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Antigenic Relationships and Rapid Identification of *Peptostreptococcus* Species

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Antisera against whole cells of each *Peptostreptococcus* species (*P. anaerobius*, *P. micros*, *P. parvulus*, and *P. productus*) were produced in rabbits. When these antisera were reacted against sonically disrupted cells and culture supernatant fluids in Ouchterlony tests, lines of identity were obtained among the antigens from all the species and uninoculated culture medium. When the antisera were subsequently absorbed with the dehydrated culture medium used to grow the peptostreptococci, all cross-reactions in heterologous antigen-antibody combinations were eliminated, leaving only species-specific precipitin arcs. These absorbed antisera, specific for each *Peptostreptococcus* species by Ouchterlony tests, were used for rapid identification studies. *Staphylococcus aureus*-bearing protein A was sensitized with each absorbed antiserum. These reagents produced specific coagglutination reactions with suspensions of each *Peptostreptococcus* reference strain and with 16 clinical isolates. No cross-reactions occurred with the *Streptococcus intermedius*, *Peptococcus magnus*, or *Peptococcus asaccharolyticus* strains tested.

Peptostreptococci (*P. anaerobius*, *P. micros*, *P. parvulus*, and *P. productus*) are commonly present as normal microbial flora in humans. They are often isolated from wounds and abscesses, pelvic inflammatory disease, empyema, peritonitis, puerperal fever, and subacute bacterial endocarditis (10, 14). However, their significance in infections is unclear because: (i) differentiation of anaerobic gram-positive cocci has been complicated by the absence of clear genus definitions and a uniformly accepted classification scheme for species identification; (ii) specimens from infected sites have contained *Peptostreptococcus* species mixed with other anaerobic organisms, causing difficulty in determining their role in the disease process; and (iii) a rapid, reliable method for identifying these organisms is not available.

Conventional methods for identifying anaerobic bacteria, including gas-liquid chromatography and inoculation of differential biochemical media, are time-consuming and costly. In miniaturized substrate systems (15, 16) many anaerobic gram-positive cocci grow so poorly that interpretations of tests are difficult. In addition, gas-liquid chromatography of cellular fatty acids (9, 18) differentiates some of the anaerobic cocci, but this information is inadequate for a complete *Peptostreptococcus* differentiation.

Because of the problems noted with conventional methods, *Staphylococcus* protein A coagglutination (SPAC) and Ouchterlony double-diffusion precipitin tests were selected for study as possible practical clinical procedures to identify these organisms and to study their taxonomic relationships. Reference strains and clinical isolates were identified by both serological and conventional differential biochemical tests and gas-liquid chromatography profiles. We found unique antigens and no common genus antigens among the *Peptostreptococcus* tested with both serological methods. SPAC was a rapid and accurate method able to differentiate among the *Peptostreptococcus* species tested and to distinguish the genus *Peptostreptococcus* from other similar genera.

MATERIALS AND METHODS

Cultures. Reference cultures of *Peptostreptococcus* obtained from Virginia Polytechnic Institute (VPI) and from the American Type Culture Collection (ATCC) included: *P. anaerobius* ATCC 27337; *P. micros* VPI 10430 and VPI 10497; *P. parvulus* VPI 11041 and VPI 0546-1; and *P. productus* ATCC 27340. Clinical isolates of *Peptostreptococcus* collected from the California State Department of Health Services, Santa Clara Valley Medical Center, Wadsworth Veterans Administration Hospital, University of California-Los Angeles Clinical Laboratories, and San Francisco General Hospital Medical Center were identified by Virginia Polytechnic Institute anaerobic methods using

gas-liquid chromatography and prereduced, anaerobically sterilized differential media (7). Cultures were maintained by biweekly transfers on Schaedler blood agar or Brucella blood agar; both were supplemented with 10 μg of vitamin K_1 per ml, and all incubations were done in an anaerobic jar or anaerobic glove box. Stock cultures in prereduced, anaerobically sterilized chopped-meat broth were transferred bimonthly (7).

Antigen preparation and immunization protocol. Reference cultures were cultivated in Roux bottles of Schaedler medium containing 0.1% Tween-80, 10 μg of vitamin K_1 per ml, and 2% agar at 35°C for 4 to 7 days in an anaerobic glove box, harvested, washed, and suspended in physiological saline. The supernatant fluid from the first centrifugation, referred to as culture supernatant fluid, was stored at −70°C for later studies. For immunization, 0.5 ml of a homogeneous mixture containing equal volumes of physiological saline containing 1.5×10^9 organisms per ml and Freund complete adjuvant was injected subcutaneously and intramuscularly into both sides (2.0 ml total) of two New Zealand white rabbits three times at 2-week intervals. One week after the end of the immunization schedule, sera were collected and checked for antibody activity by agglutination and Ouchterlony tests. If necessary, two additional weekly intravenous injections of 0.5 ml containing 9.0×10^7 organisms per ml in saline were performed to boost the titers; sera were collected 1 week later.

Serum harvesting and storage. Blood was aseptically harvested from rabbits by exsanguination. Whole blood was allowed to clot; the serum, separated by centrifugation, was stored at −70°C.

Absorption of antiserum. To remove extraneous antibodies, a tube containing an equal volume of dehydrated culture medium (solid powder) and antiserum was mixed and incubated at 25°C for 30 min and then centrifuged. Polyvalent *Peptostreptococcus* (pooled antisera, prepared by mixing serum obtained from single rabbits inoculated with one bacterial species) and *P. parvulus* antisera required three to five absorptions; the other *Peptostreptococcus* antisera required one absorption.

Antigen preparations for serological tests. To prepare soluble antigens, bacterial suspensions (6.0×10^8 organisms per ml) and glass powder were sonicated in an ice bath with a sonifier (Branson, model 350; Danbury, Conn.). A microprobe tip, at the maximal output setting and 30% pulsations, was used for 6 min. After treatment, sonicated extracts (supernatant fluids of the sonicated suspensions) were used undiluted as antigens in Ouchterlony and coagglutination tests. Also, bacterial saline suspensions from Schaedler blood agar or Brucella blood agar were adjusted to contain approximately 10^8 organisms per ml for coagglutination tests.

Bacterial agglutination tests. Qualitative slide agglutination tests were performed by mixing 1 drop of bacterial saline suspension with 1 drop of antiserum on a glass slide; the test was positive if agglutination was seen macroscopically within 2 min. If the slide test was positive, quantitative tube agglutinations were performed by incubating tubes containing 0.5-ml volumes of various dilutions of antiserum with 0.5-ml volumes of 4.5×10^7 bacterial cells per ml at 35°C for 24 and 48 h and by reading tubes for macroscopic agglutination after centrifugation. The titer was the greatest dilution of antiserum producing macroscopic agglutination (3).

Ouchterlony tests. Ouchterlony tests were performed on glass slides coated with 5 ml of 0.7% agarose and 0.1% sodium azide in 0.05 M barbital buffer (pH 7.8). Both sonicated extracts and culture supernatant fluids were used as antigens. The slides were incubated for 4 days at 35°C and checked daily. To obtain stronger precipitin reactions (if necessary), antigens and antisera were concentrated two- to fivefold by ultrafiltration.

Preparation of SPAC reagent. An overnight Trypticase soy broth culture of *Staphylococcus aureus*, Cowan I strain, was inoculated into three 1-liter Roux bottles of Trypticase soy medium containing 2% agar and was grown at 35°C for 48 h. After the cells were harvested and washed, the reagent was prepared by: (i) stabilizing protein A-producing staphylococci by heating a 10% (vol/vol) suspension at 80°C for 30 min with continuous agitation on a magnetic stirrer, followed by 0.5% formaldehyde treatment for 3 h at room temperature to inactivate autolytic enzymes; (ii) coating 1 ml of 10% staphylococci with 0.1 ml of specific antiserum for 1 h at 35°C; (iii) washing the cells with 0.1 M phosphate-buffered saline (pH 7.4); and (iv) adjusting the concentration of staphylococci to 1% with 0.1% sodium azide in phosphate-buffered saline (pH 7.4) (8).

SPAC procedure. One drop of antigen (sonicated extract or bacterial saline suspension) was placed in a well of a VDRL (Venereal Disease Research Laboratory) slide, and 1 drop of coagglutination reagent was added. The drops were mixed with an applicator stick over the entire surface of the well, and the slide was rocked continuously for 2 min. We used a microscope with a 10× objective and transmitted light; agglutination was read as positive, and no agglutination was read as negative. Direct agar plate coagglutinations were performed in a paraffin well made on the surface of an agar plate in an area where there were several colonies of the isolate being tested using the above method. Species identification by this method was not practical on primary isolation plates because paraffin rings could not contain enough colonies to make a heavy suspension.

RESULTS

Bacterial agglutination tests. Direct tube and slide agglutination tests were impractical for species identification of *Peptostreptococcus* because *P. micros*, *P. parvulus*, and *P. productus* autoagglutinated. The titers for *P. anaerobius* were greater than or equal to 1:10,240 for one rabbit and 1:2,560 for the second rabbit, indicating that at least this *Peptostreptococcus* species was a good immunogen in rabbits.

Ouchterlony tests. Ouchterlony assays initially led to the detection of two precipitin lines between *P. parvulus* antiserum and a sonicated extract of this organism (Fig. 1). One line showed complete identity with uninoculated culture me-

dium and with antigens prepared from other peptostreptococcal species, and the second line was unique. After absorption with dehydrated culture medium, the precipitin line showing complete identity with the culture medium and antigens from other species was eliminated (Fig. 2). Only unabsorbed antisera showed these cross-reactions (Table 1); after absorption, only the species-specific precipitin arcs remained (Fig. 2). No precipitin lines were found in any heterologous system between absorbed antiserum and either sonicated extracts or culture supernatant fluids, indicating that no antigenic relationships among the *Peptostreptococcus* species were detectable by this test system. Identification of clinical isolates by this procedure was not practical, however, because it required sonic treatment of the isolates as well as up to 4 extra days for their cultivation.

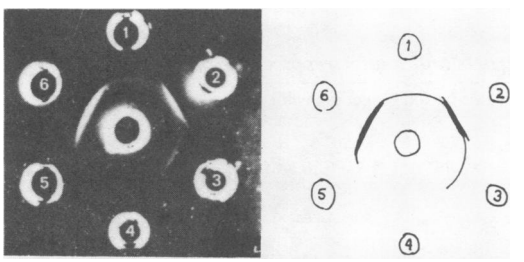

FIG. 1. *Photograph and tracing of Ouchterlony double-diffusion tests using unabsorbed P. parvulus antiserum with (1) P. parvulus culture supernatant; (2) P. parvulus sonic extract; (3) P. micros culture supernatant; (4) P. micros sonic extract; (5) P. productus culture supernatant; (6) Schaedler medium.*

SPAC tests. Because Ouchterlony tests showed that antisera were species specific for reference cultures of *Peptostreptococcus*, the simple and rapid SPAC serological method was investigated using both sonicated extracts (Table 2) and bacterial saline suspensions (Tables 3 and 4) as antigens. Identification of reference strains and clinical isolates of *Peptostreptococcus* by coagglutinations agreed with the identification of each species using biochemical and gas-liquid chromatography methods listed in the Virginia Polytechnic Institute *Anaerobe Laboratory Manual* (7). Tables 3 and 4 show the coagglutination results of reference and control cultures and clinical isolates with absorbed and unabsorbed antisera. Twenty-one strains of *Peptostreptococcus*, including five of *P. anaerobius*, nine of *P. micros*, four of *P. parvulus*, and three of *P. productus*, reacted with only the homologous specific antisera and polyvalent coagglutination reagents containing homologous antisera. No cross-reactions were seen among the *Peptostreptococcus* species. All absorbed antisera were species specific, and all strains within a species reacted with specific coagglutination reagent. Cultures other than *Peptostreptococcus* (Table 3), including three strains of *Peptococcus magnus*, one strain of *Peptococcus asaccharolyticus*, and one strain of *Streptococcus intermedius*, did not react with any of the absorbed

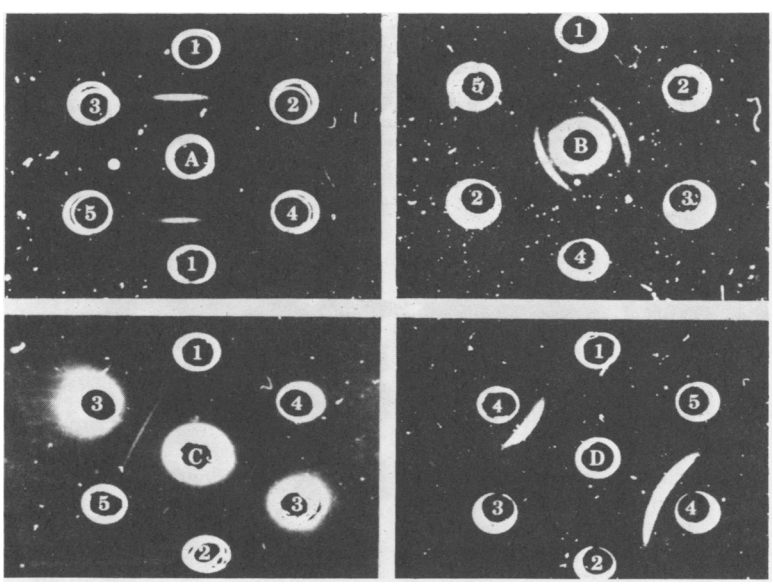

FIG. 2. *Ouchterlony tests using absorbed antisera (center wells) and sonicated extract antigens (outer wells). Antisera: (A) P. anaerobius; (B) P. micros; (C) P. parvulus; (D) P. productus. Antigens: (1) P. anaerobius; (2) P. micros; (3) P. parvulus; (4) P. productus; (5) culture medium.*

TABLE 1. *Ouchterlony tests using concentrated unabsorbed antisera against sonicated extracts and culture supernatant fluids*

Antiserum	Antigen[a]								Uninoculated medium
	P. anaerobius		*P. micros*		*P. parvulus*		*P. productus*		
	CS	SE	CS	SE	CS	SE	CS	SE	
P. anaerobius, rabbit no. 1	+[b]	+[c]	+[d]	−	+[d]	−	+[d]	−	+
P. anaerobius, rabbit no. 3	+[c]	+[c]	+[d]	−	−	−	−	−	+
P. micros	−	−	+[c]	+[c]	−	−	−	−	V
P. parvulus	−	−	−	−	+[d]	+[e]	−	−	+
P. productus	−	−	−	−	−	−	+[c]	+[c]	−

[a] CS, culture supernatant fluid; SE, sonicated extract. +, Positive reaction; −, negative reaction; V, variable reaction.
[b] Three precipitin lines: one is in complete identity with *P. anaerobius* sonicated extract, one is in complete identity with uninoculated culture medium, and one is unique.
[c] One precipitin line in complete identity with homologous culture supernatant fluid and sonicated extract.
[d] One precipitin line in complete identity with uninoculated culture medium.
[e] One unique precipitin line.

TABLE 2. *SPAC results with sonicated extracts and Peptostreptococcus antisera*

Strain (no. of isolates)	Reaction[a] with coagglutination reagent specific for:					Biochemical identification
	P. anaerobius[b]	*P. micros*[c]	*P. parvulus*[d]	*P. productus*[e]	Pooled *Peptostreptococcus*[f]	
Clinical isolates						
7PA	3+	−	−	−	1+	*P. anaerobius*
8PA	3+	−	−	−	1+	*P. anaerobius*
9PM	−	−	−	−	1+	*P. micros*
1PCM	−	−	−	−	W	*P. magnus*
2PCM	−	−	−	−	W	*P. magnus*
3PA	2+	−	−	−	1+	*P. anaerobius*
4PA	1+	−	−	−	1+	*P. anaerobius*
Reference cultures						
P. micros VPI 10497	−	1+	−	−	W	*P. micros*
P. parvulus VPI 11041	−	−	3+	−	−	*P. parvulus*

[a] −, Negative reaction; 1+ to 3+, degree of positive reaction; W, weak reaction.
[b] Absorbed antiserum prepared from immunization with *P. anaerobius* ATCC 27337.
[c] Unabsorbed antiserum prepared from *P. micros* VPI 10430.
[d] Unabsorbed antiserum prepared from *P. parvulus* VPI 0546-1.
[e] Unabsorbed antiserum prepared from *P. productus* ATCC 27340.
[f] Unabsorbed pooled *Peptostreptococcus* antiserum.

specific reagents or the polyvalent coagglutination reagents.

DISCUSSION

Our study was designed to classify these organisms into logical groups based upon serological data. Stone (17) concluded from his extensive biochemical and immunological studies with 24 clinical isolates of anaerobic gram-positive cocci that a classification based on biochemical characteristics did not correlate with results obtained from tube precipitin tests. Although good titers were seen in homologous systems, inconsistent cross-reactions were seen in heterologous systems. He hypothesized that more than one group antigen was present among these organisms. These inconsistent cross-reactions could perhaps have been due to medium components. Dehydrated culture media from various companies contain dead bacteria (2). Gram-positive rods and gram-negative coccobacilli were present in some samples of dehydrated Schaedler medium. However, other samples contained no stainable bacteria. In our study, these reactions were eliminated in Ouchterlony tests (Fig. 2) and SPAC tests (Tables 3 and 4) by absorbing

TABLE 3. *Coagglutination of Staphylococcus protein A-producing cells, sensitized by antisera absorbed with culture medium (A) or unabsorbed (UA), against heavy bacterial suspensions of reference and control cultures*

Strain	Reaction[a] with coagglutination reagent:								
	P. anaerobius	*P. micros*		*P. parvulus*		*P. productus*		Pool	
	A	UA	A	UA	A	UA	A	UA	A
Reference cultures									
Peptostreptococcus anaerobius ATCC 27337	4+	ND	–	ND	–	ND	–	ND	4+
P. micros VPI 10497	–	3+	3+	ND	–	–	–	4+	4+
P. micros VPI 10430	–	1+	4+	ND	–	–	–	2+	4+
P. parvulus VPI 11041	–	–	–	4+	4+	–	–	3+	3+
P. parvulus VPI 0546-1	–	–	–	4+	4+	–	–	3+	3+
P. productus ATCC 27340	–	–	–	–	–	3+	3+	4+	4+
Control cultures									
Streptococcus intermedius ATCC 27335	–	–	–	ND	–	–	–	–	–
Peptococcus magnus WAL 2508	–	–	–	ND	–	–	–	2+	–
P. asaccharolyticus D-1-74	–	–	–	ND	–	–	–	2+	–
P. magnus Ch 1PM	–	–	–	ND	–	–	–	2+	–
P. magnus Ch PM2	–	–	–	ND	–	–	–	2+	–

[a] –, Negative reaction; 1+ to 4+, degree of positive reaction; ND, not done. Reagent antisera were prepared by immunization with *P. anaerobius* ATCC 27337, *P. micros* VPI 10430, *P. parvulus* VPI 0546-1, and *P. productus* ATCC 27340, respectively; Pool antiserum was prepared from *Peptostreptococcus* antisera.

TABLE 4. *Coagglutination of Staphylococcus protein A-producing cells, sensitized by antisera absorbed with culture medium (A) or unabsorbed (UA), against heavy bacterial suspensions of clinical isolates*

Isolates	Reaction[a] with coagglutination reagent:								
	P. anaerobius (A)	*P. micros*		*P. parvulus*		*P. productus*		Pool	
		UA	A	UA	A	UA	A	UA	A
P. anaerobius									
7PA	3+	ND	–	ND	–	ND	–	ND	3+
8PA	3+	ND	–	ND	–	ND	–	ND	3+
Le 3PA	3+	2+	–	1+	–	1+	–	ND	3+
Le 4PA	3+	–	–	–	–	1+	–	2+	3+
P. micros									
9PM	–	1+	4+	–	–	–	–	2+	3+
10PM	–	–	2+	–	–	2+	–	4+	4+
11PM	–	3+	3+	–	–	–	–	4+	4+
12PM	–	3+	3+	–	–	2+	–	3+	3+
13PM	–	ND	2+	ND	–	ND	–	ND	3+
14PM	–	3+	3+	–	–	1+	–	3+	3+
15PM	–	2+	3+	–	–	1+	–	3+	3+
P. parvulus									
WAL 4503	–	ND	–	ND	4+	ND	–	ND	4+
WAL 4504	–	ND	–	ND	4+	ND	–	ND	2+
P. productus									
WAL 3761	–	ND	–	ND	–	ND	3+	ND	1+
WAL 4501	–	ND	–	ND	–	ND	2+	ND	1+

[a] –, Negative reaction; 1+ to 4+, degree of positive reaction; ND, not done. Reagent antisera were prepared from immunization with: *P. anaerobius* ATCC 27337, *P. micros* VPI 10430, *P. parvulus* VPI 0546-1, and *P. productus* ATCC 27340, respectively; Pool antiserum was prepared from *Peptostreptococcus* antisera.

the antisera with dehydrated culture medium.

In addition to studying the taxonomic relationships among the anaerobic gram-positive cocci, Porschen and Spaulding (11) attempted to develop a fluorescent-antibody method for identifying these organisms to species. They had varying results in their studies on strain specificity of known species and on reactions of clinical

isolates within a species using antisera prepared against a reference strain of that species. For example, seven of nine strains of *P. anaerobius* did not react with the antiserum prepared against one of the strains. They also showed strain specificity for *Peptococcus* species. In our coagglutination studies, all strains (five *P. anaerobius*, nine *P. micros*, four *P. parvulus*, and three *P. productus*) reacted with homologous absorbed antisera prepared against a reference culture of the respective species (Tables 3 and 4). Also, SPAC may be more sensitive than the fluorescent-antibody technique. In the latter method, a high concentration of antibody is required for a small concentration of bacteria, whereas a low concentration (routinely, a 1:100 dilution) of antiserum will react with a high concentration of bacteria in the SPAC test.

Bahn et al. (1), using cell wall agglutination tests developed by Cummins and Harris (4), studied the taxonomic relationships of three species of *Peptostreptococcus* recognized at that time: *P. anaerobius*, *P. elsdenii*, and *P. intermedius*. Cell wall analyses revealed that the patterns of amino acids, amino sugars, and sugars present in these three species were different; also, cell wall agglutinations showed no cross-reactions, suggesting the absence of a common genus antigen. They concluded that these organisms should be reclassified into three different genera. Rogosa (12) has suggested that *P. elsdenii* be classified in a new genus, *Megasphaera elsdenii*; the Virginia Polytechnic Institute *Anaerobe Laboratory Manual* (7) and *Bergey's Manual* (13) refer to *P. intermedius* by its older name, *Streptococcus intermedius*, because of its ability to produce lactic acid as a major end product and to tolerate oxygen after several subcultures. Our serological studies also confirm and extend these findings by showing that there were no common antigens among the *Peptostreptococcus* species and *S. intermedius* (Table 3).

Graham and Falkler (5) studied the serological reactions among the *Peptostreptococcus* species by immunodiffusion, immunoelectrophoresis, indirect fluorescent-antibody, and passive hemagglutination tests. They found that antisera prepared against sonicated reference strains of *Peptostreptococcus* showed no common genus antigen. Sonicated cells would be likely to contain more antigens than those found on the cell surface, and the use of antisera formed against sonicated extracts might complicate a serological identification. Therefore, we consider it an advantage to use whole organisms, rather than sonicated extracts, to prepare the *Peptostreptococcus* antisera. In our Ouchterlony tests, one precipitin line was seen in each homologous system using sonicated extracts of reference strains against absorbed antisera. Graham and Falkler (5) found two to three precipitin lines in each homologous system, using antisera prepared against extracts of sonicated bacterial cells. These additional lines may have been due to cytoplasmic antigens. In passive hemagglutination tests, some cross-reactions were seen with their *P. productus* antisera and sheep erythrocytes sensitized to a dialyzed sonicated extract from *P. parvulus*. In our studies, unabsorbed antisera concentrated by ultrafiltration reacted with sonicated extracts, producing lines of identity with uninoculated culture medium in Ouchterlony tests. In a later study, Graham and Falkler (6) found that *P. anaerobius* antisera correctly identified six clinical isolates and two reference strains of *P. anaerobius* and did not cross-react with antigens extracted from other species of gram-positive cocci.

We found that coagglutination tests using antisera sensitized to protein A-producing *Staphylococcus* were more rapid than Ouchterlony tests. Double-diffusion tests required 24 to 96 h of incubation, whereas coagglutination tests required 10 to 15 min. Also, SPAC reagent was more sensitive than the agar diffusion test. Low concentrations of serum were adequate for production of the SPAC reagent, whereas four- to fivefold concentrations were necessary for agar diffusion tests. Often precipitin lines were faint (Fig. 1), but SPAC reagents gave clearer reactions. Furthermore, the bacterial saline suspension used in the coagglutination test was much easier to prepare, gave stronger reactions, and produced fewer cross-reactions with unabsorbed antisera than did sonicated extracts (Table 3).

In addition to results with reference cultures, we found that all clinical isolates of *Peptostreptococcus* classified according to the Virginia Polytechnic Institute *Anaerobe Laboratory Manual* (7) could be differentiated by the SPAC method. Cross-reactions were not observed between species, and all strains within a species reacted with antiserum prepared against a reference culture of that species.

Biochemically, *Peptostreptococcus* is very difficult to differentiate from *Peptococcus*. Some differentiating characteristics exist, but they overlap between the two genera. Examples include the following. (i) *Peptostreptococcus* occur singly, in pairs, and in short or long chains; *Peptococcus* occur in pairs and short chains, as well as singly, in tetrads, or in irregular masses. (ii) Nitrates are not reduced by *Peptostreptococcus* but are reduced by *Peptococcus*, except *Peptococcus anaerobius*. (iii) Strong carbohydrate

fermentations occur with *P. parvulus* and *P. productus*; carbohydrate fermentations are limited or absent in *P. anaerobius* and *P. micros*, just as they are with *Peptococcus*. (iv) Catalase reaction is negative for *Peptostreptococcus*, but weak or variable for *Peptococcus*. (v) Indole production is generally negative for *Peptostreptococcus*, but is sometimes positive for *Peptococcus* (13). Gas-liquid chromatography can be used as a partial means to differentiate *Peptostreptococcus* from *Peptococcus*. In particular, large formate or butyrate peaks are indicative of certain *Peptococcus* species.

Our study indicated that the members of the genus *Peptostreptococcus* were serologically distinct from the genera *Streptococcus* and *Peptococcus* and could be distinguished from each other without extensive biochemical data. More taxonomic data are needed, however, to establish the relationships among the *Peptostreptococcus* species; these should include cell wall analyses, capsular studies, ultrastructural analyses, and deoxyribonucleic acid homologies.

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