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Morphological and Biochemical Differentiation of Three Types of Small Oral Spirochetes

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Thirty strains of human oral anaerobic spirochetes were isolated in three different media: veal heart infusion-ascitic fluid, Spirolate-Brain Heart Infusion-rabbit serum, and supplemented PPLO broth. The morphological and biochemical characteristics of the isolates permitted their differentiation into three distinct species: *Treponema denticola*, *T. macrodentium*, and *T. oralis* (proposed new species). These species could be differentiated as follows. Organisms of the *T. denticola* type had a "2-4-2" axial fibril relationship as determined by electron microscopy, required serum for growth, did not utilize glucose or lactate, and produced indole, ammonia, acetate, and lactate as end products. *T. macrodentium* had a "1-2-1" axial fibril relationship, did not require serum, utilized glucose but not lactate, did not produce indole or ammonia, and produced formate, acetate, lactate, and succinate as acid end products. *T. oralis* had a "1-2-1" axial fibril relationship, required serum for growth, utilized lactate but not glucose, produced indole but not ammonia, and produced propionate and acetate as acid end products.

Host-associated anaerobic spirochetes, including those resident in the human oral cavity, are rather poorly classified. The current classification schemes (1, 9) depend primarily upon the source of the microorganism, its staining characteristics, and its morphology when viewed by the light microscope. The chief reason that classification schemes have been restricted to these criteria has been the difficulty in cultivating anaerobic spirochetes. In the past few years, a simplified culture medium (supplemented PPLO) was developed which supported abundant growth of certain types of oral spirochetes (16). This culture medium did not support the growth of spirochetes isolated in the typical veal heart infusion-ascitic fluid medium used by other investigators (3, 10, 13), and the veal heart infusion-ascitic fluid medium in turn did not support growth of the spirochetes isolated in the supplemented PPLO medium. At about the same time, Listgarten and Socransky (6) suggested the use of the electron microscope as an aid in the taxonomic differentiation of certain oral spirochetes. They described small, intermediate, and large spirochetes which could be differentiated on the basis of the size of the protoplasmic cylinder, number of axial fibrils, and structure of the outer envelope. In the group of small spirochetes, two types of organisms were seen. One type had two axial fibrils, originating at each end of the protoplasmic cylinder, which overlapped in the center to form a "2-4-2" axial fibril relationship. This organism grew on veal heart infusion-ascitic fluid medium. The second small spirochete had one axial fibril, originating at each end of the protoplasmic cylinder, which overlapped in the center to form a "1-2-1" axial fibril relationship. This type of spirochete would grow in the supplemented PPLO medium but not on veal heart infusion-ascitic fluid medium. It was suggested at that time that these were two different types of spirochetes which differed not only nutritionally and morphologically, but possibly biochemically as well.

The purpose of the present investigation was to isolate and characterize strains of the "1-2-1" and "2-4-2" spirochetes, and to compare their morphological and biochemical characteristics.

MATERIALS AND METHODS

Isolation and maintenance of strains of spirochetes. Three types of media were employed to isolate the spirochetes studied. Twelve strains of spirochetes were isolated in PPLO agar without crystal violet or serum (BBL) supplemented with 1 mg of glucose per ml, 1 mg of L-cysteine per ml, 400 μg of nicotinamide per ml, 150 μg of spermine tetrahydrochloride per ml, 20 μg of sodium isobutyrate per ml, and 5 μg of thiamine pyrophosphate per ml (16). Ten strains were isolated in a veal heart infusion-ascitic fluid medium

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prepared as described by Fitzgerald and Hampp (3). Six strains were isolated in Spirolate (BBL)-Brain Heart Infusion (Difco)-rabbit serum medium described by Hanson and Cannefax (4), and two strains (TRRD and FM) were kindly supplied by A. W. Hanson, Communicable Disease Center, Atlanta, Ga.

Spirochetes were separated from other gingival crevice bacteria by inoculation of gingival debris into a well plate (12) or onto the surface of a 0.22-μm filter (Millipore Filter Corp., Bedford, Mass.) which had been placed on the surface of an agar-containing medium (7). After the spirochetes had been separated from other bacteria, single strains of spirochetes were separated by means of pour plates or by surface streaking on 3% agar plates as previously described (17). Each of the 30 strains isolated in this investigation was derived from the gingival crevice area of a different individual. The organisms were adapted to grow in broth by sequential transfer in media containing 0.8, 0.6, 0.4, 0.2, 0.1, and 0% Ionagar no. 2 (Oxoid). It was found that all strains would grow in the supplemented PPLO medium when this medium was enriched with 10% ascitic fluid or rabbit serum or 0.05% α_2-globulin (15). The PPLO medium supplemented with 0.05% α_2-globulin was used for maintenance and biochemical testing. All strains were grown at 35 C for 2 to 7 days in Brewer jars containing 95% hydrogen and 5% carbon dioxide.

Characterization of organisms. All strains were tested for their ability to grow in the absence of serum or ascitic fluid. To test carbohydrate fermentation, sufficient filter-sterilized carbohydrate was aseptically added to the supplemented PPLO medium (without glucose) to make a final concentration of 1%. The *p*H of the medium was determined after 2 weeks of incubation. A *p*H fall of less than 0.5 was considered a negative fermentation, whereas a *p*H fall of 0.5 or more was considered a positive fermentation. Gelatinase, catalase, indole, and hydrogen sulfide production and nitrate reduction were determined by using methods described in the *Manual of Microbiological Methods* (14).

Glucose utilization was determined by the use of the glucostat reagent (Worthington Biochemical Corp.). Acid end products were determined as described by Loesche, Socransky, and Gibbons (8).

All cultures were examined by electron microscopy during the stationary phase, since log-phase cells have irregular numbers of fibrils owing to overlap of fibrils of dividing cells (5). Cells were harvested and negatively stained with phosphotungstic acid as previously described (6).

RESULTS

Morphology. All strains of spirochetes appeared identical by light microscopy. The organisms varied from 6 to 16 μm in length and occasionally occurred in chains. The coiling of the organisms was variable; sometimes the organisms were tightly coiled, and at other times the organisms were loosely wound and irregularly coiled. End granules were frequently observed in all cultures. The organisms were generally not motile except in very early cultures. The organisms were gram negative and stained only weakly with the counter stain.

The electron microscopic appearance of spirochetes has been previously described (5). The 12 strains isolated on the supplemented PPLO medium all showed a "1-2-1" axial fibril relationship by electron microscopy. Five of the strains isolated in the veal heart infusion-ascitic fluid medium had a "1-2-1" axial fibril relationship. The remaining five strains isolated in the veal heart infusion-ascitic fluid medium, the six strains isolated in the Spirolate-Brain Heart Infusion-rabbit serum medium, and the two strains provided by A. W. Hanson also had a "2-4-2" axial fibril relationship (Table 1). The morphology of the 30 strains as revealed by the electron microscope was otherwise identical.

Biochemical characteristics. The biochemical characteristics of the 30 spirochete strains tested are shown in Table 1. The characteristics of the isolates allowed their separation into three distinct groups. The 12 strains isolated in the supplemented PPLO medium (i) fermented D-glucose, D-ribose, D-fructose, D maltose, and sucrose, (ii) were variable in regard to fermentation of D-xylose, D-galactose, and cellobiose, and (iii) did not ferment D-mannose, L-rhamnose, L-sorbose, lactose, D-arabinose, D$^+$trehalose, starch, inulin, mannitol, sorbitol, and salicin. None of the 18 remaining strains fermented any of the carbohydrates tested. Only strains isolated in the supplemented PPLO medium utilized glucose as determined by the glucose oxidase test. These strains required a carbohydrate for growth. None of the strains tested produced catalase or reduced nitrate. All strains produced hydrogen sulfide. The strains isolated in the supplemented PPLO medium actively produced gelatinase, whereas the remaining strains were weakly gelatinase-positive. Indole was not produced by strains isolated in the supplemented PPLO medium but was produced by the remaining organisms.

The strains isolated in supplemented PPLO broth formed approximately 0.2 μmole of formic acid, 0.2 μmole of acetic acid, and 0.6 μmole of lactic acid per 0.4 μmole of glucose utilized. The "2-4-2" strains produced acetic acid and lactic acid presumably from proteinaceous materials, since they released large amounts of ammonia and did not utilize glucose. The "1-2-1" strains isolated in veal heart infusion-ascitic fluid broth produced acetic and propionic acids. These latter organisms utilized lactate as shown by lactic acid determinations of the medium before and after spirochetal growth. As mentioned above, only the "2-4-2" spirochetes produced large amounts

TABLE 1. *Biochemical and morphological characteristics of 30 strains of small oral spirochetes*

Characteristics	Axial fibril relationship		
	1-2-1[a]	2-4 -2[b]	1-2 -1[c]
Fermentation of			
D-Glucose, D-ribose, D-fructose, D+ maltose, sucrose	+	0	0
D-Xylose, D-galactose, cellobiose	Variable	0	0
D-Mannose, L+ rhamnose, L-sorbose lactose, D-arabinose, D+ trehalose starch, inulin, mannitol, sorbitol, salicin	0	0	0
Glucose utilization	+	0	0
Catalase production	0	0	0
Nitrate reduction	0	0	0
Hydrogen sulfide production	+	+	+
Gelatinase production	+	+	+
Indole production	0	+	+
Acid end products[d]			
Formic	1	0	0
Acetic	1	10	3
Propionic	0	0	2
Lactic	3	8.5	0
Succinic	Trace	0	0
Lactate utilization	0	0	+
Ammonia production	0	+	0
Serum requirement	0	+	+

[a] Twelve strains isolated on supplemented PPLO medium.

[b] Thirteen strains isolated on veal heart infusion or Spirolate-Brain Heart Infusion.

[c] Five strains isolated on veal heart infusion.

[d] Approximate average end products, in micromoles per milliliter of culture.

of ammonia whereas the "1-2-1" organisms did not. The organisms isolated in supplemented PPLO broth (without serum, ascitic fluid, or α_2-globulin) did not require serum or ascitic fluid in contrast to the remaining strains.

DISCUSSION

Rosebury (11) in reviewing the classification of the spirochetes indigenous to man found the situation to be one of "near chaos." This situation is true in the case of spirochetes because of the very large numbers of organisms which have been named and the very small numbers of strains or characteristics on which these names have been based. He suggested that a "tentative minimum classification of the indigenous spirochetes be set up in which two species are temporarily recognized: *Treponema dentium* and *Borrelia refringens*."

The present authors concur with Rosebury's suggestion in principle; however, *T. dentium* is considered to be an illegitimate name (2).

Since *T. denticola* appears to have priority for the cultivated mouth spirochetes, we have designated the name *T. denticola* to the organisms of the "2-4-2" axial fibril types which are isolated in the veal heart infusion-ascitic fluid medium, because we believe that these organisms are probably similar to those isolated on this type of medium since the early 1900's. Obviously, this type of conjecture can never by proven, but 14 strains isolated on similar media by Marion Williams (Washington University, St. Louis, deceased) and Robert Richardson (University of Iowa, Iowa City) were all of the "2-4-2" type (*unpublished data*). This investigation has demonstrated that organisms similar to these veal heart infusion-ascitic fluid isolates can be isolated in simple media such as the Spirolate-Brain Heart Infusion-rabbit serum medium (4) or the supplemented PPLO medium enriched with ascitic fluid or serum products.

The "1-2-1" spirochete isolated on veal heart infusion-ascitic fluid medium seems to be less commonly isolated. In fact, all isolates were made in one batch of veal heart infusion base, which allowed the growth of this organism but not of the "2-4-2" type. Variations in the composition of this medium may account not only for selection of different spirochetes but differences in results between different laboratories on seemingly identical media. The "mysterious" losses of strains which so frequently plague workers in this area may also be due to the poor reproducibility of this medium. The "1-2-1" spirochetes isolated on veal heart infusion-ascitic fluid medium have been designated *T. oralis* sp. n. This organism appears to utilize lactic acid and produces acetic and propionic acids as principal acid end products.

The organism designated *T. macrodentium* is clearly different from the first two species in that it does not require ascitic fluid or serum, does require glucose, and produces a different array of end products. Table 2 lists characteristics useful in distinguishing the three species of spirochetes.

The base composition of deoxyribonucleic acid isolated from six strains of spirochetes in this investigation has been determined by Spinell, Gordon, and Clark (*personal communication*). The per cent guanine plus cytosine (GC) values

TABLE 2. *Differential characteristics of species of small oral spirochetes*

Characteristics	*T. macrodentium*	*T. denticola*	*T. oralis*
Serum requirement	0	+	+
Axial fibril relationship	1-2-1	2-4-2	1-2-1
Glucose utilization	+	0	0
Lactate utilization	0	0	+
Indole production	0	+	+
Ammonia production	0	+	0
Acid end products			
Formic	+	0	0
Propionic	0	0	+
Lactic	+	+	0

for three strains of *T. denticola* ranged from 37.1 to 38.1%. The GC values for two strains of *T. oralis* were 37.3 and 37.6%, and the GC value of the type strain TM1 of *T. macrodentium* was 39.1%. The species descriptions are as follows.

Treponema macrodentium. Twelve oral strains of which TM1 is the type strain are placed in this species. It is a gram-negative, helically coiled organism approximately 0.1 to 0.25 μm wide and 6 to 16 μm long. It is single-contoured by dark-field illumination. The spirochete has a "1-2-1" axial fibril relationship. The organism does not grow well as surface colonies. Growth in liquid medium is evenly dispersed. Strict anaerobe. The final *p*H in PPLO broth containing 1% glucose is 5.0 to 5.4. Acid fermentation products detected in broth containing glucose include lactic, acetic, formic, and traces of succinic acids in the following average percentages: 60, 20, 20, and trace, respectively. No gas is produced. Glucose is utilized. Glucose, ribose, fructose, maltose, and sucrose are fermented. Mannose, rhamnose, sorbose, lactose, arabinose, trehalose, mannitol, sorbitol, and salicin are not fermented. Starch is not hydrolyzed. Gelatin is liquified. Nitrate is not reduced. Catalase and indole are not produced. Hydrogen sulfide is produced as determined by lead acetate paper strips. Source: the gingival crevice of man.

Treponema denticola. Thirteen oral strains of which TD1 is the type strain are placed in this species. This species consists of helically coiled organisms approximately 0.1 to 0.25 μm in width and 6.0 to 16.0 μm in length. The organism is single-contoured by dark-field microscopy. End granules are frequently detected in culture. The spirochete has a "2-4-2" axial fibril relationship. The organism does not grow well on surface cultivation. Growth in liquid medium is evenly dispersed. Strict anaerobe. Final *p*H in PPLO broth with 1% glucose is 6.8 to 7.2. Acetic acid and lactic acid are detected as fermentation products in glucose broth. No gas is produced. Carbohydrates are not fermented. Glucose is not utilized. Starch is not hydrolyzed. Gelatin is liquefied weakly. Nitrate is not reduced. Catalase is not produced. Ammonia and indole are produced. Hydrogen sulfide is produced as detected by lead acetate paper strips. Source: the gingival crevice area of man.

Treponema oralis sp. n. Five strains of which strain TO1 is the type strain are placed in this species. Helically coiled organisms 0.1 to 0.25 μm in width and 6.0 to 16.0 μm in length. Occasionally forms chains. Frequently end granules seen in broth culture. Single-contoured by dark-field microscopy. The spirochete has a "1-2-1" axial fibril relationship. Does not grow well on surface cultivation. Growth in liquid medium is evenly dispersed. Strict anaerobe. Final *p*H in PPLO broth with 1% glucose is 6.8 to 7.2. Acetic and propionic acids detected as fermentation products in broth. No gas is produced. No carbohydrates are fermented. Glucose is not utilized. Starch is not hydrolyzed. Gelatin is liquefied. Nitrate is not reduced. Catalase is not produced. Ammonia and indole are not produced. Hydrogen sulfide is produced as detected by lead acetate paper strips. Source: the gingival crevice area of man.

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