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Anaerobic Production of Alginate by *Pseudomonas aeruginosa*: Alginate Restricts Diffusion of Oxygen

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Pseudomonas aeruginosa **produced alginate and elevated *algD* (encoding GDPmannose 6-dehydrogenase) transcription under strict anaerobic conditions, especially when using nitrate as a terminal electron acceptor. Purified alginate added to bacterial suspensions caused a decrease in growth, suggesting that alginate contributes to oxygen limitation for the organism and likely for patients afflicted with the inherited autosomal disease cystic fibrosis.**

The production of the viscous exopolysaccharide alginate by *Pseudomonas aeruginosa* in the upper airways of patients afflicted with the inherited autosomal disease cystic fibrosis (CF) (4) leads to the progressive deterioration of the overall clinical condition of the patients. Alginate production in vitro is increased upon nutrient deprivation (20), elevated osmolarity (3), dehydration (ethanol) (7), addition of energy inhibitors (gramicidin and DCCD [*N*,*N*′-dicyclohexylcarbodiimide]) (21), and mutation (16). Oxygen is another factor which is considered essential for alginate production (2, 14). Although classified as an obligate aerobe, *P. aeruginosa* can respire anaerobically using nitrate, nitrite, or nitrous oxide as terminal electron acceptor (10) or can ferment arginine by substrate level phosphorylation (11). In this study, the capacity of *P. aeruginosa* to produce alginate under strict anaerobic conditions was investigated.

P. aeruginosa FRD1, a mucoid CF isolate (9), and its nonmucoid revertant FRD2 (*algT18*) (8) were grown aerobically in L broth (10.0 g of tryptone, 5.0 g of yeast extract, and 5.0 g of NaCl per liter [pH 7.2]) at 37°C with shaking at 300 rpm in Erlenmeyer flasks (volume/flask ratio, 1:10). Anaerobic L broth and L broth containing either 1% potassium nitrate or 15 mM sodium nitrite were prepared by first autoclaving the medium and then bubbling sterile nitrogen gas into it for 3 h. Anaerobic L broth was also made 40 mM with crystalline L-arginine and was filter sterilized in a Coy anaerobic chamber (<1 ppm O_2). Anaerobic and static cultures were incubated at 37°C. Cell extracts were prepared from cultures harvested by centrifugation at 10,000 × *g* for 10 min at 4°C. Bacteria were washed twice in ice-cold 50 mM sodium phosphate buffer, pH 7.0, frozen at −80°C, thawed at 37°C, and sonicated in an ice-water bath for 15 to 20 s with a Fisher Scientific Sonic Dismembrator Model 50 equipped with a microtip at an output setting of 20. Sonicates were clarified by centrifugation at 13,000 × *g* for 10 min at 4°C. Alginate was purified from *P. aeruginosa* FRD1 by ethanol precipitation of culture supernatants as previously described (17). Uronic acid alginate in dialyzed culture filtrates was assayed by the method of Knutson and Jeanes (12). Bacteria harboring pKK61 (24), an *algD-cat* transcriptional fusion plasmid, were grown in L broth containing 15 μg of tetracycline per ml. Chloramphenicol acetyltransferase levels in cell extracts were determined with an enzyme-linked immunosorbent assay kit as described by the manufacturer (5 Prime-3 Prime, Inc., Boulder, Colo.). Protein concentration was estimated by the method of Bradford (5).

P. aeruginosa **maintains mucoidy under anaerobic conditions.** *P. aeruginosa* FRD1 was streaked on L agar and L agar containing 1% potassium nitrate (an alternative terminal electron acceptor) and allowed to incubate at 37°C for 48 h. Mucoid bacteria were incapable of anaerobic growth on unsupplemented L agar (Fig. 1A). In contrast, bacteria grown on L agar-nitrate plates flourished and maintained their mucoid colony morphology (Fig. 1B). Growth on L agar plates containing 15 mM nitrite, although much slower than that of nitrate-grown bacteria, also afforded maintenance of mucoidy (data not shown). In contrast, growth on L agar plates supplemented with 40 mM arginine (23) did not allow for maintenance of mucoidy (data not shown).

Mucoidy is maintained over an extended anaerobic incubation. To determine if mucoidy could be maintained over a 4-day incubation, L broth and L broth amended with nitrate, nitrite, or arginine were inoculated with mucoid bacteria and monitored for growth (CFU per milliliter) and percent mucoid colonies. Growth of *P. aeruginosa* FRD1 was most rapid in aerobic L broth, less rapid in L broth containing nitrate, and still less rapid in nitrite- and arginine-containing cultures (data not shown). Organisms inoculated into unsupplemented anaerobic L broth did not replicate but maintained viability. As shown in Fig. 2, organisms that could not replicate (unamended anaerobic L broth) maintained 100% mucoidy throughout the experiment. In addition, anaerobic nitrate-grown organisms also maintained 100% mucoidy, while nitrite-grown bacteria maintained ~95% mucoidy. Aerobically grown bacteria maintained 100% mucoidy for 2 days, a level which decreased to ~88% after 4 days. Interestingly, bacteria grown under oxygen-limited conditions (static cultures) demonstrated a marked drop in mucoidy, with only 6% of the viable bacteria maintaining mucoidy after 4 days of incubation.

Alginate levels during oxygen versus nitrate respiration. To ensure that the mucoid phenotype was attributable to alginate, uronic acid alginate levels were monitored during a 3-day incubation of aerobically and anaerobically (nitrate-) grown bacteria. As shown in Fig. 3A, alginate production was greater when organisms were grown under aerobic conditions and levels increased linearly during the experiment, consistent with the results of Zielinski et al. (25) with mucoid strain 8830. Alginate production during denitrification was less than that by

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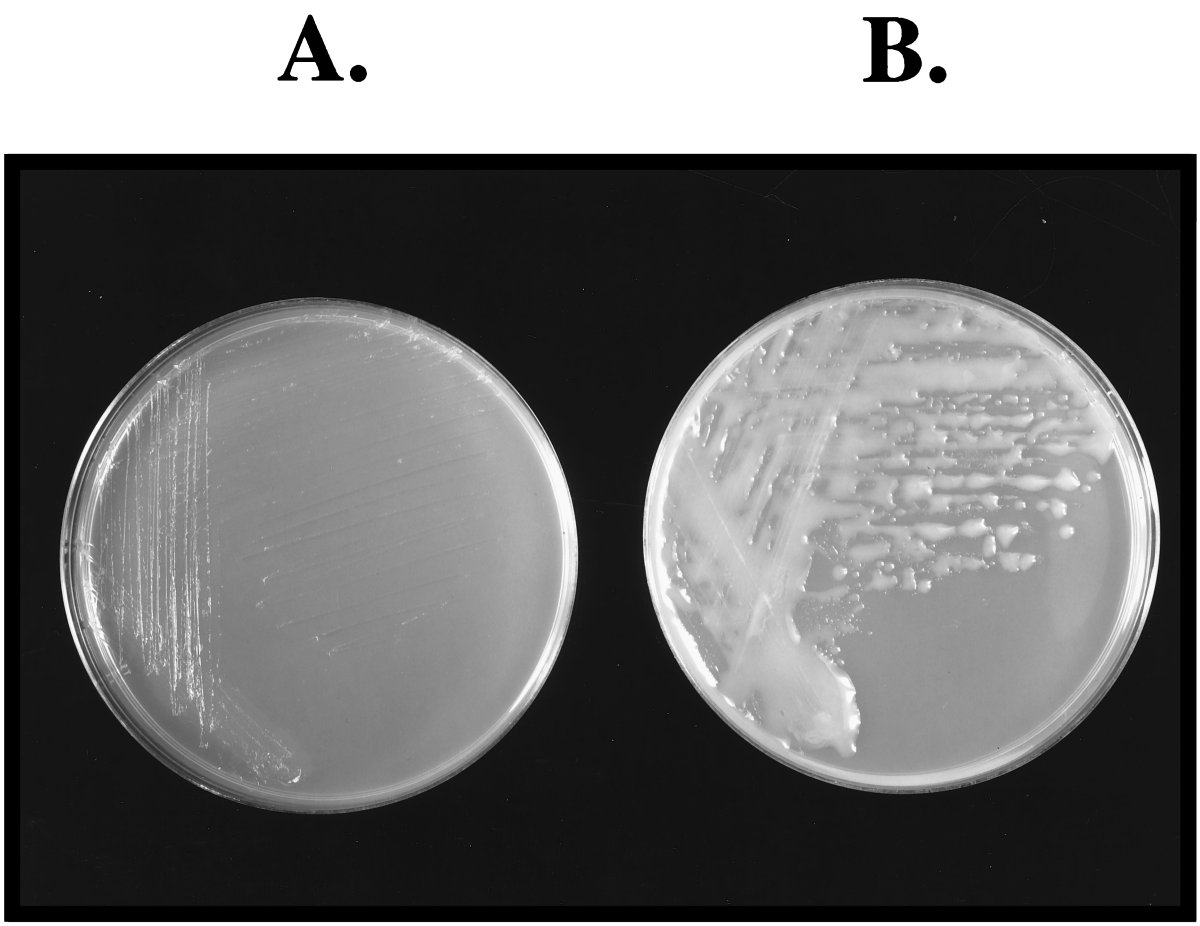

FIG. 1. Mucoid phenotype of *P. aeruginosa* FRD1 under anaerobic conditions. Mucoid bacteria were streaked on L agar (A) and on L agar amended with 1% potassium nitrate (B) and allowed to incubate in a Coy anaerobic chamber for 48 h at 37°C.

aerobically grown bacteria, but the 3-day alginate level for nitrate-grown bacteria (155 ± 10 μg/mg) was comparable to that for 1-day aerobically grown bacteria (155 ± 26 μg/mg). Alginate levels for aerobic nonmucoid bacteria (strain FRD2 [*algT18*] (8, 9) were less (~fourfold) than those for mucoid bacteria grown anaerobically in L broth containing nitrate.

To determine the influence of aerobic versus anaerobic conditions on alginate gene transcription, *algD-cat* activity was measured in bacteria grown under aerobic, static, and anaerobic conditions. As shown in Fig. 3B, *algD-cat* activity in aerobically grown mucoid bacteria was ~13,881 ng/mg (lane 1). In contrast, *algD-cat* activity for nonmucoid bacteria was only 756 ng/mg (lane 2). Interestingly, nitrate- and nitrite-grown organisms demonstrated greater *algD-cat* activity (21,161 ng/mg [lane 5] and 21,511 ng/mg [lane 6], respectively) than aerobically grown bacteria. Consistent with the rapid decrease in mucoid colony morphology of statically grown cultures (Fig. 2), the *algD-cat* activity for static cultures was only 3,792 ng/mg (lane 3) and *algD-cat* activity for anaerobic growth with L-arginine was only 4,371 ng/mg (lane 7). These data were also confirmed with *algD-xylE* fusion plasmid pAOM3 (*xylE* encoding catechol 2,3-dioxygenase) (13 and data not shown).

Alginate limits growth of *P. aeruginosa* in oxygen-limited culture. To determine whether *P. aeruginosa* alginate could limit diffusion of oxygen to the organism, thereby restricting growth, *P. aeruginosa* FRD1 was grown under static conditions in L broth containing either saline (control), a suspension into

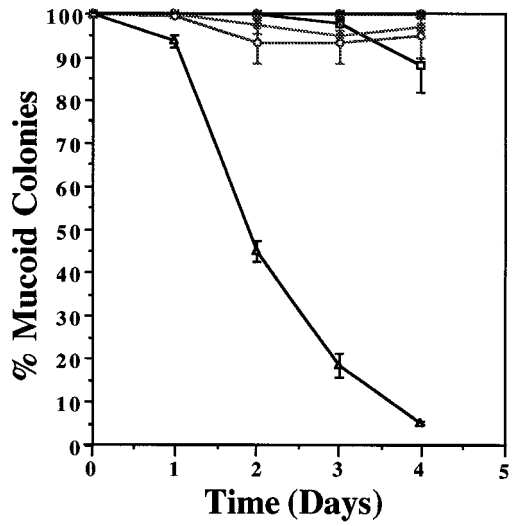

FIG. 2. Effect of a 4-day aerobic versus anaerobic incubation on mucoid colony morphology. Serially diluted L broth suspensions were assessed for percent mucoid colonies. The results are from a typical experiment performed in triplicate and are expressed as the means ± standard errors. □, aerobically grown bacteria; △, statically grown bacteria; ■, anaerobically grown bacteria; ▲, bacteria grown anaerobically with nitrate; ●, bacteria grown anaerobically with nitrite; ○, bacteria grown anaerobically with arginine.

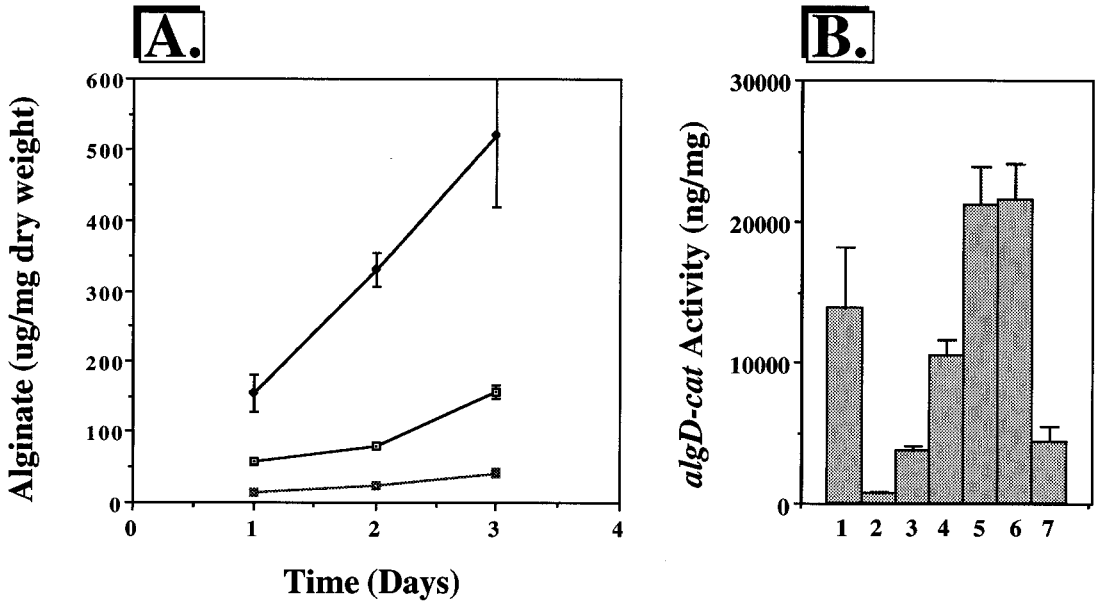

FIG. 3. Alginate production and *algD* transcription by *P. aeruginosa* grown aerobically and anaerobically. (A) Bacteria were grown aerobically or anaerobically in the presence of 1% potassium nitrate. Alginate in culture supernatants was quantified by the method of Knutson and Jeanes (12) and expressed as micrograms of alginate per milligram of cell dry weight. ◆, aerobically grown bacteria; □, bacteria grown anaerobically with nitrate; ■, nonmucoid bacteria grown aerobically. (B) Bacteria were grown under aerobic and anaerobic conditions for 48 h. *algD-cat* activities in diluted cell extracts were determined by extrapolation of a standard curve after normalizing for protein. The results are the means plus standard errors of three experiments. Bacterial growth conditions were as follows: lane 1, aerobic (mucoid); lane 2, aerobic (nonmucoid); lane 3, static; lane 4, anaerobic; lane 5, anaerobic with nitrate; lane 6, anaerobic with nitrite; lane 7, anaerobic with arginine.

which purified alginate had been mixed, or a suspension with alginate layered on top. As shown in Fig. 4, the final optical density at 600 nm (OD_{600}) of the L broth-saline control mixture was 2.85 (lane 1). Mixing alginate to a concentration of 0.4 mg/ml caused a decrease in the OD_{600} to 1.59 (lane 2). The most dramatic effect was observed when alginate was layered on top of the suspension; this rapidly depleted the suspension below of oxygen, with the final culture OD_{600} reaching only 0.372 (lane 3). When the same set of experiments was performed in L broth containing 1% potassium nitrate, the effect of the added alginate was not nearly as dramatic. Control bacteria reached a final OD_{600} of 2.67 (lane 4), while alginate mixed into the suspension or layered on top of the suspension caused only a 16% (OD_{600}, 2.24 [lane 5]) or 43% (OD_{600}, 1.52 [lane 6]) drop, respectively, in the final OD_{600}.

In this study, *P. aeruginosa* was found to be capable of alginate production during growth under strict anaerobic conditions while undergoing denitrification (with nitrate and, to a lesser extent, nitrite). Because alginate biosynthesis is an energy-intensive process (18), it is not surprising that denitrification supported this process since the theoretical energy generated in the form of ATP under such conditions is approximately half that produced during aerobic respiration (22). The reduced amount of alginate produced by nitrate-grown bacteria (24 to 37% of that produced by aerobically grown bacteria; Fig. 3A) is likely a function of suboptimal activities of several enzymes involved in alginate biosynthesis in the absence of oxygen (15). Still, the elevated anaerobic *algD-cat* activity in the presence of nitrate and nitrite (Fig. 3B) may be a function of the enhanced stability of the alginate transcriptional machinery under these conditions.

A hallmark of advanced CF is that patients suffer from hypoxia, with many requiring oxygen therapy (6). Thus, unlike normal airways, the CF airways likely possess areas with substantially reduced oxygen tension. Alginate production by *P. aeruginosa*, as well as viscous DNA released by lysed neutrophils (1), likely contributes to reduced oxygen tension in the airways, similar to the inhibitory effect of alginate on bacterial growth (Fig. 4). Interestingly, nitrate levels from CF sputa (n =

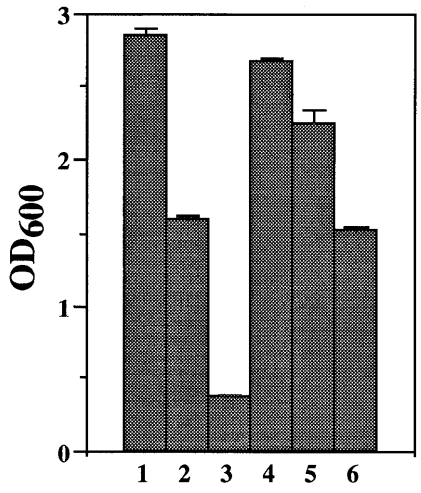

FIG. 4. Effect of alginate on growth of *P. aeruginosa* FRD1. Sterile borosilicate tubes (10 by 75 mm; Fisher Scientific) containing 1 ml of L broth (lanes 1 to 3) or L broth plus 1% potassium nitrate (lanes 4 to 6) were inoculated with 10 μl of an aerobically grown overnight culture of *P. aeruginosa* FRD1 and mixed. To one set of tubes (n = 3) was added 1 ml of 0.9% saline and the contents were mixed (lanes 1 and 4). A second set was amended with 1 ml of purified *P. aeruginosa* FRD1 alginate (0.8 mg/ml), and the contents were mixed (lanes 2 and 5). Last, 1 ml of purified alginate was layered on top of another set of bacterial suspensions without mixing (lanes 3 and 6). The tubes were incubated without agitation at 37°C for 17 h. The final OD_{600}s, after the cultures had been thoroughly mixed, were recorded, with OD_{600}s greater than 1.0 being diluted 10-fold prior to analysis.

10) were 383 ± 42 μM relative to 178 ± 42 μM detected in mucus from endotracheal tubes of patients without lung disease. Significant concentrations of nitrite (125 ± 55 μM) were also detected in CF sputa and were undetectable in normal mucus (22a). Because human neutrophils are abundant in the CF airways and are capable of producing nitrate and nitrite (19), these compounds could be used by *P. aeruginosa* for anaerobic respiration and concomitant alginate production in this arena.

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