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Role of Energy Metabolism in Conversion of Nonmucoid *Pseudomonas aeruginosa* to the Mucoid Phenotype

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Phosphatidylcholine, the major component of lung surfactant, when supplied as the sole source of phosphate for *Pseudomonas aeruginosa* PAO1, resulted in conversion of as much as 2% of the population to the mucoid phenotype under continuous culture conditions over a 24-day culture period. In addition, growth in phosphatidylcholine resulted in the highest yields of extracellular alginate compared with other environmental conditions. Iron limitation, another environmental condition relevant to the lungs of patients with cystic fibrosis, also resulted in conversion to mucoid. Since both conditions suggested the likelihood of an energy-deprived growth environment as a common variable, the effect of direct inhibition of energy generation by *N,N'*-dicyclohexylcarbodiimide or gramicidin on the conversion of nonmucoid *P. aeruginosa* to the mucoid phenotype was examined. Both inhibitors resulted in mucoid subpopulations (0.5 and 0.8%, respectively). Severe energy stress imposed by the combination of phosphate limitation and *N,N'*-dicyclohexylcarbodiimide treatment resulted in conversion of 55% of the population to mucoidy during a 7-day growth period. A growth advantage of the mucoid over the nonmucoid phenotype was observed under severe nutrient deprivation by growth on unsupplemented Noble agar or in a 1/2,500 dilution of a chemically defined medium. These results clearly demonstrate a significant role for the energy state of the cell in conversion to mucoid and in selection for the mucoid phenotype.

Subsequent to colonization of the lungs of patients with cystic fibrosis (CF) and others with chronic obstructive lung disease, *Pseudomonas aeruginosa* typically undergoes alteration from the classical nonmucoid form to an atypical mucoid form, which correlates with production of the exopolysaccharide alginic acid. Detection of alginate-producing *P. aeruginosa* strains is usually associated with a poor patient prognosis (10), although some patients with CF may harbor mucoid organisms for years without a serious decrease in pulmonary function (16). In any case, alginate is generally regarded as a significant virulence factor in chronic lung disease caused by *P. aeruginosa* by impeding phagocytosis (20) and contributing to immune complex-mediated tissue damage (13) and possibly acting as a barrier to diffusion of antibiotics to microcolonies in the lungs (5), although this has been questioned recently (24).

Through a variety of genetic and physiological studies, the host and bacterial environmental factors which contribute to emergence of the mucoid phenotype are beginning to be understood. Recent studies of *P. aeruginosa* grown in static acetamide broth cultures (30) or in a chemostat under various environmental conditions (32) have emphasized the significance of slow growth rates, as well as the requirement for a nutritionally poor environment. For example, P_i-limited nonmucoid *P. aeruginosa* PAO1 exhibited mucoid subpopulations only at dilution rates (D) of $<0.347\ h^{-1}$. In addition, energy-rich sources of carbon and nitrogen, such as gluconate and glutamate, respectively, failed to yield mucoid organisms at any growth rate (32). In contrast, energy-poor carbon (acetate or glycerol) or nitrogen (nitrate or phosphorylcholine [PC]) substrates consistently produced mucoid subpopulations. Thus, a unifying hypothesis for these studies suggests that the energy demands of the bacterial cell play a central role in mediating conversion to the mucoid phenotype. The present study extends these findings to include a major component of lung surfactant, L-α-phosphatidylcholine dipalmitoyl (DPPC), as a sole source of phosphate during continuous culture, as well as growth limitation imposed by iron restriction. Limitation of growth through partial inhibition of energy metabolism by *N,N'*-dicyclohexylcarbodiimide (DCCD) or gramicidin also resulted in mucoid subpopulations, directly linking energy production and conversion to mucoidy. In addition, evidence is presented that indicates that mucoid organisms adapt, grow, and survive more efficiently in a severely limited nutritional environment than do those with the nonmucoid phenotype.

MATERIALS AND METHODS

Maintenance of bacteria. Nonmucoid *P. aeruginosa* PAO1 was obtained from B. Iglewski, Department of Microbiology and Immunology, University of Rochester Medical Center, Rochester, N.Y. Strain J1385, a nonmucoid CF isolate, and strain J1532, its isogenic mucoid variant isolated from the same patient, were obtained from J. R. W. Govan, Department of Bacteriology, University of Edinburgh Medical School, Edinburgh, United Kingdom. Environmental nonmucoid isolates JSA-1, isolated from a Jacuzzi, and F1, isolated from an outdoor fountain, were obtained from V. Deretic, Department of Microbiology, University of Texas Health Science Center at San Antonio. Strain SRM3-R, a nonmucoid revertant of a mucoid CF isolate, was obtained in this laboratory by batch culture of a mucoid CF strain (18). Strain L8778, a mucoid strain isolated from a chronically infected patient who did not have CF, was obtained from the San Antonio State Chest Hospital. Nonmucoid isolate L78-R, a nonmucoid revertant of L8778, was obtained in the laboratory as a spontaneous nonmucoid variant by growth of

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the mucoid form in batch culture. Mucoid isolate 25C250, a mucoid variant of PAO1, was obtained from a continuous culture supplied with a growth-limiting amount of PC as the sole source of carbon, nitrogen, and phosphate (32). All stocks were maintained at −80°C in 10% skim milk and subcultured on mucoid maintenance agar (MMA; MacConkey agar base modified by addition of 50 g of glycerol per liter) plates (8).

Culture media. A modification of the chemically defined alginate-promoting (AP) medium of Mian et al. (23), which has been previously described (32), was the primary growth medium used for batch and continuous cultures. The medium contained 100 mM sodium gluconate, 100 mM monosodium glutamate, 7.5 mM NaH_2PO_4, 16.3 mM K_2HPO_4, and 10 mM $MgSO_4 \cdot 7H_2O$. The pH was adjusted to 7.0 with 2.5 N NaOH. For growth under various environmental conditions, further modifications of this medium were made as described below.

(i) Phosphate limitation (P medium). AP medium was modified to contain 0.05 mM K_2HPO_4 as the sole phosphate source (32), and 10 mM MOPS served as the buffer. The pH was adjusted to 7.0 with 6 N HCl.

(ii) Phosphatidylcholine (lecithin) limitation. Lecithin, or DPPC, was evaluated as a growth-limiting phosphate source. For phosphate-limited growth, P medium contained 2 μM DPPC instead of K_2HPO_4.

(iii) Iron limitation of growth. For growth under iron-limited conditions, AP medium was depleted of iron by a modified desferration procedure adapted from Ohman et al. (25). All glassware used for medium preparation and growth experiments was presoaked overnight in 1 N HCl. All chemostat tubing was presoaked overnight in 0.1 N HCl. Following the acid soak, all materials were extensively rinsed with deionized water immediately prior to use. AP medium was made up as a 1-liter volume of eightfold-concentrated medium without $MgSO_4 \cdot 7H_2O$. A 0.5-ml aliquot of the concentrated medium was assayed for total iron by use of a ferrozine-based iron assay kit (Sigma Diagnostics, St. Louis, Mo.) in accordance with the procedure supplied by the manufacturer. A 20-ml volume of concentrated AP medium was diluted eightfold in deionized water. A 40-μl volume of sterile 20% $MgSO_4 \cdot 7H_2O$ was added to the diluted medium. The medium was then sterilized by filtration and stored at 4°C until use, and the remaining AP medium was desferrated by addition of 20 g of sodium Chelex-100 (minus-400-mesh; Bio-Rad Laboratories, Richmond, Calif.). The flask was covered with Parafilm, and the mixture was vigorously stirred at room temperature for 6 h. The Chelex was removed by filtration, and the concentrated medium was reassayed for total iron content. A 20-ml volume of the treated medium was then diluted eightfold with deionized water and prepared in the same manner as the dilute untreated medium. Confirmation that the treated medium was indeed iron limited was accomplished by growth of 24-h batch cultures in the diluted medium. Iron limitation was demonstrated by decreased growth (<60%) of *P. aeruginosa* in desferrated AP (0.3 to 0.4 μM Fe) relative to the growth achieved in untreated AP (5 μM) and the lack of free iron remaining in culture supernatant fluids from cultures grown in desferrated medium. Conversely, free iron was still detectable in cultures grown in untreated AP medium.

Growth in the presence of inhibitors of energy metabolism. P medium or unmodified AP medium was used for continuous culture of nonmucoid *P. aeruginosa* PAO1 in the presence of gramicidin D (100 μg/ml) or DCCD (5 mM). These concentrations reflected a 50% reduction in cell viability in batch cultures compared with untreated cultures.

Continuous culture conditions. Continuous cultures were grown in a Bioflo C30 benchtop chemostat (New Brunswick Scientific Co., Inc., Edison, N.J.) with either a 350-ml or a 1.2-liter working volume. Culture conditions were the same as those described by Terry et al. (32). Addition of medium from the reservoir was initiated when batch cultures reached the mid-exponential growth phase. Samples were obtained from the culture vessel at various times during the growth of nonmucoid *P. aeruginosa* strains. Appropriate dilutions were made in AP medium, and 0.1-ml aliquots of these samples were spread onto MMA plates for determination of growth in CFU per milliliter. The phenotypic expression of the culture was determined by examining the number of each colony type on MMA plates. The stability of mucoid colonies was determined by a minimum of three passages on MMA.

Extracellular polysaccharide determinations. Culture effluents from the chemostat were collected in 1-liter volumes in flasks kept in ice baths. Whole cells were separated from culture supernatant fluids by centrifugation at 14,000 × *g* for 20 min at 4°C in an refrigerated centrifuge. Supernatant fluids were decanted carefully into dialysis tubing (Arthur Thomas Co., Philadelphia, Pa.) with an indicated retention size of >12 to 14 kDa. Dialysis was carried out at 4°C for 2 days against three changes of deionized water containing 0.02% sodium azide. The dialyzed material was concentrated by rotary evaporation and lyophilization. The dried material was suspended to 10 mg (dry weight) per ml in deionized water. Polysaccharides were extracted from this material by a procedure based on the hot water-phenol method of Westphal Jann (36) for lipopolysaccharide (LPS). All aqueous phases were dialyzed against several changes of deionized water until the odor of phenol was no longer apparent. Dialyzed samples were then concentrated by rotary evaporation and lyophilized. Samples were dissolved (5 to 10 mg/ml) in 50 mM Tris buffer (pH 7.3) containing 100 mM $MgSO_4 \cdot 7H_2O$. DNase and RNase (100 μg of each per ml) were added and incubated at 37°C for 2 h. Following nuclease treatment, sodium dodecyl sulfate (0.5%) was added at a final concentration of 0.5%, followed by proteinase K (50 μg/ml). After incubation for 2 h at 60°C in a water bath, samples were dialyzed for 2 days at 4°C against several changes of deionized water, lyophilized, and suspended in deionized water to a final dry-weight concentration of 5 to 10 mg/ml. LPS and alginic acid were then isolated by DEAE-Sephacel anion-exchange chromatography as described by Krieg et al. (19), by using a continuous 0.05 to 0.8 M $(NH_4)_2CO_3$ gradient.

Chemical assays. The anthrone assay (21) was used for determination of hexoses, with glucose as the standard. 2-Keto-3-deoxyoctonate (KDO), used as a marker for LPS, was estimated by the thiobarbituric acid assay (2), with KDO (Sigma Chemical Co., St. Louis, Mo.) as the standard. The presence of uronic acids was determined by the harmine assay (34), with D-mannuronic acid (Sigma) as the standard. The alginate yields from all samples were based upon chemical estimation of the total micrograms of uronic acid present per milligram of cell dry weight.

Growth of paired mucoid and nonmucoid *P. aeruginosa* isolates on Noble agar. Three sets of paired mucoid and nonmucoid isolates of *P. aeruginosa* were selected for a comparative growth study in a nutritionally limited environment. Six isolates, J1385, J1532, L8778, L78-R, PAO1, and 25C250, were grown overnight as shaken batch cultures in

AP medium. Aliquots (50 μl) from each culture were added to individual culture tubes (16 by 125 mm) containing 4.95 ml of sterile MOPS (morpholinepropanesulfonic acid)-buffered saline (MBS; 10 mM MOPS, 0.14 M NaCl, 3 mM KCl), pH 7.4. Samples were then diluted appropriately and plated onto MMA plates for viability and colony type determinations. The same diluted samples were also used for inoculation of plates containing 1.5% Noble agar (Agar Noble; Difco Laboratories, Detroit, Mich.) with no added nutrients. All inoculations were performed in triplicate. Two comparisons were made with respect to growth of mucoid and nonmucoid *P. aeruginosa* strains under nutrient-limited conditions. The first was a measure of relative plating efficiency of mucoid and nonmucoid organisms on Noble agar, using their respective growth on MMA as the standard. The second comparison was determination of the number of viable organisms per colony in samples picked from Noble agar plates. Three to five colonies of each *P. aeruginosa* isolate were picked at random with sterile Pasteur pipettes by taking agar plugs from Noble agar plates. The plugs were expelled into sterile culture tubes (13 by 100 mm) containing 1 ml of MBS. The tubes were vigorously vortexed and kept in a cold room (4°C) for 2 to 3 h. The cold incubation was carried out with occasional vortexing to allow for adequate elution of organisms from the agar plugs. At the end of the incubation period, each sample was diluted in MBS and 0.1-ml volumes were spread onto MMA plates. These plates were incubated for 24 h at 37°C. Colonies were then counted and the mean number of CFU per Noble agar-grown colony was determined for each isolate. This process was repeated at several intervals with the same Noble agar cultures for a 14-day period. The results of the relative plating efficiency studies and those from the colonial growth determinations were compared by a two-tailed Student *t* test for independent means.

Growth of paired mucoid and nonmucoid *P. aeruginosa* isolates in mixed batch cultures. The same isolates used in the preceding section for the Noble agar experiments were employed in nutrient-limited liquid batch cultures. The six isolates of *P. aeruginosa* were first grown separately overnight in AP medium in 50-ml shaken batch cultures. Aliquots (50 μl) of each culture were transferred to 4.95 ml of sterile MBS. A 0.1-ml volume of each diluted sample was then transferred to 9.9 ml of sterile MBS to give a final dilution of each original overnight culture of 10^{-4}. Appropriate volumes of each pair of mucoid and nonmucoid isolates were then mixed to yield approximately a 1:1 mixture of the two types of organisms. This starting mixture was confirmed by inoculation of MMA plates with these mixtures and counting of the mucoid and nonmucoid colonies which grew. An appropriate volume of each starting mixture equal to 0.5% of the volume of medium in a chemostat culture vessel was added to each of three culture vessels, which contained AP medium diluted to 1/2,500 of the original concentration. These batch cultures were aerated at 0.5 liter of air per min and mixed at an impellor speed of 250 rpm. The cultures were monitored several times over a 10-day period. Growth and viability were determined as CFU per milliliter on MMA plates. The percentages of mucoid and nonmucoid colonies were determined at the same time.

RESULTS

Emergence of mucoid *P. aeruginosa* under continuous culture conditions with DPPC as the limiting source of phosphate. In a previous study (32), PC, an energy-poor substrate and

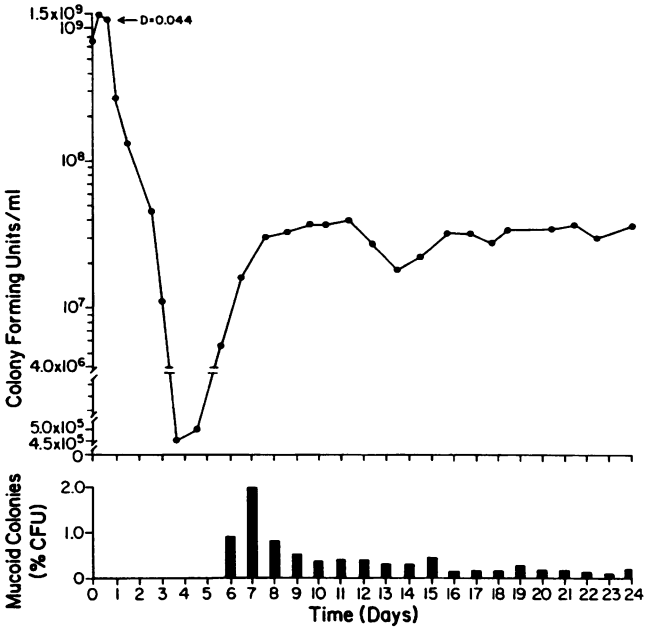

FIG. 1. Phosphate-limited growth (D = 0.044) of *P. aeruginosa* PAO1 with DPPC (2 μM) as the phosphate source. The culture was aerated (0.5 liter/min) and maintained at 37°C.

product of phospholipase C activity on DPPC (3), supplied the necessary carbon, nitrogen, and phosphate for continuous cultures of *P. aeruginosa*. These restricted growth conditions also resulted in conversion of a subpopulation to the mucoid phenotype. In the present study, DPPC (2 μM) was used as the sole source of phosphate, which required phospholipase C activity to release PC. At 6 days of continuous culture, mucoid colonies were detected; they reached a maximum of 2% and remained in the population through the remainder of the culture period (Fig. 1). These results firmly establish the relevance of DPPC as a nutrient source which promotes emergence of the mucoid phenotype during long-term continuous culture with extended growth rates.

Iron limitation of growth and emergence of mucoid *P. aeruginosa*. The correlation between iron limitation and decreased cell growth is well recognized in many bacteria, including *P. aeruginosa* (35). Since free iron is severely limited in vivo and this would therefore promote an energy-poor environment, the effect of iron limitation on conversion to mucoidy was also examined. As indicated in Fig. 2, mucoid organisms were apparent at day 5 and throughout the remainder of the 18-day study with a range of 0.5 to 2.5% mucoid organisms in the culture population. These results further demonstrate the role of a limiting nutritional environment, imposed by a variety of diverse conditions, on the emergence of a mucoid subpopulation.

Effects of energy inhibitors on conversion of *P. aeruginosa* to the mucoidy phenotype. To assess the role of energy metabolism in conversion to mucoidy directly, two inhibitors which decrease cellular energy, DCCD (5 mM) (binds subunit c of F_0 complex of ATP synthase [12]) and gramicidin D (100 μg/ml) (inhibits proton translocation [28]), were examined for effects on conversion to the mucoidy phenotype in complete AP medium during continuous culture. The concentrations selected for study were based on preliminary batch cultures which demonstrated a 50% reduction in cell growth during a 24-h batch culture in AP medium. As

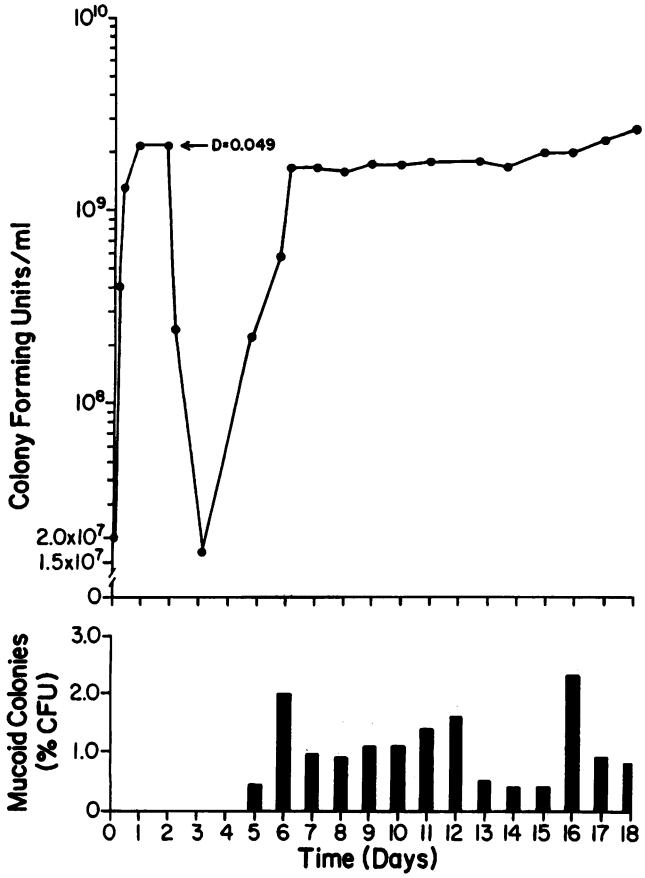

FIG. 2. Iron-limited continuous culture (D = 0.049) of *P. aeruginosa* PAO1. The growth medium was desferrated (0.3 to 0.4 μM Fe) complete AP medium. The growth conditions and symbols are the same as in Fig. 1.

indicated in a previous study (32), growth in complete AP medium did not result in isolation of mucoid variants (Table 1). Growth under phosphate-limiting conditions alone resulted in conversion of 0.3 to 1.8% of the population to the mucoid phenotype. Cultivation of strain PAO1 in AP medium plus 5 mM DCCD yielded 0.1 to 0.5% mucoid organisms. Similar results were obtained when gramicidin D (100 μg/ml) was used in a separate experiment. However, the combination of phosphate limitation and DCCD treatment resulted in conversion of 55.1% of the viable population to the mucoid phenotype during the course of a 7-day experiment. These data indicate that partial inhibition of energy metabolism directly resulted in conversion of a subpopulation to the mucoid phenotype and that severe inhibition imposed by the combination of phosphate limitation and DCCD treatment resulted in a >50% rate of conversion to mucoidy. These data further suggest that the energy state of the cell contributes directly to conversion to the mucoid phenotype.

TABLE 1. Effects of inhibitors of energy metabolism on mucoid conversion of *P. aeruginosa* PAO1 in continuous cultures

Growth condition	D (h^{-1})	% Mucoid colonies[a]
AP alone	0.140	0.0[b]
PO_4 limitation	0.067	0.1–2.0
AP + DCCD (5 mM)	0.060	0.1–0.5
AP + gramicidin D (100 μg/ml)	0.078	0.3–1.8
PO_4 limitation + 5 mM DCCD	0.090	0.3–55.1

[a] Range of percentages of colonies which expressed the mucoidy phenotype during the course of continuous culture.
[b] No mucoid colonies detected during the growth experiment.

TABLE 2. Extracellular polysaccharide yields[a] of *P. aeruginosa* PAO1 grown under various continuous culture conditions[b]

Growth condition	D (h^{-1})	Extracellular polysaccharides (μg/mg of cell dry wt)	
		Uronic acid	KDO
Carbon limitation			
Gluconate	0.038	<0.10[c]	0.12
Succinate	0.046	8.93	0.64
Glycerol	0.046	0.58	0.26
Acetate	0.058	2.67	0.33
Nitrogen limitation			
Glutamate	0.032	<0.10[c]	0.18
Nitrate	0.046	24.30	0.62
PC	0.044	4.19	0.29
Phosphate limitation			
K_2HPO_4	0.347	<0.10[c]	0.41
K_2HPO_4	0.173	2.16	0.35
K_2HPO_4	0.116	3.52	0.24
K_2HPO_4	0.069	5.06	0.59
PC	0.111	0.41	0.30
DPPC	0.044	102.81	3.14
Iron limitation	0.094	3.10	0.20
NaCl (0.3 M)	0.094	2.48	0.54
Complete AP	0.140	<0.10[c]	0.20

[a] Averages of at least three samples during each growth experiment.
[b] All samples were from culture supernatant fluids from a previous study (32), except for the present DPPC study and iron limitation.
[c] None detected.

Extracellular polysaccharide production. As confirmation of production of mucoid alginic acid polysaccharide by *P. aeruginosa* PAO1 during continuous culture under various environmental conditions, the supernatant fluids from these cultures were assayed for the presence of alginate (as uronic acid) and KDO, a constituent of LPS. A previous study indicated that most of the alginate was released into the supernatant fluids during continuous culture (19). In addition to the environmental conditions examined in the present study, data are also included on polysaccharide production during carbon, nitrogen, and phosphate limitation from a previous study (32). The average total yields of uronic acid and KDO from *P. aeruginosa* PAO1 continuous cultures are listed in Table 2. The range of average yields varied greatly with each growth condition. However, in cultures grown in complete AP medium, limited for gluconate or glutamate, or limited for K_2HPO_4 but grown at a relatively fast rate (D = 0.347 h^{-1}), alginic (uronic) acid was not detected (Table 2). These were also growth conditions which yielded no mucoid PAO1 variants. Carbon-limited cultures yielded amounts of uronic acid which ranged from undetectable levels in gluconate-limited cultures to 8.93 μg/mg of cell dry weight under succinate limitation. Results obtained from nitrogen-limited cultures also varied with the nature of the growth-

TABLE 3. Conversion to mucoidy in phosphate-limited continuous cultures of nonmucoid *P. aeruginosa* isolates

Isolate	D (h^{-1})	% Mucoid[a] colonies	Avg extracellular polysaccharide yield (μg/mg of cell dry wt)	
			Uronic acid	KDO
SRM-3R	0.035	3.7	32.6	1.64
J1385	0.031	1.3	5.8	0.35
JSA-1	0.051	1.1	1.7	0.44
F1	0.030	0.8	4.8	0.53

[a] Percentage of colonies which expressed the mucoid phenotype at the end of the growth experiment.

limiting substrate, ranging from undetectability (glutamate limitation) to 24.3 μg/mg of cell dry weight (nitrate limitation). The uronic acid yields measured from the supernatant fluids taken during the K_2HPO_4 limitation experiments decreased slightly with increasing culture growth rate (Table 2), ranging from 5.06 μg/mg of cell dry weight at $D = 0.069$ h^{-1} to undetectable levels at $D = 0.347$ h^{-1}. This relationship was similar to the correlation between growth rate and expression of the mucoid phenotype in these cultures (32). The highest average yield of uronic acid was obtained from phosphate-limited *P. aeruginosa* PAO1 supplied with DPPC as the sole phosphate source. These organisms produced an average of 102.81 μg/mg of cell dry weight (Table 2). Iron-limited cultures and cultures grown in AP supplemented with 0.3 M NaCl produced similar average yields of uronic acid, i.e., 3.10 and 2.48 μg/mg of cell dry weight, respectively.

Average extracellular uronic acid yields were also determined for continuous cultures of four other nonmucoid isolates of *P. aeruginosa*, which were grown under phosphate (K_2HPO_4)-limited conditions. Phenotypic expression of mucoidy and average yield of uronic acid differed with each isolate (Table 3). Isolate SRM-3R, a nonmucoid revertant of a mucoid CF isolate, had the highest level of mucoid variants, i.e., 3.7% of the population, and the greatest average yield of uronic acid, i.e., 32.6 μg/mg of cell dry weight. Nonmucoid environmental isolates JSA-1 and F1 and nonmucoid isolate J1385 had similarly lower levels of mucoid variants and lower yields of uronic acid.

While extracellular alginate production correlated with conversion to a mucoid subpopulation, there was no corresponding increase in LPS production (Table 1) or change in LPS profiles examined by polyacrylamide gels (31). Interestingly, there was a 16-fold increase in LPS release by continuous cultures grown in DPPC as the sole source of phosphate.

Growth of paired mucoid and nonmucoid *P. aeruginosa* isolates in nutritionally limited environments. The results of the several previously mentioned experiments seemed to indicate the importance of limited nutrients in the environment upon the emergence of mucoid subpopulations in continuous cultures. Three sets of paired mucoid and nonmucoid isolates were utilized to determine whether the mucoid form of *P. aeruginosa* might have a growth advantage in an environment greatly lacking in nutrients. The paired isolates were evaluated for the ability to grow on a solid medium composed only of Noble agar with no added nutrients. It was found that the mucoid form of *P. aeruginosa* had a significantly higher relative plating efficiency on Noble agar than did the nonmucoid form (Table 4). Furthermore, over a 2-week period, the mucoid form also was able to grow to a significantly higher average number of CFU per colony when random colonies were picked from the Noble agar plates and viable bacteria in each colony were enumerated (Table 5).

TABLE 4. Relative plating efficiencies of paired mucoid and nonmucoid isolates of *P. aeruginosa* on Noble agar

Isolate	Phenotype[a]	Relative plating efficiency (mean % ± SEM)[b]
PAO1	N	14.8 ± 5.2
25C250	M	37.9 ± 4.1[c]
L78-R	N	12.6 ± 0.5
L8778	M	30.5 ± 2.3[d]
J1385	N	12.7 ± 1.0
J1532	M	28.5 ± 1.9[e]

[a] N, nonmucoid; M, mucoid.
[b] Mean percentage of colonies on Noble agar plates relative to the number of colonies which grew on MMA plates after inoculation with identical samples.
[c] $P < 0.02$ compared with PAO1.
[d] $P < 0.01$ compared with L78-R.
[e] $P < 0.01$ compared with J1385.

A second test performed was that of growing mixed mucoid and nonmucoid batch cultures of *P. aeruginosa* in an extremely dilute liquid medium and determining which phenotype would predominate in these cultures. The same paired mucoid and nonmucoid isolates used in the previous Noble agar experiment were used for the batch culture experiment. Cultures were inoculated so that the initial mixture of mucoid and nonmucoid organisms would be equally divided between the two phenotypes. The medium used was a 1:2,500 dilution of complete AP medium. By day 3 of growth, all cultures were predominantly mucoid. Indeed, the culture composed of nonmucoid PAO1 and its mucoid variant 25C250 was 96% mucoid (Table 6). However, by day 10 of growth, the nonmucoid organisms had recovered somewhat. Nevertheless, the batch culture experiment also gave an indication that mucoid organisms might be better suited to nutritionally limited environments than are nonmucoid *P. aeruginosa* organisms.

TABLE 5. Growth of paired mucoid and nonmucoid *P. aeruginosa* strains on Noble agar

Isolate	Phenotype[a]	Mean CFU/colony ± SEM, 10^{-4}			
		Day 2	Day 5	Day 9	Day 14
PAO1	N	4.5 ± 0.3	3.9 ± 0.4	4.5 ± 0.8	4.4 ± 0.9
25C250	M	5.1 ± 0.4	27.1 ± 1.4[b]	20.4 ± 0.3[b]	19.0 ± 4.4[c]
L78-R	N	3.1 ± 0.1	4.8 ± 0.2	4.3 ± 0.3	3.5 ± 0.5
L8778	M	13.6 ± 1.7[d]	23.4 ± 0.6[b]	13.3 ± 0.7[b]	14.8 ± 2.5[e]
J1385	N	3.2 ± 0.1	5.4 ± 0.8	5.5 ± 0.6	4.0 ± 1.0
J1532	M	10.1 ± 1.0[d]	17.9 ± 0.2[b]	14.0 ± 1.5[d]	12.3 ± 1.5[d]

[a] N, nonmucoid; M, mucoid.
[b] $P < 0.001$.
[c] $P < 0.05$.
[d] $P < 0.01$.
[e] $P < 0.02$.

TABLE 6. Phenotypic expression in mixed batch cultures of mucoid and nonmucoid *P. aeruginosa* strains grown under nutrient-limited conditions[a]

Isolate	Phenotype[b]	Inoculum ratio (%)	Phenotypic ratio (% M:% N)			
			Day 1	Day 3	Day 5	Day 10
PAO1 25C250	N M	50:50	75:25	96:4	83:17	58:42
L78-R L8778	N M	50:50	81:19	—[c]	75:25	70:30
J1385 J1532	N M	50:50	75:25	79:21	77:23	66:34

[a] Cultures were grown in dilute (1:2,500) AP medium.
[b] N, nonmucoid; M, mucoid.
[c] —, not done.

DISCUSSION

Chronic persistence of *P. aeruginosa* on mucosal surfaces in the lungs without extensive tissue invasion would require prolonged survival in an environment limited for nutrients. Nutrient limitation would likely affect the physiological status of *P. aeruginosa*, including intracellular energy metabolism. Growth rates would decrease and organisms would need to become more efficient at utilization of scarce nutrients. New proteins involved in scavenging specific nutrients and conferring a more stress-resistant phenotype for prolonged survival would be synthesized (22). Another event that occurs during nutrient deprivation or starvation is synthesis of exopolysaccharides (1, 27, 37). These polysaccharides are known to alter the adhesive properties of bacteria undergoing a shift to starvation survival, aiding in either attachment and microcolony growth (1, 27) or detachment from inanimate hydrophobic surfaces (37). Perhaps mucoid colonies of *P. aeruginosa* arise in chronically infected lungs in response to nutrient deprivation and fortuitously as a defense against host clearance mechanisms (29). The results of the present and previous chemostat studies of nutrient limitation of nonmucoid *P. aeruginosa* (32) appear to agree with the above-described studies.

One nutrient available in extremely limited supply to any pathogenic bacterium is iron (35). Iron limitation of cultures of strain PAO1 resulted in expression of the mucoid phenotype by a subpopulation, as indicated in Fig. 2. This result is consistent with the previous batch culture studies of Boyce and Miller (4) and the continuous culture of Ombaka et al. (26), who demonstrated the importance of iron limitation on stable maintenance of the mucoid phenotype.

A second environmental condition examined in the present study relevant to the lung environment concerned scavenging of phosphate from the major lung surfactant, phosphatidylcholine (Fig. 1), via the enzymatic activity of bacterial phospholipase C (3). A mucoid subpopulation emerged under these conditions, resulting in the highest yield of alginate of any environmental condition examined (Table 2). Thus, the environment of the lungs appears to provide a potential growth substrate for *P. aeruginosa* which yields mucoid variants with the potential for elaborating high levels of the alginic acid polysaccharide.

Since the various nutrient limitation studies of nonmucoid *P. aeruginosa* suggested a correlation between energy demand and conversion to mucoidy, inhibitors of energy metabolism were examined. Conditions were selected that resulted in an energy downshift, as determined by a 50% reduction in cell yield compared with untreated cultures. As indicated in Table 1, growth of PAO1 in complete AP medium with either DCCD or gramicidin yielded results similar to those produced by phosphate limitation. With combined phosphate limitation and DCCD, conversion to the mucoid phenotype was greatly increased, suggesting severe energy stress. While other growth conditions yielded detectable mucoid variants only after several days of growth, cultures grown in the presence of energy inhibitors exhibited mucoid colonies within as little as 4 h after addition of the inhibitor. This would probably be compatible with the time required to synthesize new proteins in response to the extreme stress imposed by partial inhibition of energy metabolism.

The correlation between the energy state of the cell expressed as [ATP/ADP] ratios, DNA supercoiling, and possible regulation of gene expression has been made recently for several environmental conditions, such as salt shock (15) and anaerobic shift (14), for *Escherichia coli*. It is proposed that the supercoiling physiological response to environmental conditions may be attributable to the sensitivity of gyrase to changes in intracellular [ATP/ADP] ratios. For nonmucoid *P. aeruginosa* PAO1, growth in AP medium containing 0.3 M NaCl resulted in conversion to a mucoid subpopulation, and a switch from anaerobic to aerobic growth conditions during phosphate limitation also resulted in the emergence of mucoid organisms (32). The correlation between [ATP/ADP] and supercoiling in PAO1 under these conditions is under study. However, there are several widely dispersed genes involved in alginate synthesis (6, 7, 9, 11, 17), and regulation of expression at the genetic level may be quite complex, depending on the specific environmental condition.

The marked growth advantage of the mucoid phenotype of *P. aeruginosa* over the nonmucoid form under severely limited nutritional conditions (Tables 4, 5, and 6) suggests that the mucoid form may have acquired characteristics that greatly enhance scavenging of scarce nutrients in the environment through chelation and/or ionic attraction due to the negatively charged alginic acid polysaccharide. Another explanation for the growth advantage of the mucoid form might be conservation of cellular energy through energy recycling by end product excretion of organic acids, thereby creating an electrochemical gradient (33). Other explanations are equally plausible, such as an increase in specific binding or transport proteins, synthesis or modification of porins, and an increase in ATP synthase activity, among many others. In any case, these physiological alterations, readily monitored in the chemostat model, may provide an explanation for the emergence and survival of mucoid *P. aeruginosa* under growth conditions likely to exist on mucosal surfaces.

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