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Phenotypic Conversion of *Pseudomonas aeruginosa* in Cystic Fibrosis

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Pseudomonas aeruginosa **strains isolated from cystic fibrosis patients were tested for production of exoenzymes, sensitivity to pooled normal human serum, and colony morphology. Strains isolated from patients exhibiting a severe form of the disease were seen to produce a decreased range of exoenzymes, to show an increase in their serum sensitivity, and to be predominantly mucoid in colonial character compared with strains isolated from patients with a milder form of the disease. These results suggest that *P. aeruginosa* undergoes phenotypic changes with respect to exoenzyme secretion, serum sensitivity, and colony form as the clinical condition of the cystic fibrosis patient changes.**

Cystic fibrosis (CF) is the most common lethal genetic recessive disease afflicting the Caucasian population (36). Respiratory tract colonization and infection in CF patients by bacterial pathogens is often associated with deterioration and eventually death resulting from pulmonary failure (3). The disease process has many curious aspects, including the propensity of a single pathogen, *Pseudomonas aeruginosa*, to be associated with the CF lung when the patient experiences pulmonary failure (5) and the inability of the host to clear the organism despite a competent immune system. The emergence of mucoid forms of *P. aeruginosa* after initial colonization with nonmucoid forms of the bacterium (14) is another novel aspect of the disease.

Once acquired, *P. aeruginosa* is almost never eradicated, with some patients being colonized for many years while remaining relatively well, yet with others rapidly declining within months of infection by the organism (16). It is not known at which point in the disease *P. aeruginosa* becomes a significant contributor to the disease state or what host or bacterial factors are important in the decline of patient status. *P. aeruginosa* does, however, produce a wide range of exoenzymes which may contribute to the pathogenicity of the bacterium in the lung. Exoenzymes such as alkaline protease, elastase, phospholipase C, and DNase may aid in the colonization and evasion of host defenses (12, 18, 23, 37). *P. aeruginosa* strains isolated from the CF lung also exhibit an increased proportion of serum-sensitive strains (9) and a wide range of colony types.

A study of colonial forms, enzyme profiles, and serum sensitivities of *P. aeruginosa* strains isolated from patients exhibiting differing severities of CF is reported here, and the results suggest a phenotypic change in the bacterium with changing clinical condition.

MATERIALS AND METHODS

Patient group. Thirty-eight children attending the CF clinic, Royal Children's Hospital, and ranging in age from 4 to 17 years were studied. Sputum specimens were obtained from outpatients and inpatients over a 6-month period. The clinical conditions of the patients were assessed by using the criteria of pulmonary function (forced expiratory volume), chest radiograph results, sputum production, and cough as described by Hudson and Phelan (15). By using a modification of their 0-to-4 grading system, our patient set was divided among three groups, mild consisting of grades 0 and 1, moderate consisting of grade 2, and severe consisting of grades 3 and 4 of Hudson and Phelan (15).

Identification of *P. aeruginosa*. Untreated sputa were plated onto *Pseudomonas* agar base supplemented with 200 mg of cetrimide and 15 mg of nalidixic acid per liter of medium (PSA) (Oxoid Ltd.) and incubated at 37°C for 18 h. A sweep of primary growth was subcultured onto PSA, and colonies were picked for identification. Two hundred and thirty-four *P. aeruginosa* strains were thus isolated. Gram-negative, rod-shaped, cytochrome *c* oxidase-positive organisms that utilized glucose oxidatively, gave a positive arginine dihydrolase reaction, produced gas from nitrate, and grew at 41°C were identified as *P. aeruginosa*. Colony forms were classified by the method of Phillips (26).

Serum sensitivity tests. Serum sensitivity was determined by the method of Penketh et al. (25).

Enzyme profile. The enzyme activities of isolates were evaluated by spot inoculations of a nutrient broth culture (adjusted to a 0.5 McFarland standard) onto test agar plates.

Protease activity was determined on a dialyzed brain heart infusion-skim milk agar plate (34). Proteolytic activity was indicated by clearing around the inoculum spot after 18 h of incubation at 37°C. Elastase activity was determined by the method of Sbarra et al. (28); plates were incubated at 37°C for 3 days and at room temperature for a further 5 days. Elastolytic activity was indicated by clearing of the opaque medium around the inoculum. Hemolysis of human and sheep erythrocytes was determined on Tryptone soya agar (Oxoid) supplemented with 5% of the appropriate erythrocytes incubated at 37°C for 3 days. Gelatinase production was determined on gelatin agar plates (7). After incubation at 37°C for 18 h, the plates were flooded with saturated ammonium sulfate and examined for zones of clearing around the inoculum. Lecithinase production was measured on nutrient agar (Oxoid) supplemented with 10 ml of egg yolk emulsion (10 ml of egg yolk plus 40 ml of sterile 5% saline solution). Lipolytic activity was indicated by the appearance of a turbid halo around the inoculum on a 1% Tween 80 agar plate (32). DNase test agar (Oxoid) supplemented with 0.01% toluidine blue O (18) was used to determine DNase production. After 72 h of growth at 37°C, DNase activity was

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TABLE 1. Percentage of strains expressing virulence-associated enzymes isolated from CF patients of different disease status

Disease state (no. of strains isolated)	% of strains producing[a]:							
	Prot	Lipase	Lec	DNA	Ela	Gel	SH	HH
Mild (53)	86	98	88	78	73	94	94	78
Moderate (75)	81	83	93	70	68	100	96	77
Severe (78)	78	84	87	42[b]	29[b]	95	71[c]	69

[a] Abbreviations: Prot, protease; Lipase, Tween 80 hydrolysis; Lec, lecithinase; DNA, DNase; Ela, elastase; Gel, gelatinase; SH, hemolysis of sheep blood; HH, hemolysis of human blood.
[b] $P < 0.001$, according to the chi-square test of independence between two variables.
[c] $P < 0.005$, according to the chi-square test of independence between two variables.

indicated by the formation of a pink halo around the inoculum.

Statistics. The data were analyzed by using the chi-square test of independence between two variables with Yates' correction for continuity (38).

RESULTS

Enzyme production and disease state. A comparison of enzymatic activities of the isolates of *P. aeruginosa* from CF patients exhibiting different clinical states of the disease is shown in Table 1. There is no significant difference in production of the enzymes gelatinase and lecithinase. For most of the enzymes tested, a slight decrease in the proportion of strains displaying the ability to produce a particular enzyme is apparent between strains isolated from mild and severe CF. This slight difference can be seen with respect to the enzymes protease, lipase, and human erythrocyte hemolysin, but it is not statistically significant. However, the proportion of strains displaying DNase ($P < 0.001$), elastase ($P < 0.001$), and sheep erythrocyte hemolysin ($P < 0.005$) production was significantly lower in isolates from patients with severe CF compared with strains isolated from patients with mild and moderate forms of the disease.

Colony form and disease state. From patients exhibiting mild CF, nonmucoid colony forms predominated on the primary isolation plates in 70% of cases. In contrast, for 100% of severe CF patients a predominance of mucoid colony forms was seen on the primary isolation plates.

Serum sensitivity. Serum sensitivity testing showed a high proportion of serum-sensitive strains (47%) among all the *P. aeruginosa* isolated from the CF lung. When strains were classified according to disease state, the percentage of serum-sensitive strains increased as the severity of the disease increased (Table 2). This trend, however, was not statistically significant.

DISCUSSION

The data presented here suggest a transition of the colonizing *P. aeruginosa* strain from an initial nonmucoid, serum-resistant phenotype exhibiting a large range of exoenzymes to a mucoid, serum-sensitive organism producing a more restricted range of exoenzymes as the patient status deteriorates.

TABLE 2. Relationship of serum sensitivity of *P. aeruginosa* to disease state of CF patients

Disease state (no. of strains isolated)	% of strains with a serum sensitivity of[a]:		
	S	I	R
Mild (53)	38	28	34
Moderate (75)	49	27	24
Severe (78)	51	5	44

[a] Abbreviations: S, sensitive; I, intermediately sensitive; R, resistant.

Most of the exoproducts of *P. aeruginosa* have been implicated in the pathogenesis of the organism. The most widely studied of these products have been the proteases. In this study, fewer strains from patients with severe disease produced protease compared with isolates from mild and moderate diseases. Likewise, fewer strains from patients with severe disease exhibited elastase activity compared with strains from patients with mild and moderate forms of the disease. Heck et al. (10, 11) have described an ability of proteases to degrade the soluble type III and IV collagens and to deplete the tissue-associated basement membrane laminin. Hingly et al. (12) found that both protease and elastase may inhibit the ciliary beating by degrading ciliary components necessary for maintaining structure, hence giving localized ciliary dysfunction which may increase the chance of colonization. Work carried out by Schultz and Miller (30) suggests that *P. aeruginosa* elastase is destructive for complement components, thus altering the phagocytic indexes of polymorphonuclear leukocytes. Elastase has also been shown to affect the immune system by cleaving immunoglobulin G and hence inhibiting its opsonic effect (6). Woods et al. (35) demonstrated that *P. aeruginosa* adheres readily to buccal epithelial cells which have had their proteinaceous coats removed by trypsinization. Therefore, the evidence supports a role for proteases, including elastase, in the initial colonization of the CF lung. In light of the postulated role of proteases in initial colonization, a decrease in proteolytic strains due to counterselection during chronic infection may be expected. The results presented here, particularly with respect to elastase, are consistent with this model, which stresses the role of proteases only in the initial stages of adherence and evasion of the immune response necessary for colonization of the respiratory tract. Jagger et al. (17) reported a study of protease and elastase production by *P. aeruginosa* from CF patients. The percentage of protease-positive isolates (both total protease and alkaline protease) was greater in patients with an early form of CF than in patients exhibiting a more advanced lung disease; no difference in elastase production was seen in strains from the different disease states. However, in the present study a significant difference in proteolytic enzyme production between strains isolated from the different disease states was seen only for elastase production, not protease production, thus contrasting with the results of Jagger et al. (17). As suggested by the trend observed in our study (albeit not statistically significant), Jagger et al. (17) and Luzar and Montie (21) demonstrated a decrease in proteolytic activity between strains of *P. aeruginosa* from colonized patients or patients in good condition compared

with strains isolated from patients that were infected or in poor condition. The patient classification scheme used by Jagger et al. (17) differs from the scheme used here. Their scheme relies only on the colonization status of the patients, whereas our classification relies on criteria related to clinical condition rather than the period since colonization. This difference could explain the disparity between elastase-patient status relationships found for *P. aeruginosa* strains in each study. For example, the criterion of colonization used by Jagger et al. (17) does not separate mild chronicity from severe chronicity, yet both types of conditions occur. The disparity between the statistical significance of protease production-patient status relationships between our study and the previous studies (17, 21) may be partly due to different methods of characterization of disease state.

DNase production was also seen to show a statistically significant decrease as judged by the proportion of positive strains isolated from patients with severe CF compared with strains isolated from patients with less severe CF. The role of DNase as a virulence factor in CF is obscure, however. Janda and Bottone (18) speculated that DNase may inactivate the genetic machinery of phagocytes once they have engulfed the bacteria. In estuarine environments it is recognized that cell-associated and extracellular nucleases hydrolyze DNA present in the environment. The nucleic acids thus produced find their way into the intracellular nucleotide pool of DNase-producing aquatic bacteria (24). *P. aeruginosa* may utilize the DNA in the aqueous environment as a nutrient, but this may not be advantageous in the CF lung; DNase synthesis control may also be adapted to low-nutrient or low-ion environments different from that in the CF lung.

Pier et al. (27) suggest that once the bacteria become established in the lung, a class of serum-sensitive, mucoid bacteria develops under the selective pressures placed on the organisms by the host. This is supported by the results obtained here, in which 51% of strains isolated from severe CF patients showed sensitivity to human serum, whereas only 38% of strains from mildly affected CF patients were sensitive to human serum. Resistance to normal human serum has been related to smoothness of the cell wall and amount of lipopolysaccharide on the bacterium, that is, a serum-sensitive bacterium exhibits a rough cell wall because of the loss of part of its lipopolysaccharide (13, 22). Serum-sensitive strains have been demonstrated not to activate complement as effectively as serum-resistant strains, hence conferring a possible selective advantage in the host lung (29).

Reference to recent genetic evidence may aid in understanding the dominance of serum-sensitive and protease-deficient *P. aeruginosa* in the CF lung. Analysis of the regulatory genes for alginate synthesis suggests that high osmolarity of the CF sputum (19) could activate the *algD* gene, which encodes for the enzyme guanosine diphosphate mannose dehydrogenase (1, 4). This enzyme is required for the production of alginate and hence for the mucoid phenotype. The alginate exopolysaccharide produced by mucoid *P. aeruginosa* in the presence of the high cationic levels in the CF lung would also produce a stable gel which might protect the lipopolysaccharide-deficient serum-sensitive bacteria in addition to conferring the ability to form microcolonies (20, 31) and possibly biofilms (2). Once the pseudomonad is present in the lung, the protective role of the proteases in avoiding the immune system during colonization may instead be performed by the alginate exopolysaccharide outer coat, which inhibits phagocytosis of the bacteria it surrounds (33). Producers of redundant protease and elastase would be selected against under such conditions. If biofilm formation occurs, this could explain the evasion of the immune system and would aid in the development of the smoldering, chronic infection common to CF (2, 8).

Our results demonstrate a decrease in the proportion of isolates expressing elastase and DNase in strains isolated from patients with severe disease. The isolates from severely affected CF patients also show a greater percentage of serum-sensitive strains than those from patients with mild CF. These results suggest that in the chronic colonization of the CF lung with *P. aeruginosa*, the pseudomonad population may undergo an adaptation with respect to production of exoenzymes, especially elastase and DNase, and with respect to sensitivity to human serum. We are undertaking a longitudinal study to investigate further the apparent decrease in production of elastase and DNase in *P. aeruginosa* strains as patient status declines.

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