A STUDY OF TREPONEMAL ANTIGENS

by

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(ABSTRACT)

The antigenic relationship between T. denticola, T. socranskii subsp. socranskii, T. socranskii subsp. buccale, T. socranskii subsp. paredis, Treponeme D, T. vincentii, T. phagedenis biotype Reiter, T. minutum, and T. refringens was studied using fluorescent antibody staining and those organisms were found to contain a group of common antigens. The cross-reactivity was removed when the antisera was adsorbed with Reiter cells. There was a second group of common antigens that were shared by the subspecies of T. socranskii, Treponeme D and T. pectinvorum.

Triton-extracts from different treponemal species were found to contain very few cross-reacting antigens as demonstrated by immunodiffusion, crossed immunoelectrophoresis, ELISA, and Western blots. An immunodiffusion test using treponemal Triton-extracts and antisera against five treponeme species was developed for the routine identification of oral treponemes that were in either pure or mixed cutures.

T. denticola cells were found to bind to fibronectin

coated microscope slides. Proteins, that bind fibronectin, in the Triton-extracts of T. denticola, were detected in a fibronectin-capture ELISA. The Triton-extracts also reacted with rabbit fibronectin antiserum and rabbit albumin antiserum in immunodiffusion and ELISA assays. Three proteins with molecular weights of 60K, 47K, and 21K, were eluted from a agarose-gelatin-fibronectin column and reacted in Western blots with T. denticola antiserum.

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INTRODUCTION

Treponemes are microaerophilic or anaerobic helically coiled rods. The microaerophilic treponemes are the known pathogens _T. pallidum_ subsp. _pallidum_, _T. pallidum_ subsp. _pertenue_, and _T. pallidum_ subsp. _endemicum_ that cause syphilis, yaws, and nonvenereal endemic syphilis in man. These organisms cannot be cultivated in artificial medium. _T. hyodysenteriae_ is also a microaerophile and causes swine dysentery. The strict anaerobes are indigenous to the genital tract, intestinal tract, and oral cavity of man and animals and can be grown in artificial medium . _T. denticola_, _T. socranskii_ subsp. _socranskii_, _T. socranskii_ subsp. _buccale_, _T. socranskii_ subsp. _paredis_, Treponeme D, _T. pectinovorum_, and _T. vincentii_ are part of the normal oral flora of man. These organisms may be associated with periodontal disease in man.

The isolation and identification of oral treponemes is very time consuming and labor intensive. It takes one to two months to identify oral treponmes after single colony isolation from a dental sample. Therefore; it is desirable to develop a serologic assay that can be used to rapidly and accurately identify treponemes in pure or mixed cultures within several days. The difficulty with a serologic approach is that the treponemes share several

common antigens. Therefore, a serologic test that is species specific and does not detect the cross-reacting antigens in other species, is needed to identify all the strains within a species.

It has been found that T. pallidum attaches to tissue cells (8, 10). Thomas et al. (44) proposed that T. pallidum attaches to tissue cells by the binding of three proteins with molecular weights of 72K, 28K, and 26K. These proteins are found in the outer envelope of T. pallidum and bind to fibronectin (Fn) which is found on the surface of many mammalian cells. Binding of these proteins is blocked when cover slips that have been coated with Fn were pre-incubated with Fn-antiserum (10). These proteins are present in detergent extracts (Zwittergent) of T. pallidum cells.

The oral spirochetes T. denticola and T. vincentii have been reported to attach to human epithilial cells (23). These organisms, like T. pallidum, seem to attach to cell surfaces at the tips of the treponemal cells (27). Because T. denticola and T. vincentii bind to tissue cells in the same manner as T. pallidum, it is possible that T. denticola and T. vincentii contain outer envelope proteins that bind Fn on the surface of human epithelial cells. The proteins may also be present in Triton X-100 extracts of T. denticola.

Specific objectives of this study were: i) to study the antigenic relationships between T. denticola, the subspecies of T. socranskii, Treponeme D, T. pectinovorum, T. vincentii, T. phagedenis biotype Reiter, T. minutum, and T. refringens, ii) to develop and to perfect a serological test that will specifically identify the above organisms, ii) to determine if T. denticola binds to Fn and, iv) to identify the Fn binding proteins and to determine their approximate molecular weights.

LITERATURE REVIEW

Organisms that belong to the genus _Treponema_ are Gram negative, helically coiled rods, that are 0.1 to 0.2 um in diameter and are 5 to 20 um in length (34). Treponemes have an outer envelope (outer sheath), a cytoplasmic cylinder and they posses one or more periplasmic flagella that are located in the periplasmic space, between the outer envelope and cytoplasmic cylinder. The outer envelope of oral treponemes consist of 49.7% protein and 30.8% total lipid while (95% of the total lipid is phospholipid), and 11% of the outer envelope is carbohydrate (21).

Members of the genus _Treponema_ consist of pathogenic and non-pathogenic bacteria (24). They are part of the normal flora of the genital tract, intestinal tract, and oral cavity of man and animals. Known human pathogens, are probably microaerophilic and can only be maintained in rabbit testes. Pathogenic treponemes include _T. pallidum_ subsp. _pallidum_ which is the causative agent of syphilis. _T. pallidum_ subsp. _pertenue_ and _T. pallidum_ subsp. _endemicum_ cause yaws and nonvenereal endemic syphilis in man. _T. carateum_ is the etiologic organism of pinta. _T. pallidum_ subsp. _pallidum_, _T. pallidum_ subsp. _pertenue_ and _T. pallidum_ subsp. _endemicum_ are all related antigenically and by DNA homology. Sera from

patients with either yaws or nonvenereal endemic syphilis give positive results for the serological tests for syphilis. Hamsters that have been infected with T. pallidum subsp. endemicum are resistant to reinfection when challenged with T. pallidum subsp. pallidum, and T. pallidum subsp. pertenue. Treponemes pathogenic for animals are T. paraluis-cuniculi and T. hyodysenteriae. T. paraluis-cuniculi causes rabbit syphilis; whereas, T. hyodysenteriae is the causative agent of swine dysentery.

The non-pathogenic treponemes can be cultivated in artificial media and are strict anaerobes (34). Normal female and male genital flora contain, T. minutum, T. refringens, and T. phagedenis. T. phagedenis biotype Reiter has been shown to share common antigens with T. pallidum (37). T. denticola, T. socranskii subsp. socranskii , T. socranskii subsp. buccale, T. socranskii subsp. paredis , T. pectinovorum, T. vincentii, and the unnamed Treponeme D are all part of the normal flora of the oral cavity in man and may be associated with gingivitis and periodontal disease. T. denticola has also been isolated from the oral cavity of chimpanzees (34).

Antigenic Relationship Between Treponemes

The study of the antigenicity and the host immune response to T. pallidum has been hampered because of the

inability to cultivate T. pallidum in vitro. Extensive research has been done on the antigenic relationship between T. pallidum and the non-pathogenic, cultivatable, T. phagedenis biotype Reiter. Pedersen and associates have done exhaustive research on Reiter antigens that were immunologically related to T. pallidum antigens. Pedersen et al. (37) reported the presence of antibodies in serum from patients with secondary syphilis that reacted with five antigens out of forty soluble Reiter antigens.

Lukehart et al. (20) analyzed Western blots of T. pallidum antigens and reported the presence of antibodies in sera from T. pallidum infected rabbits that reacted with eight T. pallidum antigens with molecular weights ranging from 45K to 15K. Lukehart et al. showed that five out of the eight protein antigens were shared with the Reiter treponeme. When immune rabbit serum was adsorbed with Reiter sonicates, three protein bands with molecular weights of 48K, 12K, and 14K remained. These protein bands reacted with T. pallidum immune serum but not with Reiter hyperimmune serum.

Wos and Wicher (46) also used Western blots and reported that serum from rabbits that were artificially immunized (hyperimmune) with T. pallidum contained antibodies against 34 T. pallidum antigens, of which

approximately 30 antigens were shared with the Reiter treponeme. Adsorption of the serum with Reiter, T. refringens strain noguchi, and T. vincentii whole cells removed the antibodies to all but 3 protein antigens with molecular weights of 36K, 34K, and 27K.

Several antigens of the Reiter strain of T. phagedenis have been isolated and characterized by Petersen and colleagues (30, 31, 32). Soluble protein antigens isolated were called TR-b, TR-c, and TR-o and had molecular weights of 610K-630K, 630K-730K, and 456K-550K respectively. Sodium dodecyl sulfate polyacrylamide gel electrophoresis showed that TR-b consisted of two 70K subunits (31) , TR-c had two 48K subunits (30), and TR-o consisted of two 66K polypeptides (32). T. pallidum showed strong fluorescence when reacted against monospecific TR-b, TR-c, and TR-o antiserum. Crossed immunoelectrophoresis showed the presence of antibodies in human syphilitic serum against the TR-b and TR-c antigens, but not the TR-o antigen.

Other Reiter antigens that have been purified and were shown to cross-react with human syphilitic serum were the periplasmic flagella and a ribonucleic acid antigen (39,29). Enzyme linked immunosorbent assays were developed and showed the presence of antibodies in sera from patients with secondary syphilis against the

periplasmic flagella and RNA antigens (40,38). The ELISA using Reiter periplasmic flagella seemed to be more sensitive than the Treponema pallidum immobilization test; therefore was the best suited for the serodiagnosis of syphilis.

Cross-reactivity was not only seen between the Reiter treponeme and T. pallidum but also between other members of the family Spirochaetaceae. Zander and Lukehart (3), using a sensitive Western Blot method, reported that T. paraluis-cuniculi, T. hyodysenteriae, Borrelia hermsi, and Leptospira interrogans had antigens that were immunologically related to T. pallidum antigens. T. paraluis-cuniculi was the most similar antigenically to T. pallidum, and shared 21 antigens with T. pallidum. T. hyodysenteriae, B. hermsi, and L. interrogans shared 10 to 11 antigens with T. pallidum. Western Blot analysis done by Wos and Wicher (46) showed that cultivated human normal flora treponemes, T. vincentii and T. refringens strain noguchi shared common antigens with T. pallidum.

Little is known about the antigenic cross-reactivity between the cultivated non-pathogenic treponemes, particularly the oral treponemes. Using fluorescent antibody staining, Meyer et al. (22) investigated the antigenic relationship between 14 strains of treponemes

that were representatives of four species of treponemes and one species belonging to the genus Spirocheta. Four of the 14 strains were oral treponemes (T. denticola and T. vincentii) while the other strains represented the genital species T. phagedenis and T. refringens. The study showed that all 14 treponemes shared a common antigen(s) that could be removed by adsorption of the antiserum with T. phagedenis biotype Reiter cells. Five serogroups were obtained when the antiserum was adsorbed with cells of the Reiter strain of T. phagedenis. Reiter, English Reiter, Kazan, and Kazan's 2, 4, 5, all of which are now known to be strains of T. phagedenis belonged to serogroup I. Serogroup II consisted of the Nichols and Noguchi strains of T. refringens. The oral treponemes MRB, FM and N-39 which are strains of T. denticola, made up serogroup III. In addition, these three treponemes demonstrated serologic heterogenicity between each other. MRB and FM possesed antigens not present in N-39 and N-39 contained antigens that were not seen in neither MRB nor FM. Treponema zuelzerae, which is a free living spirochete now classified as Spirochaeta zuelzerae, was placed in serogroup IV. Finally serogroup V consisted of the oral treponeme T. vincentii.

Tall and Nauman (23) used microscopic agglutination and SDS polyacrylamide gel electrophoresis to show the

similarities between strains of T. denticola, T. pectinovorum, and T. vincentii. Microscopic agglutination which was less sensitive than immunofluorescence, showed, in contrast to Meyer's data, that there was no cross-reactivity between T. denticola, T. pectinovorum, and T. vincentii. They also demonstrated that one T. pectinovorum antiserum agglutinated all strains of T. pectinovorum and one T. vincentii antiserum agglutinated all T. vincentii strains. On the other hand, no one single T. denticola antiserum agglutinated all strains of T. denticola. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) demonstrated distinct protein banding patterns for each species. There was some variation in minor protein bands among strains of T. denticola and the two strains tested of T. vincentii.

Cheng et al.(7) used slide agglutination and fluorescent immunoassays to evaluate the serologic relationship among several oral treponemes. Both assays demonstrated serologic heterogenicity among strains of T. denticola. Six isolates a, b, c, e, d, and e', of oral treponemes were determined by DNA homology to be strains of T. denticola. Immunoadsorption of the antiserum against each of these strains with the heterologous organism showed that the strains were divided into two

serogroups. Strains a, b, e, and e′ belonged in serogroup a and strains c and d were placed in serogroup c.

Isolation of Treponemal Structures

Outer Envelope Isolation: Antigens and proteins on the outer envelope are important, particularly in T. pallidum because they are exposed to the extracellular environment of the host and are directly involved in antigen antibody reactions in the host. Several methods have been published that involved the removal of the outer envelope of treponemes. Most of the procedures required solubilization of the membrane with detergents. Klaviter and Johnson (16) resuspended and incubated sedimented B. hermsi cells with 0.005 % SDS. After incubation the cells were centrifuged and the supernatant containing the outer envelope was collected. Electron micrographs showed that solubilization of the outer envelope with low concentrations of SDS removed the outer envelope with very little damage to the cell wall and cytoplasmic membrane.

Penn et al. (25) extracted the outer envelope of T. pallidum with 0.2% Triton X-100. Washed cells were resuspended in 0.2 % Triton X-100 in the presence of Mg^{2+} and were incubated, then the cells centrifuged, and the supernatant fluid was saved. Electron micrographs showed

the removal of the outer envelope with minimal leakage of cytoplasmic contents due to the damage of the cell wall and cytoplasmic membrane.

Laughon (19) incubated washed treponemal cells in 8% butanol-water solution to remove the outer envelope of T. phagedenis. The cell suspension was centrifuged and the supernatant fluid was collected. Butanol-water extracts contained, in addition to outer envelope proteins, the cytoplasmic enzymes glutamate dehydrogenase and diaphorase and the cytoplasmic membrane associated enzyme fumarate reductase. This indicated the removal of both the outer envelope and cytoplasmic membrane.

Low pressure cell disruption was also used by Laughon (19) for the removal of T. phagedenis outer envelope. Cells were passed through a French pressure cell at 640 psi. The disrupted cells were centrifuged twice at 12,000 x g for 15 minutes then once at 100,000 x g for 2.5 hours. The final pellet was resuspended and centrifuged in a sucrose gradient (20-60%). A single band was observed and contained the outer envelope.

Periplasmic Flagella Isolation: Hardy et al. (11) isolated T. pallidum periplasmic flagella by lysing the cells by alkaline shock at pH 11.6. Cell ghosts were sedimented, followed by the removal of the outer envelope by incubation in 0.03 % solution of SDS. The cell bodies

were sedimented, washed, and sheared in a blender. The periplasmic flagella were purified by density gradient centrifugation.

Petersen et al. (28) described an easier way to isolate the periplasmic flagella of treponemes. Supernatant fluid from sonicated cells was applied to a diethylaminoethyl-cellulose column and eluted with a stepwise salt gradient. The fractions containing periplasmic flagella were collected, concentrated and gelfiltered on a Sepharose CL-2B column .

Penn et al. (24) and Petersen et al. (28) reported that axial fibrils of T. pallidum and T. phagedenis biotype Reiter produced a linear precipitin line in crossed immunoelectrophoresis slides that was slightly below the agarose containing antibody layer and moved only a few mm away from the antigen well. T. pallidum periplasmic flagella consisted of 3 polypeptides with molecular weights of 37K, 43K, and 33.5K respectively (24). Petersen (48) reported that T. phagedenis biotype Reiter periplasmic flagella consisted of three major polypeptides with molecular weights of 34K, and two 30K proteins that migrated very close to each other, and a faint band at 28K.

Binding of Treponemal Cells to Fibronectin

Fibronectin, Structure and Function: Fibronectin (Fn)

is an asymmetric, dimeric, glycoprotein with subunit weights ranging from 200K to 250K (47). The subunits are joined by disulfide bridges at the carboxyl terminus. Carbohydrates associated with Fn are oligosaccharides that consist of an N-acetylglucosamine and a mannose backbone that is linked by N-glycosidic bonds to asparagine. Terminal residues include sialic acid or fucose which are bound to the galactose. There are 4 to 6 oligosaccharides per Fn molecule. Each polypeptide is subdivided into domains that are highly coiled, resistant to proteolytic digestion, and are involved in the binding of Fn to proteins and membranes (14). Some of these domains include fibrin, collagen, heparin, and a cell binding domain.

There are two kinds of Fn, cell surface Fn and plasma Fn (47). Cell surface Fn, also known as galactoprotein, microfibrillar protein, fibroblast surface antigen (SFA), large external transformation-sensitive (LETS) protein, cell surface protein (CSP) or Zeta (Z) protein, comprises 1% to 3% of the total cellular protein. The subunits of cell surface Fn have molecular weights between 200K to 250K. Cell surface fibronectin is found between cells, on basement membranes, fibroblasts, epithelial cells, blood vessel endothelial cells, extracellular fibrils in connective

tissue, amniotic fluid, and in cerebrospinal fluid. The main function of cell surface fibronectin is the cell to cell and cell to substratum adhesiveness. Cell surface Fn also seems to be involved in maintaining normal cell morphology, mediating the pattern of cell alignment, and in the regulation of growth.

The second type of Fn is plasma Fn. Synonyms for plasma Fn include; cold insoluble globulin, anti-gelatin factor, opsonic protein, cell attachment factor (CAF, c-CAP), and cell spreading factor (47). Plasma Fn is found in the blood at concentrations of 0.3 mg/ml. However, the concentration is lower in serum because plasma Fn binds to fibrin. The subunits of plasma Fn are slightly smaller than cell surface Fn subunits. Plasma Fn subunits have molecular weights of 200K and 220K. Both cell surface Fn and plasma Fn bind to heparin, collagen, gelatin, and fibrin. One of the functions of plasma Fn is the removal of collagenous debris from the blood. Plasma Fn also has opsonin properties, in that it is required for macrophages to ingest denatured collagen. In the presence of plasma Fn, mammalian cells migrate faster. Cell surface and plasma Fn are very similar immunologically and in function. However they are not identical compounds.

Binding of Bacteria to Fn: In 1978 Kuusela (17) reported

the binding of Staphylococcus aureus to Fn. Since then Switalski (42) reported the binding of several staphylococcal species to Fn. Fibronectin binding staphylococci include; S. haemolyticus, S. warneri, S. hyicus, S. hominis, S. saprophyticus, S. epidermidis, and S. simulan. It was suggested that the ability of staphylococci to bind to Fn may contribute to their virulence.

Binding of Treponemal Cells to Fn: Organisms that are constantly being bathed in body fluids need to adhere to tissue before it can establish an infection or become part of the indigenous flora. Therefore, several studies have been initiated to determine if T. pallidum binds to cell surfaces. Hayes et al. (12) reported that virulent T. pallidum binds, by their tips, to monolayers of normal rabbit testicular cells and HEp-2 cells. The adherence of T. pallidum to the cells was reduced when T. pallidum was first incubated with serum from rabbits that were infected with T. pallidum. On the other hand, the non-pathogenic T. phagedenis biotype Reiter did not adhere to tissue culture cells. Fitzgerald et al. (8) demonstrated that T. pallidum did also bind to several different types of cultured cells, such as; cells derived from testis, kidney, spleen, lung, epidermis, cervix, urethra, and nerve tissue. He also reported that non-pathogenic

treponemes such as T. denticola, T. refringens, T. scoliodontum, and T. vincentii did not bind to the above mammalian cell surfaces.

Baseman and Hayes (4) initiated a study to determine what was the receptor on mammalian cells for T. pallidum. Competitive binding assays determined that there was a limited number of receptor sites of the surface of HEp-2 cells for the attachment of T. pallidum. Peterson et al. (4) incubated T. pallidum with radiolabeled Fn. After washing the cells , Peterson observed the presence of radioactive Fn associated with T. pallidum. Alderete and Baseman (1) have also shown the association of T. pallidum with various host serum proteins such as, albumin, macroglobulin, transferrin, ceruloplasmin, immunoglobulin G, immunoglobulin M, and C3. Fitzgerald et al. (10) reported the binding of T. pallidum to Fn, lamin, collagen IV, and collagen I coated cover slips. The adherence was blocked when T. pallidum was pre-incubated with T. pallidum immune serum.

Baseman and colleagues, in a series of papers, reported the isolation and characterization of three outer envelope proteins that bind to Fn. Baseman and Hayes (4) first reported that T. pallidum had 3 outer envelope proteins with molecular weights of 72K, 28K, and 26K (P1, P2, P3) that bind to HEp-2 cells. Antibodies

against the proteins were seen in syphilitic sera and yaws sera. Thomas et al. (43,46) later reported that P1, P2, and P3 bound to Fn through the cell binding domain.

Alderete et al. (2) developed an ELISA assay that used P1, P2, and P3, to detect the presence of antibodies to T. pallidum in patient's serum. Fibronectin was used to capture the Fn binding proteins (P1, P2, P3) from a detergent (Zwittergent 3-12) extract of T. pallidum. Syphilitic serum or antiserum against T. phagedenis biotype Reiter, was added to the captured P1, P2, and P3 to determine if there were antibodies present in the serum to these proteins. The results showed that syphilitic serum reacted with the proteins; however, Reiter antiserum did not react with the T. pallidum Fn binding proteins.

While Fitzgerald et al. (8) reported that the oral treponemes T. denticola, and T. vincentii did not bind to tissue cells, Reojntjens et al. (27) and Olsen (23) reported that T. denticola and T. vincentii had a polar attachment to human epithelial cells. Reojntjens et al. (27) reported that incubation of epithelial cells with T. denticola or T. vincentii cells or their culture filtrate induced increased vacuolation, loss of cytoplasmic details, rounding of the cells, and detachment of the epithelial cells from the cover slips.

Shenker et al. (33) measured the incorporation of tritiated thymidine into human lymphocytes that were been exposed to sonicated extract of *T. denticola* and *T. vincentii*. They reported that *T. denticola* contained a heat labile protein with a molecular weight of 100K, that suppressed human lymphocyte proliferation. The inhibition was dose dependant and involved the alteration of DNA, RNA, and protein synthesis. On the other hand, *T. vincentii* showed no suppression of lymphocyte proliferation. Boehringer and colleagues (6) demonstrated the presence of a 50K heat labile component of *T. denticola* that suppressed human and mouse fibroblast proliferation.

MATERIALS and METHODS

General Procedures

Bacterial Cultures: Treponeme cultures were obtained from the culture collection of Dr. Robert M. Smibert.

T. denticola : strains, D39DPP-1, T32A, ST-10, FM, oralis, Ambigua, Ichelson # 2, IPP, TRRD, D3A-1, Jethro (chimpanzee), and Fugi (chimpanzee)

T. vincentii : strain N-9

T. phagedenis : biotype Reiter

T. minutum : strain minutum

T. refringens : strain refringens C

T. socranskii subsp. socranskii : strain D43BR-1 (Type strain)

T. socranskii subsp. buccale : strain D2B-8 (Type strain)

T. socranskii subsp. paredis : strain D46CPE-1 (Type strain)

Treponeme D : strain D40DR-2

T. pectinvorum : strain D39DR-2 (Type strain)

Bacterial Growth Conditions : All media were prepared as described in the Anaerobe Laboratory Manual (13). Stock cultures of T. denticola, T. vincentii, T. phagedenis, T. minutum, and T. refringens were maintained by weekly transfer in Peptone Yeast Glucose Serum (PYGS) semisolid medium. PYGS medium contained (g/750 ml) : 3.75 g of

heart infusion broth, 15 g of pepticase, 7.5 g of yeast extract, 7.5 g of glucose, 7.5 g of gelatin, 0.96 g agar, 0.375 g ammonium sulfate, 375 ml of salts solution (g/400 ml, 0.08 g of $MgSO_4$, 0.9 g of K_2HPO_4, 0.4 g of KH_2PO_4, and 0.8 g of NaCl), 3 ml of a 0.025% resazurin solution, and 375 ml of distilled water. The medium was boiled until the resazurin was colorless and it was cooled under a flow of nitrogen. After cooling, 3.75 g of sodium bicarbonate and 0.375 g of cysteine hydrochloride was added to the medium. Heat inactivated rabbit serum containing 0.75 mg / 100 ml of thiamine pyrophosphate was added to a final concentration of 10 %. Broth medium that was used to grow cultures for cells used in outer envelope extraction and serologic tests was the above medium without agar and gelatin.

Stock cultures of _T. socranskii_ subsp. _socranskii_, _T. socranskii_ subsp. _buccale_, _T. socranskii_ subsp. _paredis_, and Treponeme D were maintained by weekly transfer in Oral Treponeme Isolation (OTI) medium (36). OTI medium consisted of 5 g of polypeptone, 5 g of heart infusion broth, 5 g of yeast extract, 0.8 g of pectin, 0.8 g of glucose, 0.8 g of starch, 0.8 g of sucrose, 0.8 g of maltose, 0.8 g of sodium pyruvate, 0.8 g of xylose, 0.8 g of ribose, 0.8 g of fructose, 2 g of K_2HPO_4, 5 g of NaCl, 0.1 g of $MgSO_4$, 2 g of $(NH_4)_2SO_4$, 3 ml of 0.025 % resazurin solution, 0.68 g of cysteine hydrochloride, 500

ml of bovine rumen fluid, 500 ml of distilled water and 0.16 % agar for the semisolid media. Filter sterilized, heat inactivated slightly hemolyzed rabbit serum containing 0.75 mg /100 ml thiamine pyrophosphate were added to the sterilized media to yield a final concentration of 10 %. Fresh filter sterilized yeast extract (1 ounce of Brewers yeast (Fleischmann's yeast) was added to 100 ml of distilled water, mixed and incubated at 60°C for 4 days. Yeast cells were sedimented by centrifugation and the supernatant fluid (yeast extract) was filtered sterilized) was added to the medium at a final concentration of 5%. Broth OTI medium was the above medium without agar.

T. pectinovorum cultures were maintained by weekly transfer, in modified OTI medium. The modified OTI medium contained : 3.75 g of pepticase, 3.75 g of peptone, 3.75 g of heart infusion broth, 7.5 g of yeast extract, 7.5 g of pectin, 0.6 g of glucose, 0.6 g of ribose, 0.6 g of starch, 0.6 g of fructose, 0.6 g of maltose, 0.6 g of xylose, 375 ml of salt solution, 225 ml of rumen fluid, 150 ml of distilled water, and 3.0 ml of a 0.025 % solution of resazurin. After boiling and cooling the medium 7.5 ml of hemin, 0.15 ml of Vitamin K, 0.375 g of cysteine hydrochloride and 3.75 g of sodium bicarbonate were added. Serum, yeast extract, and thiamine

pyrophosphate were added in the same concentrations as previously described above. Modified OTI broth was modified OTI medium without agar.

Mass Cultivation of Bacterial Cells: T. denticola, T. vincentii, T. phagedenis, T. minutum and T. refringens were grown in four 25 X 124 mm tubes that contained 25 ml of PYGS broth medium. T. socranskii and its subspecies and Treponeme D were grown in four, 25 X 124 mm tubes containing 25 ml of OTI broth medium. T. pectinvorum cultures were grown in four, 25 X 124 tubes containing 25 ml of modified OTI broth.

All cultures were incubated for 4 days at 37 °C, the cells were harvested by centrifugation at 10,000 rpm for 30 minutes and washed 3 times with 30 ml volumes of phosphate buffered saline (FTA buffer, BBL, Cockeysville, Md.). After washing, the cell pellet was resuspended in FTA buffer containing various detergents.

Treponemal Antisera: Two tubes containing 25 ml of broth culture were used as inoculum for 500 ml of prereduced broth medium. The cultures were incubated under anaerobic conditions for 4 days at 37 C and the cells were harvested and washed 3 times by the procedure described above. The cell pellet was resuspended in 15 ml of buffer and divided into 1 ml aliquots, and each sonicated for 5 seconds with a Sonifier Cell Disruptor

(Heat Systems-Ultrasonics, Inc., Planeview, N.Y.) at 60 watts, with a 50 % pulse cycle.

New Zealand White rabbits were subcutaneously injected with 0.2 ml of a mixture of 0.5 ml of Freund complete adjuvant (Sigma, St. Louis, Mo.) and 0.5 ml of sonicated cells in each of 5 separate places. Booster injections were given 2 weeks later with a 0.2 ml mixture of 0.5 ml of Freunds incomplete adjuvant (Sigma, St. Louis, Mo.) and 0.5 ml of sonicated cells in each of 5 locations. One week after booster injections, the rabbits were bled and then once a week for 3 weeks, and a final bleeding on the fourth week. Titers and specificity of the antisera were assayed by slide agglutination. The antiserum for each strain was pooled and frozen.

Slide Agglutination: The procedure for slide agglutination was described by Smibert and Burmeister (35). Briefly, cells were grown in 7 ml of broth media for 4 to 5 days at 37 C. The cells were sedimented and washed once in FTA buffer. The cells were resuspended in 0.5 ml to 1 ml of FTA buffer. The test was performed by mixing 2 drops of cell suspension and 2 drops of antiserum on a Diagluto slide (Beckman Diagnostics, Fullerton, Calf.). The mixture was allowed to react for 5 minutes and agglutination was read as $+_4$ (a heavy

agglutination) to negative (no agglutination).

Flourescent Antibody Staining: Cells from a 7 ml broth culture were sedimented, washed 3 times and resuspended in FTA buffer containing 1% Tween 20 (FTA-Tween 20) until the turbidity was barely visable. A drop of the cell suspension was placed on a slide, air dried, and was heat fixed. One drop of anti-treponemal serum was added and the slide was incubated at 37 C in a moist chamber for 30 minutes, and washed in FTA buffer for 10 minutes and in distilled water for 2 minutes. A 1:100 dilution of Protein A-FITC conjugate (Boehringer Mannheim Biochemicals, Indianapolis, Indiana) was placed on the slide and the slide incubated and washed as described before. Buffered glycerol mounting fluid and a cover slip were placed on the slide and the slide was examined under a Leitz Orthoplane microscope equiped with Leitz Ploem epifluorescence system. A K510 barrier filter was used with a 63X fluorescence oil immersion objective yielding a final magnification of 984X. Fluorescent antibody reactions were read on a $+_4$ to negative basis. A $+^4$ was a very bright fluorescence, while a negative was no fluorescence.

Adsorption of Treponemal Antisera: One ml of washed treponemes were centrifuged for 5 minutes in an Eppendorf microcentrifuge at 12,000 rpm. The cell pellet was resuspended in 1 ml of either undiluted or diluted

treponemal antiserum and incubated at 37 C for at least 3 hours. The suspension was centrifuged and the adsorbed antisera was used for fluorescent antibody staining.

Preparation of Sonicated Extract: Washed cells from a 25 ml broth culture were resuspended in 1.5 ml of FTA buffer and sonicated for 1 minute with a Sonifier Cell Disruptor (Heat Systems-Utrasonics, Inc., Planeview, N.Y.) at 60 watts, with a 50 % pulse cycle. After sonication, 16 drops of 2% Triton X-100 in FTA buffer was added to the extract.

Preparation of Sodium Lauryl Sulfate (SDS) Extracts: The following procedure was based on the method used by Klaviter and Johnson (16). Cell pellets from four, 25 ml broth cultures were combined and washed 3 times in FTA buffer. FTA buffer was added to the cell pellet until a concentration of 10^8 cells/ml was reached. SDS was added to make final concentrations of either 0.005% or 0.01% SDS. The suspension was incubated at 37 C for 20 minutes, then centrifuged at 10,000 rpm for 30 minutes. The supernatant fluid was filtered through a membrane filter (pore size 0.2 µm) and concentrated 2 times.

Preparation of Sodium Deoxycholate (DES) Extract: The extraction of the DES-antigen from **T. denticola** was done according to the procedure described by Jacob et al. (15). Washed cells from a 25 ml broth culture were

resuspended in 10 ml of FTA buffer. Sodium deoxycholate was added in the amount of 0.5 g/ 10 ml of cells and the suspension was incubated at 37 C for 2 hours and centrifuged. The suspension was cooled on ice and 90 ml of cold 95 % ethanol was added. The suspension was incubated overnight in the refrigerator and then centrifuged at 10,000 rpm for 30 minutes. The pellet was resuspended in 10 ml of FTA buffer.

Preparation of Butanol-Water Extract: The procedure used for the butanol-water extract was a modification of the procedure described by Laughon (19). Sedimented cells from four, (25 ml) broth cultures were washed 3 times with FTA buffer and the cells were resuspended in 2 ml of 10 % butanol (water saturated with butanol). The suspension was incubated at 37 C for 2 hours with occasional shaking. The cells were centrifuged and the supernatant fluid was collected and the butanol was evaporated under a stream of nitrogen.

Preparation of Low Pressure (380 Psi) Cell Disruption: Cells from four, (25 ml) broth cultures were harvested by centrifugation and washed 3 times with 30 ml of FTA buffer. After the third washing, the pellet was resuspended in 10 mls of 10mM Tris - 10mM EDTA buffer. The suspension was placed in a Mini-Bomb Cell Disruption Chamber (Kontes, Vineland, New Jersey) that was attached

to a nitrogen tank. The pressure cell containing a cell suspension was held at 380 psi for 30 minutes. The valve on the pressure cell was opened and the cells were collected in a polypropylene centrifuge tube. The disrupted cells were centrifuged at 10,000 rpm for 30 minutes, the supernatant fluid was collected, and concentrated to 1 ml. Finally, 1 ml of a detergent solution (1% Triton X-100 in FTA buffer) was added to the concentrated supernatant.

Preparation of Triton-Extract: The procedure for the preparation of Triton extracts was a modification of the method described by Penn et al.(25). Cells from four, 25 ml broth cultures were harvested and washed with 30 ml of FTA buffer containing 20 mM $MgCl_2$ (FTA-$MgCl_2$). The washed pellet was resuspended in 2 ml of 1% Triton X-100 in FTA-$MgCl_2$ buffer and incubated at 37 C for 2 hours with occasional shaking. After incubation the cells were centrifuged and the supernatant fluid was collected.

The preparation of Triton extracts that were used in the routine identification of oral treponemes was as follows: cultures were grown at 37 C in 7 ml of modified OTI broth for 4-5 days. Cultures were adjusted to an optical density between 0.7 to 0.9 at 590 nm. This corresponded to a final protein concentration of 7.1 to 10.2 mg / ml of protein in the final Triton extract. The

cells were sedimented and washed once with 5 mls of the $FTA-MgCl_2$ buffer. The pellet was resuspended in 1 ml of 1% Triton X-100 in $FTA-MgCl_2$, incubated at 37 C for 2 hours, and centrifuged in a microcentrifuge at 12,000 rpm for 10 minutes. The supernatant fluid was collected and saved.

Negative Staining of Treponemes: Whole cells and Triton treated cells were washed 4 times in distilled water. Two hundred-mesh copper grids were carbon and Parlodion coated and preincubated with 0.01% BSA. One drop of cell suspension was added to the grid and incubated at room temperature for 5 minutes. Immediately after washing off the unbound bacteria, the grid was stained with 1% uranyl acetate for 1 minute. Stained grids were examined in a Joelco 100C electron microscope.

Protein Assay: Protein concentrations of Triton extracts were determined by the BCA Protein Assay (Pierce, Rockford, Illinois) Fifty parts of Reagent A (sodium carbonate, sodium bicarbonate, BCA detection reagent, and sodium tartrate in 0.1 N NaOH) was mixed with 1 part of Reagent B (4 % $CuSO_4 * 5H_2O$). Two ml of the reaction mixture was pipetted into a test tube and 100 µl of a known concentration of bovine serum albumin, unknown protein sample or sample buffer was added to the reaction mixture. The solution was incubated at 60 C for 30 minu-

tes. Absorption at 562 nm was read using a Gilford spectrophotometer model 250.

Immunodiffusion (ID): Molten 1% agarose (Type I, Low EEO) in FTA buffer was poured onto a 50 x 75 mm glass slide and allowed to solidify. Wells, 5 mm in diameter and 3 mm (edge to edge) apart, were cut into the agarose. The wells were filled with either 45 µl of antiserum or 45 µl of various extracts. The slides were incubated at room temperature in a moist chamber for 24 hours and precipatate lines were read using a darkfield box.

Crossed Immunoelectrophoresis (CIE): Molten 1% agarose in electrophoresing buffer (Tris-tricine g/l : 9.9 g of Tris base, 0.106 g of calcium lactate, 0.2 g of sodium azide, 4.3 g of tricine at pH 8.6) was poured onto a clean 50 x 50 mm glass slide. The agarose was allowed to solidify and two wells were punched into the agarose with a 3 mm gel punch (BioRad Laboratories, Richmond, Calf.). Ten µl of sample were added to one well and 10 µl of tracking dye (0.5% bromphenol blue, 30% sucrose, 0.04 M tris-glycine pH 8.9, BioRad Laboratories, Richmond, Calf.) was added to the other well. Electrophoresis, in a horizontal electrophoresis chamber (BioRad Laboratories, Richmond, Calif.), of the first dimension was done at 150 V and 19 C until the tracking dye was on the opposite side of the slide. The slide was turned

ninety degrees and the agarose layer above the antigen was removed. Five hundred ul of T. denticola (D39DPP-1) antiserum was added to 3.4 ml of melted 1% agarose in Tris-tricine buffer. The mixture was poured onto the slide and allowed to harden. The antigen was electrophoresed into the antiserum containing agarose at 40 V for 16 to 17 hours.

After electrophoresis the gels were soaked in FTA buffer for 15 minutes. The slides were then flooded with distilled water and several layers of blotting paper were placed on the gels and the slide was pressed under a weight for 15 minutes. The blotting paper was changed and the slide was pressed for another 15 minutes. The washing and pressing procedure was repeated 6 more times. After the final pressing, the gels were dried, then stained with coomassie blue (0.5% coomassie blue, 81% ethanol, 11% glacial acetic acid) for 10 minutes. The gels were destained in the staining solution without coomassie blue until the backround was clear.

Standard Enzyme Linked Immunosorbent Assay (Standard ELISA): Extracts, antisera, and reagents were dispensed in 100 μl amounts accept as noted. Diluted Triton extracts were allowed to bind to the wells of polyvinylchloride microtiter plates (Becton Dickinson, Oxnard, Calf.) by overnight incubation at 37 C. Unbound

sites on the wells were blocked by incubation for 2 hours at 37 C with 200 µl of 4% bovine serum albumin in FTA buffer. T. denticola (D39DPP-1) antiserum was diluted 1:500 in FTA buffer containing 1% BSA and 100 µl was added to the wells and incubated for 30 minutes at 37 C. After incubation the wells were washed six times with 200 µl of FTA-0.1% Tween 20 (FTA-TWEEN 20) buffer. Protein A linked to urease (Allelix, Ontario, Canada) was diluted 1:400 in FTA buffer containing 1% BSA and was added to the wells, followed by incubation for 30 minutes at 37 C. The wells were washed three times with 200 µl of distilled water. Development of the color was accomplished by adding UREIASE substrate and color reagent (Allelix, Ontario, Canada) to the wells. The plates were incubated at 37 C for 1.5 hours to 2 hours. The ELISA results were read on a $(+_4)$ to (-) basis. A $(+_4)$ was a deep purple, a (+/-) was light grey and a (-) was yellow.

Detection of Treponemal Proteins Binding to Fibronectin

Binding of Treponemes to Fibronectin (Fn) Coated Slides: The procedure used for the binding of treponemes to Fn coated slides was described by Peterson et al. (26). Fifty µl of either a 1 mg/ml fibronectin or a solution of 4% BSA in FTA was spread on a marked area on a microscope slide and air dried. One hundred µl of treponemal cell

suspension that contained 10^8 cells/ml was added to the slide and the slide incubated in a moist chamber for 1 hour at 37 C. The slide was gently immersed in FTA buffer 5 times to remove the unbound organisms. Treponemal attachment was observed under a Leitz darkfield microscope at 450 magnification.

Fn-capture ELISA: The procedure used was a modification of the procedure described by Alderete et al. (2). Binding of human Fn (Sigma, St. Louis, Mo.) to the microtiter wells was done by adding 100 µl of a solution containing 0.01 mg/ml of Fn to each well, followed by overnight incubation at 37 C. Unbound Fn was removed by washing with 200 µl of FTA-Tween 20 buffer. Blocking was accomplished with 200 µl of 4% BSA-FTA buffer added to each well and incubation of the plates at 37 C for 2 hours. Serial dilutions of Triton extracts were added to the wells in 100 µl amounts and incubated at 37 C for 30 minutes. The wells were washed 3 times with 200 µl of FTA-Tween 20 buffer. One-hundred µl of a 1:500 dilution in 2% BSA buffer of anti-*T. denticola* (D39DPP-1) serum. The plates were incubated for 30 minutes at 37 C. After incubation the wells were washed 6 times with 200 µl of FTA-Tween 20 buffer, followed by the addition of 100 µl of a 1:400 dilution of Protein A urease in 2% BSA buffer. The plates were incubated for 30 minutes at 37 C and

washed 3 times with 200 µl of FTA-Tween 20 buffer and 3 times with 200 µl of distilled water. Urease subtrate - color reagent was added in 100 µl amounts to each well and incubated the plates at 37 C for 1.5 to 2 hours. The ELISA results were read the same way as the test described above.

Detecting the Binding of Fn to Treponemal Protein: This procedure was similar to the standard ELISA. Triton extracts were bound to the microtiter wells and blocked with 4% BSA - FTA buffer. One hundred µl of undiluted normal rabbit serum was added to each well and the plate was incubated at 37 C for 30 minutes and finally washed with 200 µl of FTA-Tween 20 buffer. Incubation with normal rabbit serum was done to ensure saturation of Fn-binding protein in the treponemal Triton extract with Fn. After washing, 100 µl of a 1:400 dilution of anti-rabbit Fn (Cappel,Cochranville,Pa) in 1% BSA in FTA buffer was added, and the plate incubated at 37 C for 30 minutes. Color development was as described in other ELISA tests.

Isolation and Identification of the Fn Binding Protein

Sodium lauryl sulfate polyacrylamide gel electrophoresis: Samples were electrophoresed in the SDS-PAGE system decribed by Laemmli (18). A sample of Triton extract was prepared in the sample buffer (5.4 ml of distilled water,

2 ml of 0.5 M Tris - HCl buffer at pH6.8, 2.0 ml of glycerol, 2.5 ml of 10% SDS, 0.2 ml of beta-mercaptoethanol and 0.05% bromphenol blue) and boiled for 3 minutes. One hundred to two hundred μg of protein was loaded into the wells of a 3% stacking gel (6.3 ml of distilled water, 2.5 ml of 0.5 M Tris - HCl pH 6.8 buffer, 0.1 ml of 10% SDS, 1 ml of acrylamide : Bis (30% : 0.8%) stock solution, 0.01 g of ammonium persulfate, and 8.0 ul of TEMED). The gel was electrophoresed with cooling at 19 C in a protein slab gel electrophoresing cell (BioRad Laboratories, Richmond, Calf) with an initial current of 30 mA until the dye front reached the separating gel. The proteins were separated in a 12% acrylamide gel (10.1 ml of distilled water, 7.5 ml of 1.5 M Tris-HCL pH 8.8 buffer, 0.3 ml of 10% SDS, 12.0 ml of acrylamide : bis stock solution, 0.01 g of ammonium persulfate, and 8.00 ul of TEMED) at 60 mA until the dye front was approximately 1 cm from the bottom of the gel. The gel was removed and stained overnight in a solution of 0.05% coomassie brilliant blue, 10% acetic acid, and 25% isopropyl alcohol. The gel was destained in a gel destainer (BioRad Laboratories, Richmond, Calf.) containing 10% acetic acid until the backround of the gel was clear.

Agarose Gelatin: Twenty-five ml of hemolyzed rabbit

serum was slowly dripped through a 4 ml gelatin-agarose (Sigma, St.Louis, Mo.) column, to insure binding of Fn to the gelatin. The column was washed with FTA buffer until there was no absorbance of the eluate at 280 nm. At least 4 ml of Triton-extract was slowly passed through the Fn-gelatin-agarose column and the column was washed with FTA buffer until there was no absorbance in the eluate at 280 nm. The Fn binding treponemal protein and Fn were eluted from the column with a buffer containing 1.0 M NaBr and 0.05 M sodium acetate at pH 5.0.

Western Blot: Electrophoretic transfer of proteins from SDS PAGE gels to nitrocellulose (BioRAD Laboratories, Richmond, Calf.) was performed by the method described by Towbin (45). Transfer of the proteins was done in a Transphor Unit (Hoefer Scientific, San Francisco, Calf.) containing a transfer buffer composed of 0.192 M glycine, 0.025 M Tris, and 20% v/v methanol pH 8.3 Proteins were transferred at 800 mA at 19 C for 6 to 7 hours. Efficiency of the transfer was determined by blotting a pre-stained molecular weight standard (BioRad Laboratories, Richmond, Calf.). The blotted membrane was blocked in 4% BSA-FTA buffer, with gentle shaking for at least 1 hour at 37 C. After blocking the membrane was incubated at 37 C with gentle shaking in either a 1:50 or 1:100 dilution of antiserum in 3 % BSA-FTA buffer.

Unbound antibody was removed from the membrane, by three 15 minute washes in FTA-Tween 20 buffer. Antigen-antibody complex staining was accomplished by incubation of the membrane with gentle shaking at 37 C for 4 to 5 hours in Protein A Gold (BioRad Laboratories, Richmond, Calf.). The membranes were washed in FTA-Tween 20 buffer as described above. Silver lactate enhancement (Protein A Gold enhancement kit, BioRad Laboratories, Richmond, Calf.) of the proteins was performed to make the protein bands more visible.

RESULTS

Fluorescent Antibody Staining: Immunofluorescent staining of T. socranskii subsp. socranskii, T. socranskii subsp. buccale, T. socranskii subsp. paredis, Treponeme D, T. pectinovorum, T. denticola strains T32A and FM, T. vincentii and T. phagedenis biotype Reiter with both homologous and heterologous antisera showed that all these species cross-reacted. Tables 1 and 2 showed the extent of antigenic cross-reactivity that was found between these treponemes. Cross-reactivity between T. denticola, T. vincentii, and T. phagedenis was eliminated when the antisera were adsorbed with T. phagedenis biotype Reiter whole cells (Tables 3 and 4). However, there was still extensive cross-reactivity between T. socranskii subsp. socranskii, T. socranskii subsp. buccale, T. socranskii subsp. paredis, and Treponeme D, but slight cross-reactivity with T. pectinovorum.

Monospecific T. socranskii subsp. socranskii, T. socranskii subsp. buccale, T. socranskii subsp. paredis, Treponeme D, T. pectinovorum, T. denticola strain T32 A and strain FM and T. vincentii antisera were obtained by various adsorptions with heterologous organisms (Tables 5 and 6). To obtain monospecific T. socranskii subsp. socranskii antiserum, the antiserum was adsorbed twice

Table 1: Fluorescent Antibody[a] Staining of Oral and Genital Treponemes

Cells		Antisera			
		T. socranskii			Treponeme D
		ss. socranskii D43BR-1	ss. buccale D2B-8	ss. paredis D46CPE-1	D40DR-2
T. socranskii					
ss. socranskii	D43BR-1	+4[b]	+3	+	+3
ss. buccale	D2B-8	+2	+4	+2	+2
ss. paredis	D46CPE-1	+2	+3	+3	+3
Treponeme D	D40DR-2	+3	+3	+2	+4
T. pectinovorum	D39DR-2	+4	+3	+3	+3
T. denticola					
	T32A	+3	+3	+3	+3
	FM	+	+	+	+
T. vincentii	N-9	+3	+3	+3	+3
T. phagedenis	Reiter	+	+3	+3	+3

[a] An indirect fluorescent antibody technique was used with Protein A-Fluorescein as the staining reagent.

b Fluorescent antibody reactions were read on a +4 to negative basis. A +4 was a very bright fluorescence, while a negative was no fluorescence.

Table 2: Fluorescent Antibody Staining of Oral and Genital Treponemes

Cells		Antisera				
		T. pectinovorum	*T. denticola*		*T. vincentii*	*T. phagedenis*
		D39DR-2	T32A	FM	N-9	Reiter
T. socranskii						
ss. *socranskii*	D43BR-1	+2	+2	+	+2	+2
ss. *buccale*	D2B-8	+	+	+	+	+3
ss. *paredis*	D46CPE-1	+3	+2	+	+	+3
Treponeme D	D40DR-2	+2	+2	+2	+2	+3
T. pectinovorum	D39DR-2	+4	+3	+3	+2	+3
T. denticola						
	T32A	+3	+4	+4	+	+4
	FM	+	+3	+4	+4	+4
T. vincentii	N-9	+3	+4	+3	+4	+4
T. phagedenis	Reiter	+3	+3	+3	+	+4

a An indirect fluorescent antibody technique was used with Protein A-Fluorescein as the staining reagent.

b Fluorescent antibody reactions were read on a +4 to negative basis. A +4 was a very bright fluorescence, while a negative was no fluorescence.

Table 3: Fluorescent Antibody[a] Staining of oral treponemes with T. socranskii subsp. socranskii, T. socranskii subsp. buccale, T. socranskii subsp. paredis, Treponeme D, and T. pectinovorum Antiserum Adsorbed with T. phagedenis Biotype Reiter Cells.

Cells		Adsorbed Antisera[b]				
		T. socranskii			Treponeme D	T. pectinovorum
		ss. socranskii (D43BR-1)	ss. buccale[c] D2B-8	ss. paredis D46CPE-1	D40DR-2	D39DR-2
T. socranskii						
ss. socranskii	D43B2-1	+4[d]	+	+3	+	+
ss. buccale	D2B-8	+	+4	+3	+2	-
ss. paredis	D46CPE-1	+	+	+4	+	+
Treponeme D	D40DR-2	+2	+	+2	+2	+
T. pectinovorum	D39DR-2	+	-	+2	+2	+3
T. denticola						
	T32A	-	-	-	-	-
	FM	-	-	-	-	-
T. vincentii	N-9	-	-	-	-	-
T. phagedenis	Reiter	-	-	-	-	-

a An indirect fluorescent antibody technique was used with Protein A-Fluorescein as the staining reagent.

b T. socranskii subsp. socranskii, T. socranskii subsp. paredis, Treponeme D, and T. pectinovorum antisera were adsorbed 2 times with Reiter whole cells.

c T. socranskii subsp. buccale antiserum was adsorbed 2 times with T. socranskii subsp. socranskii whole cells.

d Fluorescent antibody reactions were read on a +4 to negative basis. A +4 was a very bright fluorescence, while a + was faint fluorescence and a negative was no fluorescence.

Table 4: Fluorescent Antibody[a] Staining of oral treponemes with T. denticola and T. vincentii Antiserum Adsorbed with T. phagedenis Biotype Reiter Cells.

Cells		Adsorbed Antisera[a]		
		T. denticola		T. vincentii
		T32A	FM	N-9
T. socranskii				
ss. socranskii	D43BR-1	-	-	-
ss. buccale	D2B-8	-	-	-
ss. paredis	D46CPE-1	-	-	-
Treponeme D	D40DR-2	-	-	-
T. denticola				
	T32A	+3	+3	-
	FM	-	+3	-
T. vincentii	N-9	-	+	+3
T. phagedenis	Reiter	-	-	-

An indirect fluorescent antibody technique was used with Protein A-Fluorescein as the staining reagent.

a T. denticola strain T32A and FM antisera were adsorbed twice with Reiter whole cells. T. vincentii antiserum was adsorbed once with Reiter cells.

b Fluorescent antibody reactions were read on a +4 to negative basis. A +4 was a very bright fluorescence, while a + was faint fluorescence and a negative was no fluorescence.

Table 5: Fluorescent Antibody Staining of the T. socranskii subspecies and Treponeme D with Monospecific Antisera

Adsorbed Antisera	Cells			
	T. socranskii			Treponeme D
	ss. socranskii D43BR-1[a]	ss. buccale D2B-8	ss. paredis D46CPE-1	D40DR-2
T. socranskii ss. socranskii/ Reiter, D2B-8, D40DR-2[b]	+4[c]	-	-	-
T. socranskii ss. buccale/ D43BR-1	-	+4	-	-
T. socranskii ss. paredis/ Reiter, D43BR-1	-	-	+3	-
Treponeme D/Reiter, D43BR-1	-	-	-	+
T. pectinovorum/Reiter,D46CPE-1	-	-	-	-
T. denticola (T32A)/Reiter	-	-	-	-
T. denticola (FM)/Reiter, T32A	-	-	-	-
T. vincentii/Reiter	-	-	-	-

a Strain number.

b The notation used to show what organisms were used to adsorb the treponemal antisera (i.e., T. socranskii subsp. socranskii antiserum was adsorbed with T. phagedenis biotype Reiter, T. socranskii subsp. buccale and Treponeme D whole cells.

c Fluorescent antibody reactions were read on a +4 to negative basis. A +4 was a very bright fluorescence while a + was faint fluorescence and a negative was no fluorescence.

Table 6: Fluorescent Antibody Staining of T. pectinovorum, T. denticola and T. vincentii whole cells with Monospecific Antisera

Adsorbed Antiserum	Cells			
	T. pectinovorum	T. denticola		T. vincentii
	D39DR-2[a]	T32A	FM	N-9
T. socranskii ss. socranskii/ Reiter, D2B-8, D40DR-2[b]	-[c]	-	-	-
T. socranskii ss. buccale/ D43BR-1	-	-	-	-
T. socranskii ss. paredis/ Reiter, D43BR-1	-	-	-	-
Treponeme D/Reiter, D43BR-1	-	-	-	-
T. pectinovorum/Reiter, D46CPE-1	+3	-	-	-
T. denticola (T32A)/Reiter	-	+3	-	-
T. denticola (FM)/Reiter, T32A	-	-	+2	-
T. vincentii/Reiter	-	-	-	+3

a Strain number.

b The notation used to show what organisms were used to adsorb T. socranskii subsp. socranskii antiserum (i.e., T. socranskii subsp. socranskii antiserum was adsorbed with T. phagedenis biotype Reiter, T. socranskii subsp. buccale, and Treponeme D whole cells.

c Fluorescent antibody reactions were read on a +4 to negative basis. A +4 was a very bright fluorescence while a + was faint fluorescence and a negative was no fluorescence.

with Reiter cells and then a 1:10 dilution of that serum was adsorbed once with T. socranskii subsp. buccale (D2B8) and once with Treponene D (D40DR-2) cells. T. socranskii subsp. buccale antiserum was made monospecific by adsorbing the serum 3 times with T. socranskii subsp. socranskii (D43BR-1) cells. Monospecific T. socranskii subsp. paredis and Treponeme D sera were both made by, first adsorbing each antiserum twice with Reiter cells, then adsorbing a 1:10 dilution of that antisera once with T. socranskii subsp. socranskii (D43BR-1) cells. T. pectinovorum antiserum not only needed to be adsorbed twice with Reiter cells but also with T. socranskii subsp. paredis (D46CPE-1) cells. T. denticola strain T32A only needed to be adsorbed three times with Reiter cells, whole T. denticola strain FM antiserum was adsorbed twice with Reiter cells and twice with T. denticola strain FM antiserum was adsorbed twice with Reiter cells and twice with T. denticola strain T32A cells. Finally monospecific T. vincentii antiserum was prepared by two adsorptions with Reiter cells.

The monospecific T. socranskii subsp. socranskii and T. socranskii subsp. buccale (D2B-8) antisera were used in identifying oral treponemes directly from smears of periodontal samples. The samples were stained with monospecific antisera, only one sample showed a +2

fluorescence with monospecific T. socranskii subsp. buccale (D2B-8) antiserum and faint fluorescence with monospecific T. socranskii subsp. socranskii antiserum. The stained slides were difficult to read because the cells were covered with a thick film that masked the fluorescence of the cells.

Serologic Reactivity of Several Cell Extracts: Several methods were used to extract the outer envelope from treponemes. An extraction procedure that removed antigens from treponeme cells and resulted in the least amount of cross-reactivity with other species was desired. It was also desired to develop an extraction method that would remove antigens from treponemal cells that were common to all strains within a species. Immunodiffusion (ID) reactions of various types from extracts of T. denticola cells against antisera against T. denticola strains D39DPP-1, ST-10, T32A and FM, as well as, T. vincentii and T. phagedenis biotype Reiter antisera are in Table 7. The sonicated extract of T. denticola strain D39DPP-1 cells reacted with all antisera tested. Sodium deoxycholate (DES) extract from T. denticola strain FM cells reacted strongly with FM antiserum, but weakly with D39DPP-1, T32A, T. vincentii and Reiter antisera. Sodium lauryl sulfate (0.005%) extract of D39DPP-1 cells was a very gentle way of

Table 7: Immunodiffusion Analysis of Various Kinds of Cell Extracts From T. denticola Strain D39DPP-1 Against T. denticola, T. vincentii, and T. phagedenis Antisera

Extract	Antiserum					
	T. denticola				T. vincentii	T. phagedenis
	D39DPP-1	ST-10	T32A	FM	N9	Reiter
Sonicated	+	+	+	+	+	+
Deoxycholate (FM)[a]	+/-		+/-	-	+/-	+/-
0.005% SDS	+	-	-	+	-	-
0.010% SDS	+	+	-	+	-	+
Butanol water	+	+	-	+	-	+
Low Pressure Cell Disruption	+	+	+	+	+/-	+/-
1% Triton-X100	+	+	+	+	+/-	+/-

Immunodiffusion tests were rated from 1-5 heavy immunoprecipitation lines (+), to 1-2 faint lines (+/-). Negative reactions had no visible lines.

[a] T. denticola strain FM cells were extracted instead of D39DPP-1 cells.

removing the outer envelope. This extract reacted only with anti-D39DPP-1 serum and anti-FM serum. On the other hand, the extract using 0.01% SDS not only reacted with the four T. denticola antisera, but also with T. vincentii and Reiter antisera. The butanol water extract reacted with T. denticola strain D39DPP-1, ST-10, FM and T. phagedenis biotype Reiter antisera but not T. denticola strain T32A and T. vincentii antisera. A method was also developed that physically removed the outer envelope from T. denticola strain D39DPP-1. The low pressure (380 psi) cell (BOMB) disruption extract reacted with D39DPP-1, ST-10, weakly with T32A, and weakly with T. vincentii and Reiter antisera. Finally, the Triton X-100 extract of T. denticola strain D39DPP-1 reacted strongly with the four T. denticola antisera and only weakly with T. vincentii and Reiter antisera.

The BOMB extract and Triton extract were examined to see which procedure removed the outer envelope with minimum contamination with cytoplasmic, and cytoplasmic membrane constituents. This was accomplished by adsorbing D39DPP-1 antiserum with D39DPP-1 whole cells, to remove antibody against surface antigens. The adsorbed antiserum was used in crossed immunoelectrophoresis experiments with the BOMB and Triton extracts. Figure 1 shows crossed

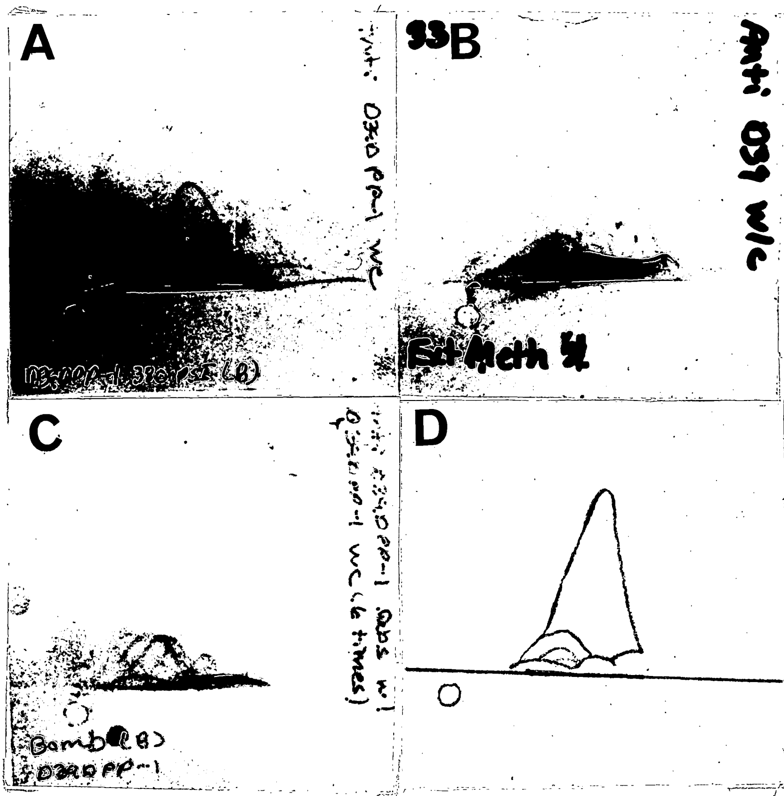

Figure 1. Coomassie blue stained crossed immunoelectrophoresis slides of T. denticola D39DPP-1 BOMB extract and T. denticola strain D39DPP-1 Triton-extract against adsorbed and unadsorbed T. denticola (D39DPP-1) antiserum. Slide A is T. denticola (D39DPP-1) BOMB extract with unadsorbed D39DPP-1 antiserum. SLide B is T. denticola D39DPP-1 Triton-extract with unadsorbed D39DPP-1 antiserum. Slides C is T. denticola (D39DPP-1) BOMB extract with D39DPP-1 adsorbed antiserum. Slide D is a drawing of T. denticola (D39DPP-1) Triton-extract with adsorbed D39DPP-1 antiserum. The immunoprecipitin arcs were too faint to photograph. The T. denticola (D39DPP-1) antiserum was adsorbed 6 times with D39DPP-1 whole cells.

immunoelectrophoresis (CIE) slides of the reaction between D39DPP-1 BOMB extract and unadsorbed and adsorbed D39DPP-1 antiserum (a and b). Slides c and d show the immunoprecipitin arcs from the reaction of D39DPP-1 Triton extract against unadsorbed (c) and adsorbed (d) D39DPP-1 antiserum. The Triton extract produced a smaller number of arcs than the BOMB extract, when run against the adsorbed D39DPP-1 antiserum.

Figure 2 is an electron micrograph of negatively stained untreated (cells not incubated with 1% Triton X-100) *T. denticola* D39DPP-1 cells, with an intact outer envelope. Figure 3 shows the effect of Triton X-100 on *T. denticola* strain D39DPP-1. The detergent caused the removal of the outer envelope and the release of the free end of the periplasmic flagella.

Serologic Reactivity of Triton Extracts: Table 8 shows that slide agglutination using washed *T. denticola* cells, reacted with only 11 of 16 *T. denticola* strains. However, *T. refringens*, *T. minutum*, *T. vincentii*, *T. phagedenis*, *T. socranskii* and its subspecies, Treponeme D and *T. pectinovorum* were not agglutinated by *T. denticola* antiserum. Immunodiffusion and crossed immunoelectrophoresis assays using sonicated cells showed all 16 strains of *T. denticola* reacted with anti-D39DPP-1 serum. In addition, sonicated extracts of *T. refringens*,

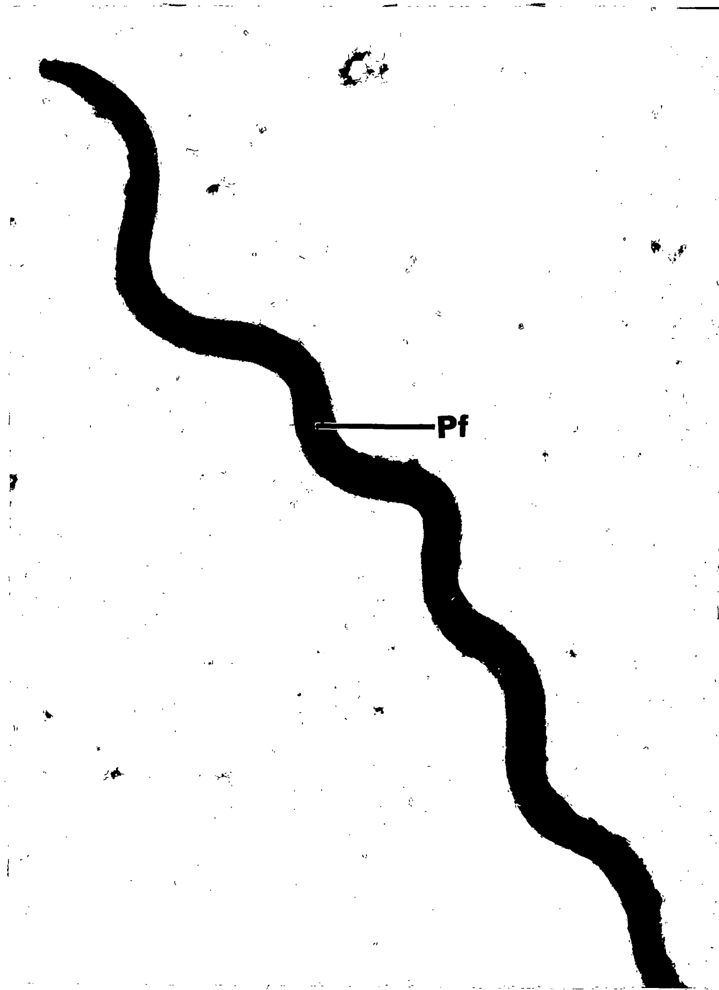

Figure 2. Electron micrograph of negatively stained T. denticola strain D39DPP-1 cells. The cells were stained with 1% uranyl acetate for 1 minute. Pf is the periplasmic flagella. The magnification is 24,447X.

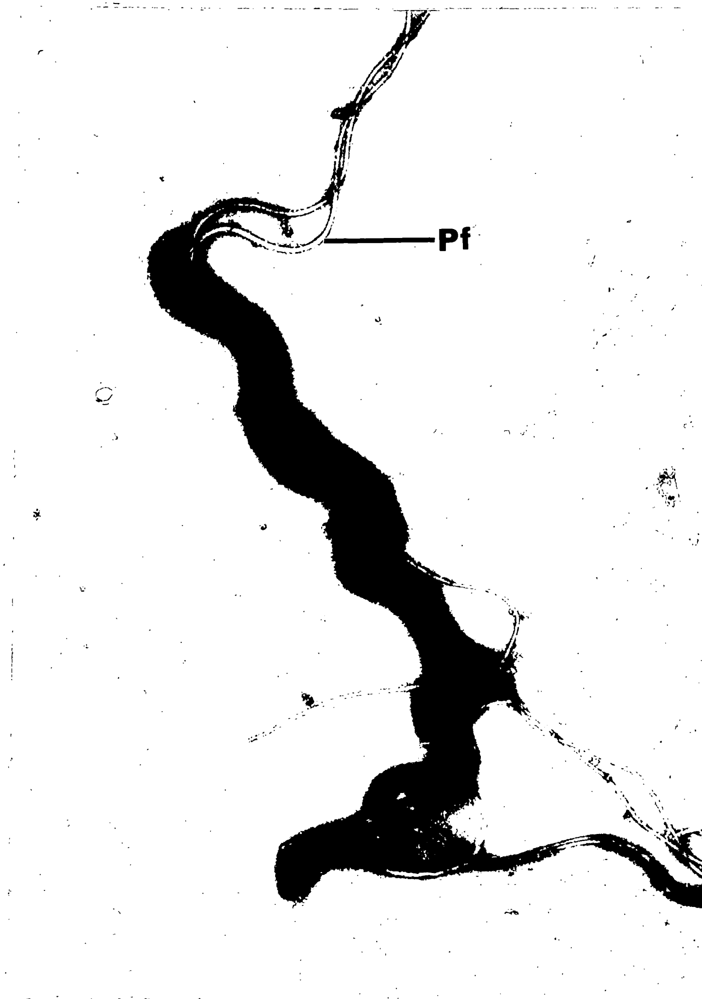

Figure 3. Electron micrograph of negatively stained T. denticola strain (D39DPP-1) cells that were incubated for 2 hours in the presence of 1% Triton X-100. The cells were stained with 1% uranyl acetate for 1 minute. Pf is the periplasmic flagella. The magnification is 58,232X.

Table 8: Results of Different Serologic Tests Using Whole Cell and Sonicated Extracts of Several Treponeme Species Against _T. denticola_ strain D39DPP-1 Antiserum

Organism	Slide Agglutination	Sonicates	
	whole cell	ID[b]	CIE[c]
T. denticola			
D39DPP-1	+4[a]	+	+
D3A-1	+2	+	+
Oralis	+3	+	+
Fuji	+3	+	+
Jethro	+2	+	+
Ichelson #2	+4	+	+
FM	+3	+	+
ST-10	+	+	+
T32A	+	+	+
IPP	+	+	+
TD-2	+	+	+
TRRD	+/-	+	+
N-39	-	+	+
D65BR-1	-	+	+
Ambigua	-	+	+
MRB	+/-	+	+
T. refringens			
refringens C	-	+	+/-
T. minutum	-	+	+/-
T. vincentii			
N-9	-	+	+/-
T. phagedenis			
Reiter	-	-	+/-
T. socranskii			
ss. _socranskii_ (D43BR-1)	-	+/-	+/-
ss. _buccale_ (D2B-8)	-	+/-	+/-
ss. _paredis_ (D46CPE-1)	-	+/-	+/-
Treponeme D (D40DR-2)	-	+/-	+/-
T. pectinovorum (D39DR-2)	-	-	+/-

a Slide agglutination tests were rated from heavy agglutination (+4) to very slight almost feathery agglutination (+/-). No agglutination was recorded as a (-).

b Immunodiffusion. These tests were rated from 1-5 heavy (+) immunoprecipitin lines to 1-2 faint lines (+/-).

c Crossed immunoelectrophoresis. A positive reaction was the presence of 15-20 immunoprecipitin arcs. A (+/-) reaction was the presence of 2-5 immunoprecipitin arcs.

T. minutum, and T. vincentii reacted strongly with anti-D39DPP-1 antiserum, while T. socranskii subsp. socranskii, T. socranskii subsp. buccale, T. socranskii subsp. paredis and Treponeme D reacted weakly with D39DPP-1 antiserum in the immunodiffusion test. Figure 4 shows examples of CIE slides of two positive reactions produced by; (a) a sonicated extract of T. denticola T32A versus T. denticola D39DPP-1 antiserum, (b) T. denticola T32A Triton extract against T. denticola D39DPP-1 antiserum and (c) Reiter sonicated cells against T. denticola D39DPP-1 antiserum.

The results summarized in Table 9 show that Triton-extracts from all T. denticola strains produced a precipitin line in ID plates and immunoprecipitin arcs in CIE slides, with T. denticola D39DPP-1 antiserum. The Triton extracts from the other species did not produce a precipitin line. However, crossed immunoelectrophoresis of Triton-extracts showed that T. refringens, T. minutum, T. vincentii, and T. phagedenis, reacted weakly with T. denticola D39DPP-1 antiserum. Extracts from all strains of T. denticola had 15-20 precipitin arcs in CIE slides, while extracts from cross-reacting species had only 2-4 faint precipitin arcs. ELISA titers showed that Triton-extracts from all T. denticola strains tested reacted with T. denticola strain D39DPP-1 antiserum. There was

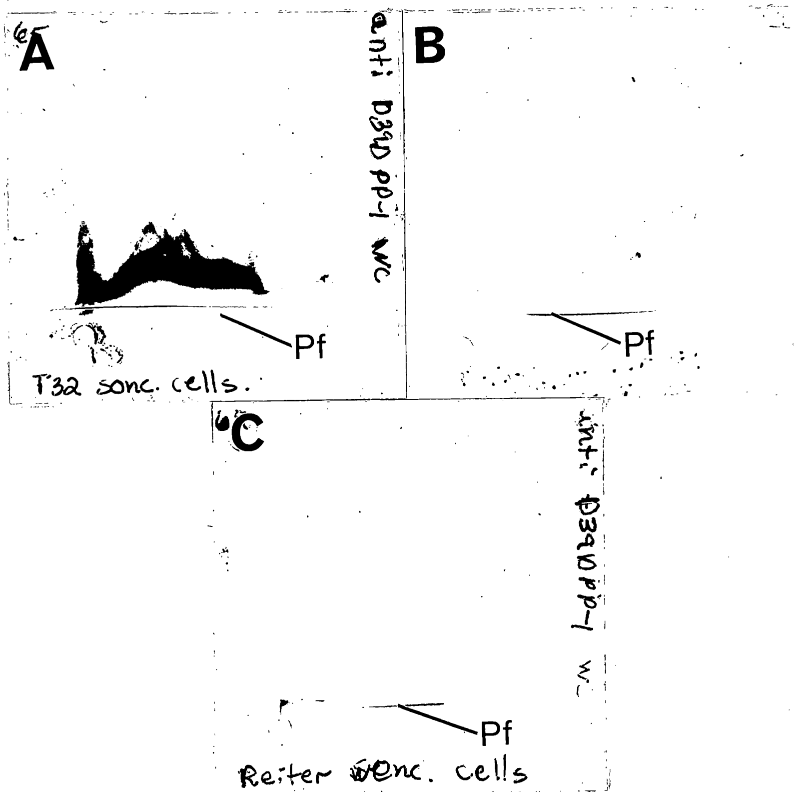

Figure 4. Coomassie blue stained crossed immunoelectrophoresis slides of T. denticola strain T32A sonicated extract (A), T32A Triton-extract (B), and T. phagedenis biotype Reiter sonicated extract (C) against T. denticola strain D39DPP-1 antiserum. Pf is the periplasmic flagella.

Table 9: Results of Different Serologic Tests of Treponemal Triton Extracts Against T. denticola Strain D39DPP-1 Antiserum

Organism	Serologic Tests		
	ID[a]	CIE[b]	ELISA Titer[c]
T. denticola			
D39DPP-1	+	+	16,392
D3A-1	+	+	4,098
Oralis	+	+	32,784
Fuji	+	+	1,024
Jethro	+	+	8,096
Ichelson #2	+	+	256,000
FM	+	+	4,098
ST-10	+	+	1,024
T32A	+	+	4,098
IPP	+	+	8,196
TD-2	+	+	16,392
TRRD	+	+	4,098
N-39	+	+	16,392
D65BR-1	+	+	
Ambigua	+	+	4,098
MRB	+	+	4.098
T. refringens			
refringens C	-	+/-	8
T. minutum	-	+/-	2
T. vincentii			
N-9	-	+/-	2
T. phagedenis			
Reiter	-	+/-	2
T. socranskii			
ss. socranskii (D43BR-1)	-	-	-
ss. buccale (D2B-8)	-	-	-
ss. paredis (D46CPE-1)	-	-	-
Treponeme D (D40DR-2)	-	-	-
T. pectinovorum (D39DR-2)	-	-	-

a Immunodiffusion. These tests were rated from 1-5 heavy (+) immunoprecipitin lines to 1-2 faint lines (+/-).

b Crossed immunoelectrophoresis. A positive reaction was the presence of 15-20 immunoprecipitin arcs. A (+/-) reaction was the presence of 2-5 immunoprecipitin arcs.

c The ELISA titer was the highest dilution of extract that still gave a purple color. A blank indicates the strain was not tested.

only slight cross-reactivity as seen by low ELISA titers with T. refringens, T. minutum, T. vincentii, and T. phagedenis and no reactivity with T. socranskii, Treponeme D, and T. pectinovorum.

Immunodiffusion of Triton-Extracts: Triton-extracts from several strains of T. denticola, T. vincentii, T. socranskii subsp. socranskii, T. socranskii subsp. buccale, T. socranskii subsp. paredis, Treponeme D, T. pectinovorum, Treponeme F and untypable strains of T. socranskii were tested against various oral treponeme species and groups. Tables 10 and 11 show the total number of strains of the different treponeme species examined. The Triton-extracts were prepared by using the procedure devised for the routine identification of oral treponemes. Untypable treponemes were strains of T. socranskii that cannot be identified to the subspecies level by the slide agglutination test. These organisms were either T. socranskii subsp. socranskii or T. socranskii subsp. buccale. These subspecies cannot be differentiated biochemically. T. socranskii subsp. paredis can be identified by phenotypic characteristics. There was a total of 139 oral treponemes tested. Results of all the isolates from the 139 treponemes are in Tables 12 and 13. Fifty-one of the 52 strains of T. denticola tested reacted only with T. denticola antiserum. The

Table 10: Total Number of Strains of Oral Treponemes Used in Immunodiffusion of Their Triton Extracts[a] and Their Reactions with Various Treponemal Antisera.

Extracts	Antisera		
	T. denticola	T. vincentii	T. pectinovorum
T. denticola (52)[b]	52[c]	1	d
T. vincentii (4)		4	
T. socranskii			
ss. socranskii (21)			
ss. buccale (10)			
ss. paredis (14)			
untypable (10)[e]			
Treponeme D (15)			
T. pectinovorum (7)			7
Treponeme F (6)			

a The Triton extracts were prepared by using the procedure used in routine identification of oral treponemes.

b Total number of strains tested.

c Number of strains that were positive for that antiserum.

d Blank; the extracts did not react with the antiserum.

e Treponemes that cannot be identified by slide agglutination. These organisms were either T. socranskii, ss. socranskii or ss. buccale.

Table 11: Total Number of Strains of Oral Treponemes Used in Immunodiffusion of Their Triton Extracts[a] and Their Reactions with T. socranskii and Treponeme D Antisera.

Extracts	Antisera			
	T. socranskii			
	ss. socranskii	ss. buccale	ss. paredis	Treponeme D
T. denticola (52)[b]	c			
T. vincentii (4)				
T. socranskii				
ss. socranskii (21)	21	21	21	21
ss. buccale (10)	10	10	10	10
ss. paredis (14)	14	14	14	14
untypable (10)[d]	10	10	10	10
Treponeme D (15)	15	15	15	15
T. pectinovorum (7)				
Treponeme F (6)				

a The Triton extracts were prepared by using the procedure used in routine identification of oral treponemes.

b Total number of strains tested.

c Blank; the extracts did not react with the antiserum.

d Treponemes that cannot be identified by slide agglutination. These organisms were either T. socranskii, ss. socranskii or ss. buccale.

Table 12: Immunodiffusion[a] of Oral Treponeme Triton-extracts Against T. denticola, T. vincentii, and T. pectinovorum Antisera

Extracts	Antisera		
	T. denticola D39DPP-1 & FM	T. vincentii N-9	T. pectinovorum D39DR-2
T. denticola			
MN2B	+[b]	-	-
D39DPP-1	+	-	-
FM	+	-	-
TD2	+	-	-
ST-10	+	+	-
TRRD	+	-	-
T32A	+	-	-
IPP	+	-	-
Ichelson #2	+	-	-
D3A-1	+	-	-
Oralis	+	-	-
Fuji	+	-	-
Jethro	+	-	-
N-39	+	-	-
MRB	+	-	-
Ambigua	+	-	-
D118MR-3	+	-	-
D56B2-1	+	-	-
D79ZR-1	+	-	-
D83ACR-1	+	-	-
D113CR-1	+	-	-
D113ERI-1	+	-	-
D135ARII-4	+	-	-
D137ARI-2	+	-	-
D137CR3	+	-	-
D138AR-1	+	-	-
D139BR-5	+	-	-
D142DR-1	+	-	-
D141ARI-1	+	-	-
D65BR-1	+	-	-
D132AR-1	+	-	-

[a] Preparation of Triton-extracts was done with the method devised for the routine identification of oral treponemes.

[b] Immunodiffusion tests were rated from 1-5 heavy (+) immunoprecipitin lines to 1-2 faint lines (+/).

Table 12 continued[a]

Extracts	Antisera		
	T. denticola D39DPP-1 & FM	*T. vincentii* N-9	*T. pectinovorum* D39DR-2
T. denticola			
D47APP-1	+[b]	-	-
D47DPP-1	+	-	-
D62DPP-1	+	-	-
D66BPP-1	+	-	-
D73DPPI-1	+	-	-
D75APP-2	+	-	-
D3A4	+	-	-
D36FR-2	+	-	-
D38APP-3	+	-	-
D39DPP-2	+	-	-
D40CPP-1	+	-	-
D43BPPII-2	+	-	-
D52AR-1	+	-	-
D53DPP-2	+	-	-
D61DPP-1	+	-	-
D61ARII-4	+	-	-
D63BR-2	+	-	-
D64BR-1	+	-	-
D67BPPI-1	+	-	-
D70DR-2	+	-	-
D79GR-2	+	-	-
T. vincentii			
N-9	-	+	-
D148CR	-	+	-
D138BR	-	+	-
D138CR	-	+	-

[a] Preparation of Triton-extracts was done with the method devised for the routine identification of oral treponemes.

[b] Immunodiffusion tests were rated from 1-5 heavy (+) immunoprecipitin lines to 1-2 faint lines (+/).

Table 12 continued[a]

Extracts	Antisera		
	T. denticola D39DPP-1 & FM	T. vincentii N-9	T. pectinovorum D39DR-2
T. socranskii			
ss. socranskii			
DR56BRIII-6	-[b]	-	-
D82AER-1	-	-	-
D79VR-1	-	-	-
D86HR-1	-	-	-
DH56CR-1	-	-	-
D39AR-1	-	-	-
D43BR-1	-	-	-
D30BR-2	-	-	-
D53AR-1	-	-	-
D20A-2	-	-	-
DH56CR-4	-	-	-
D146HR-1	-	-	-
D146BR-1	-	-	-
D146DR-1	-	-	-
D132ARII-4	-	-	-
D137AR-1	-	-	-
D974R-1	-	-	-
D128BR-1	-	-	-
D112DR-1	-	-	-
D136ARI-1	-	-	-
D143BR-1	-	-	-
ss. buccale			
D2B-8	-	-	-
D68BR-1	-	-	-
D40DPE-1	-	-	-
D22A-5	-	-	-
D11A-1	-	-	-
D4A-1	-	-	-
D34B-4	-	-	-
D36FEP-4	-	-	-
D25A-1	-	-	-
D96AAR-1	-	-	-

[a] Preparation of Triton-extracts was done with the method devised for the routine identification of oral treponemes.

[b] Immunodiffusion tests were rated from 1-5 heavy (+) immunoprecipitin lines to 1-2 faint lines (+/).

Table 12 continued[a]

Extracts	Antisera		
	T. denticola D39DPP-1 & FM	*T. vincentii* N-9	*T. pectinovorum* D39DR-2
T. socranskii			
ss. *paredis*			
D46CPE-1	-[b]	-	-
D51BR-5	-	-	-
E4EPE-1	-	-	-
E2M-1	-	-	-
E5BBR-5	-	-	-
D71CR-4	-	-	-
D51ARII-3	-	-	-
D148AR-1	-	-	-
D128AR-1	-	-	-
D148DR-1	-	-	-
D150AR-1	-	-	-
D140BR-5	-	-	-
D126AR-2	-	-	-
D131AR-1	-	-	-
Untypable			
D80CR-1	-	-	-
D52CR-1	-	-	-
D147DR-2	-	-	-
D96NR-2	-	-	-
D96NRI-2	-	-	-
D122AR-1	-	-	-
D131BR-1	-	-	-
D137ARII-4	-	-	-
D962RII-3	-	-	-
D136CRI-1	-	-	-
D80CR-1	-	-	-
D52CR-1	-	-	-
D147DR-2	-	-	-

a Preparation of Triton-extracts was done with the method devised for the routine identification of oral treponemes.

b Immunodiffusion tests were rated from 1-5 heavy (+) immunoprecipitin lines to 1-2 faint lines (+/).

Table 12 continued[a]

Extracts	Antisera		
	T. denticola D39DPP-1 & FM	*T. vincentii* N-9	*T. pectinovorum* D39DR-2
Treponeme D			
D40DR-2	-	-	-
D118ERII-4	-	-	-
D118MRII-4	-	-	-
D96ABR-2a	-	-	-
D96ADR-1	-	-	-
D116AR-1	-	-	-
D135ARI-2	-	-	-
D96FR-3	-	-	-
D96XR-2	-	-	-
D96VR-4	-	-	-
DH56AR-1	-	-	-
D70BR-5	-	-	-
D51ARII-2	-	-	-
D96NR-5	-	-	-
D96PR-2	-	-	-
T. pectinovorum			
D39DR-2	-	-	+
D96SR-1	-	-	+
D96ABR-1	-	-	+
D97WR-4	-	-	+
D141BRI-2	-	-	+
D141CR-1	-	-	+
D141DRI-2	-	-	+

[a] Preparation of Triton-extracts was done with the method devised for the routine identification of oral treponemes.

[b] Immunodiffusion tests were rated from 1-5 heavy (+) immunoprecipitin lines to 1-2 faint lines (+/).

Table 13: Immunodiffusion[a] of Oral Treponeme Triton-extracts Against T. socranskii subsp. socranskii, T. socranskii subsp. buccale, T. socranskii subsp. paredis, and Treponeme D Antisera.

Extract	Antisera			
	T. socranskii			Treponeme D
	ss. socranskii D56BRIII-6	ss. buccale D2B-8	ss. paredis D46CPE-1	D40DR-2
T. denticola				
MN2B	-[b]	-	-	-
D39DPP-1	-	-	-	-
FM	-	-	-	-
TD2	-	-	-	-
ST-10	-	-	-	-
TRRD	-	-	-	-
T32A	-	-	-	-
IPP	-	-	-	-
Ichelson #2	-	-	-	-
D3A-1	-	-	-	-
Oralis	-	-	-	-
Fuji	-	-	-	-
Jethro	-	-	-	-
N-39	-	-	-	-
MRB	-	-	-	-
Ambigua	-	-	-	-
D118MR-3	-	-	-	-
D56BR-1	-	-	-	-
D79ZR-1	-	-	-	-
D83ACR-1	-	-	-	-
D118CR-1	-	-	-	-
D118ERI-1	-	-	-	-
D135ARII-4	-	-	-	-
D137ARI-2	-	-	-	-
D137CR-3	-	-	-	-
D138AR-1	-	-	-	-
D139BR-5	-	-	-	-
D142DR-1	-	-	-	-
D141ARI-1	-	-	-	-

a Preparation of Triton-extracts was done with the method devised for the routine identification of oral treponemes.

b Immunodiffusion tests were rated from 1-5 heavy (+) immunoprecipitin lines to 1-2 faint lines (+/-).

Table 13 continued[a]

Extract	Antisera			
	T. socranskii			Treponeme D
	ss. socranskii D56BRIII-6	ss. buccale D2B-8	ss. paredis D46CPE-1	D40DR-2
T. denticola				
D65BR-1	-[b]	-	-	-
D132AR-1	-	-	-	-
D47APP-1	-	-	-	-
D49DPP-1	-	-	-	-
D62DPP-1	-	-	-	-
D66BPP-1	-	-	-	-
D73DPPI-1	-	-	-	-
D75APP-2	-	-	-	-
D3A-4	-	-	-	-
D36FR-2	-	-	-	-
D38APP-3	-	-	-	-
D39DPP-2	-	-	-	-
D40CPP-1	-	-	-	-
D43BPPII-2	-	-	-	-
D52AR-1	-	-	-	-
D53DPP-2	-	-	-	-
D61DPP-1	-	-	-	-
D61ARII-4	-	-	-	-
D63BR-2	-	-	-	-
D64BR-1	-	-	-	-
D67BPPI-1	-	-	-	-
D70DR-2	-	-	-	-
D79GR-2	-	-	-	-
T. vincentii				
N-9	-	-	-	-
D148CR	-	-	-	-
D138BR	-	-	-	-
D138CR	-	-	-	-

[a] Preparation of Triton-extracts was done with the method devised for the routine identification of oral treponemes.

[b] Immunodiffusion tests were rated from 1-5 heavy (+) immunoprecipitin lines to 1-2 faint lines (+/-).

Table 13 continued[a]

Extract	Antisera			
	T. socranskii			Treponeme D
	ss. *socranskii* D56BRIII-6	ss. *buccale* D2B-8	ss. *paredis* D46CPE-1	D40DR-2
T. socranskii				
ss. *socranskii*				
DR56BRIII-6	+[b]	+	+	+
D82AER-1	+	+	+	+
D79VR-1	+	+	+	+
D86HR-1	+	+	+	+
DH56CR-1	+	+	+	+
D39AR-1	+	+	+	+
D43BR-1	+	+	+	+
D30BR-2	+	+	+	+
D53AR-1	+	+	+	+
D20A-2	+	+	+	+
DH56CR-4	+	+	+	+
D146HR-1	+	+	+	+
D146BR-1	+	+	+	+
D146DR-1	+	+	+	+
D132ARII-4	+	+	+	+
D137AR-1	+	+	+	+
D97YR-1	+	+	+	+
D128BR-1	+	+	+	+
D112DR-1	+	+	+	+
D136ARI-1	+	+	+	+
D143BR-1	+	+	+	+
ss. *buccale*				
D2B-8	+	+	+	+
D68BR-1	+	+	+	+
D40DPPE-1	+	+	+	+
D22A-5	+	+	+	+
D11A-1	+	+	+	+

[a] Preparation of Triton-extracts was done with the method devised for the routine identification of oral treponemes.

[b] Immunodiffusion tests were rated from 1-5 heavy (+) immunoprecipitin lines to 1-2 faint lines (+/-).

Table 13 continued[a]

Extract	Antisera			
	T. socranskii			Treponeme D
	ss. *socranskii* D56BRIII-6	ss. *buccale* D2B-8	ss. *paredis* D46CPE-1	D40DR-2
ss. *buccale*				
D4A-1	+[b]	+	+	+
D34B-4	+	+	+	+
D36FEP-4	+	+	+	+
D25A-1	+	+	+	+
D96AAR-1	+	+	+	+
ss. *paredis*				
D46CPE-1	+	+	+	+
D51BR-5	+	+	+	+
E4EPE-1	+	+	+	+
E2M-1	+	+	+	+
E5BBR-5	+	+	+	+
D71CR-4	+	+	+	+
D51ARII-3	+	+	+	+
D148AR-1	+	+	+	+
D128AR-1	+	+	+	+
D148DR-1	+	+	+	+
D150AR-1	+	+	+	+
D140BR-5	+	+	+	+/-
D126AR-2	+/-	+/-	+/-	+/-
D131AR-1	+/-	+/-	+/-	+/-
Untypable				
D80CR-1	+	+	+	+
D52CR-1	+	+	+	+
D147DR-2	+	+	+	+
D96NR-2	+	+	+	+

[a] Preparation of Triton-extracts was done with the method devised for the routine identification of oral treponemes.

[b] Immunodiffusion tests were rated from 1-5 heavy (+) immunoprecipitin lines to 1-2 faint lines (+/-).

Table 13 continued[a]

Extract	Antisera			
	T. socranskii			Treponeme D
	ss. *socranskii* D56BRIII-6	ss. *buccale* D2B-8	ss. *paredis* D46CPE-1	D40DR-2
Untypable				
D96NRI-2	+[b]	+	+	+
D122AR-1	+	+	+	+
D131BR-1	+	+	+	+
D137ARII-4	+	+	+	+
D962RII-3	+	+	+	+
D136CRI-1	+	+	+	+
D80CR-1	+	+	+	+
D52CR-1	+	+	+	+
D147DR-2	+	+	+	+
Treponeme D				
D40DR-2	+	+	+	+
D118ERII-4	+	+	+	+
D118MRII-4	+	+	+	+
D96ABR-2a	+	+	+	+
D96ADR-1	+	+	+	+
D116AR-1	+	+	+	+
D135ARI-2	+/-	+/-	+	+
D96FR-3	+	+	+	+
D96DR-2	+	+	+	+
D96XR-2	+	+/-	+	+
D96VR-4	+	+/-	+/-	+/-
DH56AR-1	+	+	+	+
D70BR-5	+	+	+	+
D51ARII-2	+	+	+	+
D96NR-5	+	+	+	+
D96PR-2	+	+	+	+

[a] Preparation of Triton-extracts was done with the method devised for the routine identification of oral treponemes.

[b] Immunodiffusion tests were rated from 1-5 heavy (+) immunoprecipitin lines to 1-2 faint lines (+/-).

Table 13 continued[a]

Extract	Antisera			
	T. socranskii			Treponeme D
	ss. *socranskii* D56BRIII-6	ss. *buccale* D2B-8	ss. *paredis* D46CPE-1	D40DR-2
T. pectinovorum				
D39DR-2	-	-	-	-
D96SR-1	-	-	-	-
D96ABR-1	-	-	-	-
D97WR-4	-	-	-	-
D141BRI-2	-	-	-	-
D141CR-1	-	-	-	-
D141DRI-2	-	-	-	-

a Preparation of Triton-extracts was done with the method devised for the routine identification of oral treponemes.

b Immunodiffusion tests were rated from 1-5 heavy (+) immunoprecipitin lines to 1-2 faint lines (+/-).

exception was strain ST-10. This strain, obtained from Dr. S. S. Socransky (Forsyth Dental Center) also reacted with T. vincentii antiserum.

A combination of T. denticola anti FM and anti D39DPP-1 serum (1:1) was used so all T. denticola strains would give heavy precipitin lines in immunodiffusion tests. Some strains gave a strong precipitin line with anti- D39DPP-1 but gave only a faint line with anti-FM serum. Other strains reacted heavily with anti-FM but weakly with anti-D39DPP-1. The five strains of T. vincentii reacted only with T. vincentii antiserum. The other species tested, reacted only with their homologous antisera. However, Triton-extracts from T. socranskii subsp. socranskii, T. socranskii subsp. buccale, T. socranskii subsp. paredis and Treponema D reacted with each T. socranskii and Treponeme D antiserum. In addition, the untypable strains of T. socranskii reacted with each of the T. socranskii and Treponeme D antiserum.

The preparation of the Triton-extracts was standardized by inoculating 7 ml of modified OTI broth containing 0.25 ml of normal rabbit serum and thiamine pyrophosphate with 0.5 ml of culture. The culture was incubated for 4 days at 37 C. The optical density of the culture was read at 590 nm. The cells were centrifuged and washed once, and the cells extracted in 1ml of buffer

containing 1% Triton X-100, using the procedure developed for the routine identification of oral treponemes. The protein concentration of the extracts was determined, using the BCA reagent (Pierce, Rockford, Illinois) and the extracts were tested by ID with the homologous antiserum and T. vincentii and T. pectinovorum antisera. It was found that extracts from cultures of T. denticola (a medium sized treponeme) with an O.D. between 0.6 and 0.9 (590 nm) had a final protein concentration of 7.8 to 10.2 mg/ml of protein while extracts from cultures of the small treponeme T. socranskii (O. D. Of 0.6 to 0.9) had a protein concentration of 9.0 to 17.0 mg/ml. These parameters were found to give the heaviest immunoprecipitin lines with no cross-reactivity against the heterologous antisera. An extract with a protein concentration of 2.6 mg/ml (O. D. of 0.1 and 5×10^6 cells per ml) yielded only a very faint precipitin line against the homologous antiserum.

SDS-PAGE Gels: Triton-extracts from strains of T. denticola, T. socranskii subsp. socranskii, T. socranskii subsp buccale, T. socranskii subsp. paredis, Treponeme D, T. pectinovorum, T. vincentii, T. phagedenis biotype Reiter, T. minutum, and T. refringens were loaded onto 12% SDS-polyacrylamide gels. Protein patterns of Triton-extracts from T. denticola strains are shown in

Figure 5. The extracts contained 32 bands with molecular weights ranging from 3.2K to 155K. Most of the strains showed similar banding patterns, except N39, TRRD, and oralis which produced a slightly different pattern. T. denticola strain ST-10 also had a different pattern from the other strains of T. denticola, but was similar to the T. vincentii strain N-9 pattern (Figure 6). A culture of T. denticola strain ST-10 was streaked and 10 single colonies were picked and subcultured. Cells from each of these cultures agglutinated T. denticola antiserum, but not T. vincentii (N-9) antiserum. The Triton-extracts from these cultures consistently reacted with both T. denticola antiserum and T. vincentii antiserum in the immunodiffusion test. It is believed that ST-10 is an odd strain of T. denticola that is very closely related antigenically and by SDS-PAGE protein pattern to T. vincentii strain N-9.

Figure 6 shows the 12% SDS-polyacrylamide gel of Triton-extracts of the subspecies of T. socranskii, Treponeme D, T. pectinvorum, T. vincentii, T. phagedenis biotype Reiter, T. minutum, and T. refringens. Each species had a distinct protein pattern; whereas, the subspecies of T. socranskii and Treponeme D produced very similar patterns.

T. vincentii produced a pattern that was distinct

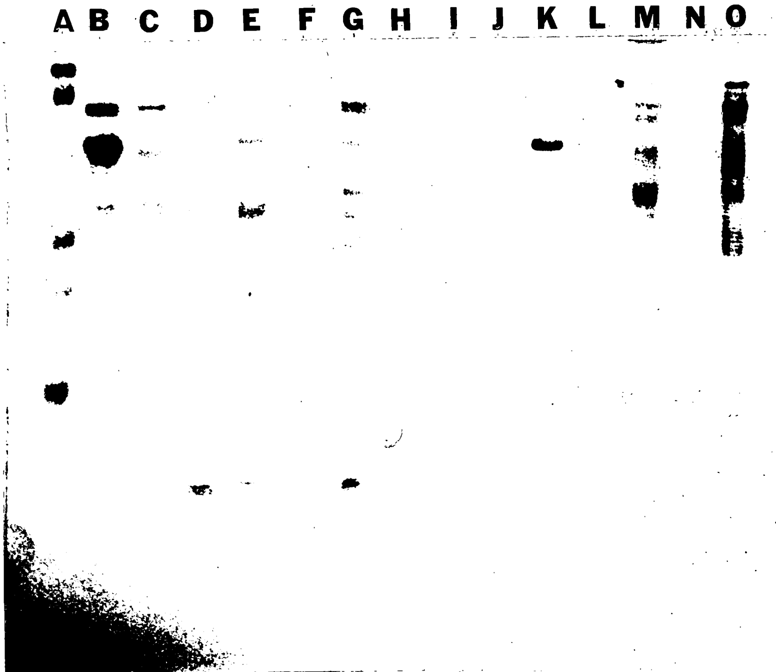

Figure 5. 12% SDS-polyacrylamide gel of Triton-extracts from several strains of T. denticola. Lane A contains a prestained molecular weight standard (Phosphorylase b 130K, Bovine serum albumin 75K, Ovalbumin 50K, Carbonic anhydrase 39K, Soybean trypsin inhibitor 27K, and Lysozyme 17K). Lanes B trough O are Triton-extracts of T. denticola strains: D39DPP-1, Ambigua, T32A, FM, IPP, N39, TD-2, ST-10, MRB, Jethro, Fuji, TRRD, D3A-1, and oralsi respectively. The proteins were stained in with coomassie blue.

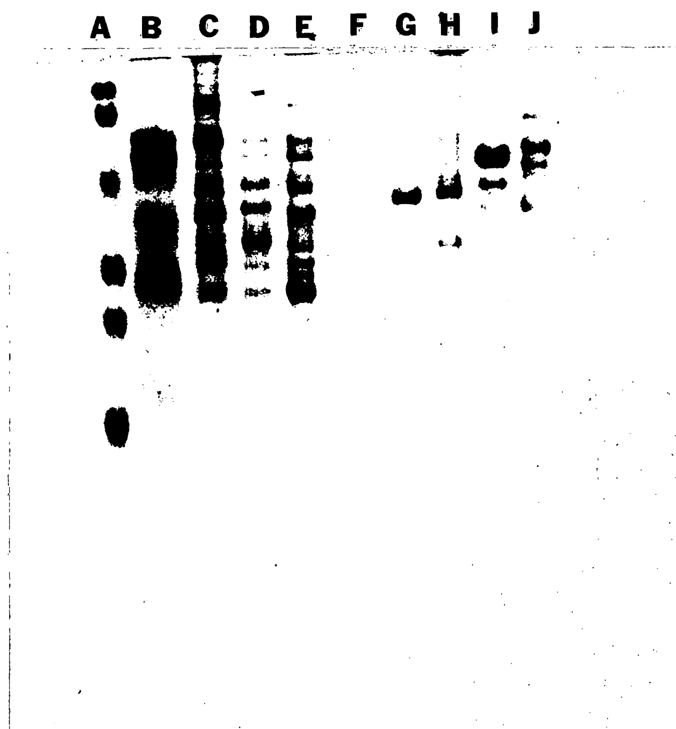

Figure 6. 12% SDS-polyacrylamide gel of the Triton-extracts of oral and genital treponemes. Lane A contains a prestained molecular weight standard(Phosphorylase b 130K, BSA 75K, Ovalbumin 50K, Carbonic anhydrase 59K, Soybean trypsin inhibator 27K, and Lysozyme 17K. Lanes B through J contain T. socranskii subsp. socranskii (D43BR-2), T. socranskii subsp. buccale (D2B-8), T. socranskii subsp. paredis (D46CPE-1), Treponeme D (D40DR-2), T. pectinovorum (D39DR-2), T. vincentii (N9), T. phagedenis biotype Reiter, T. minutum (minutum), and T. refringens (refringens C). The proteins were stained of coomassie blue.

from that of the other treponemes. However the pattern was closer to that of T. phagedenis biotype Reiter than any other and was also similar to T. denticola strain ST-10. T. vincentii Triton-extracts contained approximately 11 protein bands with molecular weights ranging between 10K and 140K. Triton-extracts of T. minutum contained 12 protein bands with molecular weights between 23K and 120K. The protein banding pattern was distinct from that of the other treponemes, but was close to the pattern of proteins in Triton extracts of T. refringens.

Western Blot Analysis: Triton-extracts from representative strains of T. denticola, T. socranskii, T. vincentii, T. phagedenis biotype Reiter, T. minutum, and T. refringens were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Common antigens were detected with T. denticola strain FM antiserum. Western blot analysis results are shown in Figure 7. T. denticola strain FM had approximately 23 protein bands in a 12% SDS-polyacrylamide, coomassie blue stained gel. Approximately 19 of these bands were shown by Western blots to react with the homologous antiserum. The molecular weights of the FM antigens ranged from 12K to 96K. T. socranskii susp. socranskii strain D43BR2 produced approximately 34 protein bands in a 12% SDS-polyacrylamide gel, 2 of which reacted with FM antiserum.

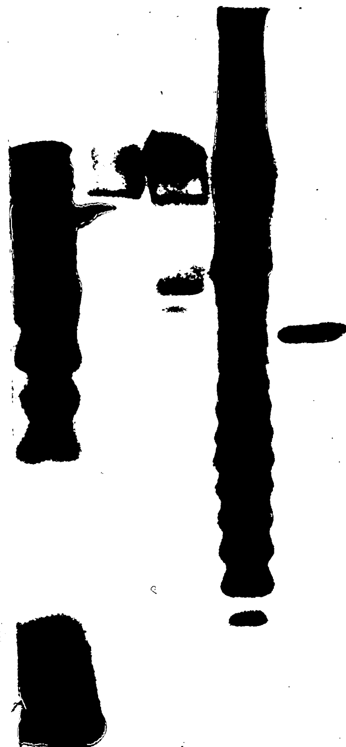

Figure 7. Western blot of Triton-extracts of oral and genital treponemes against T. denticola strain FM antiserum. Lane A contains a prestained molecular weight standard (Phosphorylase b 130K, BSA 75K, Ovalbumin 50K, Carbonic anhydrase 39K, Soybean trypsin inhibitor 27K, and Lysozyme 17K. Lanes B through G contain Triton-extracts of T. denticola (FM), T. socranskii subsp. socranskii (D43BR-2), T. vincentii (N9), T. phagedenis biotype Reiter, T. minutum, and T. refringens (refringens C). The proteins were separated in a 12% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane. The protein bands were made visible by the use of silver lactate enhancement of Protein-A Gold stained membranes.

There was only one moderately cross-reacting band at 80K and one faint band at 56K. The SDS-polyacrylamide gel of T. vincentii strain N-9 Triton extract showed 27 protein bands, of which eleven bands cross-reacted with T. denticola strain FM antiserum. Major cross-reactive proteins had molecular weights of 135K, 120K, 96K, 90K, 80K, 60K, 56K, and 50K. Minor cross-reacting bands had molecular weights of 70K, 35.5K, and 24K. T. phagedenis biotype Reiter Triton-extracts showed 28 protein bands in a 12% SDS-polyacrylamide gel, 20 of which were antigenically similar protein bands to T. denticola strain FM. Cross-reacting Reiter protein bands that coincided with T. denticola strain FM protein bands had molecular weights of 96K, 90K, 80K, 72K, 63K, 56K, 35K, 24K (very faint in FM extracts), and 12K. Approximately 6 cross-reacting Reiter protein bands with molecular weights of 32K, 26K, 22.5K, 19K, 16.5K, 14K, and 10.5K did not have corresponding proteins with the same molecular weights in the FM extract. Coomassie blue stained SDS-PAGE gels of Triton-extracts of T. minutum showed 26 bands, 5 of which reacted with T. denticola strain FM antiserum. The molecular weights of these proteins were 96K, 90K, 64K (faint), 45K, and 41K. Finally, T. refringens strain refringens C Triton-extract had 27 protein bands in a 12% SDS-polyacrylamide gel and

was shown to share 7 antigens with T. denticola strain FM, with molecular weights of 90K, 74K, 66K, 56K, 50K, 41k, and 30K.

Western blot analysis was performed on the Triton-extracts of T. socranskii subsp. socranskii, T. socranskii subsp. buccale, T. socranskii subsp. paredis, Treponeme D, and T. pectinovorum, to determine the number of antigens shared with T. socranskii subsp. socranskii. The common antigens were detected with T. socranskii subsp. socranskii (D56BRIII6) antiserum. The results are shown in Figure 8. SDS-polyacrylamide gels of T. soncranskii subsp. socranskii showed approximately 23 protein bands; 17 of these protein bands, with molecular weights ranging from 25K to 160K, reacted with T. socranskii subsp. socranskii antiserum. T. socranskii susp. buccale SDS-polyacrylamide gel had 20 protein bands, 11 of these protein bands reacted with T. socranskii subsp. socranskii antiserum. Five T. socranskii subsp. buccale protein bands with molecular weights of 85K, 68K, 60K, 29K, and 24K, coincided with T. socranskii subsp. socranskii proteins. There were 6 T. socranskii subsp. buccale protein bands that did not have corresponding protein bands in the blots of T. socranskii subsp. socranskii extract. Their molecular weights were 74K, 43K, 35K, 33K, 19.5K, and 13K. Compared to the

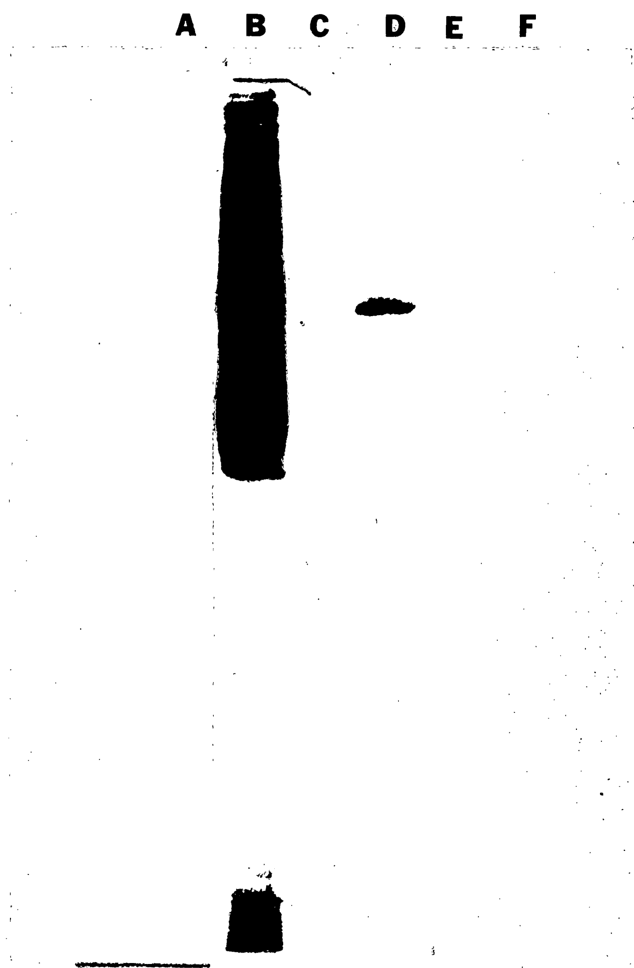

Figure 8. Western blot of Triton-extracts of oral treponemes against T. socranskii subsp. socranskii antiserum. Lane A contains a molecular weight standard (Phosphorlyase b 130K, BSA 75K, Ovalbumin 50K, Carbonic anhydrase 39K, Soybean trypsin inhibator 27K, and Lysozyme 17K. Lanes B through F contain Triton-extracts of T. socranskii subsp. socranski (D43BR-1), T. socranskii subsp. buccale (D2B-8), T. socranskii subsp. paredis (46CPE-1), Treponeme D (D40DR-2),and T. pectinovorum (D39DR-2). The proteins were separated in a 12% SDS-polyacrylamide gel, transferred to nitrocellulose. The protein bands were made visible by the use of silver lactate enhancement of Protein A-Gold stained membranes.

other subspecies *T. socranskii* subsp. *paredis* had the smallest number of cross-reacting protein bands. Only 4 bands were visible out of the 13 protein bands seen in the 12% SDS-polyacrylamide gel. The molecular weights of these protein bands were 98K, 79K, 70K, and 29K. The unnamed Treponeme D shared 7 antigens with *T. socranskii* subsp. *socranskii*. These molecular weights were 92K, 79K, 76.5K, 74K, 45K, 40K (faint), and 13K (faint). Finally, *T. pectinovorum* had 25 proteins in a 12% SDS-polyacrylamide gel and shared only one very faint protein band with *T. socranskii* subsp. *socranskii* that had an approximate molecular weight of 58K.

Binding of Treponemes to Fibronectin (Fn)

Fn Coated Slides: One hundred μl of a washed cell(10^8 cells/ml) suspension of *T. denticola* strain oralis was placed on a microscope slide coated with either bovine serum albumin (BSA) or Fn and incubated for 30 minutes at 37 C in a moist chamber. The slides were washed and examined for treponemes adhering to the slides, by darkfield microscopy. There was an average of 26 cells/oil immersion field at (675X magnification), bound to the BSA coated slides and an average of 110 cells/oil immersion field bound to the Fn coated slides (Figure 9).

Fluorescent Antibody Detecting Fn Associated with Treponemes: The washed cell pellets of *T. denticola*

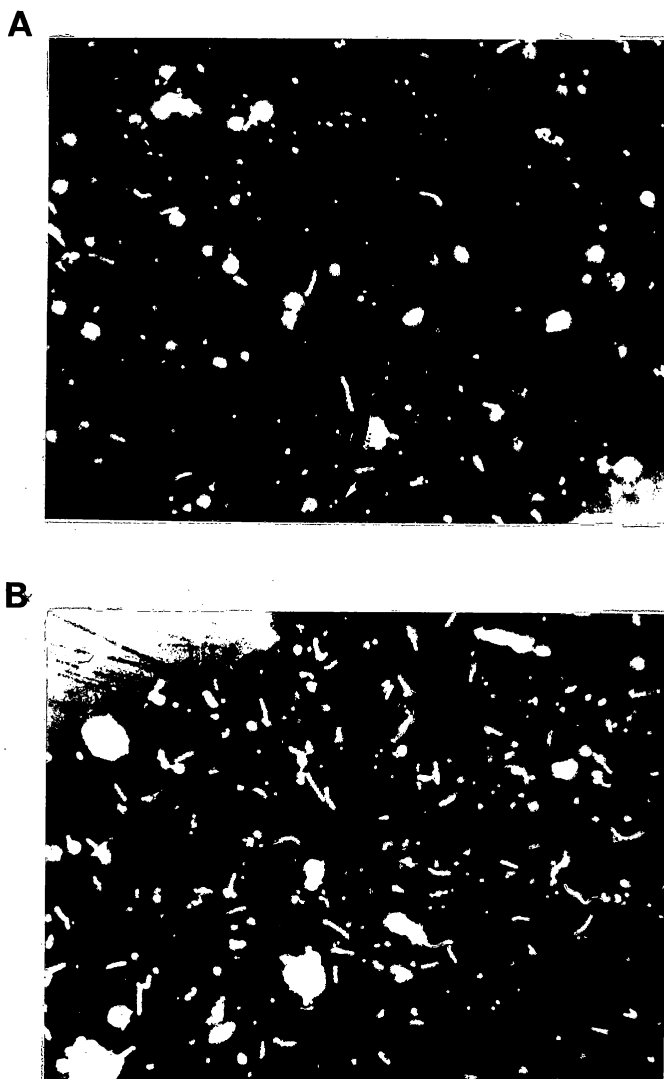

Figure 9. Darkfield micrograph of T. denticola strain oralis cells on BSA-coated (A) microscope slides and (B) Fn-coated microscope slides. T. denticola cells were incubated for 30 minutes on either BSA or Fn-coated microscope slides, then washed gently in FTA buffer. The attached cells were viewed under a darkfield microscope at a magnification of 675.6X.

strains D39DPP-1, IPP, Fugi, Jethro, N39, oralis, Ichelson #2 , Ambigua, T. vincentii strain N-9, and T. phagedenis biotype Reiter were resuspended in a solution of 20 ug/ml of human Fn, and incubated at 37 C for 2 hours. The cells were washed and the fluorescent antibody staining was done as previously described, with anti-human Fn serum. Cells of all the T. denticola strains, T. vincentii, and T. phagedenis biotype Reiter fluoresced brightly. The Fn primed cells when stained with anti-human fibrinogen (Fb) also fluoresced brightly. Immunodiffusion analysis was performed on normal rabbit serum, human Fn, bovine Fn, and human Fb against human Fn antiserum and human Fb antiserum. The results showed anti-human Fb serum gave a precipitin line with human Fb and human Fn but not with bovine Fn of normal rabbit serum. In addition, there was no fluorescence when washed cells not pre-incubated with human Fn were stained with the anti-human Fb serum. The anti-human Fn serum gave precipitin lines with human and bovine Fn, rabbit serum and human Fb.

Detection of Rabbit Serum Proteins in Triton Extracts: Wells of an agarose immunodiffusion slide were filled with T. denticola strain oralis Triton-extract and anti-rabbit Fn serum, anti-rabbit albumin serum, anti-rabbit whole serum, and anti-rabbit alpha- 2-macroglobulin serum

and allowed to diffuse overnight. The wells were refilled with the treponeme cell extract and antisera and incubated overnight. Immunoprecipitin lines developed between the Triton-extract of T. denticola strain oralis and anti-rabbit Fn serum, anti-rabbit albumin serum, and anti-rabbit whole serum, but not between the extract and anti-rabbit alpha-2-macroglobulin.

Fn-Capture ELISA: An ELISA test was developed to detect the presence of treponemal proteins in Triton-extracts that bind Fn. Fibronectin was bound to the microtiter plate wells, then the Triton extracts were added to let the treponemal Fn binding proteins be captured and bound to the wells by the Fn. T. denticola (D39DPP-1) antiserum (1:500 dilution) was used to detect the presence of the treponemal Fn binding proteins. Extracts from several strains of T. denticola, T. vincentii, T. phagedenis, T. minutum, T. socranskii and its subspecies, and T. pectinovorum were examined, using the Fn capture ELISA, for the presence of Fn-binding proteins. The results are shown in Table 14. Using T. denticola antiserum common Fn-binding proteins were detected in the Triton-extracts from T. denticola. However, T. denticola strain oralis proteins were not detected by human syphilitic serum. Extracts from T. minutum, T. refringens, T. vincentii, T. phagedenis biotype Reiter,

Table 14: Detection of Fn-binding Proteins in Triton-extracts of Various Species of Treponemes, by Enzyme Linked Immunosorbent Assays

Organism	Type of ELISA	
	FN-Capture[a]	Detection of Fn in Extracts[b]
T. denticola		
D39DPP-1	128	4,098
D3A-1	32	
Oralis	8,192	4,098
Jethro	256	
Ichelson #2	256	
FM	32	
ST-10	128	
T32A	32	
IPP	32	
TD-2	64	
TRRD	32	
N-39	512	
Ambigua	128	2,048
MRB	16	
T. refringens		
refringens C	-	1,048
T. minutum	-	4,098
T. vincentii		
N-9	-	
T. phagedenis		
Reiter	-	4,098
T. socranskii		
ss. socranskii (D43BR-1)	-	64
ss. buccale (D2B-8)	-	4,098
ss. paredis (D46CPE-1)	-	-
Treponeme D (D40DR-2)	-	-

The titer of the extracts was the highest dilution of extract that still gave a purple color (+3) after incubation.

a The antisera used in the Fn-capture ELISA was against T. denticola strain D39DPR-1.

b The antiserum used to detect Fn in the extracts was goat anti-rabbit Fn serum.

T. socranskii subsp. socranskii, T. socranskii subsp. buccale, T. socranskii subsp. paredis, and Treponeme D did not react with the T. denticola strain D39DPP-1 antiserum.

Binding of Fn to Treponemal Protein (s): Fibronectin is one of the many proteins found in normal rabbit serum. All of the treponemes used in this study were grown in a medium containing 10% rabbit serum. If the treponemes bind Fn in the serum, it is possible Fn could be detected in the triton extracts. A second ELISA assay was designed to detect rabbit Fn in the Triton-extract of selected T. denticola strains. Extracts were bound to microtiter wells and blocked with BSA. Normal rabbit serum was added to make sure all the treponemal Fn-binding proteins in the extract were saturated with Fn. Anti-rabbit Fn serum was used to detect the presence of Fn in the extract. The results are shown in Table 14. Fibronectin was detected in Triton-extracts from all the T. denticola strains examined; as well as, Triton-extracts from T. phagedenis biotype Reiter, T. socranskii subsp. socranskii, T. socranskii subsp. buccale, T. refringens strain refringens C and T. minutum. Results of a slide immunodiffusion test also showed that Triton-extracts from T. denticola contained rabbit fibronectin.

Isolation and Identification of the Fn Binding Protein

Agarose-gelatin : The method used to isolate and identify the number of proteins that bound Fn in Triton-extracts of T. denticola strain oralis, involved the binding of Fn from rabbit serum to a agarose-gelatin column. The Triton- extract was passed through the column to bind the treponemal protein to the FN. The protein was eluted from the column, electrophoresed in a 12% SDS-polyacrylamide gel, and then stained with coomassie blue. The results are shown in Figure 10. The first lane contained Triton-extract that was not passed through the agarose-gelatin-Fn column (unadsorbed). The second lane was the protein pattern of oralis Triton-extract that was passed through the column (adsorbed extract). The protein pattern for the eluted proteins from the agarose-gelatin-Fn column is shown in lane 3. The protein bands above 100K are thought to be Fn proteins. There was one heavily staining band at 64.5K and a very faint band at 47K. These bands were thought to be the treponemal Fn binding proteins and were identified later by Western blots.

Protein from the agarose-gelatin-Fn column was tested for the presence of treponemal protein. The standard ELISA using anti-T. denticola strain D39DPP-1 serum to detect treponemal protein was used. The material gave a positive ELISA reaction (1:128 titer)

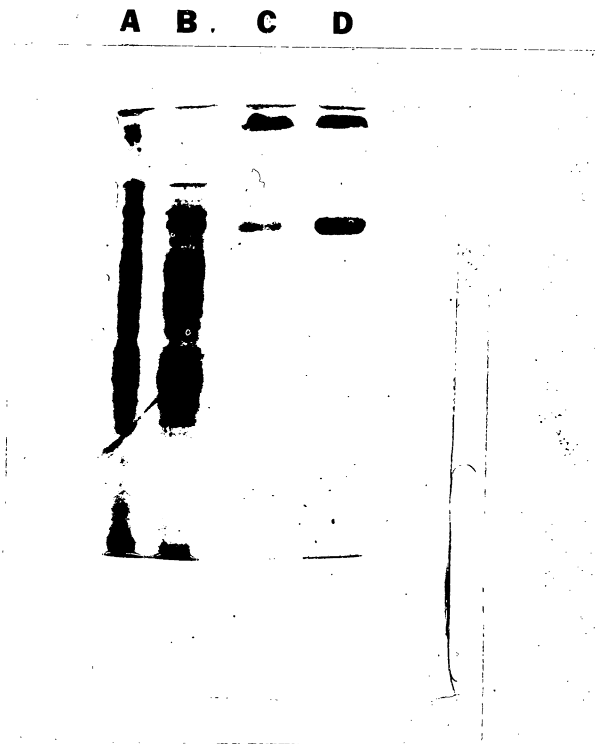

Figure 10. 12% SDS-polyacrylamide gel of lane (A) unadsorbed T. denticola strain oralis Triton-extract, (B) and adsorbed oralis Triton-extract, (C) eluted treponemal protein from an agarose-gelatin-Fn column, and (D) eluted rabbit serum proteins from the agarose-gelatin column. Adsorbed triton-extract was passed through an agarose-gelatin-Fn column. The gels were stained with coomassie blue.

with the T. denticola antiserum.

An electrophoresed 12% SDS-polyacrylamide gel containing treponemal protein eluted from the agarose-gelatin-Fn column, was transferred to nitrocellulose and the membrane incubated in T. denticola strain D39DPP-1 antiserum (1:50 dilution). The results are shown in Figure 11. Protein bands with molecular weights of 60K, 47K, and a faint band at 21K were seen. These proteins were thought to be the Fn-binding proteins. To detect rabbit serum proteins other than Fn that might have adsorbed to the gelatin-agarose column, rabbit serum was passed through the column. The column was washed with FTA buffer until no absorbance at 280 nm was seen and the material eluted with buffer at pH 5.0. The eluate was concentrated, electrophoresed and transferred to nitrocellulose. The membrane was incubated with anti-T. denticola (D39DPP-1) serum, anti-rabbit Fn serum, and anti-rabbit albumin serum. The lane that was incubated with rabbit albumin antiserum had no bands (not shown). However, the lane that was incubated with anti-D39DPP serum produced 2 faint bands with molecular weights of 75K and 59K (Figure 11). Approximately 7 protein bands with molecular weights of 145K, 130K, 105K, 85K, 62K, 49K, and 39K were seen when the membrane was incubated with anti-rabbit Fn serum.

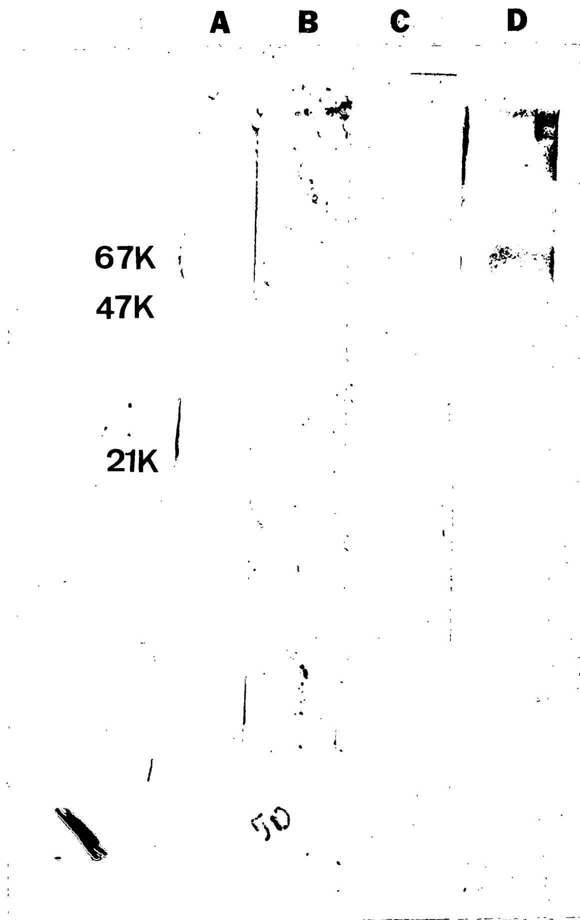

Figure 11. Western blot of (B) eluted treponemal protein from an agarose-gelatin-Fn column against T. denticola strain D39DPP-1 antiserum, and eluted rabbit serum proteins against (C) T. denticola strain D39DPP-1 antiserum and (D) rabbit Fn antiserum. Lane A contains a prestained molecular weight standard (Phosphorylase b 130K, BSA 75K, Ovalbumin 50K, Carbonic anhydrase 39K, Soybean trypsin inhibitor 27K, and Lysozyme 17K). The protein bands were made visible by the use of silver lactate enhancement of Protein A-Gold stained membranes.

DISCUSSION

Antigenic Relationship Between Oral and Genital Treponemes

One of the objectives of this study was to investigate the antigenic relationship between several species of oral and genital treponemes. Fluorescent antibody (FA) staining was used to determine the antigenic relationship between the subspecies of *T. socranskii*, Treponeme D, *T. pectinovorum*, *T. denticola*, *T. vincentii*, and *T. phagedenis* biotype Reiter. All the species examined cross-reacted with the homologous and heterologous antisera. The cross-reactivity was removed when the antisera was incubated with Reiter whole cells, which resulted in species specific antisera for *T. denticola* and *T. vincentii* but not *T. socranskii*, Treponeme D, and *T. pectinovorum*. This was in agreement with the findings of Meyer et al. (22). They reported that *T. denticola*, *T. vincentii*, *T. phagedenis*, *T. refringens*, and *S. zuelzerae* cross-reacted in FA staining. The cross-reactivity was removed when the antisera was adsorbed with *T. phagedenis* biotype Reiter cells.

In addition to the common antigen or antigens shared with *T. phagedenis*, there was also an additional group of antigens common to the subspecies of *T. socranskii*, Treponeme D, and *T. pectinovorum*. These cells still

showed cross-reactions with Reiter adsorbed antisera. Additional adsorptions with appropriate T. socranskii cells made the antisera species and subspecies specific. Antisera could also be made subspecies specific by adsorption of sera with only the appropriate T. socranskii cells. Thus immunoadsorption of treponemal antisera can be used to make species and subspecies specific antisera.

Monospecific antisera was used in the application of FA staining of smears of oral material taken directly from the mouth and was found not to work very well. The treponemal cells had faint fluorescence. The slides seemed to be coated with a film, possibly saliva, that masked the fluorescence of the cells, and may have blocked the staining of the cells. While identification of treponemes by FA staining of cells from pure cultures worked well, direct staining of oral samples worked poorly for the identification of treponemes.

The direction of the study was then turned towards the investigation of solubilized outer envelope proteins as the source of antigens that could be used in serologic tests such as immunodiffusion and ELISA, for the identification of oral treponemes. Several extraction methods were tried. The low pressure cell disruption ("BOMB") and Triton-extracts were found to have the

desired specificity for the use as antigens in the identification of treponemes to the species level. However, the Triton-extract was chosen for two reasons. First, the Triton extraction procedure was easy to perform and many treponeme cultures could be extracted at once. Therefore, Triton extraction of treponemes was a more practical procedure to use as a routine laboratory procedure. The second reason for choosing the Triton extraction was because the procedure removed the outer envelope with the least removal of non-envelope antigens. Electron micrographs of negatively stained Triton treated T. denticola cells showed that the outer envelope was removed. This was consistent with the report of Penn et al. (25) that incubation of T. pallidum with Triton X-100 removed the outer envelope of the treponemal cell. Because there were a large number of immunoprecipitin arcs in the crossed immunoelectrophoresis slides and a large number of protein bands in SDS-polyacrylamide gels, there was probably some extraction of additional cytoplasmic membrane proteins and cytoplasmic proteins by the Triton X-100 procedure. Some contamination of Triton-extracts with periplasmic flagella could be demonstrated by crossed immunoelectrophoresis. Crossed immunoelectrophoresis of Triton-extracts showed a linear immunoprecipitin line that was slightly under the agarose

antibody containing layer and moved only a few mm away from the antigen well. A similar line was identified by crossed immunoelectrophoresis and was reported by Penn et al. (24) and Petersen et al. (28) to be the periplasmic flagella in T. pallidum and T. phagedenis biotype Reiter cells.

The Triton-extracts from several different species of treponemes were tested in crossed immunoelectrophoresis (CIE), immunodiffusion, and ELISA against T. denticola strain D39DPP-1 antiserum to determine the specificity of the extract in the serologic assays. All the strains of T. denticola examined reacted strongly with T. denticola antiserum in CIE. There was only slight cross-reactivity with the extracts from T. vincentii, T. phagedenis biotype Reiter, T. minutum, T. refringens, the subspecies of T. socranskii, Treponeme D, and T. pectinovorum. One of the cross-reacting immunoprecipitin lines in all the extracts was the periplasmic flagella. In the immunodiffusion method, all the T. denticola strains reacted with the anti-T. denticola strain D39DPP-1 serum and there was no cross-reactivity between the species. The exception was T. denticola strain ST-10,which reacted with both T. denticola and T. vincentii antisera. The ELISA test again showed that all the strains of T. denticola reacted

with T. denticola antiserum, while T. refringens, T. vincentii and T. phagedenis biotype Reiter showed only minimal cross-reactivity. The cross-reactivity could be removed by diluting the extract by one or more dilutions. All the strains used in this study have been identified by phenotypic characteristics as listed in Bergey's Manual of Systematic Bacteriology (34) and the Anaerobe Laboratory Manual (13), while some strains (appendix B) also were previously subjected to DNA/DNA homology studies (36).

The antigenic relationship between the Triton-extracts of the different treponemal species was studied further by SDS-polyacrylamide gel electrophoresis and Western blots. SDS-polyacrylamide gel electrophoresis protein patterns showed that each species produced its own distinct protein pattern. T. denticola strains produced two slightly different patterns. Tall and Nauman (7), using sonic disrupted cells also, observed that T. denticola, T. pectinvorum, and T. vincentii produced distinct protein patterns. However, slight differences could be seen in minor protein bands in different strains of T. denticola.

The one strain of T. denticola that had a very different protein pattern from the other T. denticola strains, was ST-10. The protein pattern was very similar

to the protein pattern of T. vincentii strain N-9. ST-10 was the only T. denticola Triton-extract that reacted with both T. denticola and T. vincentii antisera in the immunodiffusion test. T. denticola strain ST-10 is probably an odd strain of T. denticola that has many properties of both species. According to phenotypic characteristics and slide agglutination results, ST-10 was identified as a strain of T. denticola.

Western blots were performed to determine the approximate number of cross-reacting bands in the Triton-extracts. The Western blots showed there were few protein bands in the Triton-extracts of T. socranskii subsp. socranskii, T. vincentii, T. minutum, and T. refringens that cross-reacted with T. denticola strain FM antiserum. The one extract that had many cross-reacting bands was T. phagedenis biotype Reiter. However, the Reiter Triton-extract did not give a visible precipitin line with T. denticola antiserum in the immunodiffusion test and only cross-reacted weakly in CIE and ELISA. There were several Reiter bands that did not have corresponding protein bands in the FM extract. A plausible explanation was that the antiserum was detecting common epitopes that were present on proteins of different molecular weights and different electrophoretic mobilities. Wos and Wicher (46) used

Western blots and reported that T. phagedenis biotype Reiter had 30 antigens that reacted with hyperimmune T. pallidum antiserum. Western blot analysis performed by Baker and Lukehart (3) reported that not only did the treponemes share common antigens, but they were shared with other members of the family Spirochaetaceae. They showed that syphilitic serum also reacted with sonicated cells of T. parluis-cuniculi, T. hyodysenteriae, Borrelia hermsi, and Leptospira interrogans.

There were few bands in Triton-extracts of T. socranskii subsp. socranskii, T. socranskii subsp. buccale, T. socranskii subsp. paredis, Treponeme D and T. pectinovorum that cross-reacted with T. socranskii subsp. socranskii antiserum. As a matter of fact T. pectinovorum had only one cross-reacting band and it was very faint. T. socranskii subsp. buccale had cross-reacting bands that did not have coinciding protein bands in the T. socranskii subsp. socranskii extract. Again the assay was probably recognizing common epitopes on different proteins.

Immunodiffusion, ELISA, and crossed immunoelectrophoresis assays are possible assays that can be used with Triton-extracts for the identification of treponemes. There are advantages and disadvantages for each test. Triton-extracts were shown to contain few

cross-reacting antigens. Therefore it was selected as the antigen source for use in a serologic assay for the identification of treponemes. The assay chosen for the routine serologic identification of oral treponemes was immunodiffusion because the assay did not detect any cross-reactivity between the species and was a simple and easy test to run. However, the immunodiffusion test using the Triton-extracts was not subspecies specific. Extracts from T. socranksii subsp. socranskii, T. socranskii subsp. buccale, T. socranskii subsp. paredis, and Treponeme D cross-reacted. At the present time, the immunodiffusion test does not differentiate between the three subspecies of T. socranskii and the unnamed Treponeme D. At this point, one must still rely on the slide agglutination test and phenotypic characteristics to differentiate between the T. socranskii subspecies and Treponeme D.

Immunodiffusion analysis of Triton-extracts was also used to serologically identify the "untypable" strains of T. socranskii. "Untypable" treponemes cannot be identified using the slide agglutination tests because the cell antigen autoagglutinates. Phenotypically the "untypable" treponemes are either T. socranskii subsp. socranskii or T. socranskii subsp. buccale. These subspecies have the same phenotypic characteristics and

can only be differentiated by the slide agglutination test. The immunodiffusion test serologically identified the "untypable" treponemes as T. socranskii. Thus it would not be necessary, in identifying unknown isolates to inoculate many tubes of media to determine the phenotypic characteristics. The immunodiffusion test was also easy to perform and was well suited for the routine identification of oral treponemes. Results are usually obtained in 24 hours. The tests could be used to identify treponemes that were in either pure or mixed cultures.

The ELISA assay is another method that can be used in the identification of treponemes. However, the procedure for the ELISA is more involved than the ID tests. There was slight cross-reactivity with the T. refringens, T. minutum, T. vincentii, and T. phagedenis. This could be removed by diluting the Triton-extracts by one or more dilutions. The ELISA has the sensitivity to be used to identify treponemes in extracts of direct oral samples.

Crossed immunoelectrophoresis is another possible identification method. However there is still slight cross-reactivity between the species. This method is much more time consuming and labor intensive than the other assays and is not suitable as a routine laboratory

procedure.

Future research needs to be directed towards perfecting the immunodiffusion tests so it will differentiate between the subspecies of T. socranskii and Treponeme D. This could be done by adsorbing antiserum of each species with Triton-extracts from heterologous strains of different subspecies to remove the cross-reacting antibodies.

Binding of T. denticola to Fibronectin

Several papers have been published (12, 4, 1, 2) that reported the polar attachment of T. pallidum cells to host epithelial cells. The mechanism of this binding is to cellular fibronectin (Fn). T. pallidum has been reported to have three outer envelope proteins with molecular weights of 72K, 28K, and 26K that bind to Fn. There have also been reports of polar attachment of T. denticola and T. vincentii (27, 23) to human epithelial cells, followed by the destruction of the epithelial cells. Because T. pallidum and T. denticola are both treponemes and bind to host cells by their tips, it was hypothesized that T. denticola might also bind Fn by way of outer envelope proteins.

Several methods were used to determine the ability of T. denticola to bind to Fn. T. denticola cells were shown to bind to Fn coated slides. This is consistent

titers. One would expect this because the assay was detecting a small percentage of the total protein in the extracts.

T. refringens, T. minutum, T. vincentii, T. phagedenis biotype Reiter, T. socranskii extracts did not react with T. denticola antiserum in the Fn-capture ELISA. There were two possible explanations for this. These treponemes did not bind Fn, or they did bind Fn but the protein was antigenically different from the Fn-binding protein on T. denticola. Fn was detected, by a different ELISA using anti-rabbit Fn, in Triton-extracts from the above species. This suggests that T. refringens, T. minutum, T. vincentii, T. phagedenis biotype Reiter, and T. socranskii bind Fn but the proteins are antigenically different from the T. denticola Fn-binding protein. The Fn-capture ELISA was also performed using T. denticola strain oralis Triton-extract against syphilitic serum to see if the Fn binding protein in T. denticola was antigenically similar to the T. pallidum Fn-binding protein. There was no reaction; therefore, the T. denticola Fn-binding and the T. pallidum Fn-binding proteins are not antigenically related. Alderete et al.(2) reported that Reiter antiserum did not react with syphilitic serum in the T. pallidum Fn-capture ELISA.

with the findings of Thomas et al. (43) and Fitzgerald et al.(10) that _T. pallidum_ binds to Fn. Fluorescent antibody staining has also demonstrated the association of Fn with the outer envelope of several strains of _T. denticola_, _T. vincentii_, and _T. phagedenis_ biotype Reiter cells. Fitzgerald and Repesh (9) showed, by an immunofluorescent assay, the association of Fn with _T. pallidum_ cells. Fn was detected in Triton-extracts by the immunodiffusion test and ELISA assay. An Fn-capture ELISA demonstrated the presence of treponemal Fn-binding proteins in Triton-extracts.

Three Fn-binding proteins were found in a detergent (Zwittergent) extract of _T. pallidum_ (2). Because _T. denticola_ cells were bound to Fn coated slides, it was possible that the Triton-extracts of _T. denticola_ contained the Fn-binding proteins. Two ELISA assays were developed to detect the presence of Fn-binding proteins in Triton-extracts from treponemes. The first ELISA was an Fn-capture ELISA similar to the one developed by Alderete et al. (2) for _T. pallidum_. _T. denticola_ strain D39DPP-1 was used to detect treponemal proteins. Triton-extracts of 15 _T. denticola_ strains were tested and all strains contained Fn-binding proteins that reacted with a single _T. denticola_ antiserum. The titers of the Fn-capture ELISA were much lower than the standard ELISA

The second ELISA was developed to detect the presence of Fn in the Triton-extracts. Treponemes were grown in a medium containing 10% rabbit serum. Fn (0.03 to 0.07 mg) present in the medium would bind to the surface of treponeme cells and thus, Fn bound to protein would be present in the extracts. Plasma Fn is found in blood or plasma at concentrations of 0.3 mg/ml. However, the concentration of Fn in serum is 20%-50% less than the concentration in plasma. The extract material bound to microtiter plate wells was incubated with normal rabbit serum to ensure the saturation of all Fn binding proteins. Extracts from selected strains of _T. denticola_ were shown to bind Fn. Furthermore, extracts from _T. minutum_, _T. refringens_, _T. phagedenis_ biotype Reiter, _T. socranskii_ subsp. _buccale_ also contained proteins which are able to bind Fn in rabbit serum. _T. socranskii_ subsp. _socranskii_ also had the ability to bind rabbit Fn; however,not as well as the other organisms. Immunodiffusion analysis of _T. denticola_ strain oralis Triton-extract showed the presence of both rabbit Fn and rabbit albumin in the extract suggesting that serum proteins other than Fn can be avidly bound to treponemal cell surfaces. _T. pallidum_ has been shown to bind to other serum proteins such as; albumin, macroglobulin, transferrin, ceruloplasmin, immunoglobulin G,

immunoglobulin M, and C3 (1).

The next step was to try to isolate and identify the number of Fn-binding proteins in T. denticola Triton-extracts and determine their approximate molecular weights. T. denticola strain oralis extract was passed through an agarose-gelatin column that had Fn bound to it, to allow the binding of the treponemal proteins to the Fn. The proteins were eluted from the column and the eluate was found to contain treponemal proteins.

Three proteins in the eluate with molecular weights of 60K, 47K, and 21K reacted with T. denticola antiserum in Western blots. The 21K band was identified as a treponemal Fn-binding protein. This band was present only in the eluted Triton-extract protein. The band was absent in all of the controls. On the other hand the 60K and 47K bands may or may not be treponemal proteins or they could be a mixture of treponemal proteins and fragments of Fn. The T. denticola antiserum that was made in rabbits seemed to contain antibodies to two rabbit serum proteins that had molecular weights of 75K and 59K. These bands were shown by Western blots to be fragments of fibronectin. Baughn et al. (5) reported the presence of immune complexes (IC) in sera from patients with secondary syphilis. The IC consisted of an 87K treponemal protein which was thought to be the

fibronectin receptor of the organism. They also reported the presence of antibodies to Fn degradation proteins with molecular weights of 76K and 66K. It was possible that T. denticola cells bound Fn from the rabbit serum that it was grown in. The protein-Fn complex was injected into the rabbit and was recognized as foreign; therefore, antibodies were made against the Fn portion and the treponemal protein part of the complex. An immune complex could be formed by the Fn-binding proteins of T. denticola and Fn causing an autoimmune type of response. This may contribute to the inflammation and some tissue destruction associated with periodontal disease. It is also possible that the 60K and 47K bands are a mixture of Fn and treponemal protein. This can be determined by making antibodies against T. denticola cells that were grown in Fn-free serum or a serum substitute. If these bands are still present then they are treponemal proteins; however, if they do not show up when the membranes are incubated with T. denticola antiserum then the proteins are only Fn fragments.

SUMMARY

This study showed that *T. socranskii* subsp. *socranskii*, *T. socranskii* subsp. *buccale*, *T. socranskii* subsp. *paredis*, Treponeme D, *T. pectinovorum*, *T. denticola*, *T. vincentii*, and *T. phagedenis* biotype Reiter shared a group of common antigens. The common antigens were removed by the adsorption of the antisera with Reiter whole cells. The *T. socranskii* subspecies, Treponeme D, and *T. pectinovorum* shared an additional group of common antigens.

Triton extracts of oral and gentital treponemes, by immunodiffusion, crossed immunoelectrophoresis, ELISA and Western blots, were found to contain a small number of cross-reacting antigens. An immunodiffusion assay using the Triton-extracts was developed for the use in the routine identification of oral treponemes. The immunodiffusion test using Triton-extracts worked well in specifically identifying *T. denticola*, *T. vincentiii*, *T. pectinovorum* and *T. socranskii* to the species level. However, it did not differentiate between *T. socranskii* subsp. *socranskii*, *T. socranskii* subsp. *buccale*, *T. socranskii* subsp. *paredis*, and Treponeme D.

This study also showed that *T. denticola* cells contained fibronectin binding proteins on their cell surfaces and that extracts from these cells contained

these fibronectin binding proteins. Western blot analysis of treponemal proteins eluted from an agarose-gelatin-fibronectin column revealed three proteins with molecular weights of 67K, 47K, and 21K that reacted with *T. denticola* antiserum. The 21K protein band was identified as a treponemal protein; however, it is uncertain whether or not the 67K and 47K proteins are of treponemal origin or a mixture of treponemal proteins and fibronectin fragments.

LITERATURE CITED

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APPENDIX A

Source of Commercial Antisera, Human Fn and Human Fb

Sigma, St. Louis, Mo.: Human Fn
Human Fb
Goat anti-human Fn serum
Goat anti-human Fb serum

Cappel, Cochranville, Pa.: Goat anti-rabbit Fn serum
Goat anti-rabbit albumin serum
Goat anti-rabbit whole serum
Goat anti-rabbit 2-
macroglobulin serum

APPENDIX B

Treponeme Strains Subjected to DNA-DNA Homology

T. denticola : strains, T32-A, FM, Ichelson #2, Ambigua

T. vincentii : strains N-9

T. refringens : refringens C

T. phagedenis : biotype Reiter

T. socranskii subsp. *socranskii* : strains, DR56BRIII6, D43BR1, D20A2, DH56CR1

T. socranskii subsp. *buccale* : strains, D2B-8, D11A1, D40DPE1, D34B4, D22A5, D36FEP4

T. socranskii subsp. *paredis* : strains, D46CPE1, E2M1, D71CR4, D51BR5

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