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# Microbiological and Biochemical Characterization of Spirochetes Isolated from the Feces of Homosexual Males

M. JEANETTE JONES,[1,2] JAMES N. MILLER,[3] AND W. LANCE GEORGE[1,4,5]*

Research[1] and Medical[4] Services, West Los Angeles Veterans Administration Medical Center, Wadsworth Division, Los Angeles, California 90073; Department of Microbiology and Immunology[3] and Department of Medicine,[5] University of California, Los Angeles, School of Medicine, Los Angeles, California 90024; and Department of Microbiology, California State University, Long Beach, California 90840[2]

Spirochetes were isolated from the feces of 11 homosexual males who had diarrhea. The anaerobic organisms were isolated from a selective medium that consisted of Trypticase soy agar supplemented with either 5% horse or human blood, 400 µg of spectinomycin per ml, and 5 µg of polymyxin B per ml. Nonselective media that permitted good growth of these fastidious organisms were developed, and selected biochemical tests were performed. The tests included carbohydrate utilization, detection of certain enzymes, and determination of volatile fatty-acid end products of metabolism. Two growth patterns were noted on solid media, a haze of growth and production of small colonies. Based on the results of biochemical tests, patterns of preformed enzymes, and volatile fatty-acid production, we believe that the 11 isolates represent a heterogeneous group of spirochetes. The data suggest that the human colon may harbor unique strains of cultivable spirochetes; additional study of the taxonomy of the organisms and assessment of their virulence for humans are needed.

An association between spirochetes and the human intestinal mucosa was first documented by Escherich in the late 19th century (7, 8). Since that time, scientific interest in this association, often referred to as intestinal spirochetosis, has been rather limited. It has been well established that an infection of the intestinal mucosa of swine, termed swine dysentery, is caused by the spirochete, Treponema hyodysenteriae (10). The role, if any, of intestinal spirochetes in either health or disease of humans is not known. Spirochetelike organisms have been observed in either the gut or feces of both healthy individuals and patients with a variety of gastrointestinal ailments (1, 3–5, 9, 11, 12, 14–17, 19). Very little is known about the biology of these organisms; most reports have focused primarily on morphological characterization as assessed by either light or electron microscopy of colonic mucosa (1–5, 9, 11, 12, 14, 16, 17). In our laboratory, we cultivated spirochetes from the feces of homosexual males who had diarrhea. In this report, we describe our method of culture and selected morphological and biochemical characteristics of the first 11 isolates.

## MATERIALS AND METHODS

**Isolation of organisms.** Stool specimens were screened by dark-field microscopy for the presence of spirochetes, and those that were positive were inoculated onto selective medium composed of Trypticase soy agar (TSA; BBL Microbiology Systems, Cockeysville, Md.) supplemented with 400 µg of spectinomycin per ml, 5 µg of polymyxin B per ml, and either 5% citrated human blood or 5% defibrinated horse blood (GIBCO Laboratories, Chagrin Falls, Ohio) (3, 12, 14, 15). No attempt was made to obtain control specimens from patients who did not have diarrhea. The plates were incubated anaerobically for 5 to 7 days at 37°C in a GasPak jar

(BBL). If a haze of growth was detected after 5 to 7 days of incubation and motile organisms were seen on dark-field microscope examination, then the area of haze was subcultured to a fresh selective-agar plate and incubated under the above conditions. Isolation of spirochetes in pure culture from the selective agar containing human blood required the use of TSA pour plates supplemented with 10% human serum. Specimens inoculated onto the selective TSA-horse blood medium usually yielded isolates in pure culture by the third passage on the selective-agar medium; thus it became unnecessary to use the pour-plate method described above. Motility (and, presumably, viability) of all isolates was determined by dark-field microscope examination. Purity of the cultures was determined by dark-field microscope examination and subculture to three TSA-horse blood plates that did not contain antibiotics; these plates were incubated at 37°C under aerobic, microaerophilic, and anaerobic conditions and examined for the presence of nonspirochetes.

**Growth studies.** Growth of the isolates in thioglycolate broth and brucella broth (BBL) without serum and on brucella blood agar supplemented with hemin and vitamin $K_1$ (18) was nil; growth on TSA-horse blood of most isolates was, at best, only fair. To optimize growth, we tested several media known to support spirochetes. These included Trypticase soy broth (BBL), PPLO medium (BBL), and thioglycolate without $E_h$ indicator (BBL) to which serum was added. Human serum, horse serum (GIBCO), fetal calf serum (GIBCO), and fresh or frozen (Pel-freeze Biological, Rogers, Ark.) rabbit serum were heat inactivated in a water bath at 56°C for 60 min and added as growth supplements to each of the media at concentrations of 10, 20, and 30% (vol/vol). Each broth was reduced by steaming before the addition of serum and inoculation with an isolate. For each isolate, a 10-ml tube of test medium was inoculated with 5 to 7 drops of a thioglycolate maintenance culture and incubated in an anaerobic chamber (Coy Laboratory Products, Ann

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* Corresponding author.

Arbor, Mich.) at 37°C. At 5, 10, and 14 days, the cultures were examined for motility by dark-field microscopy, for degree of visible turbidity as an indication of growth, and for purity by subculture to solid media as described above.

**Biochemical testing.** The following thioglycolate-based media were prepared as described previously (6): glucose, fructose, lactose, maltose, sucrose, mannitol, and ribose (all at final concentrations of 1%). A modified Sim medium was used for the detection of $H_2S$ production (13). All media were reduced anaerobically overnight in an anaerobic chamber (Coy). Each tube contained 5 ml of medium and was supplemented with 15% fetal calf serum (vol/vol) and inoculated with 10 drops of a viable 10-day-old spirochete broth culture or sterile broth (negative control). Purity and motility of the inoculum were determined in each case. The cultures were incubated in an anaerobic chamber at 37°C for 10 days and then examined for purity, for motility by dark-field microscope examination, and for pH. Three separate cultures of each isolate were tested, and the pH values were expressed as an average. "*Treponema vincentii*" acquired from R. M. Smibert, Virginia Polytechnic Institute, and "*Treponema phagedenis*" biotype Reiter were used as control strains.

**Enzymatic reactions.** Enzymatic activity was assessed by use of API ZYM (Analytab Products, Plainview, N.Y.). Briefly, each isolate was inoculated heavily onto three TSA plates enriched with 10% fetal calf serum; the plates were incubated anaerobically at 37°C for 10 days. Organisms were then scraped off the agar surface with a sterile applicator stick and added to 2.5 ml of sterile distilled water to produce turbidity equivalent to that of a McFarland standard 6. The enzyme kits were then inoculated, incubated aerobically for 5 h at 37°C, and then processed by the recommendations of the manufacturer. We used a 4-min exposure to sunlight to develop the reaction. Three separate cultures of each isolate were tested.

**Volatile fatty-acid end-product analysis.** Reduced thioglycolate basal medium supplemented with 1% glucose and 15% fetal calf serum was inoculated as described above and incubated anaerobically for 10 days, and the cultures were tested for purity and motility. The pH of each inoculated tube and of the control tube was also measured. The medium was then acidified by the addition of 2 drops of 50% $H_2SO_4$, centrifuged to remove particulate matter, and stored at 4°C until gas-liquid chromatography was performed. A Hewlett-Packard model 5830A gas chromatograph with a flame ionization detector was used for the volatile fatty-acid analysis. The parameters for volatile fatty-acid analysis were as follows: injector temperature, 175°C; oven maximum temperature, 200°C; and flame ionization detector temperature, 175°C. The column was a 15% SP-1220–1% $H_3PO_4$ on Chrom WAW, 100/120 mesh (Supelco Inc., Bellefonte, Pa.). The volatile fatty-acid standard contained acetic, propionic, isobutyric, *n*-butyric, isovaleric, *n*-valeric, isocaproic, and *n*-caproic acids (Supelco) (18). The volume of the injectates was 1 μl.

## RESULTS

**Isolation of organisms and growth studies.** Dark-field microscope examination of the fecal specimens revealed small helical organisms approximately 0.2 μm in diameter and 7 to 10 μm long. Motility was usually sluggish and either serpentine or rotational about the longitudinal axis; very little flexing motion was seen. The number of organisms observed varied appreciably from specimen to specimen.

The appearance of each isolate was similar on the primary isolation plates. They were recognized by a haze of surface growth with faint hemolysis, the intensity of which increased with time. The hemolysis was more pronounced on TSA plates with 5% horse blood than on those with 5% human blood. Hemolysis occurred a few millimeters behind, rather than along, the leading edge of growth. None of the strains grew under aerobic or microaerophilic conditions.

After 2 weeks of incubation, certain isolates (SP4, SP7, SP8, and SP12) produced pinpoint colonies that were circular with raised, convex centers and edges which were irregular in shape, flat, and spreading. After 1 month of anaerobic incubation, the colonies were approximately 2.0 mm in diameter. The other isolates did not produce discrete colonies.

We found that thioglycolate was superior to the other broth media tested and that fetal calf serum supplement was superior to human, horse, and rabbit serum supplements. Isolates were therefore maintained in 10 ml of reduced thioglycolate containing 1% glucose, 10 to 15% fetal calf serum, and 0.5 ml of a 2-mg/ml solution of sodium bicarbonate. The maintenance cultures were incubated anaerobically at 37°C and subcultured every 10 days. Growth in the thioglycolate maintenance broth occurred approximately 2 to 3 cm from the meniscus. By day 5, visible turbidity was present; from days 5 to 14, there was progressive development of a flocculated white mass approximately 2 cm below the meniscus.

**Biochemical testing.** All of the isolates displayed poor growth, and there was no change in pH from that in the control tube in thioglycolate medium lacking a carbohydrate (Table 1). In general, there was a correlation between the utilization of carbohydrate (as assessed by a decline in the pH of the broth) and the amount of growth. None of the isolates produced $H_2S$.

**Enzymatic reactions.** Only 5 of the 20 enzymes for which we tested were produced by the test isolates (Table 2). Enzymes not detected were esterase (C4), lipase (C14), leucine aminopeptidase, valine aminopeptidase, cystine aminopeptidase, trypsin, chymotrypsin, phosphohydrolase, beta-glucuronidase, alpha-glucosidase, beta-glucosidase, *N*-acetyl-beta-glucosaminidase, alpha mannosidase, and alpha-fucosidase.

**Volatile fatty acids.** Each isolate, as well as "*T. vincentii*" and "*T. phagedenis*" biotype Reiter, produced several volatile fatty-acid end products (Table 3). A large unknown peak was also detected (Table 3). This unknown had a retention time of 3.87 min and eluted from the column between *n*-valeric acid and isocaproic acid. Because the medium used for volatile fatty-acid analysis was supplemented with serum, there were low levels of many of the fatty acids in the uninoculated control. Because of this, our results are expressed qualitatively in relation to the uninoculated control and to the volatile fatty-acid standards.

## DISCUSSION

The human intestinal spirochetes presented in this paper are similar morphologically to human intestinal spirochetes cultivated by several investigators (3, 12, 14, 15). As is the case with other investigators, we are not able to say whether these organisms are of any consequence to the human host. A major problem encountered by other investigators (and by us) is the fastidious nature of these anaerobic organisms; they grow poorly or not at all in broth or on solid media in the absence of serum supplements. We were able to achieve

TABLE 1. Growth and change in pH of 11 spirochete isolates

| Isolate | Thioglycolate without carbohydrate | Growth/change in pH in medium with[a]: | | | | | | |
| | | Glucose | Fructose | Lactose | Maltose | Sucrose | Mannitol | Ribose |
|---|---|---|---|---|---|---|---|---|
| SP2 | +/0 | +++/0.7 | ++/0.2 | ++/0.5 | +++/0.5 | +++/0.4 | ++/0.3 | +/0.1 |
| SP3 | +/0 | ++/0.5 | +/0.1 | ++/0.3 | ++/0.7 | ++/0.5 | ++/0.3 | +/0 |
| SP4 | +/0 | +++/0.5 | ++/0.3 | ++/0.3 | ++/0.2 | ++/0.4 | ++/0.2 | +/0 |
| SP6 | +/0 | ++/0.3 | +++/0.4 | ++/0.4 | ++/0.3 | +++/0.4 | +/0.1 | +/0 |
| SP7 | +/0 | +++/0.6 | +++/0.5 | ++/0.5 | +++/0.5 | ++/0.5 | ++/0.2 | +/0.1 |
| SP8 | +/0 | ++/0.5 | ++/0.2 | ++/0.4 | ++/0.4 | ++/0.4 | +++/0.3 | +/0.1 |
| SP9 | +/0 | ++/0.6 | +/0.1 | ++/0.5 | ++/0.5 | ++/0.1 | ++/0.3 | +/0 |
| SP10 | +/0 | +++/0.5 | ++/0.3 | +++/0.3 | ++/0.3 | ++/0.1 | ++/0.2 | +/0.1 |
| SP11 | +/0 | +++/0.5 | +/0.2 | ++/0.2 | +++/0.5 | ++/0.5 | +++/0.2 | +/0 |
| SP12 | +/0 | +++/0.6 | +/0 | +/0.1 | +/0.1 | ++/0.4 | +++/0.3 | +/0 |
| SP13 | +/0 | +++/0.5 | ++/0.3 | ++/0.4 | +++/0.4 | ++/0.2 | ++/0.2 | +/0.1 |
| "T. vincentii" | +/0 | +++/0.5 | ++/0.3 | +++/0.5 | ++/0.3 | ++/0.4 | ++/0.4 | +/0 |
| "T. phagedenis" biotype Reiter | +/0 | +++/0.5 | +++/0.5 | ++/0.3 | ++/0.4 | ++/0.5 | +++/0.4 | +/0.1 |

[a] Values represent averages of three tests. +, Poor growth (slight increase in turbidity of broth and motile organisms seen on dark-field microscope examination); + +, fair growth (moderate increase in turbidity of broth); + + +, good growth (marked increase in turbidity of broth).

growth in serum-supplemented broth sufficient to permit more intensive study of the biology of the organisms.

The colonial morphology of some of our isolates is very similar to that described by Tompkins et al. (19). We used a similar culture medium, incubation temperature, and atmospheric conditions to obtain growth. In contrast to Tompkins and co-workers, we detected two distinct morphological forms (colony forming and haze forming).

The isolates that produced colonies on agar closely resembled the description of "Brachyspira aalborgi" colony type A (14); unfortunately, the strain of "B. aalborgi" that had previously been deposited with the National Collection of Type Cultures, Central Public Health Laboratory, London, England, was not available for comparison. Our isolates grew in Trypticase soy broth with 10% heat-inactivated rabbit serum, whereas the "Brachyspira" isolates reportedly did not grow in this medium. The motility of the "Brachyspira" isolates (14) also resembles the motility of our isolates when seen in the initial stool specimen by dark-field microscope examination but differs when examined in pure culture. The enzymatic profiles of our isolates differ from that reported for "Brachyspira" isolates. Our strains were much more active enzymatically than

TABLE 2. Enzymatic assays of 11 spirochete isolates[a]

| Isolate | Alkaline phosphatase | Esterase lipase (C8) | Acid phosphatase | α-Galactosidase | β-Galactosidase |
|---|---|---|---|---|---|
| SP2 | + | + | + | + | + |
| SP3 | – | + | + | + | + |
| SP4 | – | + | + | + | + |
| SP6 | – | + | + | – | + |
| SP7 | – | + | + | + | + |
| SP8 | – | + | + | + | + |
| SP9 | – | + | + | + | + |
| SP10 | – | + | + | – | + |
| SP11 | – | + | + | + | + |
| SP12 | – | + | + | – | + |
| SP13 | – | + | + | + | + |
| "T. vincentii" | + | – | + | – | + |
| "T. phagedenis" biotype Reiter | + | + | + | – | + |

[a] The uninoculated control medium was negative for all enzymes tested.

"Brachyspira" isolates. All of our isolates and the "Brachyspira" isolates produced β-galactosidase and esterase lipase (C8). We had one isolate that produced alkaline phosphatase which was not detected in "Brachyspira" isolates, whereas one isolate of "Brachyspira" (but none of ours) produced phosphoamidase. Our strains did not grow on the nonselective TSA-horse blood medium when rifampin (5 µg/ml) was added (data not shown). This finding suggests that our isolates are different from oral spirochetes for which a rifampin-containing medium is used for isolation.

Data have not been published on the biochemical characteristics, carbohydrate metabolism, or volatile fatty-acid end products of human intestinal spirochetes. We developed a method by which good growth of these organisms could be achieved. Unfortunately, the basal thioglycolate medium we used has not been used for biochemical or volatile fatty-acid analysis of other host-indigenous or free-living spirochetes; thus, comparison is not possible. Inasmuch as the thioglycolate-based medium contains only small amounts of peptone, it is not unexpected that only trace amounts of the iso fatty acids were detected (strict anaerobes produce more iso acids when grown in a peptone broth than when grown in thioglycolate broth) (13, 18).

The data presented in Table 1 indicate that some strains are able to utilize certain carbohydrates, whereas others are not. The Anaerobe Laboratory Manual (13) defines strongly positive biochemical reaction as a pH decrease of 0.50 U or more. Because we used serum-supplemented media, we elected not to use this criterion but rather to show the actual values (Table 1). The data in the table suggest that growth is clearly enhanced by the addition of a carbohydrate that the isolate can utilize. We do not know whether a carbohydrate is essential because our isolates required a serum supplement for growth. The growth (albeit poor) in thioglycolate without carbohydrate (Table 1) may have been due to small amounts of carbohydrate present in the serum supplement. Based on the ability of some isolates but not others to form colonies on solid media, and on the biochemical, enzyme, and volatile fatty-acid profiles, we believe that these 11 isolates do not represent a homogeneous group.

To determine the role of such organisms in the biology of the human gut, we must first learn whether there is, in fact, more than one type of human intestinal spirochete. Hence,

TABLE 3. Analysis of 11 spirochete isolates for volatile fatty-acid end products of metabolism[a]

| Isolate | Volatile fatty acid | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Acetic | Propionic | Isobutyric | n-Butyric | Isovaleric | n-Valeric | Unknown | Isocaproic | n-Caproic |
| SP2 | + | − | − | + | Tr | tr | + | − | Tr |
| SP3 | + | − | − | + | − | − | + | − | Tr |
| SP4 | + | − | − | + | − | tr | + | − | − |
| SP6 | + | Tr | − | + | Tr | − | + | − | Tr |
| SP7 | + | Tr | − | + | + | − | + | − | Tr |
| SP8 | + | Tr | − | + | + | − | + | − | Tr |
| SP9 | + | Tr | − | + | + | − | + | − | Tr |
| SP10 | + | Tr | − | + | Tr | − | + | − | Tr |
| SP11 | + | Tr | − | + | Tr | − | Tr | − | Tr |
| SP12 | + | Tr | − | + | Tr | − | + | − | Tr |
| SP13 | + | Tr | − | + | + | − | + | − | Tr |
| "T. vincentii" | + | Tr | − | + | − | − | + | − | Tr |
| "T. phagedenis" biotype Reiter | + | Tr | − | + | − | − | + | − | Tr |

[a] +, Amount (as determined by peak height) was greater than the concentration of the fatty acid in the volatile fatty-acid standard; Tr, amount was greater than that in the thioglycolate control tube but less than in the volatile fatty-acid standard; −, amount was equal to that found in the thioglycolate control broth.

further studies of the biology of intestinal spirochetes are needed and would seem feasible by using the growth methods we have developed. Our findings suggest that the human colon may harbor unique strains of cultivable spirochetes and that several different biotypes can be discerned. DNA homology studies and ultrastructural characterization studies are in progress to determine whether these organisms represent previously undescribed species. Cellular and molecular investigations have also been designed to elucidate the potential virulence of these organisms and their structure-function relationships.

## ACKNOWLEDGMENTS

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