JOURNAL OF BACTERIOLOGY, Feb., 1965
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Vol. 89, No. 2
Printed in U.S.A.

Enzymatic Action on the Capsular Material Produced by *Pseudomonas aeruginosa* of Cystic Fibrosis Origin

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Received for publication 3 August 1964

ABSTRACT

DOGGETT, ROBERT G. (Texas Institute for Rehabilitation and Research, Houston), GUNYON M. HARRISON, RICHARD N. STILLWELL, AND EVERETT S. WALLIS. Enzymatic action on the capsular material produced by *Pseudomonas aeruginosa* of cystic fibrosis origin. J. Bacteriol. **89**:476–480. 1965.—An enzymatic study of the action of α- and β-amylases on the slime envelope produced by *Pseudomonas aeruginosa* originating from patients with cystic fibrosis (CF) has been reported. It has been shown that these two enzymes in combination prevent the formation of the abnormal ethanol-benzene insoluble mucoid present in the slime layer of the above organism of CF origin. Evidence is also given which suggests that this organism can serve as a tool for studies of chemical abnormalities in the mucous metabolism of CF individuals.

Recurrent pulmonary infection with resulting progressive respiratory insufficiency secondary to bronchial obstruction and pulmonary fibrosis represents the major threat to life of the patient with cystic fibrosis (CF) of the pancreas. In recent years Huang, Van Loon, and Sheng (1961) and Iacocca, Sibinga, and Barbero (1963) have emphasized the increased incidence of *Pseudomonas aeruginosa* in the flora of the upper respiratory airway and in the pulmonary secretions of children with this disease.

Data already published (Doggett et al., 1962; Doggett, Harrison, and Wallis, 1964) not only support these findings but give positive evidence that specific morphological and biochemical differences exist between the *P. aeruginosa* obtained from the respiratory tract of the CF patient and the same organism invading the tracheobronchial tree of a noncystic fibrosis individual with a major chronic pulmonary disease. The etiology of these dissimilarities and the full scope of the fundamental differences between these organisms obtained from different hosts is still under vigorous investigation.

Because of hereditary aspects of CF of the pancreas, it seemed logical that the metabolic errors present in this disorder might involve enzymatic catalytic processes. Therefore, this organism could be used as a tool to study the effect of various enzymes on the synthesis of this abnormal mucoid material, since we believe that this organism undergoes a metabolic change influenced by the environment of the CF host.

For this study Merck & Co., Inc., Rahway, N.J., kindly furnished samples of approximately 40 different enzyme preparations. All of these preparations were screened for possible effects on the synthesis of the abnormal slime material obtained from *P. aeruginosa* of CF origin. Two of these preparations, α- and β-amylases, showed sufficient promise to warrant a more detailed study when they were used in a mixture of equal concentrations. This paper describes results obtained in this continuation of our studies on the *P. Aeruginosa* organism isolated from the pulmonary secretions of subjects with chronic pulmonary disease both of CF and non-CF origin.

MATERIALS AND METHODS

Two groups of 10 subjects each were used in this study. Group I, acting as controls, had an active pulmonary disorder secondary to bronchiectasis, asthma, chronic emphysema, and tuberculosis. Group II represented patients from 1 to 15 years of age with CF of the pancreas; all showed a well-defined clinical picture with diagnostic chest X-rays, increased sweat sodium chloride concentrations, and depression of pancreatic enzyme activity, as manifested by decreased lipid absorption.

Cultures were obtained from the tracheobron-

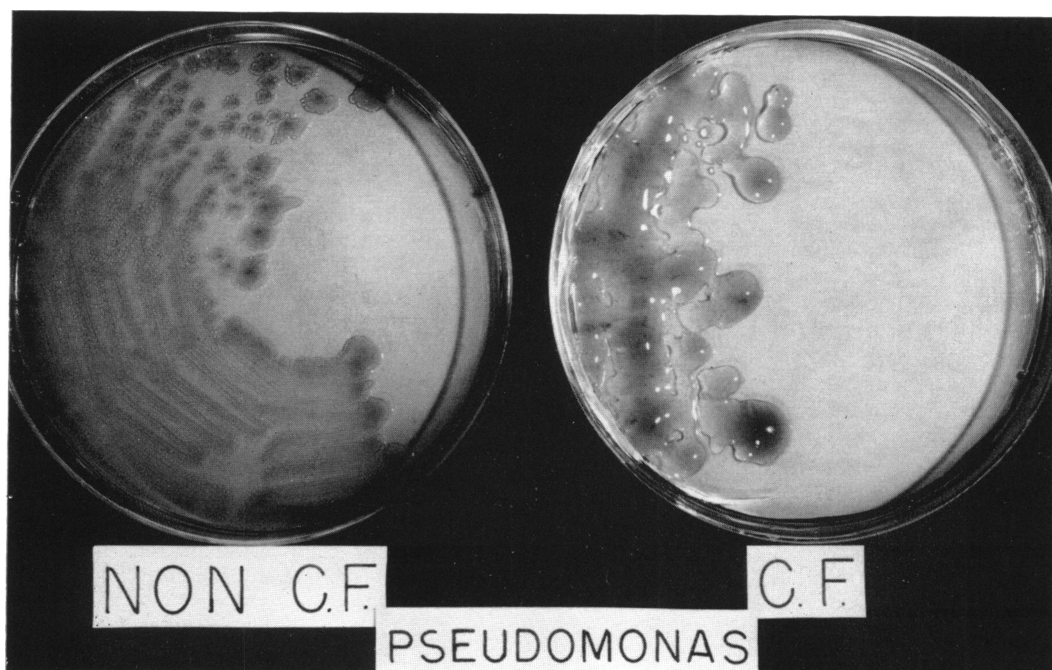

FIG. 1. *Two cultures of Pseudomonas aeruginosa illustrating differences in mucoid colonization.*

chial mucus and the *P. aeruginosa* isolated in pure culture by streak-plating and subculture of discrete colonies on differential and selective media [eosin methylene blue (EMB) and McConkey's blood agar]. Biochemical tests for oxidase (Kovacs, 1956) and pigment (King, Ward, and Raney, 1954) were carried out to establish specific identity of this organism. The *P. aeruginosa* was subcultured five times to insure complete exhaustion of the host environmental material from the slime layer. The type of growth is illustrated in Fig. 1. A standard loop containing the organism was suspended in 5 ml of sterile water, of which 0.4 ml was pipetted onto solid agar medium covered with a dialyzing membrane as described by Liu (1957). Equal amounts of a mixture of α- and β-amylases (purchased from Worthington Biochemical Corp., Freehold, N.J.) were weighed and dissolved in physiological saline solutions. Solutions containing 50, 100, and 150 mg per 0.6 ml of 0.9% saline solution were individually pipetted on the dialyzing membrane, which had been well-streaked with a subculture of CF *P. aeruginosa*.

Control cultures of CF *P. aeruginosa* were run also; the enzyme preparations were left out, and only the physiological saline (0.6 ml of 0.9% sodium chloride solution) was added. In all cases, the blood-agar plates were incubated at 37 C for 48 hr. The organisms were then harvested by removing the dialyzing membranes from the agar plates and washing them into 10 ml of distilled water.

These harvested organisms were treated according to the following procedure. The extracellular slime was separated from the soma by forcing the suspension in and out of a syringe equipped with a 20-gauge needle until the mixture appeared to be homogeneous. The solution was then centrifuged at 18,000 $\times$ g for 30 min. The supernatant layer containing the slime was placed in a flask for precipitation. The soma was desiccated to dryness and weighed.

To the slime material fraction, 9 volumes of a 1:1 mixture of ethanol and benzene were added slowly and with stirring to each volume of the supernatant liquid. The contents of the flask were allowed to stand at 4 C for 2 hr. When the precipitation was carried out in this manner, various steroids and pigments remained in solution in soluble form and thus were separated from the more insoluble precipitated mucoid. The precipitate was removed, washed twice with cold ethanol to remove traces of soluble material and benzene, and desiccated to dryness and weighed.

Part of the harvested organisms of group I individuals (non-CF) were treated with the enzyme mixture and part were not treated. The ethanol-benzene precipitate was readily soluble in water. From the harvested organisms of the group II individuals (CF not treated with the enzyme mixture), the precipitate obtained was difficult to dissolve in water and formed only a gel after a long period. The precipitated material from the enzyme-treated organisms of group II (CF) appeared similar to that obtained from the organisms of group I individuals, but was readily soluble in

water. For further studies of this material's composition, 10 mg of the white insoluble precipitate from each group were subjected to hydrolysis with 10 ml of 2 N hydrochloric acid. Paper chromatography was used to study the sugar moiety of this precipitated mucoid.

Results

P. aeruginosa from group I individuals. No difference could be demonstrated in the ethanol benzene-precipitated material obtained from *P. aeruginosa* from group I individuals (Table 1) when treated as described above or when not treated with the α- and β-amylase mixture of enzymes. The enzymes appeared to have no effect on the processes involved in the synthesis of the slime from these sources.

P. aeruginosa from Group II individuals. The precipitate from the organisms (CF, not enzyme-treated) was difficult to dissolve in water, whereas the precipitate from the organisms (CF, enzyme-treated) appeared to resemble the material obtained from the organisms of group I. Morphological differences in this case were noted between the enzyme-treated organisms and the group II controls (CF not enzyme-treated). The organisms from group II (CF individuals treated with the α- and β-amylase mixture) were rough with little or no mucoid colonization, whereas in the case of the nonenzyme-treated organisms of group II (CF), mucoid colonization was always the rule (Fig. 2). Furthermore, it was observed that the amount of the dried

TABLE 1. *Mean averages from Pseudomonas aeruginosa studies on group I and group II individuals*

P. aeruginosa source	Enzyme mixture added	Wt of soma (mg)			Wt of insoluble capsular mucoid			Decrease of insoluble mucoid
		Avg mean	Range	SD	Avg mean	Range	SD	
Group I non-CF host	Control	28.35	33.24–24.37	2.35	2.57	3.16–2.12	0.366	—
	50	28.11	33.21–23.15	2.92	2.50	3.53–1.98	0.452	—
	100	58.00	62.07–51.10	4.9	1.73	2.64–1.00	0.537	—
	150	79.71	87.12–72.48	4.3	2.39	3.01–1.37	0.51	—
Group II CF host	Control	52.38	57.27–47.01	2.9	439.02	453.20–412.91	12.8	—
	50	58.84	63.04–55.82	2.29	67.63	74.01–61.25	3.68	84.6
	100	55.06	60.01–51.02	2.43	51.45	54.27–47.11	2.1	88.2
	150	57.38	70.01–58.21	7.2	1.54	2.11–1.04	0.399	99.6

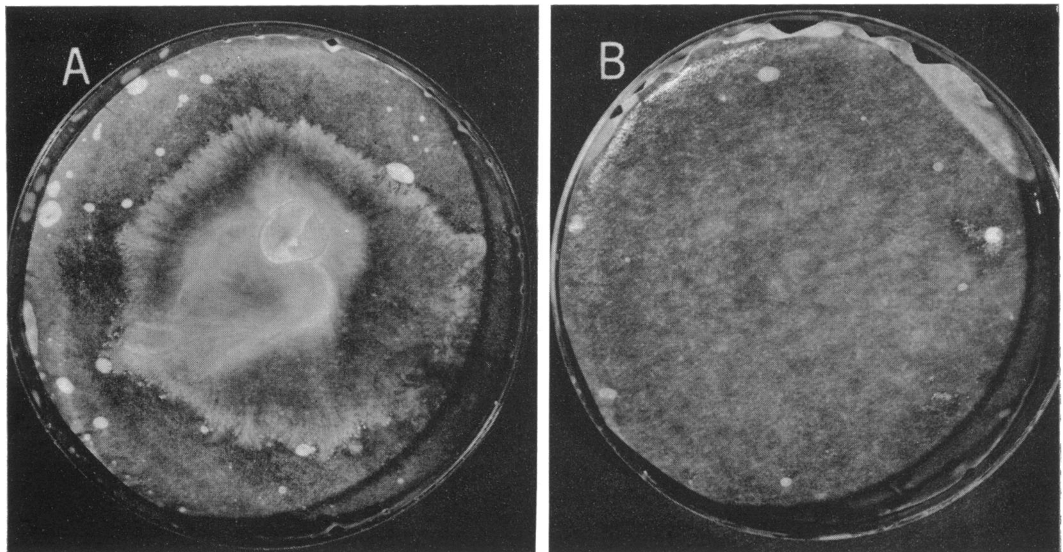

FIG. 2. *Cultures A and B representing Pseudomonas aeruginosa from group II (CF) individuals. A has not been treated with the mixture of α- and β-amylases. (Note the abundance of mucoid growth.) B has been treated with 150 mg of the enzyme mixture. (Note the lack of growth, and the rough nonmucoid form.) In both cases, the bacteria has been separated from the media by a dialyzing membrane.*

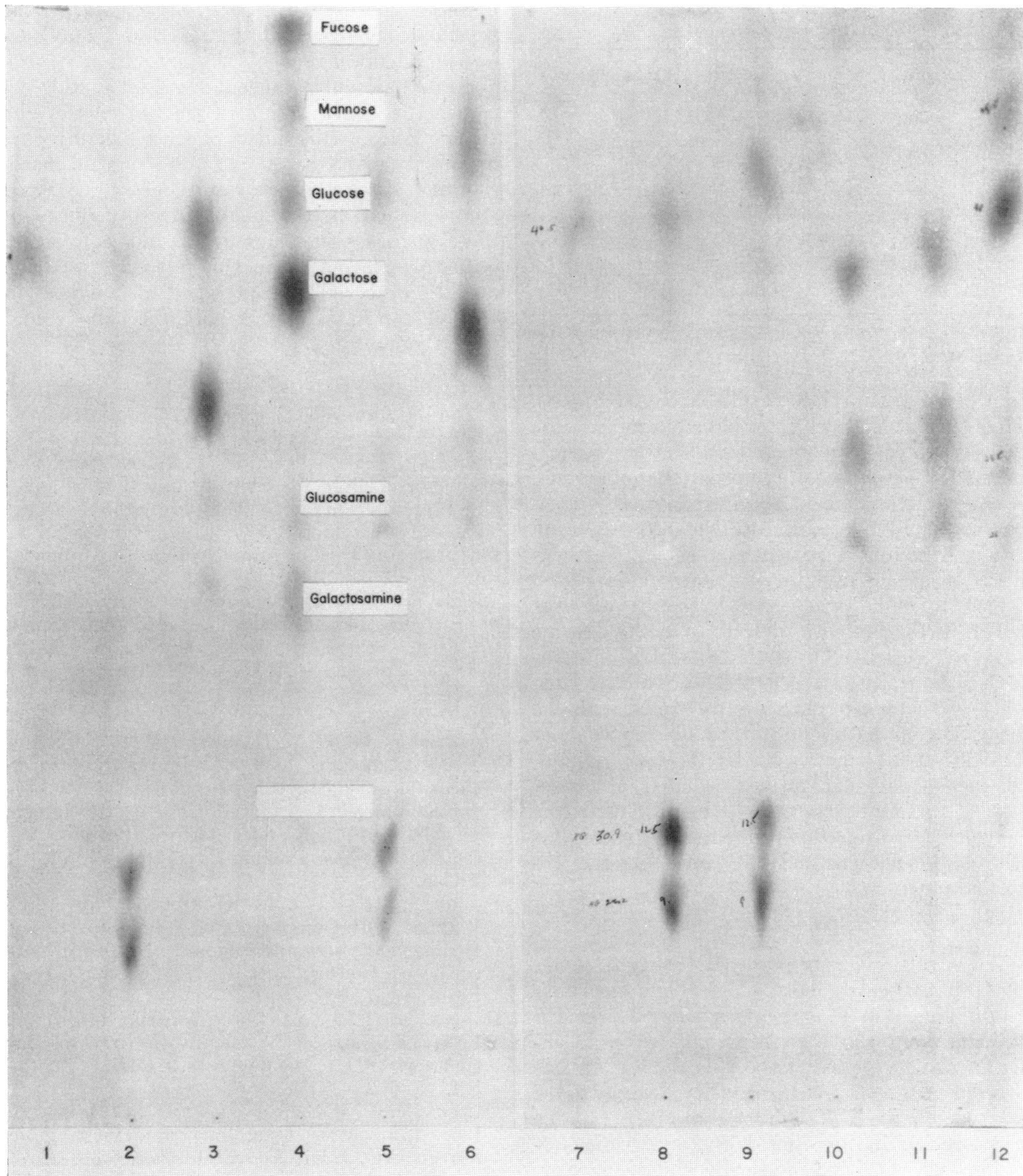

FIG. 3. *Descending paper chromatogram representing 10 µliters of samples on Whatman no. 1 chromatography paper for 20 hr at 25 C and run in a solvent system consisting of 120 ml of ethyl acetate, 50 ml of pyridine, and 40 ml of water. Numeral 1 represents glucose marker; 2, CF; 3, non-CF; 4, sugar markers; 5, CF; 6, non-CF; 7, glucose marker; 8, CF; 9, CF; 10, enzyme-treated CF 8; 11, enzyme-treated CF 9; and 12, sugar markers.*

ethanol-benzene insoluble precipitate of the enzyme-treated organisms (group II, CF) was greatly decreased, as compared with the amount obtained from the nontreated organisms (group II, CF) as shown in Table 1. Also, a chromatographic study of the carbohydrates present in the precipitated mucoid of the enzyme-treated organisms of group II revealed a disappearance of two compounds of low R_g value (Fig. 3) which were never observed in the material from group I individuals, but which were always present in high concentration in the nontreated enzyme

TABLE 2. *Group II CF Pseudomonas aeruginosa slime components**

Component	Untreated	Enzyme-treated
Fucose	1+	1+
Mannose	Trace	Trace
Glucose	2+	Trace
Galactose	1+	3+
Glucosamine	1+	3+
Galactosamine	1+	3+
First unknown	4+	Trace–absent
Second unknown	4+	Trace–absent

* Designations 1 to 4+ were determined by densitometry.

controls of group II (CF) (Table 2 and Fig. 3). Thus, in this respect it would appear that the carbohydrates of the mucoid material of enzyme-treated *P. aeruginosa* of CF origin resemble those of the capsular mucoid obtained from *P. aeruginosa* of non-CF origin. Also, in our results on group I (non-CF) patients, it should be mentioned that the weight of the soma increased nearly threefold when the *P. aeruginosa* was grown in the presence of 150 mg of the enzyme mixture. The weight of the soma was not increased in group II (CF) subjects. To determine whether the enzyme mixture interfered with the formation of the abnormal insoluble mucoid, or modified it in some way after it was produced, 10 specimens of preformed isolated abnormal mucoid material were treated in vitro with the enzymes. Examination showed no noticeable effects of such enzyme treatment on the abnormal mucus.

DISCUSSION

We have previously reported (Doggett et al., 1964) that there are morphological and biochemical differences in *P. aeruginosa* isolated from CF patients from the organisms isolated from individuals who do not have this disease. It has also been shown by the authors (*unpublished data*) that *P. aeruginosa* of CF origin is capable of producing in its slime a material which resembles closely, both physically and chemically, the mucus obtained from the tracheobronchial tree of the CF patient. Thus, it appears that this organism can serve as a good tool for studies of the chemical abnormalities in the mucous metabolism of CF individuals. In this paper we have described its use as such a tool to evaluate the effects of α- and β-amylase enzymes on the slime material produced by CF *P. aeruginosa*. So far we have demonstrated that α- and β-amylases in combination prevent the formation of the abnormal insoluble mucoid material of this organism for the duration of the activity of the enzymes. In a subsequent paper we shall report on the chemistry of this insoluble mucoid material and discuss other results of our enzyme studies, such as the quantitative amounts of these amylases in the mucoid and nonmucoid forms of this organism. Qualitatively, we have already observed the presence of amylase in varying concentrations in both the non-CF and the CF organisms.

ACKNOWLEDGMENTS

The authors wish to thank William A. Spencer for his useful suggestions in the preparation of this manuscript; Albert Canida, F. G. Nieberg, and Stanley Gunsberg for their technical assistance; and Richard Kenyon for the photographic work. Thanks are due Merck & Co., Inc., Rahway, N.J., for furnishing various samples of enzyme preparations.

This investigation was supported by a grant-in-aid from the Association for Aid to Crippled Children, New York, N.Y., and grant FR-129 from the National Institutes of Health.

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