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Phenotypic and Genotypic Heterogeneity among Cultivable Pathogen-Related Oral Spirochetes and *Treponema vincentii*

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Recent findings challenge the assumption that pathogen-related oral spirochetes (PROS) are related to *Treponema pallidum*. *Treponema vincentii*, grown in OMIZ-Pat media, cross-reacted with monoclonal antibody H9-2 against *T. pallidum*, and cultivable PROS had 16S rRNA gene sequences similar to those of *T. vincentii* (C.-B. Choi, C. Wyss, and U. B. Göbel. J. Clin. Microbiol. 34:1922–1925, 1996). Aims of the present study were to determine whether antigen phenotypes of oral treponemas were influenced by growth conditions and to evaluate the genetic relatedness of cultivable PROS to *T. pallidum* and *T. vincentii*. Results show that three *T. pallidum* monoclonal antibodies (H9-1, H9-2, and F5) cross-reacted with whole cells from four *Treponema* species grown in modified OMIZ-Pat medium, but not with treponemas grown in NOS medium. Only H9-2 reacted in immunoblots with reduced proteins from cultivable PROS and *T. vincentii*. Three of five PROS isolates were amplified by *T. vincentii*-specific PCR, and one was amplified by *Treponema medium*-specific PCR. None were amplified by *T. pallidum*-specific PCR. Three of five PROS isolates had 16S ribosomal DNA restriction fragment length polymorphism patterns identical to that of *T. vincentii*, and the patterns of two isolates resembled that of *T. medium*. Arbitrarily primed-PCR profiles from whole genomic DNA were distinct among five PROS isolates and two *T. vincentii* strains. Thus, PROS isolates represent a heterogeneous group of treponemas that share some 16S rRNA gene sequences with *T. vincentii* and *T. medium*, but not with *T. pallidum*. It is proposed that the PROS nomenclature be dropped.

Monoclonal antibodies (MAbs) H9-1 and H9-2 against the 47-kDa outer membrane protein and the 37-kDa endoflagellar sheath protein, respectively, from *Treponema pallidum* were used to identify spirochetes in dental plaque from sites of destructive periodontal diseases (12). H9-1, H9-2, and F5 (against a 15-kDa protein from *T. pallidum*) did not react with cultivable oral treponemas, including *T. denticola*, *T. pectinovorum*, *T. socranskii*, and *T. vincentii* (1, 8–10, 12). Cross-reactivity with *T. pallidum* MAbs and the ability to invade tissue (13), as did *T. pallidum* (11), suggested that these oral spirochetes might be related to *T. pallidum* (pathogen-related oral spirochetes [PROS]). However, cultivable oral treponemas identified with H9-2 were described as being closely related to *T. vincentii* on the basis of similar 16S rRNA sequences, and *T. vincentii* grown in OMIZ-Pat medium also expressed determinants that cross-reacted with H9-2 (3). These conflicting findings raise questions about the apparent antigenic phenotypes of cultivable oral treponemas grown in different synthetic media, as well as the relatedness of PROS to *T. pallidum* and *T. vincentii*. The first aim of this investigation was to assess the reactivities of three MAbs, thought to be specific for protein determinants from *T. pallidum* (6), with whole cells from oral treponemas grown in two distinct synthetic media and with reduced proteins in immunoblots. The second aim was to evaluate the 16S ribosomal DNA (rDNA) relatedness of cultivable PROS isolates to *T. pallidum* and *T. vincentii* by species-specific nested PCR and restriction fragment length polymorphism (RFLP) analysis. The third aim was to use arbitrarily primed PCR (AP-PCR) to determine whether any PROS isolates were identical to *T. vincentii*.

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MATERIALS AND METHODS

Treponemas. The following treponemas were obtained from the American Type Culture Collection (ATCC) unless otherwise noted: *T. denticola* ATCC 35405, *T. denticola* ATCC 33521, *T. denticola* ATCC 35404, *T. denticola* GM-1 (gift from Denée Thomas, University of Texas, San Antonio), *T. phagedenis* ATCC 51274, *T. vincentii* ATCC 35580 and ATCC 700013, *T. pectinovorum* ATCC 33768, *T. socranskii* subsp. *buccale* (ATCC 35534), *T. socranskii* subsp. *paredis* (ATCC 35535), *T. socranskii* subsp. *socranskii* (ATCC 35536), *T. maltophilum* ATCC 51939 (gift from Chris Wyss, University of Zurich, Zurich, Switzerland), *T. medium* G7201 (gift from Toshihiko Umemoto, Asahi University, Asahi, Japan), *T. pallidum* (D. Thomas), and cultivable PROS isolates OMZ-802, OMZ-804, and OMZ-805 (C. Wyss).

All treponemas were maintained in OMIZ-P4 broth (unpublished formula from Chris Wyss), which was a modification of OMIZ-WI (17). OMIZ-P4 differs from OMIZ-WI as follows: deletion of lecithin, 1,4-dihydroxy-2-naphthoic acid, *n*-acetyl muramic acid, and L-valyl-L-lysine HCl and addition of 1,536 mg of glutathione, 50 mg of pyridoxal HCl, 250 mg of thiamine pyrophosphate, 5 mg of spermidine, 1.4 mg of adenine, 1 mg of rifampin, and 100 mg of fosfomycin per liter, as well as 0.05% (vol/vol) yeast extract solution (Gibco BRL, Grand Island, N.Y.), and 1% (vol/vol) heat-inactivated human serum (Sigma Chemical Co., St. Louis, Mo.). We further modified the OMIZ-P4 medium by eliminating the following trace metals and carbohydrates: $MgSO_4$, $CuSO_4$, $MnSO_4$, $ZnSO_4$, $NiSO_4$, $SnCl_2$, $NaCO_3$, D-glucose, D-fructose, D-maltose, D-mannitol, glucuronic acid, and galacturonic acid. The modified OMIZ-P4 medium is referred to as mP4. Maintenance cultures were incubated at 35°C in anaerobic GasPak jars.

Isolation of cultivable PROS. OHSU 242-9 and 242-10 *Treponema* strains were isolated as follows. Subgingival plaque taken from sites of periodontitis was suspended in normal saline and combined with an equal volume of 2× mP4 broth. Suspensions were enriched for spirochetes by overnight incubation at 35°C in anaerobic GasPak jars. Enrichment cultures were viewed by ×400 dark-field microscopy to estimate spirochete numbers, and 10-fold dilutions were made to produce spirochete concentrations between 10 and 1,000 cells per ml. Pour plates were made by combining 1 ml of each dilution with 30 ml of molten mP4 agar supplemented with 1.5% SeaPlaque agar (FMC Bioproducts, Rockland, Maine) in disposable petri dishes. After 7 days of incubation at 35°C in anaerobic GasPak jars, discrete colonies were picked and suspended in 0.5 ml of mP4. PROS were defined by reactivity with H9-2 according to an established protocol (10). This

TABLE 1. Reactivity of *T. pallidum* MAb H9-2 with PROS and *T. vincentii* whole cells during sequential subculture in mP4, NOS, or combinations of mP4 and NOS

Treponema	Reactivity under indicated subculture conditions[a]			
	NOS/NOS/NOS	NOS/mP4/NOS	mP4/NOS/mP4	mP4/mP4/mP4
T. vincentii	Neg/neg/neg	Neg/neg/weak	Pos/pos/pos	Pos/pos/no growth
OMZ-802	Neg/neg/neg	Neg/neg/weak	Pos/neg/pos	Pos/pos/weak
OMZ-804	Pos/pos/no growth	Pos/pos/pos	Pos/pos/pos	Pos/pos/pos

[a] NOS and mP4 were used in the order indicated. The corresponding reactivities are similarly indicated. No stained cells were detected in negative (neg) reactions. Some lightly stained cells were detected, but most cells were not stained, in weak reactions. All, or nearly all, cells were well stained in positive (pos) reactions.

process was repeated with H9-2-positive suspensions through second and third rounds of pour plates to isolate pure cultures.

Western blots. Treponemas were washed twice in normal saline. Washed cell pellets were resuspended in 2× treatment buffer (0.125 M Tris-HCl [pH 6.8], 4% sodium dodecyl sulfate, 20% glycerol, 10% 2-mercaptoethanol). Protein concentrations were determined by the bicinchoninic acid protein assay (Pierce, Rockford, Ill.) and adjusted to 2 μg/μl. Protein was extracted by boiling at 70°C for 5 min followed by sonication on ice for 4 min. Crude extracts were then centrifuged at 10,000 × *g* for 10 min, and supernatants were electrophoresed on 4 to 20% Tris-glycine gradient gels (Novex Experimental Technology, San Diego, Calif.) at 150 V for 70 min. Experimental samples were routinely placed in alternate lanes, leaving one lane blank between each sample. Prestained molecular mass markers (6.5 to 200 kDa; Bio-Rad Laboratories, Hercules, Calif.) were introduced into lanes 1, 11, 12, and 22 in order to facilitate the interpretation of experimental and control bands.

Proteins were then electroblotted onto nitrocellulose membranes, and membranes were blocked overnight with 3% nonfat milk in Tris-buffered saline (TBS). Separate immunoblots were incubated with one of three *T. pallidum*-specific MAbs, H9-1, H9-2, or F-5 (Shiela Lukehart, Seattle, Wash.), diluted 1:10 in 1% nonfat milk–TBS, for 2.5 h at 4°C. Blots were washed in TBS and then incubated for 2.5 h at 4°C with alkaline phosphatase-labeled goat anti-mouse immunoglobulin G (IgG) (Sigma) at a dilution of 1:1,000 in blocking solution. Alkaline phosphatase color development reagents (5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt and *p*-nitroblue tetrazolium chloride; Bio-Rad) were used for detection.

Media modulation and cell staining. Log-phase cultures of *T. vincentii*, OMZ-802, and OMZ-804 were established in both modified new oral spirochete (NOS) broth (ATCC medium 1494) and mP4. Cells were subcultured three times at 48-h intervals. In another series of experiments, cells were subcultured from NOS to mP4 or from mP4 to NOS and after 48 h were subcultured back to the original medium. Cells from each subculture were washed twice in normal saline, spotted onto glass slides, and air dried. Whole-cell reactivities with H9-1, H9-2, and F-5 were determined by a biotin-amplified microscopic immunocytochemical assay (10). A pool of two MAbs, specific for *T. socranskii* subsp. *buccale* and *T. socranskii* subsp. *socranskii*, was used as the control in each experiment.

DNA isolation. One milliliter of the log-phase culture was sedimented, and cells were washed three times in sterile normal saline. Each pellet was resuspended in 25 to 50 μl of distilled water and then lysed by three cycles of exposure to dry ice for 10 min followed by 95°C for 10 min. DNA was isolated by centrifugation at 10,000 × *g* for 5 min, and supernatant was transferred and stored at −20°C until analyzed.

16S RFLP. PCR with a 100-μl reaction mixture was carried out in a buffer containing 2.5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl (pH 8.3; Boehringer Mannheim, Indianapolis, Ind.), 50 μM (each) deoxynucleoside triphosphates (Sigma), 10 pM (each) 1492R and 8F primers (4, 15), and 2.5 U of *Taq* polymerase (Boehringer Mannheim), and the mixture was overlaid with 1 drop of mineral oil (Sigma). Four microliters of DNA was added as the template. After initial denaturation at 97°C for 1 min, 26 cycles were performed as follows: denaturation at 97°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 1 min. A final extension was conducted at 72°C for 4 min. Amplified product was stored at 4°C until analyzed on a 1.0% agarose gel in 0.5% TBE buffer (89 mM Tris, 89 mM borate, and 2 mM EDTA). Three reaction mixtures for each template were combined, precipitated with 0.5 volume of ammonium acetate and 2 volumes of isopropanol, centrifuged for 20 min at 10,000 × *g*, washed twice in 70% ethanol, dried, and resuspended in 30 μl of 0.1× TE buffer (10 mM Tris and 1 mM EDTA). Ten microliters of each PCR product was digested overnight at 37°C with 5 U of *Hin*fI, *Hae*III, or *Rsa*I (Gibco BRL); dried down completely; resuspended in 4 μl of loading dye; and electrophoresed in 2% agarose and 0.5× TBE at 4 V/cm until the leading dye front ran to the 15-cm mark. Gels were stained with ethidium bromide, destained, and photographed.

Species-specific PCR. PCR was performed in 25-μl reaction volumes as previously described (16). In brief, PCR was performed as described above for RFLP except that the concentration of $MgCl_2$ was reduced to 1.5 mM and the 8F universal primer was used with species-specific reverse primers.

AP-PCR analysis. AP-PCR was performed in 25-μl reaction volumes as described previously (6), by using 10 pM random primer OPA-2 (AGTCAGCCAC; Operon Technologies, Alameda, Calif.), 7 mM $MgCl_2$, 2 μl of target DNA, and 50 μM (each) deoxynucleoside triphosphates in a buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3; Boehringer Mannheim), and 1.25 U of *Taq* polymerase. Amplifications were performed with the following parameters: 1 min of initial denaturation at 96°C followed by 45 cycles of 30 s at 95°C, 30 s at 36°C, and 100 s at 72°C and a final 3-min elongation at 72°C. Twenty-four microliters was loaded on a 1.5% agarose gel, stained, and photographed as described above.

RESULTS

MAb reactivity with whole cells grown in NOS and mP4. Table 1 shows that cultivable PROS strains OMZ-802 and OMZ-804 and *T. vincentii* ATCC 35580 varied in the expression of determinants that cross-reacted with *T. pallidum* MAb H9-2. *T. vincentii* grown in NOS did not cross-react with H9-2, and a 48-h exposure to mP4 was not sufficient to promote a cross-reaction. On the other hand, *T. vincentii* grown in mP4 did cross-react with H9-2, and cells retained their reactivity after subculture in NOS for 48 h. OMZ-802 performed in a manner similar to that of *T. vincentii*, except that it appeared to be more responsive to brief changes in medium. OMZ-802 grown in NOS acquired cross-reactivity after 48 h in mP4, and cells grown in mP4 lost cross-reactivity after 48 h in NOS. In contrast to both *T. vincentii* and OMZ-802, OMZ-804 expressed cross-reactivity with H9-2 when grown in either NOS or mP4.

Table 2 shows that reactivity of oral treponemas with *T. pallidum* MAbs H9-1 and F5 also varied according to growth conditions and that strain differences were similar to those described for H9-2. Cultivable PROS and *T. vincentii* grown in mP4 demonstrated strong cross-reactivity with all three MAbs, while only OMZ-804 retained cross-reactivity with H9-2 and F5 when grown in NOS. Strong cross-reactions were characterized by confluent staining of all, or nearly all, cells grown in mP4. Weak cross-reactions were observed in some experiments with cells grown in NOS. Weak reactions were characterized by partial staining of some, but not the majority, of the cells in representative microscopic fields.

Table 2 also shows that, in addition to *T. vincentii*, *T. maltophilum*, *T. pectinovorum*, and two strains of *T. denticola*, grown in mP4, also cross-reacted with H9-1, H9-2, and F5. However, with the exception of the weak reactivity of H9-2 with *T. maltophilum*, none of these treponemas grown in NOS cross-reacted with *T. pallidum* MAbs. In almost every instance, *Treponema* cells that cross-reacted with H9-2 also reacted with H9-1 and F5. The one exception was *T. medium*. When grown in mP4 it displayed weak cross-reactivity with H9-2 and no cross-reactivity with either H9-1 or F5. *T. medium* grown in NOS did not cross-react with any *T. pallidum* MAb.

Other treponemas were consistently nonreactive in both NOS and mP4 media. *T. phagedenis*, *T. socranskii*, and *T. denticola* strains GM-1, ATCC 35404, ATCC 35405, and ATCC 33521 never reacted with H9-1, H9-2, or F5.

TABLE 2. Reactivity of whole treponema cells with *T. pallidum* MAbs and control *T. socranskii* MAbs following continuous culture in either mP4 or NOS medium

Treponema	Reactivity with MAb after[a]:							
	mP4 culture				NOS culture			
	F5	H9-2	H9-1	Control	F5	H9-2	H9-1	Control
OMZ-804	Pos	Pos	Pos	Neg	Pos	+/−	Pos	Neg
OHSU 242-9	Pos	Pos	Pos	Neg	+/−	Neg	+/−	Neg
OHSU 242-10	Pos	Pos	Pos	Neg	+/−	Neg	+/−	Neg
OMZ-802	Pos	Pos	Pos	Neg	+/−	Neg	+/−	Neg
OMZ-805	Pos	Pos	Pos	Neg	+/−	Neg	+/−	Neg
T. maltophilum	Pos	Pos	Pos	Neg	+/−	Neg	Neg	Neg
T. denticola ST10	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg
T. denticola ATCC 33520	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg
T. pectinovorum	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg
T. vincentii ATCC 35580	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg
T. medium	+/−	Neg	Neg	Neg	Neg	Neg	Neg	Neg
T. denticola ATCC 33521	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
T. denticola ATCC 35404	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
T. denticola ATCC 35405	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
T. denticola GM-1	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
T. phagedenis	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
T. socranskii subsp. *buccale*	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Pos
T. socranskii subsp. *paredis*	Neg	Neg	Neg	ND[b]	Neg	Neg	Neg	Neg
T. socranskii subsp. *socranskii*	Neg	Neg	Neg	Pos	Neg	Neg	Neg	Pos

[a] No stained cells were detected in negative (Neg) reactions. Some lightly stained cells were detected, but most cells were not stained, in weak (+/−) reactions. All, or nearly all, cells were well stained in positive (Pos) reactions.
[b] ND, not determined.

The *T. socranskii* MAb pool control never reacted with treponemas other than appropriate *T. socranskii* target cells.

Cross-reactivity of treponema proteins with *T. pallidum* MAbs in immunoblots. All cultivable treponemas were grown in mP4. Figure 1 shows that H9-2 reacted with reduced proteins from *T. pallidum*, *T. vincentii* ATCC 35580 and ATCC 700013, and PROS strains OHSU 242-9, OHSU 242-10, OMZ-802, OMZ-804, and OMZ-805. Cross-reactive proteins from oral treponemas were slightly larger than the 37-kDa protein of *T. pallidum* that reacts with H9-2. H9-2 did not react with proteins from any other cultivable treponemas. H9-1 and F5 reacted with *T. pallidum* but not with proteins from any other treponema, including those strains displaying cross-reactivity between whole cells and MAbs.

Species-specific PCR. PCR studies are summarized in Table 3. *T. vincentii*-specific PCR generated a product of approximately 200 bp with DNA templates from OHSU 242-9, OHSU 242-10, and OMZ-802. *T. vincentii*-specific PCR did not amplify DNA from OMZ-804 or OMZ-805. OMZ-805 was amplified by *T. medium*-specific PCR, producing an amplicon of approximately 200 bp, identical in size to the product obtained with the *T. medium* template. OMZ-804 was not amplified by *T. vincentii*, *T. medium*, or *T. pallidum* species-specific primers. *T. pallidum*-specific PCR did not amplify DNA from any oral treponema, including cultivable PROS.

RFLP. Figure 2 shows RFLP patterns obtained with *Rsa*I enzyme digests of 16S rDNA PCR products. OHSU 242-9, OHSU 242-10, and OMZ-802 had RFLP patterns identical to

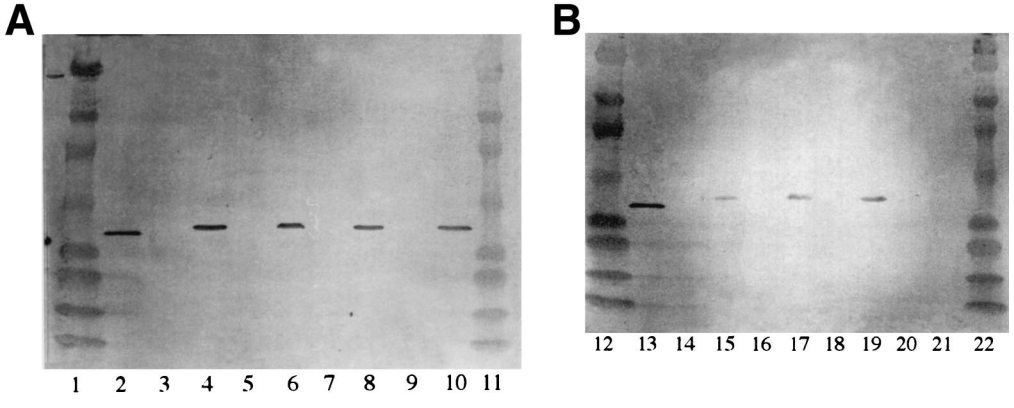

FIG. 1. Representative immunoblot for treponema following incubation with *T. pallidum* MAb H9-2. Lanes 1, 11, 12, and 22, molecular mass markers (200, 116, 97, 66, 45, 31, 21.5, and 14.4 kDa; Bio-Rad); lanes 2 and 13, *T. pallidum* band at 37 kDa (all other bands are slightly larger); lanes 4 and 6, *T. vincentii* ATCC 35580 and ATCC 700013, respectively; lanes 8 and 10, OHSU 242-9 and OHSU 242-10, respectively; lanes 15, 17, and 19, OMZ-802, OMZ-804, and OMZ-805, respectively; lane 21, *T. phagedenis*. Lanes 3, 5, 7, 9, 14, 16, 18, and 20 are blank.

TABLE 3. Amplification of cultivable PROS isolates by species-specific PCR[a]

Treponema tested	Results of species-specific PCR with[b]:		
	T. medium (192)	*T. pallidum* (198)	*T. vincentii* (193)
PROS isolates			
OHSU 242-9	Not amplified	Not amplified	Amplified
OHSU 242-10	Not amplified	Not amplified	Amplified
OMZ-802	Not amplified	Not amplified	Amplified
OMZ-804	Not amplified	Not amplified	Not amplified
OMZ-805	Amplified	Not amplified	Not amplified
Control treponema			
T. medium	Amplified	Not amplified	Not amplified
T. pallidum	Not amplified	Amplified	Not amplified
T. vincentii	Not amplified	Not amplified	Amplified

[a] Each species-specific PCR produced a characteristic and reproducible product only with the appropriate template (16). Each experiment included both positive and negative controls, and each experimental PCR product was identical in size to control amplicons.
[b] The product size in base pairs is in parentheses.

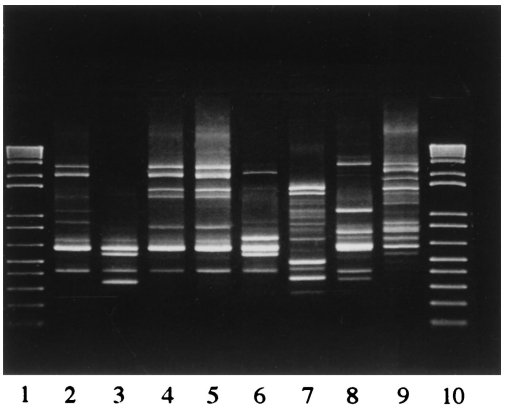

FIG. 3. AP-PCR patterns obtained from whole genomic DNA with random primer OPA-2. Lanes: 1 and 10, 1-kb DNA marker (Gibco BRL); 2, DNA from *T. vincentii* ATCC 35580; 3, DNA from *T. vincentii* ATCC 700013; 4, DNA from OHSU 242-9; 5, DNA from OHSU 242-10; 6, DNA from OMZ-802; 7, DNA from OMZ-804; 8, DNA from OMZ-805; 9, DNA from *T. medium*.

that of *T. vincentii*. The pattern of OMZ-804 was identical to that of *T. medium*, and the pattern of OMZ-805 was identical to that of *T. vincentii*.

OHSU 242-9 and 242-10, OMZ-802, -804, and -805, *T. medium*, and *T. vincentii* were found to have identical RFLP patterns when *Hin*fI and *Hae*III were used.

AP-PCR. Figure 3 shows that *T. vincentii* ATCC 35580 and ATCC 700013 and PROS isolates OHSU 242, OMZ-802, OMZ-804, and OMZ-805 all have unique AP-PCR profiles with random primer OPA-2. OHSU 242-9 and OHSU 242-10 had identical AP-PCR profiles.

DISCUSSION

The present work helps to reconcile previous contradictory reports (1, 3, 8–10, 12) by confirming that oral treponemas grown in NOS medium did not react with *T. pallidum* MAbs while cells grown in mP4 media did cross-react with H9-1, H9-2, and F5 MAbs. Furthermore, cross-reactivity was modulated when treponemas were subcultured from one medium to another. An unexpected finding was that phenotypic modulation and *T. pallidum* MAb cross-reactivity extended to oral treponemas other than cultivable PROS. *T. denticola*, *T. maltophilum*, *T. pectinovorum*, and *T. vincentii* also acquired cross-reactivity with *T. pallidum* MAbs after growth in mP4. It is unlikely that cross-reactivity was due to an artifact created by mP4 because *T. denticola* GM-1, ATCC 35404, ATCC 35405, and ATCC 33521, three subspecies of *T. socranskii*, and *T. phagedenis* failed to react with *T. pallidum* MAbs after growth in mP4.

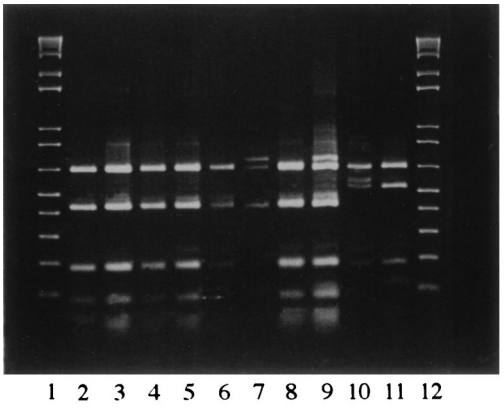

FIG. 2. RFLP patterns obtained with *Rsa*I enzyme digests of 16S rDNA PCR products. Lanes: 1 and 12, 1-kb DNA marker (Gibco BRL); 2, DNA from *T. vincentii* ATCC 35580; 3, DNA from *T. vincentii* ATCC 700013; 4, DNA from OHSU 242-9; 5, DNA from OHSU 242-10; 6, DNA from OMZ-802; 7, DNA from OMZ-804; 8, DNA from OMZ-805; 9, DNA from *T. medium*; 10, DNA from *T. phagedenis*; 11, DNA from *T. pallidum*.

While whole treponema cells cross-reacted with three *T. pallidum* MAbs, only H9-2 reacted in Western blots with reduced proteins from cultivable PROS and *T. vincentii*. We confirmed that the cross-reactive protein identified by H9-2 is slightly larger than the 37-kDa protein from *T. pallidum* that bears the H9-2 determinant (3). The nature of cross-reactive determinants identified on whole cells by H9-1 and F5 MAbs has not been determined, but unpublished observations indicate that they are preserved by extraction under nonreducing conditions (7). Cross-reactive determinants expressed by PROS in subjects with necrotizing ulcerative gingivitis may have been responsible for serum antibodies that reacted with proteins from *T. pallidum* (12). However, in a recent study (14) subjects with no detectable oral PROS were just as likely to have IgA, IgG, or IgM to 15-, 37-, or 47-kDa proteins from *T. pallidum* as were subjects with PROS. The presence of cross-reactive serum antibodies from subjects with no detectable oral spirochetes (14) suggests that cross-reactive determinants may be expressed by moieties other than oral treponemas.

Choi et al. (3) suggested that PROS were related to *T. vincentii* based upon DNA sequence homology, and the present investigation supports this close relationship for three of five PROS strains. OHSU 242-9, OHSU 242-10, and OMZ-802 were amplified by *T. vincentii*-specific PCR, and their 16S rDNA RFLP patterns were identical to that of *T. vincentii*. However, not all PROS isolates, defined by H9-2 cross-reactivity, were so easily aligned with *T. vincentii*. For example, OMZ-805 displayed MAb phenotypes like those of *T. vincentii* but was amplified by *T. medium*-specific PCR, not by *T. vincentii*-specific PCR. Furthermore, the RFLP pattern for OMZ-805 appeared to contain bands common to both *T. medium* and *T. vincentii*. OMZ-804 was also different. It was the only strain that reacted with *T. pallidum* MAbs in both NOS and mP4 media; it was not amplified by species-specific PCR for *T.*

medium or *T. vincentii*, but the RFLP profile for OMZ-804 was identical to that for *T. medium*. Thus, the available evidence suggests that OHSU 242-9, OHSU 242-10, and OMZ-802 may be most like *T. vincentii*; that OMZ-805 is related to both *T. medium* and *T. vincentii*; and that OMZ-804 is the most different, sharing RFLP patterns only with *T. medium*. Based upon these provisional relationships, it may be that PROS and *T. medium* belong to the group I treponemas described by Choi et al. (2).

PCR amplification and RFLP patterns for OHSU 242-9, OHSU 242-10, and OMZ-802 suggest that they may be closely related to *T. vincentii*. However, AP-PCR revealed not only that each PROS isolate was unique (OHSU 242-9 and OHSU 242-10 had identical profiles but differed from the others) but also that *T. vincentii* ATCC 35580 and ATCC 700013 were distinct. Thus, these three PROS isolates are not clonotypes of *T. vincentii*. OHSU 242-9 and OHSU 242-10 were not identical because they differed in morphology and invasive potential (13a).

Finally, these experiments cast aside any thoughts that PROS are related to *T. pallidum*. No PROS strain was amplified by *T. pallidum*-specific PCR, and their RFLP profiles were different (data not shown). Cross-reactivity with *T. pallidum* MAbs H9-1 and F5 appeared to be a consequence of some undefined effect of growth conditions, which created changes in secondary or tertiary protein structure, a process that was reversed when NOS was used for growth. OMZ-804 was the exception to this rule because it cross-reacted with *T. pallidum* MAbs in both mP4 and NOS media. Reactivity with H9-2 was preserved after reduction in buffer for immunoblots, but the protein bearing the determinant was larger than the 37-kDa protein from *T. pallidum*. Perhaps most telling was the observation that four oral *Treponema* species, *T. denticola*, *T. maltophilum*, *T. pectinovorum*, and *T. vincentii*, cross-reacted with *T. pallidum* MAbs if they were grown in mP4 medium. Thus, reactivity with H9-1 and F5 should be considered an artifact related to growth conditions, and H9-2 should not be regarded as specific for *T. pallidum* unless close attention is paid to the size of reactive proteins in Western blots.

In conclusion, immunologic and genetic techniques disclosed considerable diversity among five strains of cultivable PROS, including those that are very similar to *T. vincentii*, some that appear to be intermediate between *T. medium* and *T. vincentii*, and those that are somewhat more closely related to *T. medium*. The implied relatedness between *T. pallidum* MAb-reactive oral spirochetes and *T. pallidum* is not supported by molecular data. It is proposed that the PROS nomenclature be dropped.

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