Static growth of mucoid Pseudomonas aeruginosa selects for non-mucoid variants that have acquired flagellum-dependent motility

Timna J. O. Wyckoff,1† Brittany Thomas,1 Daniel J. Hassett2 and Daniel J. Wozniak1

Author for correspondence: Daniel J. Wozniak. Tel: +1 336 716 2016. Fax: +1 336 716 9928. e-mail: dwozniak@wfubmc.edu

When mucoid (alginate-producing) Pseudomonas aeruginosa FRD1 is grown under low oxygen conditions in liquid culture (static), non-mucoid variants appear and eventually predominate. This conversion is not readily observed in aerobic, shaken cultures or static cultures containing the alternative electron acceptor nitrate. In this study, it is shown that the non-mucoid variants that arise under static growth conditions are almost exclusively algT mutants. It has been shown that AlgT not only positively regulates alginate biosynthesis, but also directly or indirectly negatively regulates flagellum synthesis. Indeed, during static growth, conversion to the non-mucoid phenotype is accompanied by the acquisition of flagellum-mediated motility. Surprisingly, by using a reporter gene fusion with the fliC promoter (pfliC::xylE), it was found that fliC expression begins within hours of static growth and is reversible after returning the culture to shaking conditions. The ability of the strain to produce alginate seems to be irrelevant to this phenomenon, as an AlgT ΔalgD strain showed identical results. Thus, it is suggested that the first effect of static growth is to induce motility as an adaptive measure in the presence of wild-type algT. This may afford P. aeruginosa the ability to swim towards areas of higher oxygen concentrations. Subsequent to this, algT mutations are likely to secure the motile phenotype.

Keywords: aerotaxis, oxygen, AlgT, alginate

INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous Gram-negative organism that is an opportunistic human pathogen. It can cause serious infections in burn victims and in post-surgical wounds. P. aeruginosa also causes chronic respiratory infections in cystic fibrosis (CF) patients. Many strains of P. aeruginosa isolated from the lungs of CF patients produce a viscous exopolysaccharide called alginate. The amount of alginate-producing, or mucoid, P. aeruginosa in a particular infection correlates with the risk of mortality (Govan & Deretic, 1996). All P. aeruginosa strains carry the genes that encode the regulatory and biosynthetic machinery for alginate production, but these genes are normally not activated (Goldberg et al., 1993). Unknown conditions in the CF airway select for mutations that lead to the production of alginate. For example, the P. aeruginosa CF isolate FRD1, as well as many other mucoid CF-derived strains, is mucoid due to a mutation in mucA (Devrie & Ohman, 1994; Martin et al., 1993). The MucA protein normally antagonizes AlgT, an alternative sigma factor essential for alginate gene expression, so mucA mutations result in constitutive alginate production in many CF isolates (Govan, 1975; Mathee et al., 1997). Other alterations associated with strains isolated from CF patients include a conversion from smooth to rough lipopolysaccharide structure and loss of motility (Govan & Deretic, 1996; Luzar et al., 1985; Mahenthiralingam et al., 1994; Martin et al., 1993).

When mucoid P. aeruginosa is grown aerobically in liquid culture with shaking, the alginate phenotype is...
quite stable. However, previous studies have shown that under certain conditions, such as static growth that results in low oxygen tension, the mucoid phenotype is unstable (Govan et al., 1979; Hassett, 1996; Ohman & Chakrabarty, 1981). Under these growth conditions, conversion from mucoid to non-mucoid occurs primarily via second-site algT mutations (Devries & Ohman, 1994).

Our laboratory has shown that AlgT not only positively regulates alginate biosynthesis, but also directly or indirectly negatively regulates flagellum synthesis (Garrett & Wozniak, 1999). This led us to hypothesize that the algT mutations that convert mucoid strains to non-mucoid under low oxygen conditions also promote motility. Motility may then allow taxis toward areas of higher oxygen tension (aerotaxis), where maximal ATP production can occur. In support of this, previous studies demonstrated that the mucoid phenotype is stabilized in static growth conditions when the alternative electron acceptor nitrate is added (Hassett, 1996).

In this paper we explore the possibility that the low oxygen environment created by static growth is an important determinant that allows populations to maintain their mucoid phenotype. We first show that non-mucoid isolates emerging from the static growth of P. aeruginosa FRD1 are indeed motile. A complementation experiment verified that the majority of these motile variants are algT mutants. We then used a reporter gene fusion with the flagellin promoter (pflC::xylE) to demonstrate that the algT mutations that convert mucoid strains to non-mucoid under low oxygen conditions also promote motility. Motility may then allow taxis toward areas of higher oxygen tension (aerotaxis), where maximal ATP production can occur. In support of this, previous studies demonstrated that the mucoid phenotype is stabilized in static growth conditions when the alternative electron acceptor nitrate is added (Hassett, 1996).

**METHODS**

**Bacterial strains and growth conditions.** The P. aeruginosa strains used in this study all originate from either PA01 or FRD1 (CF isolate, muaA22; Ohman & Chakrabarty, 1981). Plasmid DNA manipulations were done using Escherichia coli strain JM109 (Promega). P. aeruginosa strains were cultured in LBNS (Luria broth, no NaCl, 10 g tryptone l⁻¹, 5 g yeast extract 1⁻¹) or on LBNS agar plates (LBNS + 15 g agar 1⁻¹). When appropriate, antibiotics were used as follows: tetracycline, 100 µg ml⁻¹; carbenicillin, 250 µg ml⁻¹; irgasan, 25 µg ml⁻¹. E. coli strains were cultured in LB (10 g tryptone l⁻¹, 5 g yeast extract 1⁻¹, 10 g NaCl l⁻¹) or on LB agar plates. When appropriate, antibiotics were used as follows: tetracycline, 10 µg ml⁻¹; ampicillin, 100 µg ml⁻¹. Media components were from Difco and antibiotics were from Sigma.

**General recombinant DNA techniques.** Recombinant DNA techniques were done as described by Ausubel et al. (1992) and Maniatis et al. (1982). Plasmid DNA was prepared using Qiagen reagents. Restriction endonucleases, Klenow, T4 DNA ligase and shrimp alkaline phosphatase were from Promega and were used according to the manufacturer’s specifications. Taq polymerase was from Perkin Elmer. DNA was extracted from gels using Qiagen Qiaquick gel extraction reagents.

**Plasmid and strain constructions.** FRD1400 (FRD1 attB::pflC::xylE) and WFPA227 (PAA1 attB::pflC::xylE) were constructed using the mini-CTX1 system as described elsewhere (Hoang et al., 1996; Wyckoff & Wozniak, 2001). Briefly, plasmid pTW13, a mini-CTX1-derived vector harbouring pflC::xylE, was developed. We used PCR with primers fliCrev (5’-GGGAGGCTCGAGGTTGCCCCGAGATTCGCG-3’) and fliCfwd (5’-GTTTCCCCAAGCTTCTGGAAGCCGG-TGGCG-3’) to amplify the 260 to +22 region of fliC based on the fliC sequence described by Stover et al. (2000). This fragment contains 5’ regulatory sequences as well as the fliC promoter (Totten et al., 1990). The xylE gene was obtained by cleavage of pX918G (Schweizer & Hoang, 1995) with XhoI and KpnI. The HindIII–XhoI fliC fragment and the XhoI–KpnI fragment harbouring the promoterless xylE and the adjacent aacC1 gene (encoding gentamicin resistance) were cloned into HindIII/KpnI-cleaved mini-CTX. PTW13 was introduced into both PA01 and FRD1 by a biparental mating of the P. aeruginosa strain, E. coli JM109 (PTW13) and E. coli helper strain HB101 (PTW13) (Figurski & Helsinki, 1979). Unwanted plasmid sequences were then removed using Flip recombinase (Hoang et al., 1998; Wyckoff & Wozniak, 2001). Appropriate PCR controls using combinations of attB or fliC or mini-CTX primers were performed as described previously (Wyckoff & Wozniak, 2001) to confirm integration of pflC::xylE at the attB site of the P. aeruginosa chromosome and removal of flanking plasmid sequences.

Plasmid pSW195, which contains an arabinose-inducible algT allele in the vector mini-CTX, was constructed by cloning an EcoRI–HindIII fragment containing algT from pWG13 (Garrett & Wozniak, 1999) into EcoRI/HindIII-treated pSW161 (Woolwine et al., 2001), resulting in pSW186. Next, a 1 kb KpnI–HindIII fragment from pSW186 encompassing araC–P<sup>BAD</sup>–algD was cloned into KpnI/HindIII-treated mini-CTX. Plasmid pSW195 was integrated at the neutral attB locus as described above and elsewhere (Hoang et al., 1998; Wyckoff & Wozniak, 2001). For complementation studies, non-mucoid variants arising from static growth were examined on LANS containing 2.5% (w/v) arabinose.

Plasmid pDJW487 (ΔalgD::Ωtet) was used for mutagenesis of algD in strain FRD1400 using allelic exchange techniques as described by Ma et al. (1998). Plasmid pDJW487 was generated by subcloning a 2 kb Smal fragment containing the Ωtet cassette from pHPS4Q-tet (Fellay et al., 1987) into a ΔalgD allele contained in the gene replacement vector pEX100T (Schweizer & Hoang, 1995). Plasmid pDJW487 was transformed into the E. coli helper strain SM10 (Hoang et al., 1998) and then transferred into FRD1400 by a biparental mating of FRD1400 and SM10(pDJW487). The ΔalgD::Ωtet allele of pDJW487 was used to replace the wild-type algD gene of FRD1400, resulting in strain FRD1402 (FRD1 attB::pflC::xylE ΔalgD::Ωtet). All allele replacements were confirmed by either PCR or Southern blot analyses.

**Static growth experiments.** For an experiment comparing shaking and static growth conditions, an overnight (shaking) culture was diluted 1:100 in LBNS and several 5 ml aliquots were transferred to 18 x 150 mm culture tubes. These 5 ml cultures were incubated at 37 °C either vertically in test tube racks (static growth) or placed in a roller (shaking growth). At each time point, a single tube from each treatment was
removed from the incubator, briefly vortexed and portions were removed for catechol-2,3-dioxygenase assays. Western blotting and/or observation by microscopy. Each time point represents a separate culture tube, i.e. static cultures, once vortexed, were not returned to the incubator – at the next time point, samples were taken from an undisturbed tube. The only exception to this was when static cultures were sampled at 4 h and then shaken for an additional 4 h.

**Western blotting.** Western blot analysis was performed using whole cells or purified flagella. Whole-cell samples were prepared from *P. aeruginosa* strains grown in 10 ml LBNS at 37 °C to an OD_{540} of 0.4 or under the conditions described for the specific experiment. Cultures were centrifuged (5000 g, 10 min) and pellets (whole-cell fraction) were resuspended in 1 ml 50 mM potassium phosphate (pH 7.1) was centrifuged and resuspended in 1 ml 50 mM 2001). Briefly, an appropriate amount of cell culture (usually 1 ml) was centrifuged and resuspended in 1 ml 50 mM potassium phosphate (pH 7.5) and 2% of the original culture volume in FB (10 mM Tris/HCl, pH 8.0, 100 mM NaCl, 1 mM MgCl₂). A 10 μl sample of this preparation was analysed by Western blotting. Anti-flagellum type B antiserum (Garrett & Wozniak, 1999) was used in Western blots at a dilution of 1:10000 with chemiluminescent reagents by procedures outlined by the manufacturer (Amer sham) and film was exposed for 30 s prior to development.

**Catechol-2,3-dioxygenase assays.** The assay of XylE activity was performed as described previously (Wyckoff & Wozniak, 2001). Briefly, an appropriate amount of cell culture (usually 1 ml) was centrifuged and resuspended in 1 ml 50 mM potassium phosphate (pH 7.5) and 10% (v/v) acetone. An aliquot (990 μl) of this sample was combined with 10 μl freshly made 100 mM catechol in water. The A_{25} was recorded for 2 min. Enzyme activity was calculated using the extinction coefficient of the reaction product 2-hydroxymuconic semialdehyde (ε_{235} = 4.4 × 10⁴ M⁻¹) and corrected for the number of cells by dividing by the OD_{540} of the sample.

**Motility assays.** Motility assays were performed by inoculating a single colony into 0.3% (w/v) LANS. Following overnight growth at 37 °C, motility was assessed qualitatively by examining colonies which spread beyond the point of inoculation (Arora et al., 1998).

**RESULTS**

**Non-mucoid variants obtained from static growth conditions acquire motility and flagellum production**

The mucoid phenotype of *P. aeruginosa* FRD1 is stable for days when the strain is maintained aerobically in liquid culture with shaking. However, when the bacteria are grown statically in liquid culture without shaking, approximately 50% become non-mucoid within 48 h (Hassett, 1996; this study). In one study, non-mucoid colonies arising from statically grown cultures acquired mutations in algT, the gene encoding an alternative sigma factor essential for alginate gene expression (Devries & Ohman, 1994). It has been proposed that the selection to maintain an energy-expensive process such as alginate production favours this conversion (Devries & Ohman, 1994; Govan, 1975). In an earlier study, we observed an inverse correlation between alginate production and flagellum-mediated motility (Garrett & Wozniak, 1999). Interestingly, the negative control of motility in mucoid strains requires AlgT. We hypothesized that static growth of mucoid *P. aeruginosa* strains selects for algT mutants that acquire motility. This may afford such variants the ability to acquire oxygen, which is essential for growth under these conditions. To test this, we examined independent non-mucoid colonies after 24, 48, 72 and 96 h static growth of FRD1. Whole-cell extracts were prepared and a Western blot was prepared from *P. aeruginosa* strains grown in 10 ml LBNS at 37 °C to an OD_{540} of 0.4 or under the conditions described for the specific experiment. Cultures were centrifuged (5000 g, 10 min) and pellets (whole-cell fraction) were resuspended in 1 ml 50 mM potassium phosphate (pH 7.1) was centrifuged and resuspended in 1 ml 50 mM 2001). Briefly, an appropriate amount of cell culture (usually 1 ml) was centrifuged and resuspended in 1 ml 50 mM potassium phosphate (pH 7.5) and 2% of the original culture volume in FB (10 mM Tris/HCl, pH 8.0, 100 mM NaCl, 1 mM MgCl₂). A 10 μl sample of this preparation was analysed by Western blotting. Anti-flagellum type B antiserum (Garrett & Wozniak, 1999) was used in Western blots at a dilution of 1:10000 with chemiluminescent reagents by procedures outlined by the manufacturer (Amer sham) and film was exposed for 30 s prior to development.

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**RESULTS**

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performed with antiserum raised against flagella purified from *P. aeruginosa* PAO1 (Garrett & Wozniak, 1999). As described previously (Garrett & Wozniak, 1999), synthesis of a flagellum is inhibited in mucoid strains of *P. aeruginosa* such as FRD1 (Fig. 1a, lane 1) whereas an isogenic algT mutant is motile and expresses a flagellum (Fig. 1a, lane 2). Flagellum expression was observed in all of the non-mucoid strains examined in this assay (Fig. 1a, lanes 3–14). In addition, all non-mucoid strains tested exhibited flagellum-dependent swimming motility on 0·3% (w/v) agar plates (Fig. 1b). Scanning electron micrographs of 12- and 48-h static cultures of FRD1 demonstrate the appearance of flagellum-producing variants at the latter time point (data not shown). Thus, growth of mucoid *P. aeruginosa* FRD1 in static conditions selects for non-mucoid variants that synthesize a flagellum and are motile.

### Non-mucoid variants that acquire motility and flagellum production during static growth conditions are predominantly algT mutants

To determine if the non-mucoid strains isolated by static growth were algT mutants, an arabinose-inducible algT allele from pSW195 was introduced into each of the non-mucoid variants described above. This plasmid contains a mini-CTX origin, so integration occurs through site-specific recombination of the att sequences present in pSW195 and chromosomal att sequences (Hoang *et al.*, 1998; Wyckoff & Wozniak, 2001). Ectopic expression of AlgT can then be achieved by growth on plates containing 2.5% (w/v) arabinose. Based on several independent static growth experiments we determined that 93% of the non-mucoid colonies arising during static growth were algT mutants (data not shown). Further analyses of the remaining class of mutants (7% of the population) will be the subject of another study. Data from representative non-mucoid variants isolated from each day of static growth (24, 48, 72 and 96 h), as well as a spontaneous non-mucoid algT mutant, FRD2, or the algT::Tn501 mutant FRD440 (Flynn & Ohman, 1988; Ohman & Chakrabarty, 1981; Wozniak & Ohman, 1994), each containing a chromosomal pBAD-algT allele, were cultured in the absence or presence of 2–5% (w/v) arabinose. In all cases, alginate production (as demonstrated by the mucoid phenotype) was restored upon growth with arabinose, indicating complementation.

Figure 2. Non-mucoid variants that acquire motility and flagellum production during static growth conditions are algT mutants. (a) Representative non-mucoid variants isolated from each day of static growth (24, 48, 72 and 96 h), as well as a spontaneous non-mucoid algT mutant, FRD2, or the algT::Tn501 mutant FRD440 (Flynn & Ohman, 1988; Ohman & Chakrabarty, 1981; Wozniak & Ohman, 1994), each containing a chromosomal pBAD-algT allele, were cultured in the absence or presence of 2·5% (w/v) arabinose. In all cases, alginate production (as demonstrated by the mucoid phenotype) was restored upon growth with arabinose, indicating complementation. (b) The same representative cultures from (a) grown in the presence or absence of arabinose, were analysed for flagellum production by Western blotting with flagellum antiserum (see Methods). The non-mucoid variants express a flagellum in the absence of AlgT, while arabinose induction of AlgT reverses flagellum expression.
and flagellum production during static growth conditions are indeed algT mutants.

**Oxygen gradient sensing is likely to be the signal for inducing motility under static growth conditions**

*P. aeruginosa* is capable of growth via anaerobic respiration (denitrification) in L-broth amended with nitrate, but cannot grow in L-broth alone under anaerobic conditions due to an absence of a terminal electron acceptor. We previously showed that anaerobic incubation of *P. aeruginosa* FRD1 in L-broth or L-broth containing 100 mM KNO₃ (LBN) triplicate samples were incubated under the following conditions: 1, LBN anaerobic growth; 2, LBN aerobic static growth; 3, L-broth aerobic static growth; 4, L-broth aerobic growth at 50 r.p.m.; 5, L-broth aerobic growth at 50 r.p.m.; 6, L-broth aerobic growth at 300 r.p.m.; 7, LBN aerobic growth at 50 r.p.m.; 8, LBN aerobic growth at 150 r.p.m.; 9, LBN aerobic growth at 300 r.p.m. At 24 h intervals, the bacteria were shaken to evenly disperse the cells, dilutions were plated on L-agar plates and the percentage mucoid colonies was enumerated after 24 h growth at 37 °C.

![Graph](image)

*Fig. 3. Oxygen gradient sensing is likely to be the signal for inducing motility under static growth conditions. P. aeruginosa FRD1 was grown with vigorous aeration for 24 h at 37 °C. The bacteria were diluted 100-fold in 150 ml Erlenmeyer flasks containing 10 ml of either L-broth or L-broth containing 100 mM KNO₃ (LBN). Triplicate samples were incubated under the following conditions: 1, LBN anaerobic growth; 2, LBN aerobic static growth; 3, L-broth aerobic static growth; 4, L-broth aerobic growth at 50 r.p.m.; 5, L-broth aerobic growth at 150 r.p.m.; 6, L-broth aerobic growth at 300 r.p.m.; 7, LBN aerobic growth at 50 r.p.m.; 8, LBN aerobic growth at 150 r.p.m.; 9, LBN aerobic growth at 300 r.p.m. At 24 h intervals, the bacteria were shaken to evenly disperse the cells, dilutions were plated on L-agar plates and the percentage mucoid colonies was enumerated after 24 h growth at 37 °C.*

**pfliC promoter activity increases before non-mucoid variants appear**

The above results suggested that low oxygen tension conditions, such as those encountered during static growth, may serve as a signal to induce motility in *P. aeruginosa*. To address this, we sought evidence for flagellum expression at earlier time points during static growth. The fliC gene encodes flagellin, the major structural protein of the flagellum. The fliC promoter, pfliC, is thus a good choice for monitoring transcriptional control of flagellum synthesis. We created strain FRD1400, which is FRD1 with a pfliC::xylE fusion at the neutral attB site (see Methods). xylE encodes catechol-2,3-dioxygenase, a commonly used reporter enzyme (see Methods).

Using FRD1400 we monitored pfliC activity during 48 h of shaking or static growth. As expected, the mucoid and non-motile phenotypes of FRD1400 were stable for 48 h under shaking conditions. The results for pfliC expression in static conditions were somewhat surprising. While non-mucoid colonies did not appear until the 48 h static culture, pfliC activity was increased much earlier, even while all the cells were mucoid (100% mucoid; pfliC expression = 6.6 nmol min⁻¹ per 10⁸ cells). However, we tested numerous mucoid colonies from the 24 h statically grown culture and found no individual mucoid variants with pfliC activity. This suggested that growth under static conditions enhanced fliC expression and motility. As no non-mucoid colonies could be detected in this experiment, fliC expression was occurring even in the presence of active AlgT.

The above result led us to hypothesize that the increase in pfliC activity was due to an adaptive response rather than a mutation in algT. We tested this hypothesis by examining the time course and reversibility of the increase in pfliC activity. We grew two cultures, each of FRD1400, under static and shaking conditions. After 4 h, we assayed the pfliC activity of one culture of each treatment (FRD1400 static, FRD1400 shaking). We then placed the two 4 h static cultures under shaking conditions. After 4 h, we assayed the cultures that had been growing for a total of 8 h under the same treatment, as well as the 4 h static cultures that had then been shaking.
for 4 h. As expected, the AlgT+ strain FRD1400 had very little pflIC activity under shaking conditions (Fig. 4a). However, when FRD1400 was cultured statically for as little as 4 h, pflIC activity was detected. Most importantly, the pflIC activity observed under static growth conditions was completely reversible when the statically grown cultures were transferred back to shaking conditions (Fig. 4a).

**Increase in pflIC activity during static growth is independent of the mucoid phenotype**

We were interested in determining whether the mucoid phenotype of FRD1400 has any effect on pflIC activity during static growth. To this end, we created strain FRD1402, which is FRD1400 with a ΔalgD::Ωtet allele (see Methods). This strain has a wild-type algT, but is non-mucoid due to the ΔalgD::Ωtet mutation. During static growth, the FRD1402 pflIC activity profile is similar to that of FRD1400 (Fig. 4a). This suggests that the motile phenotype of FRD1400, which is induced upon static growth, is reversible and independent of the alginate phenotype.

For the experiments described in Fig. 4(a), at each time point that we assayed for pflIC activity, we also examined each culture for flagellum expression via Western blotting. This provides an independent assay for the observed differences in static versus shaken cultures. A representative Western blot from the samples examined in Fig. 4(a) is depicted in Fig. 4(b). The results from the Western blot correlated with the pflIC-xylE activity assay results. In addition, we observed a droplet of each culture by microscopy and observed no motility in cultures with low pflIC activity or flagellum expression, but active motility in cultures with high pflIC activity. These results taken together suggest that FRD1400 is able to adapt to static growth within hours by inducing flagellum synthesis, but is able to turn off flagellum synthesis rapidly when it is no longer needed, i.e. during growth with high oxygen tensions (shaken cultures). The short time course and reversibility of this change argues against selection for mutations under these conditions and in favour of an AlgT-independent method of flagellum regulation.

**DISCUSSION**

*P. aeruginosa* is a Gram-negative bacterium of significant medical importance. It is an opportunistic human pathogen that causes dangerous infections in the lungs of CF patients. These infections are particularly difficult to treat and one explanation may be that *P. aeruginosa* forms biofilms in CF patients’ lungs. Both flagellum synthesis and alginate synthesis have been implicated as being involved in the formation of *P. aeruginosa* biofilms (Davies et al., 1993; O’Toole & Kolter, 1998). Thus, a detailed understanding of these processes at the molecular level is potentially useful in the development of anti-biofilm treatments.

Our laboratory recently reported a link between alginate and flagellum synthesis. AlgT, a positive regulator of alginate biosynthesis, also negatively regulates flagellum synthesis (Garrett & Wozniak, 1999). It had previously been suggested that the algT mutations that accumulate in mucoid strains during static growth were selected for by the cells’ need to reduce their alginate production to better utilize the low levels of oxygen present under this growth condition (Hassett, 1996). The initial goal of this study was to determine if, rather, the algT mutations were selected for by the cells’ need to become motile to move to areas of higher oxygen tension where maximal ATP generation could occur.

We first showed that the algT mutations that lead to the non-mucoid phenotype do indeed also allow flagellum synthesis and motility (Fig. 1). At this point we could not distinguish between the possibilities that selection was for loss of alginate or gain of motility – either was equally likely. In the process of investigating our hypothesis further, however, we discovered a new regulation of flagellum synthesis.

When the mucoid strain FRD1 is grown statically, algT mutations begin to appear between 24 and 48 h (Hassett, 1996; this study). However, flagellum synthesis is evident much earlier (Fig. 4). Thus, under these condi-
tions, a flagellum is synthesized in the presence of wild-type AlgT, which is normally an inhibitor of the process. It is unlikely that under these conditions flagellum synthesis is bypassing AlgT repression via a mutation since synthesis begins in such a short time span and is reversible (Fig. 4). Thus, we suggest that flagellum synthesis can be expressed in an AlgT-independent manner to adapt to static growth conditions. Taken together, our results support a model in which non-motile, mucoid P. aeruginosa strains, when grown statically, are able to sense an oxygen gradient and rapidly turn on the synthesis of a flagellum (within minutes to hours of static growth). This would provide this population with the ability to swim towards areas where maximum ATP can be generated. This adaptive mechanism apparently occurs independent of normal AlgT inhibition. During prolonged exposure to low oxygen tension, there is a selection for algT mutations and the population eventually becomes predominated by motile, non-mucoid, algT mutants.

Of notable interest is the observation that the AlgT-independent induction of flagellum synthesis, seen during early static growth periods, does not affect the entire bacterial population. When observed by microscopy, a typical mucoid culture (e.g. strain FRD1) grown under shaking conditions has no motile cells. After 4 or 8 h of static growth, this culture shows significant pFIC activity (Fig. 4), yet only about 30–50% of the cells appear motile via microscopy. This may indicate that the AlgT-independent pathway is part of a phase-variable system of flagellum synthesis. In this regard, Deziel et al. (2001) reported that small (S) colony variants of P. aeruginosa strain 57RP that emerge during biofilm growth or under static growth conditions also have altered motility phenotypes. They concluded that these S variants result from phase variation and are selectively enriched when P. aeruginosa 57RP is grown as a biofilm or in static liquid cultures.

What is the signal that triggers flagellum biosynthesis during static growth? At the moment, the answer is unknown, yet important clues have surfaced during this study. We believe that flagellum biosynthesis in mucoid bacteria is triggered by an ability to sense and move toward an oxygen gradient, often referred to as aerotaxis. When mucoid bacteria are dispersed into static broth, they can sense an oxygen gradient where the highest levels are at the meniscus and the lowest are at the base of the tube. When the gradient is recognized, the bacteria synthesize a flagellum to swim toward the meniscus (highest pO2). This is likely to be why there is often a thick pellicle of organisms at the top of the tube after static growth. Conversely, they remain mucoid and non-motile when the oxygen gradient is dissipated, such as during static growth in the presence of nitrate. Similarly, the mucoid/non-motile phenotype is stable when mucoid bacteria are grown with shaking, since oxygen levels are essentially at equilibrium. Aerotaxis systems have been described in E. coli and P. putida (Nichols & Harwood, 2000; Rebbapragada et al., 1997; Taylor et al., 1999) and P. aeruginosa has a previously uncharacterized aer gene (PA1561) (Stover et al., 2000). We are in the process of disrupting this locus to determine if oxygen sensing is linked to aerotaxis and mucoid to non-mucoid conversion in P. aeruginosa. Alternative mechanisms, including energy taxis, chemotaxis or quorum sensing, may be involved in this process.

Finally, how might these results relate to mucoid P. aeruginosa in the CF airway? Although it is believed that the initial insult leading to alginate production and mucoidy is oxidative-stress-mediated mutations (Govan & Deretic, 1996; Hassett et al., 1993; Mathee et al., 1999), it is unknown why mucoid bacteria prevail and increase in population during the course of CF airway disease. P. aeruginosa can grow via anaerobic respiration if nitrate, nitrite or nitrous oxide is available as a terminal electron acceptor. Anaerobic growth using these compounds in vitro did not allow for mucoid to non-mucoid conversion, unlike the rapid conversion observed during aerobic static growth (Fig. 3; Hassett, 1996). Because copious amounts of nitrate and nitrite have been found in spuva of CF patients (Hassett, 1996), we postulate that strict anaerobiosis is one signal that stabilizes the mucoid phenotype and prevents mucoid to non-mucoid conversion. As mucoid populations increase with progression of CF airway disease (Govan & Deretic, 1996) and anaerobic pockets are likely to exist in the thick mucus lining the airways (Woltzsch et al., 2002), this condition may serve to maintain the mucoid phenotype for the remainder of the disease.

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