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Microscopic Agglutination and Polyacrylamide Gel Electrophoresis Analyses of Oral Anaerobic Spirochetes

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Microscopic agglutination (MA) analysis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were used to determine strain and species similarities and dissimilarities among three species of oral anaerobic spirochetes, *Treponema denticola*, *Treponema pectinovorum*, and *Treponema vincentii*. The MA analysis revealed a diversity of serologic reactivity or sharing of common antigens within each species. However, there was no cross-reactivity or sharing of common antigens among the three species. Distinct SDS-PAGE whole-cell electrophoretograms for each species were obtained. The banding patterns for 16 *T. denticola* strains revealed 30 distinct proteins, while the banding patterns for 5 strains of *T. pectinovorum* and 2 strains of *T. vincentii* revealed 26 and 35 distinct proteins, respectively. Analysis of the electrophoretograms showed that their respective banding patterns could be used to distinguish the three species from one another. In addition, strain differences within each species could be detected. There was a correlation between MA analysis and SDS-PAGE analysis. It is thus suggested that both MA and SDS-PAGE analyses be included in classification schemes for the identification of oral spirochetes.

The oral anaerobic spirochetes represent a diversified group of nutritionally fastidious microorganisms which are present as indigenous components of the oral flora of humans and other dentulous animals. There is strong evidence that an association exists between the presence, progression, and severity of periodontal diseases and the presence of oral anaerobic spirochetes (1, 7, 11, 14, 15, 18–20, 22, 23, 32, 34). Although these studies do not provide direct evidence implicating oral spirochetes as etiologic agents in periodontal diseases, they do, based on microscopic as well as immunological and cultural observations, provide evidence that spirochetes contribute to the periodontal disease state and that the periodontal pocket is a suitable ecological niche for their proliferation (14, 42).

At present, the oral anaerobic spirochetes are classified under the genus *Treponema* (35). This classification scheme, based on the ultrastructural studies of Listgarten and Socransky (21), divides the oral spirochetes into three main groups based on the diameter of the protoplasmic cylinder, the morphology of the outer membrane, and the number of periplasmic flagella originating from each cell pole. As pointed out by Breznak (3), the taxonomic status of the oral anaerobic spirochetes is confusing. Presently five species of oral anaerobic spirochetes are recognized: *T. denticola*, *T. scoliodontum*, *T. vincentii*, *T. pectinovorum*, and newly described *T. socranskii* (23, 36, 37, 42).

By using distinctive phenotype evidence of the similarity and dissimilarity of strains within a species and between species, a number of investigators have shown that polyacrylamide gel electrophoresis (PAGE) (4, 6, 9, 12, 16, 26, 33, 39), sodium dodecyl sulfate (SDS)-PAGE (5, 8, 25, 38), and urea-PAGE (13, 27, 28) of soluble proteins can be used to differentiate species of bacteria. Identifications of isolates based on these kinds of data closely agree with separations based on DNA-DNA homology studies (2, 26). Therefore, SDS-PAGE appears to be a useful and highly sensitive tool for screening multiple isolates for comparative identification.

In the present study electrophoretograms of 16 strains of *T. denticola*, 5 strains of *T. pectinovorum*, and 2 strains of *T. vincentii* were examined. In addition, the results of SDS-PAGE analysis were compared with those of microscopic agglutination (MA) testing. The protein patterns and agglutination results obtained were of value in determining the similarity among well-documented members of each species and in demonstrating differences among these three species of oral anaerobic spirochetes.

MATERIALS AND METHODS

Organisms and growth conditions. The *Treponema* strains used in this study and their origins are listed in Table 1. Stock cultures of the *T. denticola* strains were maintained on a semisolid medium consisting of PPLO broth, without crystal violet (BBL Microbiology Systems, Cockeysville, Md.) plus 15 μg of cocarboxylase (Sigma Chemical Co., St. Louis, Mo.) per ml, 10% sterile rabbit serum (GIBCO Laboratories, Grand Island, N.Y.), 0.05% sodium thioglycolate (Sigma Chemical Co.), and 0.1% agar. Stock cultures of the *T. pectinovorum* strains were maintained on the NOS medium described by Leschine and Canale-Parola (17), supplemented with 0.3% sodium glucuronate (Sigma Chemical Co.) and 0.1% agar (43). Stock cultures of *T. vincentii* N-9 and *T. vincentii* LA1 were both maintained on the medium described by Mangan et al. (24) supplemented with 15 μg of cocarboxylase per ml and 10% sterile rabbit serum. The same media lacking agar were used for liquid cultivation. Electron microscopic analysis of negatively stained specimens of *T. denticola*, *T. pectinovorum*, and *T. vincentii* indicated that they possessed the 2-4-2, 1-2-1, and 5-10-5 periplasmic flagellum arrangements, respectively. Stock cultures, as well as cultures used in the experiments, were incubated at 37°C in a Coy anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.), as previously described (14).

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TABLE 1. Spirochete strains used in the study

Species[a] and strain[b]	Source[c]
T. denticola	
TT[d]	UMDS, AP
W[d]	UMDS, AP
11[d]	UMDS, AP
MS[d]	UMDS, ANUG
DW	UMDS, ANUG
MI	UMDS, ANUG
JP428[d]	UMDS, GJP
23	UMDS, ANUG
22[d]	UMDS, ANUG
1022	UMDS, ANUG
JD_1	R. Mink, WLC; tongue isolate[e]
JD_2	R. Mink, WLC; tongue isolate[e]
JD_3	R. Mink, WLC; tongue isolate[e]
8A	R. Mink, WLC; gingivitis
2513	M. Listgarten, U. PA[e]
2516	M. Listgarten, U. PA[e]
2519	M. Listgarten, U. PA[e]
MRB[d]	E. Hunter, CDC; healthy
USA[d]	UMDS, monkey (*Macaca mulatta*)
T. pectinovorum	
P2[d]	E. Canale-Parola, U. MASS[e]
P3[d]	E. Canale-Parola, U. MASS[e]
P4	E. Canale-Parola, U. MASS[e]
P5	E. Canale-Parola, U. MASS[e]
P8[d]	E. Canale-Parola, U. MASS[e]
T. vincentii	
N-9[d]	P. Hardy, Johns Hopkins University[e]
LA1[d]	M. Listgarten, U. PA[e]

[a] Organisms labeled *T. denticola* were identified as such based on periplasmic flagellum arrangement, dark-field morphology, and gas-liquid chromatography of metabolic end products. The organisms labeled *T. pectinovorum* and *T. vincentii* were identified as such based on periplasmic flagellum arrangement, dark-field morphology, and nutritional requirements.

[b] *T. denticola* W and 11 are now ATCC 33520 and 33521, respectively, and *T. vincentii* LA1 is now ATCC 35580. The arrangements of periplasmic flagella by species were as follows: *T. denticola*, 2-4-2; *T. pectinovorum*, 1-2-1; *T. vincentii*, 5-10-5.

[c] *T. denticola* USA was isolated from a rhesus monkey (*Macaca mulatta*) with periodontal disease. Abbreviations: U. MASS, University of Massachusetts, Amherst; UMDS, University of Maryland Dental School, Baltimore; U. PA, University of Pennsylvania, Philadelphia; AP, advanced periodontitis; ANUG, acute necrotizing ulcerative gingivitis; GJP, generalized juvenile periodontitis; WLC, Warner-Lambert Company, Morris Plains, N.J.

[d] *Treponema* strains used in antiserum preparation.

[e] Oral health of the individual from whom the organism was isolated was not defined.

Antiserum preparation. Specific rabbit antiserum was prepared against eight strains of *T. denticola*, three strains of *T. pectinovorum*, and two strains of *T. vincentii* in New Zealand White rabbits by inoculating 2.5 ml of a 5- to 7-day-old broth culture (2.5×10^8 cells per ml) into the marginal ear vein. The immunization schedule consisted of a single inoculation per week for a total of 4 weeks. Blood was collected 3 to 5 days after the last inoculation by cardiac puncture, and the serum was separated and stored at −20°C until needed. Before the first inoculation, nonimmune serum was obtained from each rabbit in the same fashion as for the hyperimmune rabbit antisera.

MA analysis. MA analysis was performed by a method used for *Leptospira* analysis (10). This consisted of preparing serial 10-fold dilutions (0.5-ml volume) ranging from 1:5 to 1:500,000 of each rabbit antiserum diluted in 0.85% NaCl. This was followed by the addition of 0.5 ml of an actively growing 5- to 7-day broth culture, adjusted to 1×10^8 cells per ml as determined by direct count in a Petroff-Hausser chamber, of either the homologous or heterologous spirochetal strains. The tubes were incubated at 25°C for 2 h with intermittent agitation every 15 min. Controls included nonimmune rabbit serum reacted with each isolate as well as saline, instead of antiserum, to test for autoagglutination. After the 2-h incubation period, 1 drop of each reaction mixture was placed on a microscope slide, and agglutination was recorded at 125 diameters with a Dialux 20 microscope (Leitz/Opto-Metric Div. of E. Leitz Inc., Rockleigh, N.J.), equipped with a low-power dark-field condenser. The reciprocal of the highest dilution of each serum which resulted in agglutination of at least 50% of the spirochetal cells was considered as the endpoint.

SDS-PAGE. The gels and solutions used were modifications of those previously described by Payne (31) and Swindlehurst et al. (39). The spirochete isolates were grown in 175-ml broth cultures for 5 to 7 days and harvested by centrifugation at 16,000 × *g* for 30 min. The cell pellets were washed three times with 0.1 M Tris buffer (pH 6.8) and were suspended in the same buffer to an optical density of 1.5 at 650 nm. The cells were then frozen and thawed four times, divided into 50-μl fractions, and either stored at −20°C or frozen and thawed a fifth time and immediately used. Samples were then digested by boiling 25 μl of each cell suspension for 2 min in a mixture containing 0.6 M Tris, 2% SDS, 5% mercaptoethanol, 10% sucrose, and 0.001% bromophenol blue. After cooling to room temperature, 30 μl of each suspension was added to wells of a 1.5-mm-thick vertical discontinuous polyacrylamide gel slab composed of a 5% acrylamide stacking gel (0.125 M Tris hydrochloride, pH 6.8) and a 12.5% acrylamide separating gel (0.375 M Tris hydrochloride, pH 8.8). Electrophoresis was performed by using a vertical gel electrophoresis system (model V16, Bethesda Research Laboratories, Inc., Gaithersburg, Md.) at 150 V of constant voltage, in a Tris-glycine buffer (0.025 M Tris, 0.192 M glycine [pH 8.3]) until the bromophenol blue tracking dye approached the bottom of the gel.

The gel was removed from its glass plate sandwich and fixed for 2 h in an aqueous solution containing 10% (wt/vol) trichloroacetic acid and 5% (wt/vol) sulfosalicylic acid (Sigma Chemical Co.). The fixative was then removed, and the gel was equilibrated for 1 h in an aqueous solution of 25% (vol/vol) methanol and 5% (vol/vol) acetic acid. After equilibration, the gel was then stained for 6 h in 0.1% Coomassie brilliant R-250 blue in an aqueous solution of 25% (vol/vol) methanol and 5% (vol/vol) acetic acid and then destained by soaking the gel in the equilibration solution until the background was clear. Once the gels were destained, they were photographed with Polaroid 55 P/N film. Approximate molecular sizes were determined by the method of Weber and Osborn (44). The protein molecular size standards used to establish the molecular sizes of spirochetal proteins are shown in Fig. 1.

RESULTS

Serologic analysis of spirochetes. The MA test was used to determine whether the spirochetes *T. denticola*, *T. pectinovorum*, and *T. vincentii* shared common antigens. MA titers ranging from 10^3 to 10^6 were obtained when each strain was reacted against its homologous rabbit antiserum (Table 2). When each isolate was reacted against the heterologous antisera, several consistent patterns were found: (i) there was a variety of cross-reactivity displayed within the *T. denticola* strains, ranging from no reactivity to a titer as

TABLE 2. MA analysis of rabbit antisera reacted with homologous and heterologous strains of oral spirochetes

Antigen	MA titer[a] of antisera prepared against:												
	T. denticola								*T. pectinovorum*			*T. vincentii*	
	MS	JP428	USA	TT	11	W	22	MRB	P2	P3	P8	N-9	LA1
MS	10^6	10^2	0	10^2	0	10^2	10^1	0	0	0	0	0	0
JP428	0	10^4	0	10^1	0	10^1	10^2	—	0	0	0	0	—
USA	10^2	10^2	10^4	10^2	10^1	10^5	10^3	10^2	0	0	0	0	0
TT	10^2	10^1	0	10^3	10^1	10^1	0	10^2	0	0	0	0	0
11	10^2	10^1	0	10^1	10^4	10^1	0	10^2	0	0	0	0	0
W	10^3	10^3	10^3	10^1	10^1	10^6	10^2	10^1	0	0	0	0	0
22	10^2	10^2	10^2	10^2	0	10^3	10^3	0	0	0	0	0	0
MRB	10^3	0	0	0	0	0	10^2	10^3	0	0	0	0	0
DW	10^3	10^1	0	0	10^4	0	10^1	10^3	0	0	0	0	0
JD_1	0	0	10^2	0	0	0	0	0	—	—	—	0	—
8A	0	0	10^2	0	0	10^3	0	0	—	—	—	0	—
P2	0	0	0	0	0	0	0	0	10^4	10^3	10^3	0	—
P3	0	0	0	0	0	0	0	0	10^2	10^3	10^3	0	—
P4	0	0	0	0	0	0	0	0	10^3	10^3	10^3	0	—
P8	0	0	0	0	0	0	0	0	10^3	10^3	10^3	0	—
N-9	0	0	0	0	0	0	0	0	0	0	0	10^3	0
LA1	0	0	0	0	0	0	0	0	0	0	0	10^2	10^6

[a] Reciprocal of highest dilution resulting in agglutination of 50% of cells. —, Not tested.

high as 10^5; (ii) there was a much closer range of MA titers associated with the *T. pectinovorum* strains, indicating that these strains possess a high degree of cross-reactivity; (iii) rabbit anti-*T. vincentii* N-9 serum cross-reacted to a minimal extent with *T. vincentii* LA1 although the converse, rabbit anti-*T. vincentii* LA1 serum, showed no cross-reactivity or sharing of common antigens with strain N-9; and (iv) there was no detectable serologic cross-reactivity or sharing of common antigens among *T. denticola*, *T. pectinovorum*, and *T. vincentii*.

SDS-PAGE. Electrophoretic protein banding patterns for representative strains of *T. denticola*, *T. pectinovorum*, and *T. vincentii* are shown in Fig. 1 through 3. An electrophoretogram showing protein banding patterns of 16 *T. denticola* strains is shown in Fig. 1. The patterns were very similar, and major common protein bands were found at the 92-kDa, 87-kDa, 74-kDa (doublet), 56-kDa, 27-kDa, and 24-kDa (doublet) positions. Several obvious strain differences were visible. For example *T. denticola* MRB lacked a 92-kDa protein, while strain JP428 exhibited a greater amount of a 66-kDa protein. Another slight difference was an additional band in the 28-kDa region with strain MS. At least 30 distinct proteins were identified after staining with Coomassie blue, as shown in Fig. 1. These proteins ranged in molecular size from 200 to 14 kDa with most of the bands occurring between the 92- and 21-kDa molecular size markers.

An electrophoretogram for five strains of *T. pectinovorum* is shown in Fig. 2. At least 26 detectable protein bands were identified, and their molecular sizes ranged between 100 and

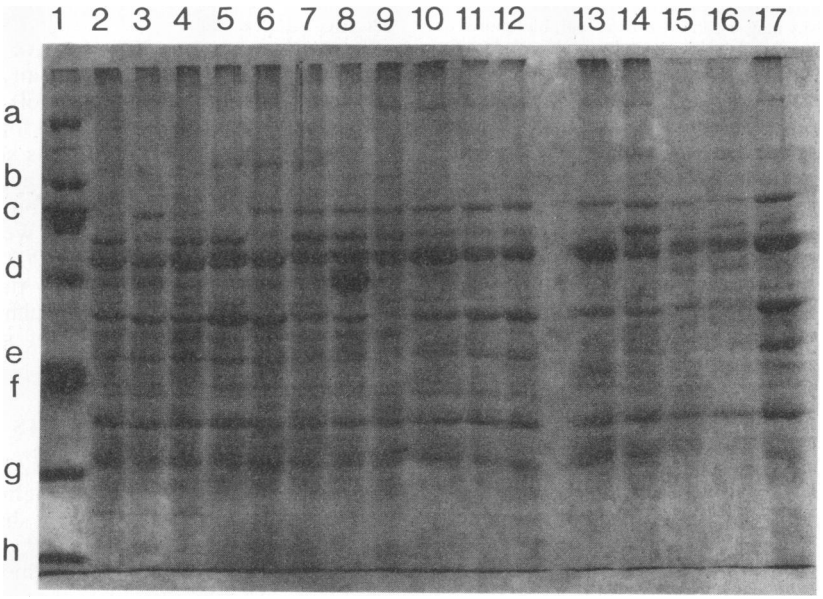

FIG. 1. SDS-PAGE electrophoretogram of whole-cell protein banding patterns of 16 *T. denticola* strains. Lane 1 contains molecular size standards (kilodaltons) as follows: (a) myosin, 200; (b) β-galactosidase, 116.2; (c) phosphorylase B, 92.5; (d) bovine serum albumin, 66.2; (e) ovalbumin, 45; (f) carbonic anhydrase, 31; (g) soybean trypsin inhibitor, 21.5; and (h) lysozyme, 14.4. Lanes 2 through 17 show patterns for *T. denticola* TT, W, 11, MRB, MS, 1022, JP428, 8A, JD_1, JD_2, JD_3, USA, 2513, 2516, 2519, and 23, respectively.

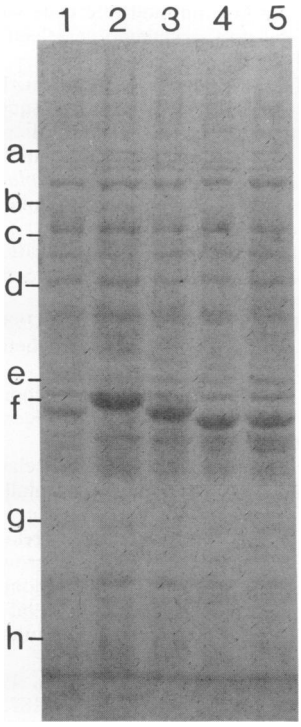

FIG. 2. SDS-PAGE electrophoretogram of whole-cell protein banding patterns of five *T. pectinovorum* strains. The positions of molecular size standards are the same as those listed in the legend to Fig. 1. Lanes 1 through 5 show patterns for *T. pectinovorum* P_2, P_3, P_4, P_5, and P_8, respectively.

21 kDa. The protein banding patterns observed for these strains were essentially identical. The only apparent difference observed was a slight variation in the banding position of a 29- to 31-kDa protein.

An electrophoretogram showing the electrophoretic protein banding patterns for two *T. vincentii* strains is shown in Fig. 3. At least 35 distinct proteins were identified, with molecular sizes ranging from 100 to 14 kDa and with most of the proteins falling between the 92- and 21-kDa molecular size markers. The two banding patterns had regions of homogeneity, but for the most part presented many differences. The protein bands present that were unique to each of the strains of *T. vincentii* are indicated by arrows in Fig. 3. The remaining bands were shared between the two strains.

The possibility existed that the protein banding patterns observed were influenced by the medium used to grow each species. To rule out this possibility, a strain of *T. vincentii* was grown in the medium used to grow *T. denticola*, and a strain of *T. denticola* was grown in the medium used for *T. vincentii*. Although the growth obtained was not ideal for each species, we were able to prepare SDS-polyacrylamide gel protein banding patterns for each species and to compare the patterns with those observed for cells grown in the original growth medium. The results obtained (data not shown) indicated that the growth medium did not change the observed protein banding patterns.

By comparing several regions of the electrophoretic protein banding patterns for the three species of spirochetes, we could distinguish each species from the others. Strains of *T. denticola* shared distinctive species protein band markers with approximate molecular sizes of 74 to 92, 46 to 56, and 24 to 27 kDa. *T. pectinovorum* strains shared distinctive species bands with approximate molecular sizes of 120, 92, 66, 56, and 25 to 31 kDa, whereas the *T. vincentii* strains shared bands at the 116-, 92-, 66-, 56-, 47-, and 24-kDa positions.

DISCUSSION

Over the past decade, SDS-PAGE has gained increasing popularity for determining taxonomic similarities and differences in a wide variety of microorganisms (5, 8, 25, 39, 40) and for revealing single- or multiple-protein components of microbial structures and antigens (29, 38, 41). The present investigation describes the use of SDS-PAGE to determine whole-cell protein banding patterns for 23 strains of oral anaerobic spirochetes, representing three species of *Treponema*. A total of sixteen *T. denticola* strains, 5 *T. pectinovorum* strains, and 2 *T. vincentii* strains were evaluated. Distinct protein banding patterns were obtained for each of the three species of oral anaerobic spirochetes, and these patterns were found to be independent of the medium which was used to grow each species. To compare MA with SDS-PAGE, a total of 11 *T. denticola* strains, 4 *T. pectinovorum* strains, and 2 *T. vincentii* strains were evaluated by MA. MA analysis revealed a diversity of serologic reactivity or sharing of common antigens within each species. However, there was no cross-reactivity or sharing of common antigens among the three species. The dissimilarity in banding patterns seen among the three species agreed with the lack of cross-reactivity determined by MA. In addition, the similarity in banding patterns observed among strains of the three spirochetal species agreed with the strain variation seen among strains by MA. The MA and electrophoretic banding patterns for *T. vincentii* N-9 and LA1 suggest that these two spirochetes may be two distinct species.

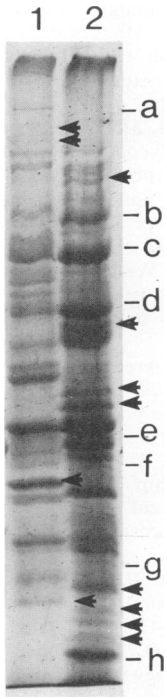

FIG. 3. SDS-PAGE electrophoretogram of whole-cell protein banding patterns of *T. vincentii* N-9 and LA1. The positions of molecular size standards are the same as those listed in the legend to Fig. 1. Arrows indicate protein bands unique to each strain of *T. vincentii*.

Our electrophoresis data were consistent with those reported by Stamm and Bassford (38) in that [^{35}S]methionine-labeled *T. denticola* W and 11 possessed some 30 to 34 identifiable labeled proteins, whereas the Coomassie blue-stained electrophoretograms of *T. denticola* presented here identified at least 30 stained proteins. The difference in total identifiable proteins observed in these two studies may be attributed to the greater sensitivity of radiolabeling techniques versus protein staining. In addition, the protein bands identified by Stamm and Bassford (38) and those reported here possessed visually similar banding patterns, with the majority of the proteins that were identified in both studies being positioned within the 21- and 92-kDa molecular size range. Lastly, our data support the rRNA oligonucleotide catalog technique of Paster et al. (30), who have shown that *T. denticola* and *T. pectinovorum* (P5) possess a single spirochetal ancestor, but that these organisms diverge separately into two distinct species.

In summary, SDS-PAGE of oral anaerobic spirochetes is a useful technique for determining species-to-species and species-to-strain relatedness, and a high degree of correlation was found between the results of MA and SDS-PAGE analyses. We recommended that SDS-PAGE and MA analyses be included in classification schemes for the identification of oral anaerobic spirochetes.

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