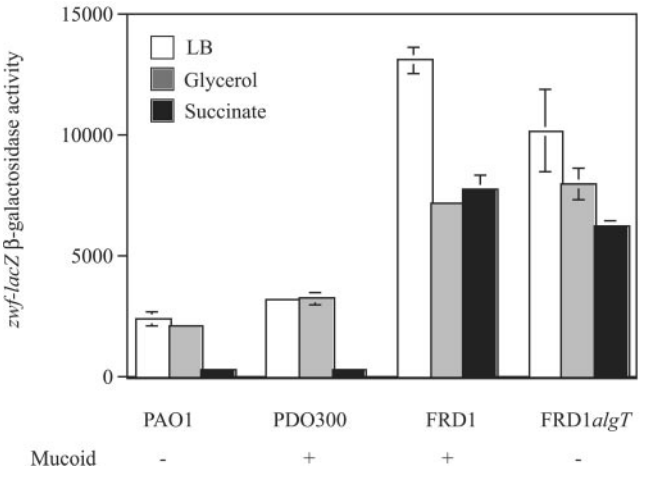

FIG. 2. Expression of *zwf-lacZ* in PAO1, FRD1, and their derivatives. A. Cells were grown in with aeration at 32°C in L broth, NCE plus glycerol or NCE plus succinate as the main carbon source. Samples were taken at OD_{600} ~1.0 and β-galactosidase activities (Miller units) were determined. The data represent the averages (±standard deviations) of three experiments.

more *zwf-lacZ* transcriptional activity than nonmucoid FRD*algT* (Fig. 2). Mucoid PDO300 contained about 40% more *zwf-lacZ* transcriptional activity than nonmucoid PAO1 (Fig. 2). Thus, alginate production correlated with a moderate increase in *zwf* expression. However, a marked increase in *zwf-lacZ* transcriptional activity was observed for FRD1 strains compared to PAO1 strains grown in L broth. This was similar to that described above with G6PDH levels.

We next used the *zwf-lacZ* fusion to examine its regulation in the PAO and FRD strain backgrounds. Previous studies on PAO1 (grown in defined minimal media) have shown that *zwf* expression is high when using the carbon source glycerol, glucose, or gluconate; glycerol elicits the highest response. The presence of a TCA cycle intermediate, such as succinate, reduces *zwf* expression via catabolite repression (18). Similar studies of carbon source utilization by CF isolates of *P. aeruginosa* have not been reported and so were examined here. Although PAO1 grew well in minimal media, FRD1 and other CF isolates grew slowly, and so 0.1% Casamino Acids were added to all defined media to permit robust growth. CF isolates may be partially deficient in the synthesis of one or more essential nutrients, which has been previously observed (43).

Expression of *zwf-lacZ* in PAO1 was approximately ninefold higher with glycerol than with succinate (Fig. 2), which was in agreement with previous studies on catabolite repression in this strain (19). Mucoid PDO300 (a *mucA22* derivative of PAO1) contained approximately 1.5-fold more *zwf-lacZ* activity than the parent when grown on glycerol but still showed strong catabolite repression of *zwf-lacZ* with succinate (Fig. 2). In contrast, *zwf-lacZ* expression in FRD1 was two- to fourfold higher than in the PAO strains with glycerol, and strong repression of *zwf-lacZ* by succinate was not observed (Fig. 2). Overall, the activation of sigma-22 activity in *P. aeruginosa* produced a minor enhancement of *zwf-lacZ* within a strain background. However, the catabolite control of *zwf* was relaxed in the CF strain, FRD1, and its derivatives. This was a phenotype much like a *crc* mutant (46).

Role of Crc in PAO1 and FRD1. *P. aeruginosa* PAO1 is known to preferentially metabolize organic acids and TCA cycle intermediates, such as succinate, before metabolizing nonorganic acids, such as glycerol, or glucose (2). Furthermore, expression of *zwf* and other genes involved in catabolism of hexoses are repressed in the presence of preferred carbon sources in PAO1 (18). Catabolite repression of *zwf* in PAO1 is mediated by the catabolite repression control protein (Crc) (46) and at the transcriptional level (19), although the mechanism has not been deduced.

To address the role of Crc in FRD1, we constructed Δ*crc* mutants of FRD1 and PAO1 (see Materials and Methods). The FRD1*crc* and PAO1*crc* mutants overproduced a blue pigment on agar plates and in liquid culture, which has been observed previously with other *crc* mutants (31). This blue coloration appears to be due to the overproduction of pyocyanin (data not shown). This phenotype was complemented by *crc* in *trans* on plasmid pLS1446. We compared G6PDH activities of the wild type and the *crc* mutant when the bacteria were grown in the presence of succinate (preferred carbon source), glycerol (nonpreferred carbon source), or a combination of succinate and glycerol. In agreement with published results, the level of G6PDH activity was high in PAO1 with glycerol and was repressed by succinate plus glycerol (Fig. 3A). As previously described (46), this catabolite repression of Zwf required Crc because G6PDH activity was not severely repressed in the PAO1*crc* mutant by succinate plus glycerol in PAO1 (Fig. 3A).

In contrast, there was little phenotypic difference between FRD1 and FRD1*crc*. G6PDH remained high in the presence of succinate plus glycerol in both strains, as if parent strain FRD1 lacked a functional Crc control mechanism (Fig. 3B). Also, G6PDH remained relatively high in both FRD1 and

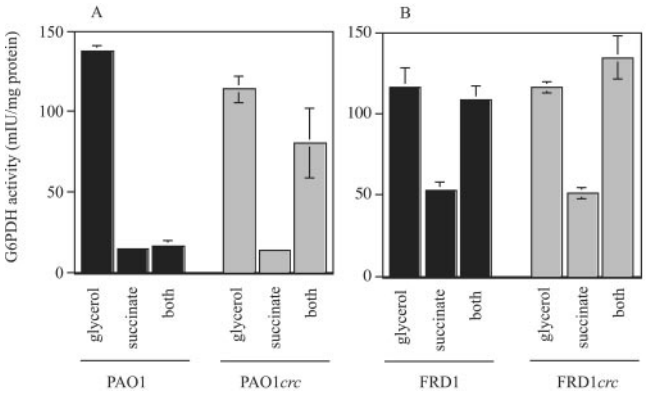

FIG. 3. Effect of a *crc* mutation in PAO1 and FRD1 on G6PDH production. G6PDH activity was measured from cells grown to an OD_{600} of approximately 1.0 in NCE supplemented with the indicated carbon source(s). The data represent the averages (±standard deviations) of three experiments.

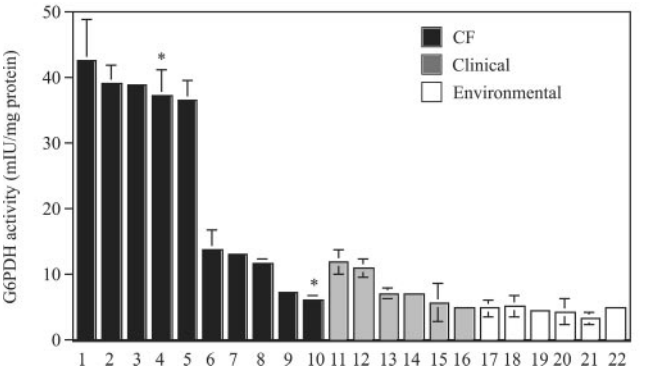

FIG. 5. Survey of G6DPH activity in *P. aeruginosa* isolates. G6PDH activity was measured from cells grown to an OD_{600} of approximately 1.0 in NCE plus succinate. G6PDH activity was normalized to protein concentrations and calculated for mIU. The data represent the averages (±standard deviation) of two experiments. The asterisks indicate nonmucoid CF isolates. Lanes: 1, FRD1; 2, 3064; 3, DO5; 4,DO62; 5, PAM57-15; 6, PA2192; 7, DO326; 8, DO133; 9, DO249; 10, DO60; 11, P2; 12, P15; 13, P16; 14, P1; 15, PAO1; 16, PA14; 17, ENV2; 18, ENV8; 19, ENV10; 20, ENV46; 21, ENV48; 22, ENV54.

FRD1*crc* when grown with succinate alone, which strongly represses *zwf* transcription in PAO1. Because Crc-mediated control of G6PDH activity appeared to be defective in FRD1, we tested for catabolite repression control of amidase. Amidase activity is derepressed in PAO1*crc* compared to PAO1 when grown in the presence of succinate (preferred substrate) and lactamide (inducing substrate) (Fig. 4) (20). Although we observed slightly higher amidase activity in FRD1 compared to PAO1, we also observed that loss of Crc in FRD1 led to increased amidase activity. Therefore, deregulation of G6PDH activity in FRD1 appears to be independent of Crc regulation when FRD1 is grown in L broth. To determine whether Crc may be altered in FRD1, a *crc-lacZ* fusion was constructed (pLS1051), but little difference in the expression levels was observed in FRD1 and PAO1 in L broth over time (data not shown). We then cloned the FRD *crc* gene and sequenced it from ~400 bp upstream to 80 bp downstream of the *crc* ORF. However, the sequence analysis revealed only two conserved changes compared to *crc* from PAO1, which did not change any amino acids, and these were located at nucleotides 606 (C to T) and 684 (A to G) in the coding sequence. This suggests that expression of *crc* in FRD1 is not defective. Studies to identify the molecular mechanism for this apparent deregulation of *zwf* in FRD1 are in progress.

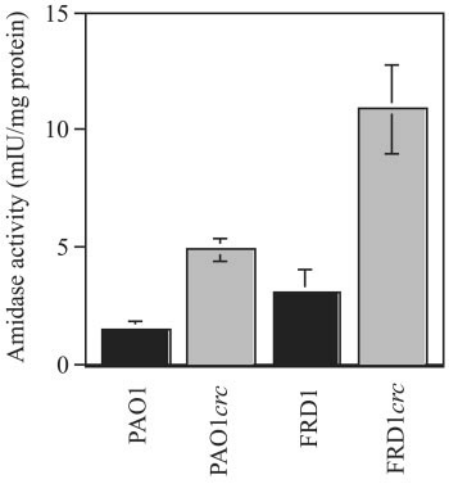

FIG. 4. Effect of a *crc* mutation in PAO1 and FRD1 on amidase. Amidase activity was measured from cells grown to an OD_{600} of approximately 1.0 in basal salts medium supplemented with 40 mM succinate plus 20 mM lactamide. The data represent the averages (±standard deviations) of two independent experiments conducted in duplicate.

High-level G6PDH activity correlates with adaptation to the CF lung. We next looked at a collection of CF isolates of *P. aeruginosa* to see if a high level of expression of *zwf* in the presence of succinate (i.e., *zwf* deregulation) was a common trait among such strains. We compared G6PDH activity following growth with succinate as a sole carbon source in a variety of *P. aeruginosa* isolates: 10 CF isolates, 5 non-CF clinical isolates, and 6 environmental isolates. Like FRD1, half of the CF isolates exhibited a high level of G6PDH activity (defined as >35 mIU/mg of protein). None of the other 5 clinical or 6 environmental strains exhibited this alteration in *zwf* control (Fig. 5). In that 8 of the 10 CF isolates tested were mucoid but not all of the mucoid isolates had high G6PDH activity, this small survey suggests that a high level of unregulated G6PDH activity is a trait acquired separately from overproduction of alginate.

We also examined alginate production by mucoid CF strain PA2192, which retained the normal catabolite repression phenotype of *zwf* when grown in succinate (Fig. 5, lane 6). It accumulated ~835 ± 125 μg alginate/ml in an L broth culture, which is comparable to FRD1. A PA2192*zwf* mutant was also constructed, and it accumulated much-reduced levels of alginate (125 ± 25 μg/ml), much like an FRD1*zwf* mutant. Thus, *zwf* was required for high-level alginate production in both classes of CF strains. Also, normally regulated G6PDH activity can still support substantial alginate production, at least under laboratory conditions. This led us to consider the possibility that deregulated G6PDH could have other selective advan-

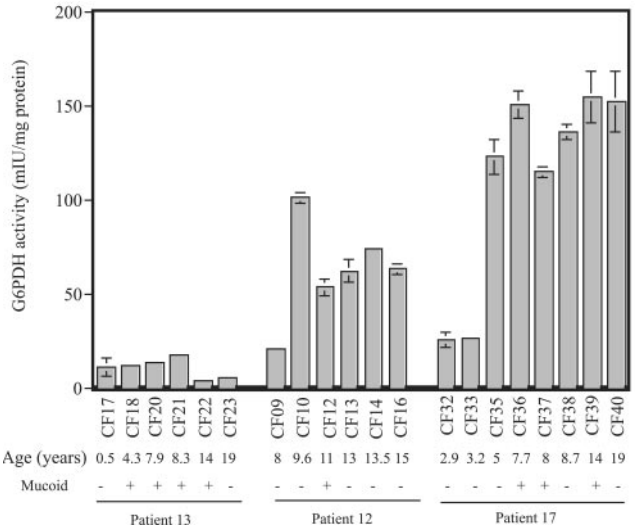

FIG. 6. Comparison of sequential isolates for unregulated G6PDH activity. G6PDH activity was measured from cells grown to an OD_{600} of approximately 1.0 in LB. The data represent the averages (±standard deviations) of two independent experiments conducted in duplicate. The approximate ages of the patients from which the isolates were collected are shown beneath the graph. The mucoid phenotypes of the isolates as observed on agar plates are shown.

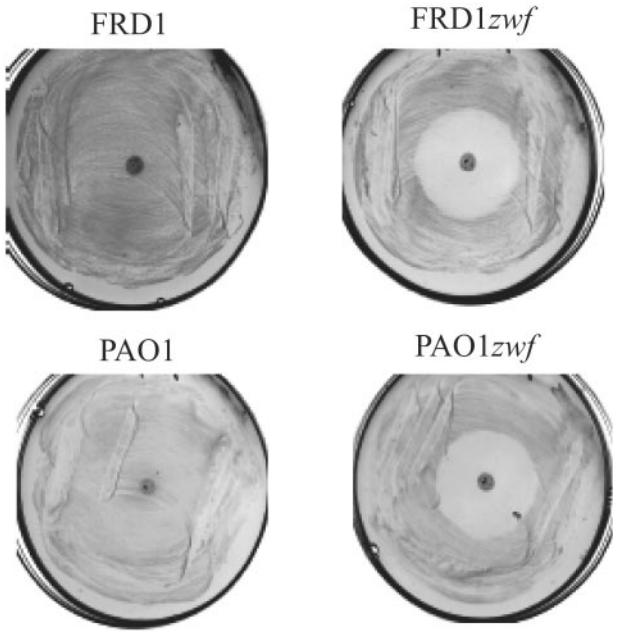

FIG. 7. Effect of sputum on the growth of *P. aeruginosa*. NCE agar plates were seeded with *P. aeruginosa* and centrally inoculated with sputum. Shown is growth of the bacteria following an overnight incubation at 32°C.

tages (besides alginate production) for *P. aeruginosa* while growing within the CF lung.

G6PDH activity in sequential CF clonal isolates shows selection in vivo for unregulated *zwf*. Three sets of sequential *P. aeruginosa* isolates that had been collected from individual CF patients were obtained (21). RAPD analysis of these isolates indicates that the isolates are clonal from each patient but different between patients (data not shown). We observed that all of the isolates recovered from patient 13 retained low levels of G6PDH activity throughout the infection (Fig. 6). In contrast, the early isolates from patients 12 and 17 displayed low levels of G6PDH activity, while the later isolates displayed high levels of G6PDH activity, suggesting a conversion in vivo to the higher production of G6PDH. From patient 12, we observed a moderate increase in G6PDH activity between the ages of 8 and 9.6 years, while from patient 17, we observed a ~5-fold increase between the ages of 3.2 and 5. In both patient 12 and patient 17, the levels of G6PDH activity remained high in isolates from subsequent years (Fig. 6) following the conversion. Interestingly, in both patients 12 and 17 the conversion to high levels of G6PDH activity may have preceded the conversion to a dominantly mucoid phenotype, suggesting that increased G6PDH confers some selective advantage to these isolates within the CF lung.

A defect in *zwf* causes sputum sensitivity. Previous studies suggest an association between G6PDH and alginate in protecting *P. aeruginosa* from oxidative stress (18, 38). However, in this study, FRD1 and FRD1*zwf* showed equal sensitivity to paraquat and hydrogen peroxide (data not shown). In that the FRD1*zwf* mutant was partially defective for alginate production, this suggests that neither excess alginate nor G6PDH activity contributed to increased resistance to oxidative stress in FRD1 under these laboratory-tested conditions.

In an attempt to explore the selection for high-level G6PDH activity in *P. aeruginosa* while in the CF lung environment, we tested the effect of lung sputum on the growth of several *P. aeruginosa* isolates. We observed that lung sputum, either isolated from CF or non-CF individuals, inhibited the growth of FRD1*zwf* and PAO1*zwf* mutants but not the parental strains (Fig. 7). Complementation of *zwf* in *cis* restored the ability of PAO1 and FRD1 to grow in the presence of sputum (data not shown). The inhibitory effect appeared to be specifically associated with loss of *zwf*, in that a variety of other FRD1 mutants were not inhibited by sputum, including *algT*, *algD*, and *crc* mutants (data not shown). In that G6PDH appears to be required for maximum protection from lung sputum, it is plausible that the selection for deregulated high-level G6PDH activity in the CF lung may be associated with the phenomenon. Identification of the specific inhibitor in sputum or whether growth on sputum leads to a build up of a toxic intermediate within the *zwf* mutants is currently under investigation.

DISCUSSION

The Entner-Doudoroff pathway is an alternative to the Emden-Meyerhof glycolytic pathway in a diverse group of bacteria, including *Pseudomonas* sp., and it provides both energy and metabolic precursors for many biosynthetic processes. Here, we established that a mutation in *zwf* (encoding G6PDH, the first enzyme of the Entner-Doudoroff pathway) leads to a ~90% reduction in alginate production in the mucoid, CF isolate of *P. aeruginosa*, FRD1. This effect was presumably due to a reduction in the pool of fructose-6-phosphate, the primary precursor of alginate. Consistent with this model is the observation that there were no differences between FRD1 and

FRD1*zwf* for expression on an *algD-lacZ* transcriptional fusion over a growth cycle (data not shown). Mucoid strains produce large amounts of alginate, which is expensive in terms of carbon and energy, and so we began to explore the possibility that mucoid strains contain increased levels of G6PDH to supply sufficient amounts of precursor for the alginate pathway. However, an *algD* mutation in FRD1, which blocked the biosynthetic pathway for alginate, did not significantly affect G6PDH levels, indicating that this drain on the pool of metabolic sugar precursors did not affect G6PDH levels. Also examined was a FRD1 derivative with a mutation in *algT*, encoding sigma-22, the master regulator of alginate biosynthesis, and this defect did reduce G6PDH by ~30%. Also tested was the effect of a *mucA* mutation in strain PAO1, which inactivated the anti-sigma factor of sigma-22 to increase the level of active sigma-22 in the cell and results in alginate gene activation. A modest increase in G6PDH activity was observed in mucoid PAO1*mucA* compared to the wild type. Thus, sigma-22 apparently has a small role in the induction of *zwf* expression in *P. aeruginosa*, which is likely to be indirect because no obvious sigma-22 consensus sequence could be identified upstream of the *zwf* coding region.

The striking observation from the data described above was that the levels of G6PDH activity and *zwf* expression were both severalfold higher in FRD1 than in PAO1 background strains when grown in L broth. Although caution is always advised when making interstrain comparisons, this led us to examine whether *zwf* in strain FRD1 was still subject to catabolite repression as it is in PAO1. As expected for PAO1, levels of *zwf* expression and G6PDH activity were high following growth with glycerol but low with succinate or glycerol plus succinate, as previously described (46). However, in FRD1, levels of *zwf* expression and G6PDH activity remained high with succinate or glycerol plus succinate, suggesting that *zwf* expression was possibly deregulated and constitutively expressed. Although a mutation affecting Crc or its expression would produce these phenotypes (46), no evidence could be found here to support a Crc defect in FRD1. However, we cannot rule out the possibility that Crc activity is modulated in *P. aeruginosa* as it is in *Pseudomonas putida* (34). Future studies have been initiated to characterize Crc in FRD1 and other CF isolates of *P. aeruginosa*.

Unexpectedly, we found that relaxed control of *zwf* was not unique to FRD1 but was actually common to CF isolates. Among the 10 CF isolates tested, 5 showed high G6PDH when grown with succinate as the major carbon source. In contrast, all of the other five clinical or six environmental isolates showed normal catabolite repression of G6PDH with succinate. Although the sample of strains was small, the results clearly suggest that adaptation to the CF lung environment selects for a defect in the repression of *zwf* expression and may extend to other genes as well. Fortuitously, several sets of clonal isolates that had been collected from individual CF patients over a number of years were available (21). Analysis of these isolates showed that in two of three patients, the resident *P. aeruginosa* strain underwent an adaptation to promote deregulated *zwf* expression. Thus, we hypothesized that a high level of G6PDH activity provides a survival advantage to *P. aeruginosa* within the lung environment because deregulated *zwf* variants predominate and persist in vivo following conversion. This is similar to the mucoid phenotype of *P. aeruginosa*, which usually arises in the CF lung by adaptive mutation in *mucA*, although its predominance is not always immediate upon conversion (21, 39).

Based on in vitro data shown here, *zwf* expression in *P. aeruginosa* is required for resistance to human sputum. A simple plate test was developed which dramatically showed that G6PDH was necessary in both the FRD1 and PAO1 strain backgrounds to prevent growth inhibition. Thus, up-regulation of *zwf* may protect *P. aeruginosa* within the CF lung from some factor found in human sputum. The FRD1*zwf* did not become hypersensitive to paraquat or hydrogen peroxide, suggesting that the selection for increased G6PDH activity in CF isolates was not related to oxidative stress resistance. Also, nonmucoid FRD1*algT* and FRD1*algD* mutants did not show increased sensitivity to CF sputum, so reduced alginate levels associated with *zwf* mutation were not associated with sputum sensitivity. Alternatively, a substance in sputum may cause the accumulation of a toxic intermediate in the *zwf* mutants. Future studies will attempt to identify the substance in sputum that produced this phenotype.

The list of reported differences between CF isolates and other clinical isolates of *P. aeruginosa* is rapidly expanding. Within the CF lung, *P. aeruginosa* acquires multiple phenotypic and genotypic changes, including alterations and attenuation of several virulence factors, such as reduced ADP-ribosylating activity of exotoxin A, loss of motility, loss of O antigen in lipopolysaccharide, increased auxotrophy, antibiotic resistance, defects in type III secretion, and reduced production of proteases and phospholipase C (4, 6, 9, 16, 17, 22, 26, 43, 48). Furthermore, CF isolates of *P. aeruginosa* appear to utilize a different set of virulence determinants and pathogenic mechanisms to cause disease than non-CF isolates (37). Presumably, the hostile environment of the CF lung not only induces mutations in *P. aeruginosa* but also selects those mutants best able to survive and persist. Therefore, it is not unreasonable to expect that basic metabolic activities, such as regulation of carbon catabolism, might also be altered in CF isolates of *P. aeruginosa*, as demonstrated in this study. However, because basic metabolic processes and not just virulence determinants are altered in CF *P. aeruginosa*, these isolates may respond differently than non-CF isolates to treatments that were developed for non-CF isolates. Thus, in order to develop more effective treatments for pulmonary infections in CF patients, it is important to further characterize the physiology and metabolism of CF isolates of *P. aeruginosa*.

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