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COMPARISON OF SOME PROPERTIES OF *PSEUDOMONAS AERUGINOSA* ISOLATED FROM INFECTIONS IN PERSONS WITH AND WITHOUT CYSTIC FIBROSIS

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ABSTRACT

DOGGETT, ROBERT G. (Texas Institute for Rehabilitation and Research, Houston), GUNYON M. GARRISON, AND EVERETT S. WALLIS. Comparison of some properties of *Pseudomonas aeruginosa* isolated from infections in persons with and without cystic fibrosis. J. Bacteriol. **87**:427-431. 1964.—*Pseudomonas aeruginosa*, isolated from the respiratory tract of a group of patients diagnosed as having cystic fibrosis (CF) of the pancreas, attained the ability to produce in its capsule a material which was insoluble in certain organic solvents, such as ethanol. The capsule obtained from *P. aeruginosa* isolated from infected individuals who did not have CF was ethanol-soluble. This alcohol-insoluble mucoid from the CF *P. aeruginosa* could be demonstrated to persist after sequential subcultures of this organism. The relative viscosity and carbohydrate moiety of this insoluble mucoid fraction of *P. aeruginosa* from individuals with CF differed from that of the non-CF *P. aeruginosa* soluble mucoid fraction.

Various investigators, including di Sant'Agnese, Dische, and Danilzenko (1957), and Bauer (1960), reported on differing chemical properties of the mucoid fraction isolated from the duodenal fluid, gastric juice, and bronchial mucus of cystic fibrosis (CF) patients and of healthy individuals. A mucoid fraction of these secretions is precipitated with a mixture of ethanol-benzene. The CF mucoid material loses its solubility to a great degree when treated in this manner, whereas the material from healthy individuals is more soluble.

The carbohydrate moiety of this mucoid fraction was studied exclusively by the same investigators. Sugars reported as present were fucose, mannose, glucose, galactose, hexosamine, and sialic acid. No direct evidence was given that these sugars, or fractions containing these sugars, cause an increase in the viscosity of the mucus secreted by the different organs of the CF patient.

The purpose of this paper is to describe some biochemical and physical properties of the bacterial organism, *Pseudomonas aeruginosa*, isolated from the respiratory tract of CF patients, and from individuals without CF who were infected with the same type of organism.

P. aeruginosa isolated from CF patients has the ability to produce, in its capsule, a material which loses its solubility when treated with a mixture of ethanol and benzene. This material is even more insoluble when treated only with ethanol. Also, there appears to be none of this ethanol-insoluble material in the capsular mucus of *P. aeruginosa* isolated from the non-CF controls. We have also shown that CF *P. aeruginosa* continues to produce the same highly viscous material after sequential subcultures on a partial nutrient-restrictive membrane.

In our studies on the composition of this ethanol-insoluble capsular mucoid, the presence of the following sugars and their derivatives was shown: fucose, mannose, galactose, glucose, and certain hexosamines. The presence of sialic acid was also demonstrated. In addition, we found at least two unidentifiable compounds not present in the mucoid of *P. aeruginosa* obtained from non-CF controls. We also found that the duration of infection in the CF patient with *P. aeruginosa* appears to affect the amount of capsular ethanol-insoluble material found.

MATERIALS AND METHODS

P. aeruginosa was isolated from the tracheobronchial mucus of ten CF patients and ten non-CF controls. The ten non-CF subjects represented patients with chronic lung disease secondary to broncheostasis, asthma with chronic emphysema, and chronic tuberculosis. *P. aeruginosa* was the predominant organism cultured in 50% of these patients. The sweat test and roentgenological examinations were used to aid in the diagnosis of CF. Pure cultures of *P. aeruginosa* were established by streak-plate isolation and by biochemi-

cal tests. Further identification was made with the oxidase test (described by Kovac, 1956) for *Pseudomonas*. The *P. aeruginosa* used in this study was a smooth strain, as determined by the method of Duguid (1953).

When working with an organism having a capsule or slime layer, it is difficult to get a maximal yield of the capsular material in the liquid media because the material diffuses. Therefore, a solid medium, blood agar covered with a dialyzing membrane, was used in our procedure. (The use of a dialyzing membrane was suggested to us by W. J. Fahlberg, Department of Microbiology, Baylor University College of Medicine.) This sheet of sterile dialyzing membrane served as a partial nutrient restriction and allowed only dialyzable nutrients to contact the organism. Because part of this investigation was designed to determine whether this ethanol-insoluble mucoid of the CF *P. aeruginosa* would continue to be produced after sequential subculture, experiments were done to establish this point. CF *P. aeruginosa* was subcultured to the blood-agar medium every 24 hr for a period of 10 days, and daily samples were taken for analysis.

The organisms from 20 petri dishes were harvested into individual 250-ml beakers containing approximately 100 ml of distilled water. The cells and water were repeatedly drawn into a 50-ml syringe fitted with a 22-gauge needle, and were then forced through the needle in the manner described by Sall (1962). This solution was then centrifuged at 18,000 $\times$ *g* at 4 C for 30 min; the supernatant liquid was saved for precipitation with ethanol-benzene or with ethanol alone. For our chromatography experiments, samples were placed in individual dialyzing bags, and were dialyzed against distilled water for 72 hr at 4 C.

Precipitation of P. aeruginosa capsular mucoid fraction with ethanol-benzene. Essentially the same procedure described by di Sant'Agnese et al. (1957) for duodenal fluid was followed. The capsular material was placed in individual 1,000-ml Erlenmeyer flasks. For each volume of the aqueous capsular material used, 9 volumes of a mixture of equal parts of 95% ethanol and benzene were added slowly, with mixing, at 4 C. The CF material formed a fibrillary elastic gel-type precipitate under these conditions, or when ethanol alone was used (Fig. 1), whereas the non-CF material formed a much smaller amount of nonfibrillary-type precipitate. With ethanol alone, no precipitate of any type was formed from the non-CF material.

The samples were stored at 4 C for 2 hr, and were washed three times with cold ethanol. Distilled water was then added to the mucoid fraction precipitate in twice the volume of the original aqueous capsular material. The non-CF precipitate went into solution immediately, but the CF *P. aeruginosa* capsular material precipitate formed a thick viscous gel-like solution. This same material could also be demonstrated in the CF *P. aeruginosa* after ten sequential subcultures on the dialyzing membrane. The samples were left at 4 C for 12 hr, with occasional mixing to obtain complete solution, and were then lyophilized.

Viscosity studies on mucoid fraction. Following the method described by Daniels et al. (1949), viscosity studies were made at 37 C with an Ostwald viscosimeter. Temperature was held constant to 0.1 C. The viscosimeters were calibrated with distilled water. A sample containing exactly 10 mg of the lyophilized precipitate was added to 10 ml of distilled water and mixed; the non-CF material went into solution with gentle mixing, whereas the CF capsular material formed a gel. Therefore, the CF *P. aeruginosa* mucoid material had to be further diluted; 1 mg of this was placed in 10 ml of water and went into solution (dilution ten times greater). Viscosity was then determined; relative viscosity values are given in Table 1.

Sugar chromatography. The carbohydrate moiety of this ethanol-benzene precipitate of the CF and non-CF *P. aeruginosa* was studied by the descending paper chromatography technique described by Smith (1960).

In a vacuum-sealed Pyrex ampule, 10 mg of each sample were hydrolyzed with 10 ml of 2 N HCl for 2 hr at 100 C. The samples were decolorized with a small amount of Norit and centrifuged. The clear supernatant liquid was placed in a desiccator containing $CaCl_2$ and KOH pellets, and was kept under vacuum until dry. Each sample was then taken up in 0.2 ml of distilled water; 10 μliters of each sample were placed on Whatman no. 1 chromatography paper for 20 hr at 25 C, and were suspended in a mixture of 120 ml of ethyl acetate, 50 ml of pyridine, and 40 ml of water. The chromatograms were air-dried for 1 hr under a hood.

The location reagents used were $AgNO_3$ and

NaOH for the monosaccharides, and the Elson-Morgan reagent for the hexosamines. Individual sugar markers were run with the samples to aid in the identification of the monosaccharides. Table 2 lists the individual sugars of the CF and non-CF capsular mucoid corresponding to the sugar markers used, and the relative amounts of each material as shown by densitometric measurements.

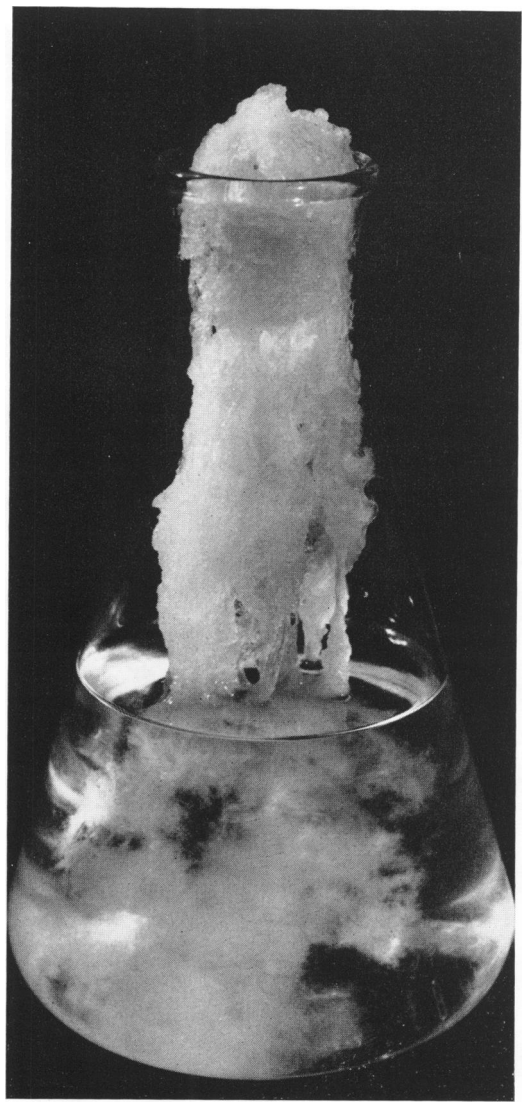

FIG. 1. *Ethanol-benzene precipitate of CF Pseudomonas aeruginosa capsular material after second washing with ethanol.*

TABLE 1. *Relative viscosity of CF and non-CF capsular precipitates (ethanol-benzene) from Pseudomonas aeruginosa*[a]

Non-CF no.	Relative viscosity[b]	CF no.	Relative viscosity[c]
	10 mg/10 ml		*1 mg/10 ml*
1065	1.79	9	4.25
938	2.80	10	2.28[d]
1522	2.70	12	5.62
940	1.17	16	3.10
1530	1.70	17	6.50
1009	2.23	18	5.10
1448	2.27	25	2.25[d]
136	1.60	27	4.68
140	2.55	28	5.72
1066	1.75	30	4.60

[a] Concentration used: CF, 1 mg of mucoid per 10 ml of water; non-CF, 10 mg of mucoid per 10 ml of water.

[b] Mean = 2.05; standard deviation = 0.28.

[c] Mean = 4.41; standard deviation = 1.79.

[d] *P. aeruginosa* infection of short duration.

RESULTS

The capsular material of the organism *P. aeruginosa* isolated from CF patients contained a mucoid fracton which was insoluble in ethanol and in ethanol-benzene. An insoluble mucoid could not be demonstrated in *P. aeruginosa* isolated from non-CF patients when ethanol alone was used.

The capsular material from such organisms contained a high concentration of this insoluble mucoid fraction, and the amount appeared to be related to the duration of the infection of the CF patient. This was supported by the viscosity studies (Table 1), which showed that the CF mucoid fraction had a high relative standard deviation viscosity compared with that of the non-CF, which was relatively low. The CF *P. aeruginosa*, after ten sequential subcultures, continued to retain its property of having a fibrillary mucoid fraction.

The mean relative viscosity for the non-CF fraction was 2.05 with a solution containing 10 mg of the dried mucoid fraction in 10 ml of water, whereas the CF relative viscosity was 4.41 with a solution containing only 1 mg of the dried mucoid fraction in 10 ml of water (Table 1). After ten sequential subcultures, the CF *P. aeruginosa* had a mean relative viscosity of 2.15 with the same dilution (1 mg of mucoid material to 10 ml of water), although less than initially.

The results obtained from the sugar chromatography are presented in Table 2. The sugars identified in both the CF and non-CF mucoid were fucose, mannose, glucose, galactose, and two hexosamines. Sialic acid was present in both the CF and non-CF fractions, as determined colorimetrically by the methods described by Glick (1960). Further investigation is needed to characterize the unidentified compounds. These compounds from CF reacted with $AgNO_3$ and yielded a yellow color with the Elson-Morgan reagent, a green color with the aniline diphenylamine reagent, and a yellow-brown color, which had fluorescent properties, with the aniline reagent. They did not react with the naphthoresorcinol reagents. They appeared in the region of the chromatogram where Bauer (1960) presumed he had located a disaccharide, lactose, from one of the body fluids of CF patients under investigation. However, lactose can be ruled out because of its property of being hydrolyzed under such acidic conditions, as was proved by a hydrolysis experiment on lactose under these conditions.

One compound (non-CF), which had a positive reaction with the Elson-Morgan reagent, was absent in the CF mucoid. This particular substance was highly concentrated in each non-CF mucoid studied, and was suspected to be an amino sugar.

TABLE 2. *Chromatographic analysis of sugars present in the CF and non-CF Pseudomonas aeruginosa capsular mucoid fraction**

Sugar	CF	Non-CF	10th subculture of CF
Fucose	1+	2+	Trace
Mannose	Trace	Trace	Trace
Glucose	2+	3+	2+
Galactose	1+	1+	1+
Glucosamine	1+	2+	1+
Galactosamine	1+	2+	1+
First unidentified compound	4+	Absent	4+
Second unidentified compound	4+	Absent	4+
Hexosamine (unknown)	Absent	4+	Absent

* Numbers 1 to 4 represent degrees of color intensity by developing reagent determined by densitometry.

DISCUSSION

The results clearly show that certain fundamental differences exist between cultured and subcultured *P. aeruginosa* isolated from the tracheobronchial mucus of CF and non-CF individuals. A preliminary report of our findings was already made. We believe that these differences lie in the chemical composition of the capsular material of the organism. Evidence of a physical and chemical nature is here presented to support this belief. The viscosities as well as the composition are strikingly different. *P. aeruginosa* mucus obtained from organisms infecting CF patients contains, in a high concentration, two unidentified compounds (probably sugar derivatives) not present in the mucus of the organism found in a non-CF individual. On the other hand, the non-CF material contains a hexosamine not present in the *P. aeruginosa* mucus of CF origin.

Investigations are underway to study in more detail the exact composition of this ethanol-benzene-insoluble material. Experiments are being conducted to obtain this material in a highly purified form so that structural studies of the pure compound can be made. Of equal importance are our studies now underway to establish the relationship between the CF *P. aeruginosa* abnormal capsular compound and physically similar viscid ethanol-insoluble mucoid secreted by the exocrine glands of CF individuals. If such a relationship can be established, and if it should be found that the two ethanol-insoluble mucoids are essentially identical, then two implications can be drawn. First, the *Pseudomonas* organism growing in the mucoid environment of the host CF child is altered by the host so that it produces a mucus resembling the host mucoid in physical and chemical properties. Thus, it is possible that the bacteria adds its mucoid to the natural, abnormal mucoid present in the CF patient's bronchial tree. From this viewpoint, it could be hypothesized that the *P. aeruginosa* undergoes an adaptation caused by the environment and provided by the CF patient's mucous-producing organs. Secondly, this abnormal *P. aeruginosa* then could be used as a unique tool to study the synthesis mechanisms involved in the abnormal mucus of the CF child. Investigations are being conducted to establish the validity of these hypotheses, and the results will be reported at a later date.

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