BIOCHEMICAL AND SEROLOGICAL REACTIONS OF AN ORAL FILAMENTOUS ORGANISM[1]

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The genus *Leptotrichia* was omitted from *Bergey's Manual of Determinative Bacteriology* (Breed, Murray, and Smith, 1957) because a wide variety of unrelated, oral filamentous microorganisms had been previously placed in this genus. The confusion concerning the classification and definition of this genus has been emphasized by Baird-Parker and Davis (1958) and Gilmour and Hunter (1958).

Two very different organisms have been termed *Leptotrichia buccalis*. According to Baird-Parker and Davis (1958), the first of these was described by several early investigators as a gram-positive, fusiform, anaerobic bacterium found in materia alba of the teeth. The cultural and biochemical reactions of this microorganism have been studied by Thjøtta, Hartman, and Bøe (1939), Bøe and Thjøtta (1944), and Hamilton and Zahler (1957). The second organism, a gram-positive, filamentous, facultative actinomycete, was described and studied by Bibby and Berry (1939) and Bartels (1943). More recently, Gilmour and Hunter (1958) and Howell and Rogosa (1958) reported improved techniques for the isolation and maintenance of these bacteria.

To resolve the confusion which resulted from the application of the name *Leptotrichia buccalis* to both of these organisms, Davis and Baird-Parker (1959) suggested that two species be recognized in the genus *Leptotrichia*. They refer to the first type (the fusiform bacillus) as *Leptotrichia buccalis*, and the second type (the actinomycete) as *Leptotrichia dentium*.

This paper is concerned with a study of the oral filamentous organism which has been designated recently as *Leptotrichia dentium*. Biochemical reactions were investigated to be certain we were dealing with the same organism described by other workers. Agglutination, cross-agglutination, and precipitin reactions were undertaken to obtain information concerning the antigenic characteristics of this microorganism. To our knowledge, this is the first instance in which serological studies have been employed.

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MATERIALS AND METHODS

Isolation and maintenance. Samples of calculus or materia alba were obtained from the lingual aspect of the lower anterior teeth or the buccal surface of the upper molars from patients in the University of Illinois dental clinics and laboratory personnel. No attempt was made to correlate the oral conditions with the microorganisms isolated.

The source material was dispensed into serological tubes containing 0.5 ml of 0.85% sterile saline and ground with a sterile glass rod which previously had been shaped to fit the bottom of the tube. One loopful of the ground suspension was streaked in a single line across the center of the surface of two plates of brain heart infusion (Difco) agar supplemented with 0.2% yeast extract. A sterile loop was then streaked through the inoculum at right angles. The plates were placed in a Brewer anaerobic jar and incubated for 3 or 4 days at 37 C. This procedure was devised after we found that it was difficult to obtain well-isolated colonies with conventional streaking methods.

The plates were observed with the aid of a stereomicroscope for the typical flat rhizoid colonies described by Gilmour and Hunter (1958). Colonies were transferred to slants of the same medium used for isolation; later, brain liver heart (Difco) agar was found to be more satisfactory for subculture and maintenance. After incubation for 3 to 4 days, the cultures were checked for purity by Gram staining and streaking on plated media. After primary isolation, all the organisms grew under both aerobic and anaerobic conditions. The isolates were assigned numbers in order of their isolation, and only one pure culture obtained from any individual was

placed in the stock culture collection. For purposes of comparison, one typical culture, designated as E, was obtained from Dr. Marion N. Gilmour of the Eastman Dental Dispensary, Rochester, New York.

Cultures were transferred monthly to slants of brain liver heart agar and stored at 4 C. Stock cultures have also been successfully frozen at −56 C in brain heart infusion broth containing 15% glycerol. These have remained viable for as long as 6 months.

Biochemical reactions. A semisolid cysteine trypticase agar base (no. 174, Baltimore Biological Laboratory) was employed as the basal medium. This base was sterilized by autoclaving at 121 C for 20 min. The carbohydrates, prepared in 20% concentration in distilled water, were sterilized separately in the same manner, and added aseptically to the basal medium. The final concentration of sugar in each tube was 1%. All fermentations were carried out in duplicate, and the tests read at intervals of 1, 3, and 7 days. Tests for nitrate reduction, indole and hydrogen sulfide production, and gelatin liquefaction were conducted by methods described by Schaub and Foley (1952). The presence of catalase was determined by the method of King and Meyer (1957).

Preparation of antigens. The organisms used either for immunization or agglutination reactions were grown in brain heart infusion broth (Difco) for 3 days at 37 C under aerobic conditions. Since the growth was granular, most of the clear supernatant broth could be siphoned off. After centrifugation, the cultures were washed 3 times with sterile saline by repeated centrifugation at 2,000 rev/min (732 × *g*) for 15 min.

For vaccine production, the organisms were ground in a Teflon tissue grinder (Arthur H. Thomas Company) to give a homogeneous suspension. After heating at 60 C for 1 hr, the antigens were standardized to the density of a no. 8 MacFarland nephelometer tube, and stored at 4 C.

For agglutination tests, the organisms were heated at 60 C for 1 hr and then ground in the Teflon tissue grinder. Heating after grinding sometimes resulted in auto-agglutination which made interpretation of the agglutination tests difficult. The ground homogenous suspension was then diluted with saline and filtered through a gauze and absorbent cotton pad. Finally, the antigens were adjusted to 70% transmission at 540 mμ with the Coleman Junior spectrophotometer, model 6A.

Preparation of immune sera. Albino rabbits (2 kg), previously trial bled and tested, were given intravenous injections of the heated, standardized suspensions of each of 11 isolates on 3 consecutive days followed by 4 days of rest. This procedure was continued for 4 weeks. The amount of antigen given per injection is indicated by the following protocol: first week, 1.0 ml, 1.0 ml, and 1.5 ml; second week, 1.5 ml, 1.5 ml, and 2.0 ml; third week, 2.0 ml, 2.0 ml, and 2.5 ml; fourth week, 2.5 ml, 2.5 ml, and 3.0 ml. Seven days after the final injection, the rabbits were bled and the sera collected and stored at −20 C.

Agglutination tests. Serial twofold dilutions of serum were made in 0.5 ml volumes of saline, and 0.5 ml of the standardized antigen was added to each tube. Normal serum and antigen controls were included in each assay. The tests were incubated in a water bath at 56 C for 1 hr and read after standing at room temperature for 10 min.

RESULTS

Twenty-six pure cultures were obtained during the course of this investigation; however, five of these were lost during subculture. Only those isolates which possessed the cellular and colonial morphology described by Gilmour and Hunter (1958) were selected for further study. For purposes of clarity, it is deemed necessary to reiterate these morphological characteristics.

Upon initial isolation, most of the plates incubated anaerobically yielded small numbers of colonies which were translucent and circular (0.5 to 1.0 mm diameter) with a slightly raised center. The periphery was flat, and tangled filamentous processes radiated to give a rhizoid appearance. They were tough, membranous, and tenaciously adherent to the medium. All pure cultures produced long, nonseptate filaments approximately 1 to 2 μ wide and 20 to 100 μ long. Rectangular bacillary bodies, averaging 1.5 by 6.0 μ, were often found at the tips of the filaments. Dichotomous branching was frequently observed. Young cells were gram-positive, but in older cultures, many small, round, purple elements were observed within a pink sheath.

To more accurately characterize the organisms which we had isolated, fermentation and other

TABLE 1

Agglutination reactions of Leptotrichia dentium

Isolate No.	Antibody Titers* with Untreated Antisera No.:										
	E	1	2	3	4	5	6	8	13	16	17
E	3,200	1,600	400	400	200	800	200	200	1,600	800	3,200
1	3,200	2,650	800	100	400	800	200	50	3,200	400	1,600
2	400	200	400	100	400	200	100	200	400	800	800
3	800	800	400	800	400	400	400	100	800	400	800
4	800	800	400	400	800	400	400	800	400	800	800
5	800	400	200	100	200	800	400	200	800	800	400
6	800	400	200	200	400	400	800	200	800	400	800
7	1,600	800	100	200	400	400	200	200	800	400	400
8	200	50	0†	0	0	0	0	800	400	400	400
9	400	100	100	100	200	200	200	200	200	400	200
11	2,650	1,600	800	400	400	400	100	100	3,200	800	1,600
12	400	200	0	0	0	50	0	100	400	400	400
13	1,600	800	400	200	200	200	100	200	1,600	400	800
14	1,600	400	100	100	200	200	100	200	800	800	800
15	400	400	0	0	100	200	0	200	200	800	400
16	200	100	0	0	0	0	0	200	200	800	200
17	1,600	800	100	100	100	100	100	100	800	400	1,600
19	400	200	200	200	400	200	50	800	800	800	800
20	800	800	100	200	400	400	400	200	800	800	400
21	800	800	200	200	400	200	200	400	800	400	800
24	800	400	100	200	400	400	200	50	800	400	800
26	1,600	800	100	100	200	200	200	400	1,600	800	800

* Titers expressed as reciprocal of highest dilution of serum showing agglutination.

† Negative reaction in lowest dilution tested, 1:50.

biochemical tests were conducted. All of the isolates tested produced acid but no gas, usually within the initial 24-hr period of incubation, on the following carbohydrates; fructose, glucose, maltose, mannose, sucrose, and dextrin. All fermented galactose at 1 to 3 days, but the phenol red indicator reverted by the seventh day of observation. Only isolate 26 fermented mannitol. None fermented arabinose, dulcitol, inulin, lactose, rhamnose, sorbitol, starch, or xylose.

All were strongly catalase-positive, and reduced nitrate. None produced indole, hydrogen sulfide, or liquefied gelatin.

To determine if these organisms could be divided into serological groups or types on the basis of acid-soluble antigens, each isolate was extracted with 0.2 N HCl according to the Lancefield technique for classifying streptococci (Schaub and Foley, 1952). The neutralized extracts were then tested by the precipitin reaction against the 11 antisera which had been prepared by the injection of whole, heat-inactivated cells. In all cases, the extract from each isolate gave a positive ring test with all of the antisera.

Agglutination studies were undertaken to detect any differences in the antigenic composition of these organisms. Preliminary experiments had indicated that it was desirable to use the antigen on the same day that it was prepared because auto-agglutination usually resulted from storage at 4 C. Freshly ground, standardized antigens were tested against serial twofold dilutions of each of the 11 antisera. The data shown in Table 1 would indicate that some antigenic differences existed. While all of the isolates tested were agglutinated by antiserum E, 1, 8, 13, 16, or 17, some definite quantitative differences were observed. Several organisms failed to react with antiserum 2, 3, 4, 5, or 6 and reacted weakly with other high-titer antiserum, for example E or 1.

Since no distinctive or consistent pattern was

TABLE 2

Serum antibody titers of rabbits injected with three preparations of isolate 26*

Treatment of Vaccine	Rabbit No.	Interval in Weeks					
		0	1	2	3	4	5
Formalin, 0.5%	a	0†	800	800	3,200	3,200	1,600
	b	0	800	1,600	1,600	6,400	3,200
	c	0	400	1,600	3,200	3,200	3,200
	d	0	400	1,600	3,200	3,200	3,200
Heat, 60 C, 1 hr	e	0	400	1,600	3,200	3,200	3,200
	f	0	800	1,600	6,400	3,200	3,200
	g	0	400	400	6,400	3,200	1,600
	h	0	400	1,600	6,400	3,200	3,200
Untreated	i	0	800	800	1,600	1,600	1,600
	j	0	200	1,600	3,200	1,600	3,200
	k	0	400	800	6,400	6,400	6,400

* Titers expressed as reciprocal of highest dilution of serum showing agglutination.

† Negative reaction in lowest dilution tested, 1:10.

evident from the agglutination reactions, the possibility existed that certain antigenic components were heat labile. To test the validity of this proposition, 11 rabbits were injected with formalin-treated, heated or living organisms prepared from washed cultures of isolate 26. Table 2 shows that there was little difference in the quantitative response of the rabbits to the 3 types of antigen preparations. Cross-agglutination studies using pooled sera revealed no detectable differences.

TABLE 3

Comparison of carbohydrate fermentations of Leptotrichia dentium*

Carbohydrate	Bibby and Berry (1939)	Davis and Baird-Parker (1959)	Beck and Gilmour (1959)	Richardson and Schmidt (1959)	Kroeger and Sibal (This Paper)
Glucose	+	+	+	+	+
Maltose	+	+	+	+	+
Fructose	+	+	+	+	+
Sucrose	+	+	+	+	+
Mannose		+	+	+	+
Dextrin	+	+		+	+
Galactose	0	v			+
Glycerol			0		0
Lactose	0	0	0		0
Inulin	0		0		0
Rhamnose			0		0
Sorbitol			0		0
Dulcitol	0		0		0
Starch			0		0
Xylose			0		0
Arabinose		v	0		0
Mannitol	0				0†
Raffinose	+		0		
Salicin		v	0	+	
Cellobiose		0	0		
Trehalose		0			
Melezitose		0			
Melibiose			0		
α-Methyl glucoside			0		

* + = Fermentation; 0 = no activity; v = variable; blank = not tested.

† One of 21 isolates fermented this carbohydrate.

DISCUSSION

Isolation from dental calculus or materia alba of a large, gram-positive, filamentous, branching organism which grows under both aerobic and strict anaerobic conditions has been accomplished by several investigators previously cited. Cultures from this laboratory as well as those obtained by other workers (Table 3) showed certain distinctive fermentation reactions. Glucose, maltose, sucrose, fructose, mannose, and dextrin were consistently fermented by all, whereas lactose, glycerol, dulcitol, xylose, inulin, arabinose, rhamnose, sorbitol, and starch were not fermented. Our isolates fermented galactose readily, whereas those of Davis and Baird-Parker (1959) appeared to be variable in this respect. Bibby and Berry (1939) reported no fermentation of this carbohydrate. The minor variations in the fermentation patterns of these microorganisms appear to complicate the definition of the species; however, not all investigators have employed the same basal media and carbohydrate concentrations. Other biochemical tests seem less variable, since our findings are in agreement with those of other investigators.

The biochemical and cultural properties may be helpful in characterizing these organisms. Our results indicate that identification may be facilitated by microprecipitin tests using acid-soluble extracts. However, cross-reactions with other oral filamentous organisms remain to be investigated before this method can be accepted

as definitive. Since we were unable to detect any serological differences with this technique, it has been concluded that all of our isolates possess some antigen or antigens in common.

The complexity of the antigenic composition has been demonstrated by the agglutination reactions. Although six of the antisera agglutinated all of the isolates tested, quantitative differences were evident. At this time, it is not known whether these differences are due to the presence of distinct antigens or to the quantity of certain antigens present in the organisms. It has been shown that the antigenic response in rabbits to the injection of heated, formalin-treated, or living organisms was quantitatively similar. Since cross-agglutination reactions revealed no difference in the antisera, it was concluded that the differences observed in the agglutination reactions (Table 2) were not the result of heat labile antigens. Agglutinin-adsorption tests are currently in progress in order to answer this question.

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SUMMARY

Twenty-one isolates of an oral filamentous organism usually termed Leptotrichia were obtained from calculus or materia alba. The cultural, morphological, and biochemical characteristics were similar to those reported by other investigators although some variations in the carbohydrate fermentations were observed.

Extraction of the organisms with dilute hydrochloric acid revealed the presence of common acid-soluble antigens which reacted with antisera in a microprecipitin test. This technique was tentatively proposed as a rapid means of identification. Agglutination reactions demonstrated that the antigenic composition was complex, but no distinct serological types have been found.

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