Treponema pectinovorum sp. nov. Isolated from Humans with Periodontitis

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A new species of *Treponema*, *Treponema pectinovorum*, which was isolated from supragingival and subgingival samples from patients with periodontitis, is described. This new anaerobic species requires rumen fluid or short-chain volatile fatty acids and a fermentable energy source. Pectin, polygalacturonic acid, glucuronic acid, and galacturonic acid are the only substrates fermented. The main end products of fermentation are acetic and formic acids; traces of pyruvic and lactic acids occasionally are found. The type strain of *T. pectinovorum* is strain VPI D-36DR-2 (= ATCC 33768). The guanine-plus-cytosine content of the deoxyribonucleic acid of the type strain is 39 mol%.

In a study of the flora of supra- and subgingival samples from human subjects with periodontitis and gingivitis, treponemes were isolated that did not conform to the description of any previously described species. The purpose of this report is to present the characteristics of some unidentified strains of human oral treponemes and propose an appropriate taxon for this organism.

MATERIALS AND METHODS

Sites sampled. Treponemes were isolated from samples from the bottom and sides of the space between the tooth and the gingiva; the sampling sites were apical to the gingival margin and had chronic destructive inflammation of the periodontium. All such sites, called subgingival sites, were classified as exhibiting either moderate, juvenile, or severe periodontitis. In the moderate disease state the sites had a 5- to 7-mm probable depth and were in subjects older than 34 years of age. The diseased sites for both juvenile and severe periodontitis samples had probable depths of 7 mm or more and were in patients 30 years of age or younger. Juvenile periodontitis subjects had sites of periodontal destruction limited to the first molar or incisor teeth or both, whereas severe periodontitis subjects had more generalized patterns of destruction involving teeth in addition to first molars and incisors. Samples were also taken from sites without destruction in juvenile periodontitis subjects. Samples were also collected coronal to the gingival margin from the same tooth surfaces and these were called supragingival samples.

In addition, subgingival samples were taken from subjects with experimentally induced gingivitis. These subjects were males less than 32 years old with healthy gingiva who stopped all dental cleaning procedures, thereby allowing an undisturbed dental flora to accumulate on the teeth and within the gingival crevices (4). No probable crevice depths exceeded 3 mm. Clinical gingivitis developed in each subject within 26

days. None of the subjects in the various periodontal states had evidence of systemic disease.

Samples. All samples were taken with a Morse scaler equipped with a replaceable nickel-plated size 00 tip. The scaler tips with the periodontal samples were placed in tubes containing prereduced peptone-yeast extract (PY) broth (2) flushed with oxygen-free CO₂. Suspensions of organisms were prepared by shaking the tubes on a Vortex mixer for 5 to 10 s. Each tube contained PY broth, a sample, a scaler tip, and glass beads (diameter, 74 to 110 μ m). Serial 10-fold dilutions of the sample were made in 0.9 ml of gelatin-salts dilution medium (2), and 0.1 ml of each dilution was inoculated onto duplicate plates of treponeme isolation agar medium. The inoculated plates were immediately placed into GasPak jars (BBL Microbiology Systems, Cockeysville, Md.) containing fresh catalyst pellets. A GasPak envelope was added to each jar, the top was placed on the jar, and the jar was evacuated twice and clamped. All cultures were placed in GasPak jars within 45 min of the time that the sample was taken. The cultures were incubated at 37°C for 2 weeks.

Isolation medium. Oral treponeme isolation (OTI) medium contained 5 g of polypeptone (BBL), 5 g of heart infusion broth (BBL), 5 g of yeast extract (Difco Laboratories, Detroit, Mich.), 0.8 g of pectin, 0.8 g of glucose, 0.8 g of starch, 0.8 g of sucrose, 0.8 g of maltose, 0.8 g of sodium pyruvate, 0.8 g of xylose, 0.8 g of ribose, 0.8 g of fructose, 2 g of K₂HPO₄, 5 g of NaCl, 0.1 g of MgSO₄, 0.68 g of cysteine hydrochloride, 500 ml of clarified rumen fluid, and 500 ml of distilled water. OTI agar contained 1.5% agar, and OTI broth contained 0.16% agar. The pH was adjusted to 7.0. The OTI basal medium was autoclaved and cooled to 45°C, and serum and thiamine pyrophosphate were added to final concentrations of 5 and 0.75 mg/100 ml, respectively. A fresh filter-sterilized yeast autolysate was added to a final concentration of 5%. This yeast autolysate was prepared by adding 56 g of dry bakers' yeast to 200 ml of distilled water and incubating the preparation at 56°C for 4 days. The

yeast cells were sedimented by centrifugation at $3,000 \times g$ for 30 min, and the clear supernatant was filter sterilized. The agar medium was poured into petri dishes and solidified. A sterile membrane filter (diameter, 50 mm; Sartorius, San Francisco, Calif.) with an average pore size of $0.15 \mu\text{m}$ was placed on the agar surface. A sterile 1- or 1.5-inch (2.54- or 3.81-cm) O ring was placed on the filter and sealed to the filter with sterile molten 3% agar in water. The inoculum was placed on the surface of the filter inside the O ring.

After 2 weeks of incubation the O ring and filter were carefully removed, and the isolation medium was observed for white hazy growth in the agar. Plugs of treponemal growth were removed and inoculated into prereduced anaerobically sterilized OTI broth in rubber-stoppered tubes (18 by 150 mm). Unless otherwise stated, all cultures were inoculated and incubated in an oxygen-free mixture containing 20% CO_2 and 80% N_2 . After subculture the treponemes were inoculated into prereduced anaerobically sterilized OTI agar in rubber-stoppered 6- or 8-ounce (180- or 240-ml) prescription bottles: 1 or 2 drops of broth culture was inoculated into molten (45°C) OTI agar medium in a prescription bottle, the inoculum was mixed into the agar, the bottle was placed on its flat side, and the agar was solidified. These bottle plates were incubated at 37°C and observed for 1 to 3 weeks for development of colonies. Single well-isolated colonies were picked and inoculated into OTI broth. All isolates were preserved in liquid nitrogen in OTI broth containing dimethyl sulfoxide.

Tests. The procedures and media used for bacteriological tests and chromatographic analyses have been described previously (2). The PY-carbohydrate media for biochemical and cultural tests were supplemented with 5% serum, 0.75 mg of thiamine pyrophosphate per 100 ml, 20% rumen fluid, 0.025% volatile fatty acid solution, 4% fresh yeast autolysate, and 0.07% hemin solution. The volatile fatty acid solution contained 5 ml of glacial acetic acid, 4 ml of *n*-butyric acid, 1 ml of *n*-valeric acid, 1 ml of isovaleric acid, 1 ml of isobutyric acid, and 100 ml of distilled water. The pH was adjusted to 7.0, and the solution was filter sterilized. The hemin solution was composed of 0.05 g of hemin that was dissolved in 1 ml of 1 N NaOH and diluted to 100 ml with distilled water. The serum, rumen fluid, thiamine pyrophosphate, volatile fatty acid, and heme solutions were combined and added to media as one component. The pectin used in these studies was obtained from Sigma Chemical Co., St. Louis, Mo., and was grade I from citrus fruits with a galacturonic acid content of 81%.

The fatty acids produced by fermentation were analyzed by using a Varian gas chromatograph equipped with a flame ionization detector and a Hewlett-Packard model 3380A integrator-recorder. The packing in the column (6 feet [1.83 m] by 0.25 inch [6.35 mm]) was Supelco 1000. The oven temperature was 130°C , and the detector and injector oven temperatures were 140°C . The carrier gas was helium (flow rate, $80 \text{ cm}^3/\text{min}$). Metabolic acids were also analyzed by high-pressure liquid chromatography, using a Bio-Rad Laboratories organic acid analysis column (type HPX-87H). The eluting solvent was 0.013 N H_2SO_4 containing 5% (final concentration) acetonitrile. The solvent was pumped through the column at a rate of 0.8 ml/min by using a Bio-Rad model 1330 high-

pressure liquid chromatography pump. Fatty acids were detected at 214 nm by using a Bio-Rad model 1305 variable-wavelength detector. A 1-ml portion of culture broth was acidified with 50% H_2SO_4 and extracted twice with 5-ml portions of ethyl ether. The ether extracts were combined, and the fatty acids were extracted as the sodium salts into 1 ml of 0.2 N NaOH. A 20- μl portion of the aqueous sample was loaded into the injection valve. The peaks were recorded with a Hewlett-Packard model 3380A integrator-recorder.

RESULTS AND DISCUSSION

A total of 52 isolates of anaerobic treponemes that required pectin, glucuronic acid, or galacturonic acid were obtained from 12 sites from 10 of 52 patients (Table 1). In patients with periodontal disease the organisms were isolated from seven subgingival sites and only one supra-gingival site. This organism was not isolated from samples from people with normal healthy gingivae and no clinical signs of periodontitis. Treponemes are usually not isolated from patients who have no signs of either gingivitis or periodontitis.

***Treponema pectinovorum* sp. nov.** *Treponema pectinovorum* (pec.ti.no'vo.rum. M.L. noun *pectinum* pectin; L.v. *voro* to devour. M.L. adj. *pectinovorum* pectin destroying or devouring). This species is an obligately anaerobic motile helically coiled treponeme (Fig. 1). In electron photomicrographs the helical protoplasmic cylinder is surrounded by an outer envelope, and in cells from cultures in the logarithmic growth phase there appear to be two periplasmic flagella (axial fibrils) which originate from each end of the cell (Fig. 2). The periplasmic flagella overlap in the central area of the cell.

The cells are 7 to $15 \mu\text{m}$ long and 0.28 to $0.30 \mu\text{m}$ wide. They are coiled and usually have straight, slightly pointed ends. Secondary coils are observed in motile cultures. Coccoid bodies occur in old cultures. The organism is motile with both rotational and translational movement. Serpentine movement can be observed in a semisolid medium.

Colonies. In OTI medium in bottle plate colonies usually appear in the agar after 4 to 5 days. Colonies grow into the agar and are white and translucent with slightly denser centers. The edges of the colonies may be entire. The colonies spread out and become larger after additional incubation.

Cultural characteristics. These organisms grow in PY-pectin broth containing either rumen fluid or a short-chain fatty acid mixture, such as the volatile fatty acid-heme supplement. Serum and thiamine pyrophosphate are not required. Growth only occurs in the presence of a fermentable energy source, such as pectin, polygalacturonic acid, galacturonic acid, or glucuronic acid. Growth is greatly stimulated by the addi-

TABLE 1. Isolation of *T. pectinovorum* from the oral flora

State of periodontitis	No. of sites sampled	No. of sites positive	% Of sites positive	No. of subjects sampled	No. of subjects positive	% Of subjects positive
Moderate	37	1	2.7	15	1	6.6
Juvenile: diseased sites	27	4	15	12	4	33.3
Juvenile: healthy sites	11	2	18	6	1	16.6
Severe	59	2	3.3	15	2	13
Experimental gingivitis	96	3	3	4	2	50

tion of a fresh filter-sterilized yeast autolysate to the medium. Optimum growth occurs at 37°C. There is no growth at 25 or 42°C. Broth cultures become turbid with a granular sediment that can be seen after 4 to 5 days of incubation. Our cultures were stored frozen in liquid nitrogen or at -85°C in a mechanical freezer.

Biochemical reactions and fermentation products. Pectin is utilized, with a final pH that ranges from 5.3 to 5.9. Polygalacturonic acid, galacturonic acid, and glucuronic acid are also fermented and may be substituted for pectin in PY-rumen fluid broth. Growth and acid production occur in PY-rumen fluid broth supplemented with either autoclaved or filter-sterilized pectin. Growth and acid production also occur in PY-rumen fluid broth containing 0.5% polygalacturonic acid. The pH of this medium is 5.0 after 5 days of incubation at 37°C. Adonitol, amygdalin, arabinose, cellobiose, dextrin, starch, dulcitol, erythritol, esculin, fructose, galactose, glycerol, glycogen, inositol, inulin, lactose, maltose, mannitol, mannose, melezitose, melibiose, mucin, raffinose, rhamnose, ribose, salicin, sorbose, sorbitol, sucrose, trehalose, xylose, and glucose are not fermented; no

growth occurs in PY-rumen fluid broth containing any of these substrates.

The organism produces neither catalase nor hydrogen sulfide. Hydrogen gas was not detected by gas chromatography of the atmospheric phase of cultures in rubber-stopper sealed tubes; gas was not detected in agar deep cultures. *T. pectinovorum* does not hydrolyze gelatin, esculin, glycogen, or starch. Indol and acetylmethylcarbinol are not produced.

The major fermentation products from PY-pectin-rumen fluid broth are acetic acid (27.9 mM) and formic acid (8.5 mM). Only traces of pyruvic and lactic acids are detected. The products from polygalacturonic acid are acetic acid (39.5 mM) and formic acid (16.7 mM) with only traces of lactic and pyruvic acids.

G+C content. The guanine-plus-cytosine (G+C) content of the deoxyribonucleic acid of strain ATCC 33768^T (= VPI D-36DR-2^T) (T = type strain) is 39 mol%, as determined by thermal denaturation.

Type strain. The type strain is ATCC 33768 (= VPI D-36DR-2), which was isolated from a subgingival sample from a patient with periodontal disease. *T. pectinovorum* does not conform to

FIG. 1. Dark-field photomicrographs of wet-mount preparations of *T. pectinovorum*. Bar = 10 µm.

FIG. 2. Electron micrograph of *T. pectinovorum* negatively stained with ammonium molybdate: typical spiral form of the treponeme. Two periplasmic flagella are present at the end of the cell. Bar = 1 μ m.

the descriptions of previously described treponeme species (1-3, 6, 7). "*Treponema denticola*" (G+C content, 37 mol%), "*Treponema vincentii*," "*Treponema skolidontum*," "*Treponema refringens*" (G+C content 39 to 43 mol%), and *Treponema minutum* (G+C content, 37 mol%) differ from *T. pectinovorum* in not fermenting carbohydrates and in having requirements for serum and long-chain fatty acids (2, 6).

"*Treponema phagedenis*" (G+C content, 38 mol%) ferments many carbohydrates but not pectin and has a requirement for serum (2, 6). We have determined that "*T. phagedenis*," "*T. denticola*," "*T. vincentii*," "*T. skolidontum*," and "*T. refringens*" do not produce acid when they are grown in a medium containing pectin. *Treponema hyodysenteriae* (G+C content, 25.7 mol%) and *Treponema innocens* (G+C content 25.7 mol%) are much larger organisms, and both require serum (3). "*Treponema macrodentium*" requires short-chain fatty acids for growth and ferments many sugars. Two other short-chain fatty acid-requiring species have been described. One is *Treponema succinifaciens* (G+C content, 36 mol%) from swine intestines, and the other is *Treponema bryantii* (G+C content, 36 mol%) from bovine rumens (1, 7). These species also ferment many carbohydrates but not pectin.

Large rumen spirochetes that ferment pectin but not glucose have been described (8, 9). The end products of this fermentation are formate and acetate. The G+C content of the deoxyribonucleic acid of strain 608 is 46 mol%, and the cells of this strain are 0.6 by 15 μ m and have 32

or more periplasmic flagella (5). This is quite different from *T. pectinovorum*, which has a G+C content of 39 mol%. In addition, strain 692, a small rumen treponeme that utilizes pectin as a sole source of energy, has been described, and this organism produces formic and acetic acids as the main fermentation products (10). This strain has four periplasmic flagella that originate from each end of the cell, whereas *T. pectinovorum* has only two periplasmic flagella. Other pectin-requiring treponemes from bovine rumens have also been described (5). The G+C contents of the deoxyribonucleic acids of these organisms DNA range from 41 to 46 mol%. Isolation from human gingival crevices of five strains of pectin-fermenting treponemes that also ferment glucuronic and galacturonic acids has been reported (F. H. Weber and E. Canale-Parola, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, I113, p. 158). The characteristics described for these isolates, including the end products of fermentation and the G+C contents of the deoxyribonucleic acids (30 mol%, as determined by thermal denaturation), are similar to those of *T. pectinovorum* described in this paper, with one difference. The strains of Weber and Canale-Parola reportedly have only one periplasmic flagellum that originates from each end of the cell, whereas *T. pectinovorum* has two periplasmic flagella. The new species described here is unique in only fermenting and growing in a medium containing pectin, polygalacturonic acid, galacturonic acid, or glucuronic acid.

Habitat. *T. pectinovorum* was found in human oral cavities. It was found mainly in subgingival samples from patients with juvenile and severe periodontal disease and in subgingival samples from patients with experimentally induced gingivitis. It was not the most frequently isolated oral treponeme because it was found in only 19% of the patients studied. However, it was found in 33% of the patients with juvenile periodontitis.

Distinguishing characteristics. *T. pectinovorum* is easily recognized because it grows only in a medium containing pectin, polygalacturonic acid, galacturonic acid, or glucuronic acid and ferments these substrates, producing acetic and formic acids as the main end products. In addition to using only these substrates, it requires rumen fluid or a mixture of volatile fatty acids for growth. It does not require serum.

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