JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 1996, p. 1922–1925 Vol. 34, No. 8
0095-1137/96/$04.00+0

Phylogenetic Analysis of Pathogen-Related Oral Spirochetes

B.-K. CHOI,[1] C. WYSS,[2] AND U. B. GÖBEL[1]*

Universitätsklinikum Charité, Institut für Mikrobiologie und Hygiene, D-10117 Berlin, Germany,[1] and Institute for Oral Microbiology and General Immunology, Center for Dentistry, Oral Medicine and Maxillofacial Surgery of the University of Zürich, CH-8028 Zürich, Switzerland[2]

Received 23 January 1996/Returned for modification 2 April 1996/Accepted 14 May 1996

Recently, Riviere et al. reported as yet uncultivable invasive oral spirochetes that cross-reacted with monoclonal antibodies (MAbs) specific for *Treponema pallidum* (G. R. Riviere, K. S. Elliot, D. F. Adams, L. G. Simonson, L. B. Forgas, A. M. Nilius, and S. A. Lukehart, J. Periodontol. 63:131–136, 1992; G. R. Riviere, M. A. Wagoner, S. A. Baker-Zander, K. S. Weisz, D. F. Adams, L. Simonson, and S. A. Lukehart, N. Engl. J. Med. 325:539–543, 1991; G. R. Riviere, K. S. Weisz, D. F. Adams, and D. D. Thomas, Infect. Immun. 59:3377–3380, 1991; G. R. Riviere, K. S. Weisz, L. G. Simonson, and S. A. Lukehart, Infect. Immun. 59:2653–2657, 1991). In an attempt to phylogenetically analyze these pathogen-related oral spirochetes, we used immunomagnetic separation, combined with comparative sequence analysis of 16S rRNA genes amplified in vitro by the PCR. The bacteria were immunomagnetically enriched from subgingival plaque samples of patients with rapidly progressive periodontitis by using MAb H9-2 specific for the 37-kDa endoflagellum sheath protein of *T. pallidum*. After PCR amplification with universal eubacterial primers 16S rRNA gene fragments were cloned into *Escherichia coli*. A total of 20 randomly selected recombinants were analyzed by sequencing about 200 to 300 bases of the 500-bp inserts. All the spirochetal 16S rRNA sequences clustered to previously described, as yet uncultivable cluster 7 treponemes of group I (B. K. Choi, B. J. Paster, F. E. Dewhirst, and U. B. Göbel, Infect. Immun. 62:1889–1895, 1994). With a sequence similarity of 96.4% the most closely related cultivable treponeme was *Treponema vincentii*, which also belongs to the group I treponemes. Subsequent immunological analysis of cultured treponemes with MAb H9-2 revealed that only *T. vincentii* strains showed specific immunofluorescence or a characteristic 37-kDa band in immunoblots. We therefore conclude that pathogen-related oral spirochetes constitute a heterogeneous population of treponemes comprising *T. vincentii* and *T. vincentii*-related organisms that have common epitopes cross-reacting with MAb H9-2.

Spirochetes are found in subgingival plaque samples of periodontitis patients at high frequencies. Large numbers of these organisms correlate well with the severity of the disease (1, 11, 22, 25). In the past a number of different treponemal morphotypes were described microscopically. However, most of them are as yet uncultivated. Hence, studies of the etiology and pathogenesis of periodontal infections were restricted to a few cultivable species, e.g., *Treponema denticola* (7, 8, 15, 22, 23, 26, 27). In order to elucidate the role of as yet uncultivable treponeme species in periodontal disease, we have applied comparative 16S rRNA sequence analysis to identify and phylogenetically classify these unknown oral treponemes. On the basis of 98% sequence similarity, 21 new treponemal "phylotypes" were identified by this approach (4).

Recently, Riviere et al. (17–20) suggested that hitherto-uncultivable spirochetes sharing immunodominant pathogen-restricted antigens (12, 14, 37, and 47 kDa) with *Treponema pallidum* subsp. *pallidum* may be involved in the pathogenesis of periodontal disease. Subsequently these organisms, named pathogen-related oral spirochetes (PROS), were found in great numbers in the majority of gingival plaques from patients with necrotizing ulcerative gingivitis and severe periodontitis (17). In addition, it was shown that patients with gingivitis developed high-level serum immunoglobulin G (IgG) against the four pathogen-restricted determinants (18). PROS invaded intact epithelia and connective tissues adjacent to ulcers (19, 20). These findings suggested an important role of PROS in the etiology of periodontal diseases.

To define the phylogenetic position of these organisms, we used comparative 16S rRNA sequence analysis of suspected PROS after applying the technique of immunomagnetic separation (IMS) combined with in vitro amplification of 16S rRNA genes. IMS has been shown to be a useful technique for the selection of pathogenic bacteria from clinical samples or food by use of magnetic polymer beads coated with antibodies (3, 13, 16, 24, 28). Treponemes were separated by using superparamagnetic beads coated with monoclonal antibody (MAb) H9-2 directed against the 37-kDa endoflagellum sheath protein of *T. pallidum*, which was shown to cross-react with PROS but not with any known cultivable treponemes (18). After PCR amplification and cloning, 16S rRNA gene sequences were analyzed. Comparative sequence analysis showed that PROS clustered to recently described group I treponemes (4) and are closely related to *Treponema vincentii*. The latter was shown to cross-react with MAb H9-2 by immunofluorescence (IF) microscopy and immunoblotting.

MATERIALS AND METHODS

Bacterial strains and patient specimens. We used the following bacteria: *T. pallidum* subsp. *pallidum*, *T. denticola* (ATCC 33521 and OMZ677), *Treponema pectinovorum* (ATCC 33768 and FO2FA), *Treponema socranskii* subsp. *socranskii* (FO1B and ATCC 35536), *T. socranskii* subsp. *buccale* (FOC6H1 and ATCC 35534), *T. socranskii* subsp. *paredis* (PFA6DB and ATCC 35535), *T. socranskii* isolates (MHPI and FO2A1), *T. vincentii* (ATCC 35580, Ritz A, MH1F3, and FOC4A), novel treponeme isolates (BR, HO2A, PFB4G, and FOC6C1), and *Borrelia burgdorferi* B31. Two intestinal spirochetes, *Serpulina jonesii* ATCC 49776 and *Brachyspira aalborgi* ATCC 43993, were also included. *T. pallidum* subsp. *pallidum* organisms, kindly provided by S. A. Lukehart (Seattle, Wash.) on slides as touch preparations of infected rabbit testis tissue (acetone fixed), were used as positive controls for IF. *B. burgdorferi* B31 was obtained from G. Stanek

* Corresponding author. Mailing address: Universitätsklinikum Charité, Institut für Mikrobiologie und Hygiene, Dorotheenstraße 96, D-10117 Berlin, Germany. Phone: 49 30 609 3247. Fax: 49 30 229 2741. Electronic mail address: goebel@rz.charite.hu-berlin.de.

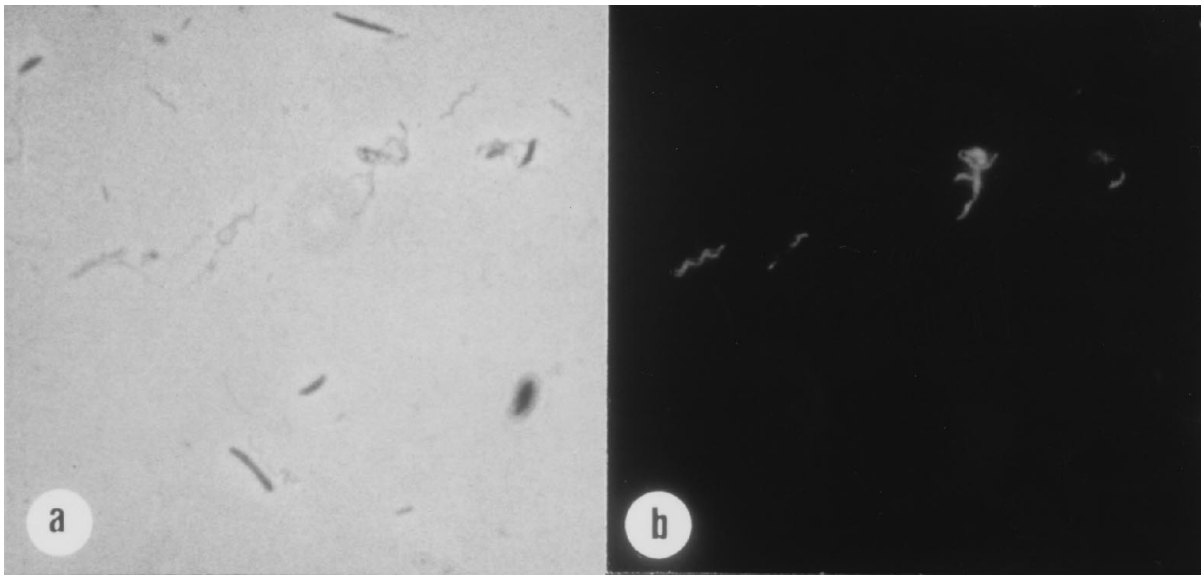

FIG. 1. Indirect IF of oral treponemes in a subgingival plaque sample from a patient suffering from rapidly progressive periodontitis using MAb H9-2. Micrographs show the results of phase-contrast (a) and epifluorescence (b) microscopy. Note that only selected spirochetal morphotypes are stained.

(Vienna, Austria), and *T. vincentii* ATCC 35580 and Ritz A were from M. Listgarten (Philadelphia, Pa.). All other strains either were our own isolates or were obtained from the American Type Culture Collection. Spirochetes were grown in OMIZ-Pat (30). For growth of *B. aalborgi* and *S. jonesii* the medium was supplemented with 10% fetal bovine serum. For *B. burgdorferi* 10% fetal bovine serum, 30 mM KCl, and 1.5 g of sodium hydrogencarbonate per liter were added to OMIZ-Pat.

Subgingival plaque samples were taken from two to four sites in five patients (white; three females and two males; 30 to 54 years of age) with rapidly progressive periodontitis by inserting sterile paper points into periodontal pockets greater than 5 mm in depth. Only patients without previous antimicrobial therapy or mechanical debridement were included in this study. Samples from different sites were pooled for each patient. Immediately after probing, samples were placed in reduced transport medium (12), and they were brought into the laboratory in an anaerobic jar within 1 h.

Indirect IF assay. Bacterial cultures and plaque samples were washed twice with phosphate-buffered saline (PBS). Pellets were resuspended in PBS, fixed in 3.7% formaldehyde for 30 min at room temperature, and stored at −20°C. Fixed cells (1 to 2 μl) were applied to the wells of glass slides (Paul Marienfeld KG, Bad Mergentheim, Germany) previously coated with 0.1% (wt/vol) gelatin and 0.01% (wt/vol) $CrKSO_4$ and then allowed to air dry. After application of MAb H9-2, the slides were incubated at room temperature in a humid chamber for 30 min and washed three times with PBS–0.1% (vol/vol) Tween 20. An aliquot (5 μl) of 1:500-diluted fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Dianova, Hamburg, Germany) was applied to each well, and the slides were incubated in a humid chamber at room temperature for 30 min. Slides were washed three times each for 3 min with PBS–0.1% (vol/vol) Tween 20. Test and control slides were washed separately to prevent any spillover. The slides were examined under oil immersion with a Neofluar 100× objective in a Zeiss (Jena, Germany) Axioskop equipped with a high-pressure mercury bulb (HBO 50) and filter set 487909 (FT 510, LP 520). Photomicrographs were made with Kodak Ektachrome 400 HC film.

IMS and PCR. MAb H9-2 as mouse hybridoma culture supernatant, directed against a pathogen-specific 37-kDa endoflagellum sheath protein from *T. pallidum* subsp. *pallidum* (14), was kindly provided by S. A. Lukehart (Seattle, Wash.). For IMS anti-mouse IgG1-coated superparamagnetic beads (Dynabeads M-450; Dynal, Hamburg, Germany) were coupled with MAb H-9 according to the manufacturer's instructions. Plaque samples were washed twice with PBS (pH 7.4) and incubated at 4°C with magnetic beads under continuous shaking for 30 min. The magnetic beads were recovered by magnetic force with a magnetic particle concentrator (Dynal). After they were washed with PBS–0.1% bovine serum albumin, DNA of bound bacteria was prepared and 16S rRNA gene fragments (about 500 bp) were amplified with universal primers RE-TPU1 and RE-RTU3, cloned, and sequenced as previously described (4).

Phylogenetic analysis. 16S rRNA gene sequences were aligned by using the sequence analysis program PILEUP of Husar 4.0 (DKFZ, Heidelberg, Germany). The TREECON software package (version 3.0) was used for construction of dendrograms. All positions were included in distance calculations. Multiple base changes at single positions were corrected by the method of Jukes and Cantor (10). Dendrograms were constructed by the neighbor-joining method of Saitou and Nei (21).

Immunoblotting. Cell extracts were separated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE) and electroblotted onto nitrocellulose as previously described (29). Before immunoblotting, blots were stained and destained for protein (2). MAb H9-2 was used at a dilution of 1:10, and alkaline phosphatase-conjugated goat anti-mouse IgG1 (Sigma) was used at a dilution of 1:1,000. Naphthol AS-E phosphate served as a substrate.

RESULTS

Indirect IF with MAb H9-2 to visualize reactive spirochetes in plaque samples. Several subgingival plaque samples from patients with rapidly progressive periodontitis were examined by indirect IF for cross-reactivity with *T. pallidum*-specific MAb H9-2, a MAb that had been used by Riviere et al. (17) for monitoring PROS in patient material. The specimens contained 30 to 55% of the spirochetal morphotypes as assessed by phase-contrast microscopy, 20 to 30% of which revealed positive signals by IF. No other bacteria showed a positive reaction (Fig. 1).

Characterization of bacteria selected by IMS. To select as yet uncultured PROS, we used paramagnetic beads coated with MAb H9-2. Immunomagnetic capturing followed by PCR was separately performed with materials from two different patients. The plaque samples were examined for reactivity with MAb H9-2 by IF prior to IMS, and no bacteria other than spirochetes showed a positive reaction. The 16S rRNA gene fragments (500 bp) of bacteria captured by the immunomagnetic beads were amplified by PCR and subsequently cloned into *Escherichia coli*. The 5′ ends (200 to 300 bases from position 28 in *E. coli* 16S rRNA) of inserts from 10 randomly selected recombinants in each experiment were sequenced. About 20% (4 of 20) of the clones analyzed contained spirochetal inserts. As shown in Fig. 2, two sequences were found to be identical to a sequence (NZM3D378) which clustered within so-called cluster 7 (group I) treponeme sequences that were retrieved from a 16S rRNA sequence library constructed from subgingival plaque material from a patient with a severe destructive periodontitis (4). On the basis of the comparison of 250 bases, the other treponemal sequences showed a 3-bp

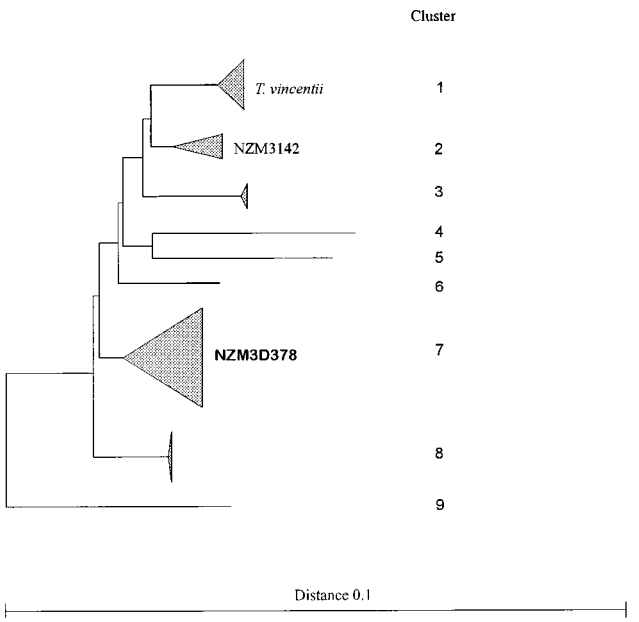

FIG. 2. Phylogenetic trees of group I treponemes (4), comprising *T. vincentii* and related organisms, based on comparative 16S rRNA gene sequence analysis. Clone sequences in some clusters are represented by a triangle. Sequences from organisms retrieved by IMS using MAb H9-2 clustered within cluster 7 and were identical or nearly identical to NZM3D378. The scale bar indicates 10% difference in nucleotide sequences as determined by taking the sum of the lengths of the horizontal lines connecting two species. The height of each triangle corresponds to the mean distance separating the sequences forming the cluster from the deepest branching point within that cluster, and the base is proportional to the number of species.

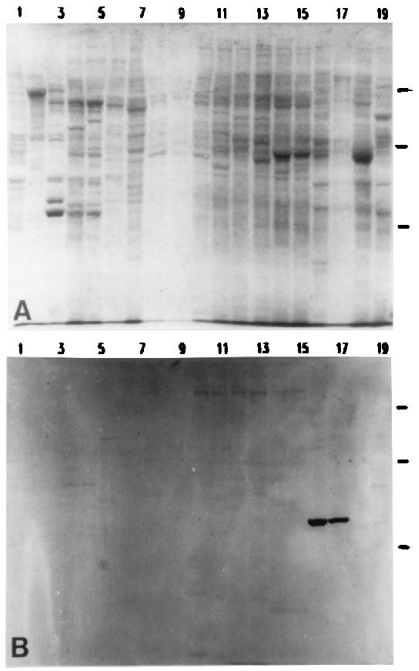

FIG. 3. Cross-reactivity of MAb H9-2 with culturable treponemes. SDS–10% PAGE analysis of whole-cell extracts of 19 cultivable human spirochete strains was performed. A Coomassie blue-stained gel (A) and an immunoblot (B) probed with MAb H9-2 detected with phosphatase-conjugated goat anti-mouse IgG and naphthol AS-E phosphate are shown. Lanes: 1, *B. aalborgi* ATCC 43994; 2, *S. jonesii* ATCC 49776; 3, *B. burgdorferi* B31; 4 to 7, novel treponeme isolates FOC6C1, PFB4G, HO2A, and BR, respectively; 8 and 9, *T. socranskii* MHP1 and FO2A1, respectively; 10 and 11, *T. socranskii* subsp. *paredis* PFA6DB and ATCC 35535, respectively; 12 and 13, *T. socranskii* subsp. *buccale* ATCC 35534 and FOC6H1, respectively; 14 and 15, *T. socranskii* subsp. *socranskii* FO1B and ATCC 35536, respectively; 16 and 17, *T. vincentii* MH1F3 and FOC4A, respectively; 18, *T. pectinovorum* FO2FA; 19, *T. denticola* OMZ677. The positions of molecular mass markers are indicated on the right (from the top, 69, 46, and 30 kDa).

mismatch with NZM3D378. The nonspirochetal sequences were heterogeneous, and comparative analysis revealed no significant similarity to 16S rRNA sequences deposited in current databases except for two sequences that showed 96 and 97% similarity to sequences of *Fusobacterium nucleatum* and *Porphyromonas endodontalis*, respectively.

Screening of cultivable spirochetes for reactivity with MAb H9-2. The fact that spirochetal sequences retrieved by IMS from two distinct subgingival plaque samples were strikingly similar to that of the cultivable treponeme *T. vincentii* (sequence similarity, 96.4%) prompted us to examine a set of cultivable spirochetes, including four strains of *T. vincentii* (ATCC 35580, Ritz A, and two patient isolates [FOC4A and MHIF3]), for their reactivity with MAb H9-2 by IF and immunoblotting. We also included previously uncultured oral treponeme strains (FOC6C1, PFB4G, HO2A, and BR) that were shown to belong to cluster 17 treponemes by 16S rRNA sequence analysis (30). Of all 23 strains tested, only those of *T. vincentii* showed positive IF and staining of a 37-kDa band in immunoblotting. Figure 3 shows the representative immunoblot containing 19 spirochete strains.

DISCUSSION

Recent findings of Riviere et al. (17–20) indicating that some invasive spirochetes reacted with MAbs previously thought to be specific for pathogenic *T. pallidum* subsp. *pallidum* and *T. pallidum* subsp. *pertenue* sparked great interest and suggested the identification of oral spirochetes (PROS) with pathogenic potential. Our attempts to further characterize these as yet uncultured spirochetes by sequence analysis of 16S rRNA genes of immunomagnetically captured organisms revealed that most of the captured organisms were not spirochetes. This result was in contrast to those of IF experiments with identical plaque samples. Here MAb H9-2 identified spirochetes exclusively. Whereas most IMS procedures reported by other groups included preenrichment with selecting organisms prior to IMS (16) or culturing of immunomagnetically captured organisms in selective medium and/or specific DNA amplification by PCR after IMS (3, 13, 24, 28), we tried to select spirochetes directly from subgingival plaque samples which contained large numbers ($>10^9$ organisms per ml) of other microorganisms. One might argue that epitopes present on the endoflagellum sheath may not be exposed at the surface. Therefore, we have performed IF using *T. vincentii* grown in culture before and after washing with PBS. Most of the washed cells were reactive with MAb H9-2, while unwashed cells were negative (data not shown). Similar observations have been reported elsewhere (5, 6, 9). The nonspecific capturing observed in our study may be due to nonspecific interaction with the surfaces of magnetic beads. Alternative explanations include the possibility that either bacterial coaggregates commonly found in subgingival plaque materials cosedimented with magnetic particles or other bacteria closely adhered to immunomagnetically captured spirochetes. However, the attempt to disrupt bacterial coaggregates by gentle homogenization and subsequent filtration through membrane filters (pore size, 5 μm) before incubation with immunomagnetic beads was

unsuccessful. Resulting sequences were as heterogeneous as those in the first experiment were, and the ratio of spirochetal sequences to nonspirochetal sequences did not increase. One might doubt the specific capture of spirochetes. Nevertheless, the fact that independent experiments revealed sequences identical or closely related to a treponemal sequence (NZM 3D378) described previously underlines the significance of our results. The observation that on the basis of sequence comparison the closest cultivable relative was *T. vincentii* and the finding that IF and immunoblotting with MAb H9-2 showed positive reactivity only for the four *T. vincentii* strains included in our study led us to conclude that the presence of the epitope recognized by MAb H9-2 may be a phenotypic trait of a heterogeneous group of treponemes clustering within group I. The reason why MAb H9-2 was nonreactive with a *T. vincentii* strain included in the study of Riviere et al. remains obscure. This discrepancy might be explained by either false identification of the *T. vincentii* strains used or differences in the laboratory protocols. All of the *T. vincentii* strains used in our study were verified by 16S rRNA sequence analysis.

While in vitro-cultured *T. vincentii* cross-reacted with MAb H9-2, *T. vincentii* was not detected by IMS from plaque samples. This result might be due to the small numbers of *T. vincentii* organisms in the plaque samples used for this study. This view is supported by recent epidemiological data (16a).

The assumption that PROS may represent a heterogeneous spirochete population including several group I treponemes was supported by the isolation of a novel treponeme belonging to a cluster 2 phylotype (NZM3142). This novel treponeme was shown to be MAb H9-2 positive by IF and immunoblotting. However, as most group I treponemes, and cluster 7 treponemes in particular, have not been cultivated yet, the confirmation of cross-reactivity with MAb H9-2 by IF or immunoblotting awaits successful isolation of hitherto-uncultured strains. Since PROS have been related to severe periodontitis previously (17–20), we are currently performing a prospective study to correlate the frequency of group I treponemes with the severity of disease. The ultimate goal is the identification by in situ hybridization of PROS in inflamed tissue.

ACKNOWLEDGMENTS

We thank S. A. Lukehart for MAb H9-2 and T. Adam for critical reading of the manuscript. This work was supported by a grant (Nr.01 KI 9318) from the Bundesministerium für Bildung, Wissenschaft, Forschung und Technologie (BMBF) to U.B.G.

REFERENCES

1. **Armitage, G. C., W. R. Dickinson, R. S. Jenderseck, S. M. Levine, and D. W. Chambers.** 1982. Relationship between the percentage of subgingival spirochetes and the severity of periodontal disease. J. Periodontol. **53:**550–556.
2. **Bickar, D., and P. D. Reid.** 1992. A high-affinity protein stain for Western blots, tissue prints, and electrophoretic gels. Anal. Biochem. **203:**109–115.
3. **Biswas, B., R. Vemulapalli, and S. K. Dutta.** 1994. Detection of *Ehrlichia risticii* from feces of infected horses by immunomagnetic separation and PCR. J. Clin. Microbiol. **32:**2147–2151.
4. **Choi, B. K., B. J. Paster, F. E. Dewhirst, and U. B. Göbel.** 1994. Diversity of cultivable and uncultivable oral spirochetes from a patient with severe destructive periodontitis. Infect. Immun. **62:**1889–1895.
5. **Cox, D. L., D. R. Akins, S. F. Porcella, M. V. Norgard, and J. D. Radolf.** 1995. *Treponema pallidum* in gel microdroplets: a novel strategy for investigation of treponemal molecular architecture. Mol. Microbiol. **15:**1151–1164.
6. **Cox, D. L., P. Chang, A. McDowall, and J. D. Radolf.** 1992. The outer membrane, not a coat of host proteins, limits the antigenicity of virulent *Treponema pallidum*. Infect. Immun. **60:**1076–1083.
7. **Ellen, R. P., M. Song, and C. A. G. McCulloch.** 1994. Degradation of endogenous plasma membrane fibronectin concomitant with *Treponema denticola* 35405 adhesion to gingival fibroblasts. Infect. Immun. **62:**3033–3037.
8. **Grenier, D., V.-J. Uitto, and B. C. McBride.** 1990. Cellular location of a *Treponema denticola* chymotrypsinlike protease and importance of the protease in migration through the basement membrane. Infect. Immun. **58:**347–351.
9. **Holt, S. C.** 1978. Anatomy and chemistry of spirochetes. Microbiol. Rev. **42:**114–160.
10. **Jukes, T. H., and C. R. Cantor.** 1969. Evolution of protein molecules, p. 21–132. *In* H. N. Munro (ed.), Mammalian protein metabolism, vol. 3. Academic Press, New York.
11. **Loesche, W. J.** 1988. The role of spirochetes in periodontal disease. Adv. Dent. Res. **2:**275–283.
12. **Loesche, W. J., R. N. Hockett, and S. A. Syed.** 1972. The predominant cultivable flora of tooth surface plaque removed from institutionalized subjects. Arch. Oral Biol. **17:**1311–1325.
13. **Luk, J. M. C., and A. A. Lindberg.** 1991. Rapid and sensitive detection of *Salmonella* (O:6,7) by immunomagnetic monoclonal antibody-based assays. J. Immunol. Methods **137:**1–8.
14. **Lukehart, S. A., M. R. Tam, J. Hom, S. A. Baker-Zander, K. K. Holmes, and R. C. Nowinski.** 1985. Characterization of monoclonal antibodies to *Treponema pallidum*. J. Immunol. **134:**585–592.
15. **Mikx, F. H. M., F. Jacobs, and C. Satumalay.** 1992. Cell-bound peptidase activities of *Treponema denticola* ATCC 33520 in continuous culture. J. Gen. Microbiol. **138:**1837–1842.
16. **Molla, B., J. Kleer, and H.-J. Sinell.** 1994. Detection of *Salmonella* in foods by immunomagnetic separation. Arch. Lebensmittelhyg. **45:**110–113.
16a. **Moter, A., et al.** Unpublished results.
17. **Riviere, G. R., K. S. Elliot, D. F. Adams, L. G. Simonson, L. B. Forgas, A. M. Nilius, and S. A. Lukehart.** 1992. Relative proportions of pathogen-related oral spirochetes (PROS) and *Treponema denticola* in supragingival and subgingival plaque from patients with periodontitis. J. Periodontol. **63:**131–136.
18. **Riviere, G. R., M. A. Wagoner, S. A. Baker-Zander, K. S. Weisz, D. F. Adams, L. Simonson, and S. A. Lukehart.** 1991. Identification of spirochetes related to *Treponema pallidum* in necrotizing ulcerative gingivitis and chronic periodontitis. N. Engl. J. Med. **325:**539–543.
19. **Riviere, G. R., K. S. Weisz, D. F. Adams, and D. D. Thomas.** 1991. Pathogen-related oral spirochetes from dental plaque are invasive. Infect. Immun. **59:**3377–3380.
20. **Riviere, G. R., K. S. Weisz, L. G. Simonson, and S. A. Lukehart.** 1991. Pathogen-related spirochetes identified within gingival tissue from patients with acute necrotizing ulcerative gingivitis. Infect. Immun. **59:**2653–2657.
21. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:**406–425.
22. **Simonson, L. G., C. H. Goodman, J. J. Bial, and H. E. Morton.** 1988. Quantitative relationship of *Treponema denticola* to severity of periodontal disease. Infect. Immun. **56:**726–728.
23. **Simonson, L. G., P. J. Robinson, R. J. Pranger, M. E. Cohen, and H. E. Morton.** 1992. *Treponema denticola* and *Porphyromonas gingivalis* as prognostic markers following periodontal treatment. J. Periodontol. **63:**270–273.
24. **Skjerve, E., L. M. Rørvik, and Ø. Olsvik.** 1990. Detection of *Listeria monocytogenes* in foods by immunomagnetic separation. Appl. Environ. Microbiol. **56:**3478–3481.
25. **Soder, P. O., L. Frithiof, and B. Soder.** 1990. Spirochetes and granulocytes at sites involved in periodontal disease. Arch. Oral Biol. **35:**197–200.
26. **Uitto, V.-J., Y.-M. Pan, W. K. Leung, H. Larjava, R. P. Ellen, B. B. Finlay, and B. C. McBride.** 1995. Cytopathic effects of *Treponema denticola* chymotrypsin-like proteinase on migrating and stratified epithelial cells. Infect. Immun. **63:**3401–3410.
27. **Weinberg, A., and S. C. Holt.** 1990. Interaction of *Treponema denticola* TD-4, GM-1, and MS25 with human gingival fibroblasts. Infect. Immun. **58:**1720–1729.
28. **Wolfhagen, M. J. H. M., A. C. Fluit, R. Torensma, M. J. J. G. Poppelier, and J. Verhoef.** 1994. Rapid detection of toxigenic *Clostridium difficile* in fecal samples by magnetic immuno PCR assay. J. Clin. Microbiol. **32:**1629–1633.
29. **Wyss, C.** 1989. *Campylobacter-Wolinella* group organisms are the only oral bacteria that form arylsulfatase-active colonies on a synthetic indicator medium. Infect. Immun. **57:**1380–1383.
30. **Wyss, C., B.-K. Choi, P. Schüpbach, B. Guggenheim, and U. B. Göbel.** 1996. *Treponema maltophilum* sp. nov., a small oral spirochete isolated from human periodontal lesions. Int. J. Syst. Bacteriol. **46:**745–752.