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Characterization, Cloning, and Binding Properties of the Major 53-Kilodalton *Treponema denticola* Surface Antigen

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Treponema denticola **surface proteins were studied for their biochemical and biological characteristics. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of detergent extracts of whole cells revealed a major protein of 53 kDa and a number of minor proteins. Antiserum raised against whole cells of *T. denticola* ATCC 35405 reacted with the 53-kDa protein and a 72-kDa protein but not with the other proteins. Immunoelectron microscopy with anti-53-kDa-protein antibodies showed that the 53-kDa protein is located on the surface of the cell. SDS-PAGE analysis of unheated samples indicated that the 53-kDa protein is the major component of oligomers with molecular masses ranging from 130 to 300 kDa. Western blot (immunoblot) analysis showed that the high-molecular-mass oligomers reacted with whole-cell antiserum and anti-53-kDa-protein antibody. The aggregates dissociated into their subunits after heating to 70°C. Isoelectric focusing followed by SDS-PAGE indicated that the 53-kDa protein was separated into several forms with apparent pI values ranging from 8.0 to 5.5. The oligomeric forms were highly resistant to proteolysis by trypsin and proteinase K, whereas the monomeric proteins were readily digested. A clone expressing a 53-kDa antigen in *Escherichia coli* was isolated from a lambda ZAP II DNA library of *T. denticola* ATCC 35405. The recombinant protein had exactly the same molecular mass as the major 53-kDa *T. denticola* surface protein and reacted with antisera raised against this protein. The role of *T. denticola* ATCC 35405 surface proteins in attachment to laminin, fibronectin, gelatin, fibrinogen, and bovine serum albumin (BSA) was studied by a modified Western blot binding assay. Fibronectin, laminin, and fibrinogen attached to the 53-kDa surface protein of *T. denticola* as well as to a 72-kDa protein, whereas no attachment to gelatin or BSA was observed. Attachment could be inhibited by pretreating the blots with fibrinogen but not with gelatin or BSA. Our results suggest that the 53-kDa major surface protein of *T. denticola* may play a role in the attachment to host proteins and may thus be an important virulence determinant of this species.**

Adult periodontitis is characterized by the presence of a highly complex microflora (13, 16). Although no single species has been shown to be solely responsible for the initiation and/or the progress of the disease, several studies have suggested that certain species are more closely related to active, destructive periodontitis than others. Among the possible key species are the oral *Treponema* spp. (1, 11, 12, 21, 22). *Treponema denticola* is one of the treponemes closely associated with periodontitis (28, 29). This spirochete possesses a wide pattern of putative virulence factors, including motility; a number of hydrolytic enzymes, including at least two proteases; factors interfering with polymorphonuclear leukocyte function; and the ability to attach to host tissues and other oral bacteria (2, 4, 6–8, 14, 18–20, 24, 30, 32). For a better understanding of the pathogenic potential of *T. denticola*, we have focused on the clarification of its adherence properties. Recently, we reported that *T. denticola* proteins appear to be the adhesins mediating binding to a number of mammalian proteins (9). In this paper, we report the isolation and biological characterization of a major *T. denticola* surface protein and the cloning of the corresponding gene.

MATERIALS AND METHODS

Bacteria. *T. denticola* ATCC 35405, ATCC 35404, and ATCC 33520 were obtained from the American Type Culture Collection. For this study, *T. denticola* was grown in broth medium and maintained by weekly transfers. One hundred milliliters of broth contained 1.25 g of brain heart infusion medium (Difco Laboratories, Detroit, Mich.), 1 g of Trypticase (BBL Microbiology Systems, Cockeysville, Md.), 0.25 g of yeast extract (Difco), 0.05 g of sodium thioglycolate (Difco), 0.1 g of L-cysteine hydrochloride (Sigma Chemical Co., St. Louis, Mo.), 0.025 g of L-asparagine (Sigma), 0.2 g of glucose (Difco), 0.6 mg of thiamine pyrophosphate (Sigma), 0.001% (vol/vol) isobutyric acid (Sigma), 0.001% (vol/vol) isovaleric acid (Sigma), 0.001% (vol/vol) valeric acid (Sigma), 0.2% (wt/vol) sodium bicarbonate (Fisher), and 2 ml of heat-inactivated horse serum (GIBCO, Burlington, Ontario, Canada). Purity of the culture was checked each time by phase-contrast microscopy. For the experiments, a two-day-old liquid culture of *T. denticola* was collected by centrifugation (10,000 × *g*), washed twice in phosphate-buffered saline (PBS), and kept on ice. In some experiments, the cells were treated with 20 mM (final concentration) *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (Sigma). For cloning and the experiments with recombinants, we used *Escherichia coli* XL1-Blue (3) (Stratagene Cloning Systems, La Jolla, Calif.). For plasmid preparation, *E. coli* was grown in Terrific broth (23). For infection with λ phages (lambda ZAP II; Stratagene) (25), *E. coli* was grown in NZY medium (5 g of NaCl, 2 g of $MgSO_4 \cdot H_2O$, 5 g of yeast extract, 10 g of Casamino Acids per liter [pH 7.5]) supplemented with 0.2% maltose. Agar concentrations were 1.5% and 0.65% (top agar).

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Preparation of *T. denticola* proteins. Freshly cultured bacterial cells were incubated in 20 mM Tris buffer (pH 8.0) in the presence of a variety of anionic and nonionic detergents: 1% Nonidet P-40 (NP-40) (Sigma), 0.1% Triton X-100 (Sigma), 0.001 to 0.5% sodium dodecyl sulfate (SDS) (Sigma), 100 mM *n*-octyl-β-D-glucopyranoside (Sigma), 0.5% 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate (CHAPS) (Sigma), 0.5% Zwittergent 3-12 and 3-16 (Calbiochem, San Diego, Calif.), 10 mM deoxycholate (Sigma). The cells were gently shaken with the detergents at 20°C for 30 min and centrifuged (16,000 × *g*, 10 min). The supernatant and the pellet were analyzed as described below. The particulate fraction of *T. denticola* was obtained as follows. The cells were disrupted in 20 mM Tris buffer (pH 8.0) by ultrasonication (3-min pulse sonication at 20% duty cycle, output of 2) (Sonifier Cell Disrupter 350; Branson Sonic Power Co., Danbury, Conn.). Unbroken cells were removed by centrifugation (8,000 × *g*, 10 min). The supernatant was centrifuged again at 16,000 × *g* for 10 min, and the upper, lighter-colored, area of the pellet was carefully collected, washed twice, and used for the experiments as the particulate fraction.

FPLC. The bacterial protein extract obtained from strain ATCC 35405 by sonication and detergent extraction was fractionated with a fast-protein liquid chromatography (FPLC) system (Pharmacia, Uppsala, Sweden) using (i) gel filtration (Superose 12 HR10/30 column; 0.75 ml/min) in 0.5% SDS–0.05 M Tris–0.15 M NaCl buffer at pH 7.4 or (ii) anion-exchange chromatography on a Mono Q HR6 column equilibrated with 1% NP-40 in 20 mM Tris (pH 7.4). The bound proteins were eluted with a 0 to 2 M NaCl linear gradient in the buffer described above. The eluted fractions were further analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

SDS-PAGE and immunoblotting. SDS-PAGE was performed in 10 and 12% (wt/vol) polyacrylamide gels as described by Laemmli (10) by using a Mini-Protean II electrophoresis apparatus (Bio-Rad) at 200 V. Samples were solubilized in sample buffer (4% SDS, 2% glycerol, 1% 2-mercaptoethanol, 0.01% bromophenol blue in 125 mM Tris-HCl [pH 6.8]) either at room temperature or heated at 100°C for 5 min prior to electrophoresis.

For immunoblotting, polypeptides were transferred to nitrocellulose membranes. Before the attachment experiments, the membranes were blocked with 3% bovine serum albumin (BSA) in Tris-buffered saline (20 mM Tris, 0.5 M NaCl [pH 7.5]). When the attachment or inhibition by BSA was studied, 1% Tween 80 (Sigma)–1% SDS in Tris-buffered saline was used for blocking. Control experiments showed that similar attachment results were obtained with both blocking methods.

Isoelectric focusing. Isoelectric focusing of the detergent (NP-40) extract of *T. denticola* ATCC 35405 and the particulate fraction of cell sonic extracts was done in the presence of 9.5 M urea and 20% NP-40 by using a Mini-Protean II two-dimensional apparatus (Bio-Rad). The first-dimension capillary gels contained 1.6% 5-8 Bio-Lyte (Bio-Rad) and 0.4% 3-10 Bio-Lyte (Bio-Rad) and were subsequently analyzed in the second dimension by SDS-PAGE as described above.

Enzyme and heat treatment of *T. denticola* proteins. The non-water-soluble protein fraction of *T. denticola* (5 mg of protein per ml) was treated with a mixture of glycosidase (100 μg/ml; Miles Laboratories Inc., Elkhart, Ind.), proteinase K (25 μg/ml; BRL, Gaithersburg, Md.), and trypsin (25 μg/ml) at 37°C for 1 h and then mixed with sample buffer for SDS-PAGE. Two parallel samples were prepared; one was heated at 100°C for 5 min, and the other one was kept at room temperature.

Heat modification of *T. denticola* ATCC 35405 and ATCC 35404 proteins was studied by incubating a sonic extract of *T. denticola* cells for 5 min at 60, 70, 80, and 100°C.

Preparation of antisera. A New Zealand White rabbit was immunized with whole cells of *T. denticola* ATCC 35405. One milligram of cells was injected intramuscularly with complete Freund's adjuvant. Subsequent intramuscular injections without adjuvant were performed after 1, 2, 3, 5, and 7 weeks. The rabbit was bled one week after the last booster. The specificity of the antiserum was determined by enzyme-linked immunosorbent assay using alkaline phosphatase-conjugated goat anti-rabbit antibody (1:3,000; BRL). The highest dilution to give an optical density at 405 nm of >0.3 (2550 EIA Reader, Bio-Rad), was 1:12,800. The control (no bacteria) and *Fusobacterium nucleatum* ESF4, used as a reference, gave values of <0.15 at all antiserum dilutions tested (1:100 to 1:25,600).

Antibody to the *T. denticola* (ATCC 35404) 53-kDa protein was prepared from material eluted from an SDS-PAGE gel of the *T. denticola* particulate fraction. Following electrophoresis, the gel was electroblotted; under our blotting conditions, the 53-kDa protein transfers poorly (approximately 20%), whereas contaminating proteins transfer with high efficiency. After blotting, the gel was sliced to remove the 53-kDa band. The gel was equilibrated with PBS, and an aliquot containing 50 to 80 μg of protein was emulsified with Freund's complete adjuvant and injected into the rabbit. Subsequent injections of the same amount of protein were administered with Freund's incomplete adjuvant at weeks 3 and 6; injections without any adjuvant were administered at weeks 9 and 12. Antibody against the 53-kDa protein was also prepared by an alternate protocol by using affinity purification from the whole-cell antiserum. In the latter procedure, a partially purified preparation of the 53-kDa protein was subjected to SDS-PAGE and transferred to nitrocellulose membranes. Two reference lanes were stained with Coomassie brilliant blue to localize the proteins. The membrane piece containing the 53-kDa protein was excised, blocked with 3% BSA, and incubated at 4°C for 3 h with 1 ml of a 1:10 dilution of *T. denticola* whole-cell antiserum. After being washed once with PBS and twice with TTBS (see below), the antibody attached to the 53-kDa protein was eluted by treatment of the membranes with 50 mM glycine–500 mM NaCl–0.5% Tween 20–200 μg of BSA per ml at pH 2.3. The eluted antibody was immediately neutralized with 100 mM Na_2HPO_4 and stored at −20°C. To obtain affinity-purified antibodies directed against recombinant proteins produced from clone 1, the proteins from one plate lysate were bound to an S&S Nytran membrane (Schleicher & Schuell, Keene, N.H.). The membrane was then blocked with 3% skim milk in 0.05% Tween 20 in Tris-buffered saline (TTBS) and incubated with *T. denticola* whole-cell antiserum (100 ml in 4 ml of TTBS–0.5% skim milk) overnight at 4°C. The membrane was extensively washed (three times, for 15 min each time) with TTBS. The absorbed antibodies were eluted with 2 ml of 8 M urea in TTBS (15 min at room temperature) and immediately diluted fourfold with TTBS–0.5% skim milk and stored on ice. The final dilution in Western blots was 1:1,000, calculated from the original amount of whole-cell serum (100 μl) used.

Electron microscopy. The location of the 53-kDa protein on *T. denticola* was studied by immunoelectron microscopy using the anti-53-kDa-protein antibody. Bound antibody was

detected with a goat anti-rabbit antibody conjugated to gold particles (10 nm; Sigma). Carbon-coated nickel grids were coated with *T. denticola* cells in 20 mM Tris-HCl, pH 8.0, washed, incubated for 1 h with primary antibody (1:20), washed again (five times for 3 min each time), and incubated with the second antibody (1:50) for 1 h. After being washed, the grids were negatively stained with 2% phosphotungstic acid and studied by transmission electron microscopy (EM 10C/CR STEM; Zeiss, Oberkochen, Germany) at 60 kV.

Preparation of chromosomal DNA from *T. denticola* and construction of a genomic library. DNA from *T. denticola* ATCC 35405 was prepared as described by Silhavy et al. (26), with the following modifications. To ensure complete and rapid cell lysis and inactivation of endogenous endonucleases, the final SDS concentration was raised to 2% and the incubation temperature during lysis was 65°C. Chromosomal DNA was then partially digested with *Alu*I, *Hae*III, and *Rsa*I (three separate digests). The digests were combined and applied to a 10 to 40% sucrose gradient (23). Fractions containing DNA in the size range from 3 to 10 kbp were pooled and concentrated by ethanol precipitation. After attachment of *Eco*RI adaptors (Regional DNA Synthesis Laboratory, University of Calgary, Calgary, Alberta, Canada), the size-fractionated *T. denticola* DNA was ligated to dephosphorylated *Eco*RI λ ZAP II arms and packaged with Gigapack II Gold packaging extract (Stratagene). A total of 1×10^6 to 2×10^6 PFU was obtained, with an insert frequency greater than 90%.

Identification of *T. denticola* recombinant clones. The library was plated on *E. coli* XL1-Blue in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at a density of approximately 10^4 plaques per plate. After growth for 6 h at 37°C, nitrocellulose filter discs (Schleicher & Schuell) were layered onto the soft agar and phage growth was continued for an additional 2 to 3 h. The filters were blocked and screened with whole-cell antiserum against *T. denticola*. Plaques reacting strongly with the antiserum were purified and further analyzed. For Western blot analysis of recombinant phages, the top agar of one plate with confluent lysis was scraped off and frozen at −20°C. The agar was thawed, and the proteins were collected in the supernatant after centrifugation. This supernatant was used for Western blot analysis and affinity purification of antibodies.

Subcloning of the 5.5-kbp recombinant fragment containing the gene for the 53-kDa protein. For subcloning, we constructed a medium-copy-number vector with the multiple cloning site of pBluescript II KS (Stratagene) flanked by the β-galactosidase and a T3 promoter on one side and a T7 promoter on the other side (see Fig. 7 [pKX10]). The *Pvu*II fragment of pBluescript II KS containing the multiple cloning site was cloned into the large *Sca*I fragment of pSUP401 (27), which removes most of the chloramphenicol acetyltransferase gene (see Fig. 7). The 5.5-kb *Not*I recombinant fragment of λ ZAP II recombinant clone 1 was ligated into the *Not*I site of the vector described above. Clones with the fragment in both possible orientations were obtained (pKX101 [clone 4] and pKX102 [clone 7]).

Preparation of plasmid DNA. Plasmid DNA was prepared by the rapid alkaline lysis method (23) with the modifications suggested by Morelle (17).

Attachment of various proteins to *T. denticola*. Proteins from whole-cell lysates of *T. denticola* ATCC 35405 were separated by SDS-PAGE and transferred to a nitrocellulose membrane. After the membrane was blocked with 3% BSA–Tris-buffered saline for 1 h and rinsed with distilled water, it was incubated for 1 h with 10 μg of laminin (Sigma), fibronectin (Sigma), or biotinylated gelatin or biotinylated fibrinogen per ml in Tris-buffered saline. This was followed by three washes with TTBS. Adherent laminin and fibronectin were detected by specific antibodies (rabbit anti-mouse laminin, Sigma; goat anti-human fibronectin, Sigma) diluted 1:2,000 and alkaline phosphatase-conjugated secondary antibodies (1:3,000). Bound biotinylated fibrinogen and biotinylated gelatin were detected with a streptavidin-alkaline phosphatase conjugate (Bio-Rad) diluted 1:20,000. The filters were developed with nitroblue tetrazolium (330 μg/ml) (Bio-Rad) and 5-bromo-4-chloro-3-indolylphosphate (167 μg/ml) (Bio-Rad) in 100 mM Tris-HCl–100 mM NaCl–50 mM $MgCl_2$, pH 9.5. Biotinylation was done as follows. Protein (10 mg in 1 ml of PBS) was incubated with 2 μl of ε-caproylamido-biotin-*N*-hydroxysuccinimide ester (50 mg/ml in dry dimethyl sulfoxide) (BRL) at room temperature for 2.5 min, and the reaction was stopped by adding 50 mM Tris (final concentration), pH 7.0. Each experiment included controls lacking the receptor protein or the first antibody.

Inhibition of attachment and receptor specificity. Previously we have shown that attachment of *T. denticola* to proteins was almost completely abolished by the sulfhydryl-blocking agents *p*-chloromercuribenzoate and oxidized glutathione (9). To investigate the effect of these substances on the binding of laminin to specific *T. denticola* proteins, nitrocellulose membranes blotted with *T. denticola* proteins and blocked with BSA were incubated for 1 h with *p*-chloromercuribenzoate (2 mM; Sigma) or oxidized glutathione (10 mM; ICN Pharmaceuticals, Cleveland, Ohio) before incubation with laminin. Receptor specificity was studied by an inhibition assay using either 1% fibrinogen or 1% gelatin for 1 h.

RESULTS

Biochemical characterization of the proteins. NP-40 detergent extracts of whole cells and the particulate fraction of sonicated *T. denticola* cells were analyzed by SDS-PAGE (Fig. 1, lanes A and B). In both samples, most of the protein was found in a 53-kDa band. Similar results were obtained when whole cells of *T. denticola* ATCC 35404, ATCC 35405, and ATCC 33520 were extracted with Triton X-100, SDS, octylglucoside, CHAPS, Zwittergent 3-12, Zwittergent 3-16, and deoxycholate. The protein profile remained the same regardless of whether reducing or nonreducing sample buffers were used. The 53-kDa protein band was absent from samples which had not been heated prior to electrophoresis, and instead, a number of protein bands appeared in the 130- to 200-kDa region. In addition, a distinct band of about 300 kDa was detected (Fig. 2A). These high-molecular-mass complexes were dissociated, and the 53-kDa protein appeared to be the major component when samples were incubated at 70 or 80°C (Fig. 2, lanes C and D). When the 300-kDa and 130- to 200-kDa proteins were eluted from the SDS gels, heated in sample buffer (100°C), and electrophoresed again, the 300-kDa protein was observed to disaggregate into proteins of 53 and 45 kDa (Fig. 2, lane E), whereas the 130- to 200-kDa complex of proteins yielded the 53-kDa protein and sometimes an 85-kDa protein (Fig. 2, lane G). These high-molecular-mass complexes were highly resistant to treatment with a wide range of proteases, including the intrinsic chymotrypsin-like protease (Fig. 3, lane H). In subunit form, the proteins were readily degraded by these proteases. That can be seen when the complexes were dissociated by heating prior to the protease treatment (see Fig. 3, lane I). Two-dimensional PAGE suggested that the

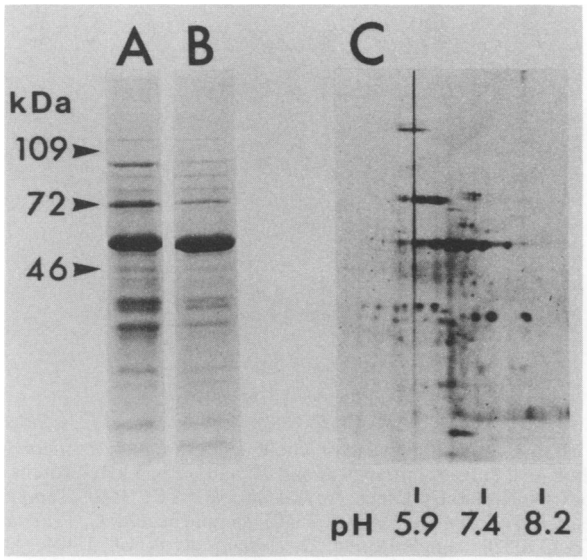

FIG. 1. SDS-PAGE and isoelectric focusing of *T. denticola* ATCC 35405 protein extracts. Lanes: A, NP-40 extract of whole cells; B, NP-40 extract of the particulate fraction of sonicated *T. denticola*; C, isoelectric focusing of proteins of the particulate fraction.

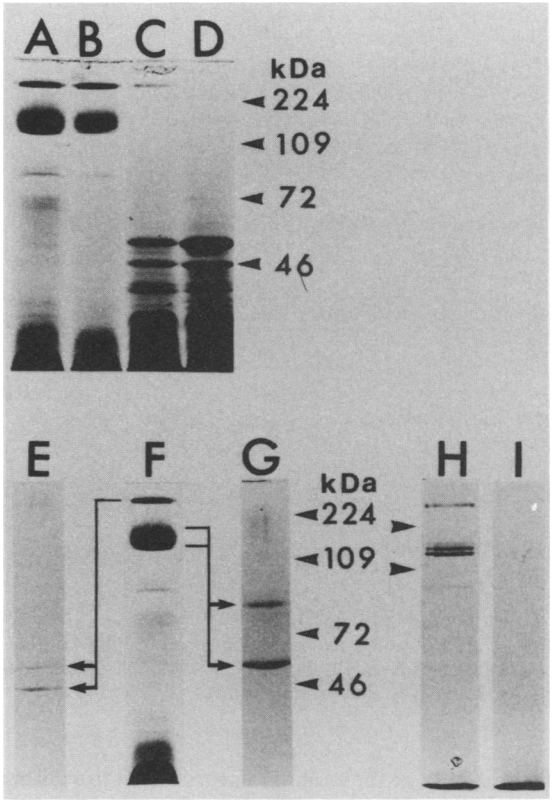

FIG. 2. Effect of heat and proteolytic treatment on *T. denticola* ATCC 35405 protein; SDS-PAGE protein profiles. Lanes: A, SDS-PAGE of unheated whole-cell extract; B to D, incubation at 60, 70, and 80°C, respectively, prior to electrophoresis; E and G, SDS-PAGE of the 300-kDa and 130- to 200-kDa proteins (shown in lane F) following their isolation from the gels and heating at 100°C; H and I, proteinase K treatment of the particulate fraction of *T. denticola* ATCC 35405 before (H) and after (I) heating at 100°C for 5 min.

53-kDa protein exists in several forms having isoelectric points ranging from 8.0 to 5.5 (Fig. 1, lane C). These may be true subunit isoforms, or they may have had similar pIs and simply have existed in oligomeric forms which had different pIs. Some lower-molecular-mass proteins also showed forms with different pI values. These lower-molecular-mass proteins were detected with the anti-53-kDa-protein antibody (see below) and are probably degradation products of this protein.

In order to partially purify the proteins, detergent-solubilized whole-cell extracts and the particulate fraction of sonicated *T. denticola* cells were subjected to gel filtration and anion-exchange chromatography using FPLC. Adding SDS (SDS was used only in gel filtration), NP-40, Triton X-100, or urea did not change the chromatographic behavior of the proteins. When the fractions collected from gel filtration were analyzed by SDS-PAGE, the 53- and 72-kDa proteins were present in several fractions covering a wide molecular mass range. Figure 3 shows separation of proteins from the particulate fraction of *T. denticola* ATCC 35405 by anion-exchange chromatography on a Mono Q column. A sharp peak containing the 53-kDa protein and the 72-kDa protein could be obtained.

The relationship between the 53-kDa protein and the high-molecular-mass proteins was investigated immunologically (Fig. 4). Proteins from whole-cell lysates separated by SDS-PAGE and transferred to nitrocellulose were reacted with antisera against *T. denticola* ATCC 35405 cells (Fig. 4, lanes A and B), the *T. denticola* 53-kDa protein eluted from a gel, or antibodies affinity purified with the recombinant proteins. The whole-cell antiserum reacted with the 53- and 72-kDa proteins in heated samples and with the >130-kDa complexes in the unheated sample. The antiserum also detected several low-molecular-mass proteins; the number and size of these polypeptides varied from preparation to preparation (Fig. 4, lanes A and B). The anti-53-kDa-protein antibody reacted with the 53-kDa band from all three strains tested (Fig. 4, lanes C to E). Weak reactions at the >130-kDa region were detected with strains ATCC 35405 and ATCC 35404 even in heated samples. The anti-53-kDa-protein antibody also reacted with several low-molecular-mass bands of the strains; the strongest reaction was with strain ATCC 35405. However, similar to experiments with whole-cell antiserum, the number and intensity of staining of the lower-molecular-mass bands varied from preparation to preparation. The 72-kDa protein showed no cross-reactivity with the anti-53-kDa-protein antiserum.

Immunogold bead labelling with anti-53-kDa-protein antibody showed that the 53-kDa protein was located on the *T. denticola* cell surface (Fig. 5). Gold beads can be seen on the cell surface and on material dissociated from the cell. The latter case was not observed with cells reacted with preimmune serum. The dissociated cell wall material is particularly noticeable when cells were reacted with whole-cell antiserum (Fig. 5A).

Cloning of DNA expressing the 53-kDa *T. denticola* antigen. To obtain a *T. denticola* gene library with a random distribution of recombinant fragments, we partially digested treponemal chromosomal DNA with three restriction enzymes having a 4-bp recognition sequence and cloned into the lambda expression vector λ ZAP II. The recombinant phages were plated onto *E. coli* XL1-Blue in the presence of IPTG to induce expression from the *lacZ* promoter. Screening with antiserum against whole cells of *T. denticola* ATCC 35405 revealed more than 100 positively reacting clones per

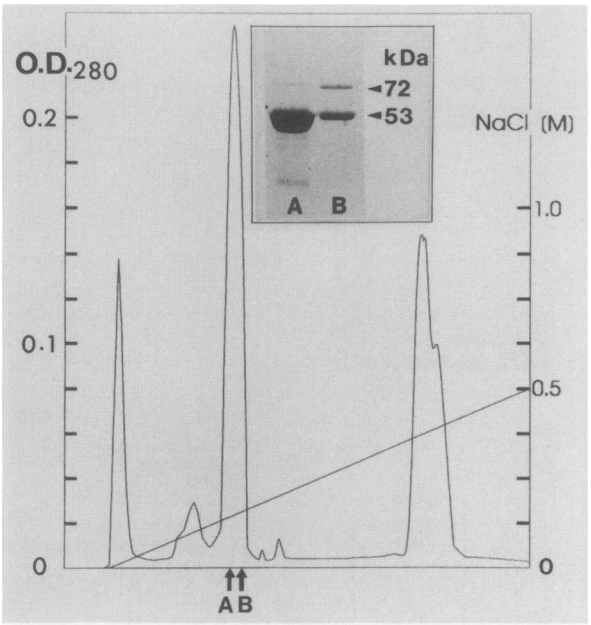

FIG. 3. FPLC anion-exchange chromatography of the water-soluble fraction of *T. denticola* ATCC 35405. SDS-PAGE analysis of fractions A and B is shown in the inset.

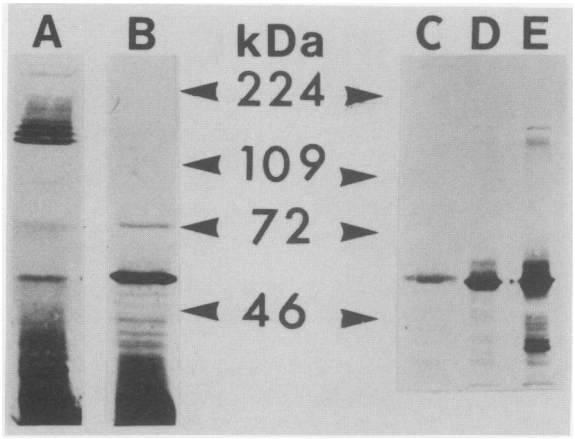

FIG. 4. Immunoblotting of whole-cell lysate of *T. denticola* with whole-cell antiserum (lanes A and B) and anti-53-kDa-protein antibody (lanes C to E). Lanes: A, *T. denticola* ATCC 35405, sample not heated; B, *T. denticola* ATCC 35405, sample heated; C, *T. denticola* ATCC 33520, sample heated; D, *T. denticola* ATCC 35404, sample heated; E, *T. denticola* ATCC 35405, sample heated.

10,000 plaques of the original library. Six strongly reacting clones were isolated, purified, and further analyzed by SDS-PAGE and Western blotting. Unique bands representing recombinant proteins were not detected by either Coomassie blue or silver stain. Immunoblotting of the clone 1 lysate showed two recombinant proteins of 53 and 44 kDa that reacted with the *T. denticola* whole-cell antiserum (Fig. 6, lane F). The 53-kDa protein comigrated with the 53-kDa surface component of *T. denticola* (Fig. 6, lanes A and B).

Subcloning of the recombinant fragment encoding the 53-kDa protein. DNA isolated from λ ZAP II clone 1 contained a 5.5-kbp *Not*I insert. The insert was subcloned into the *Not*I site of pKX10 (Fig. 7). We obtained clones with the fragment inserted in both orientations. Clones 4 (pKX101, + orientation; expression of 53-kDa protein) and 7 (pKX102, − orientation; no expression of the 53-kDa protein) were further analyzed in detail. Whole-cell lysates (samples were not heated) of XL1-Blue/pKX101 or XL-1 Blue/pKX102 grown with and without IPTG were separated by SDS-PAGE, transferred to nitrocellulose, and reacted with *T. denticola* whole-cell antiserum. Clone 4 expressed immunoreactive recombinant polypeptides of 53, 48, and 44 kDa (Fig. 6, lane D). The expression of the 53-kDa protein was increased in the presence of IPTG. The clone with the insert in the opposite orientation (pKX102) expressed no 53-kDa protein, only the 48- and 44-kDa antigens (Fig. 6, lane C). The gene for the 53-kDa protein is obviously not transcribed from its own promoter but requires the *lacZ* promoter (see restriction maps [Fig. 7]) for its expression.

Identification of the 53-kDa recombinant protein as the major surface protein of *T. denticola*. To investigate the relationship of the recombinant protein and the 53-kDa protein of *T. denticola*, we chose to use affinity-purified antibodies. Affinity-purified antibodies were prepared with the 53-kDa protein of *T. denticola* and were prepared in a similar manner with the 53-kDa recombinant protein. Both antibodies reacted with the *T. denticola* 53-kDa protein and the 53-kDa recombinant protein in a Western blot, demonstrating their immunological similarity (Fig. 6, lanes G to N). These antibodies reacted with the high-molecular-mass complexes observed in SDS-PAGE run with samples that were not heated prior to electrophoresis (data not shown). Both affinity-purified antibodies also detected several polypeptides smaller than 53 kDa in *T. denticola* whole-cell extracts, suggesting that these are proteolytic degradation products. The whole-cell antiserum (Fig. 6, lanes A to F) recognized the 53-kDa recombinant protein.

Attachment of laminin, fibronectin, fibrinogen, BSA, and gelatin to *T. denticola* proteins. The role of the 53-kDa surface protein in binding a number of "matrix proteins" was investigated in a modified immunoblot assay. Fibronectin, laminin, and fibrinogen all attached to the nitrocellulose-bound 53-kDa surface protein of *T. denticola* (Fig. 8, lanes A, B, and D). They also bound to a number of other *T. denticola* proteins; of these, only a 72-kDa protein and a 45-kDa protein reacted with whole-cell antiserum raised to *T. denticola* (Fig. 4, lane B; Fig. 6, lanes A and B). The 45-kDa protein was present in only some preparations and reacted with anti-53-kDa antibody (Fig. 6, lane N), suggesting that it is a degradation product of the 53-kDa protein. Gelatin and BSA did neither bind to nor inhibit binding of the matrix proteins described above to any *T. denticola* proteins (Fig. 8, lanes C, E, and F), although we have previously shown that they bind to whole *T. denticola* cells (9). Binding of laminin to the filter-bound *T. denticola* proteins could, however, be inhibited when the filter was incubated with 1% fibrinogen for 1 h before the addition of laminin (Fig. 8, lane G). Pretreatment of the *T. denticola* proteins bound to the nitrocellulose filter with sulfhydryl reagents, *p*-chloromercuribenzoate, and oxidized glutathione, which all inhibit binding in vivo, did not inhibit binding of laminin in this kind of assay (Fig. 8, lane H).

DISCUSSION

Recent studies have drawn attention to *T. denticola* proteins in the 52- to 54-kDa region (5, 15, 31). Cockayne et al. (5) studied protein profiles of *T. denticola* ATCC 33520 and found a major protein of 54 kDa obtained by detergent

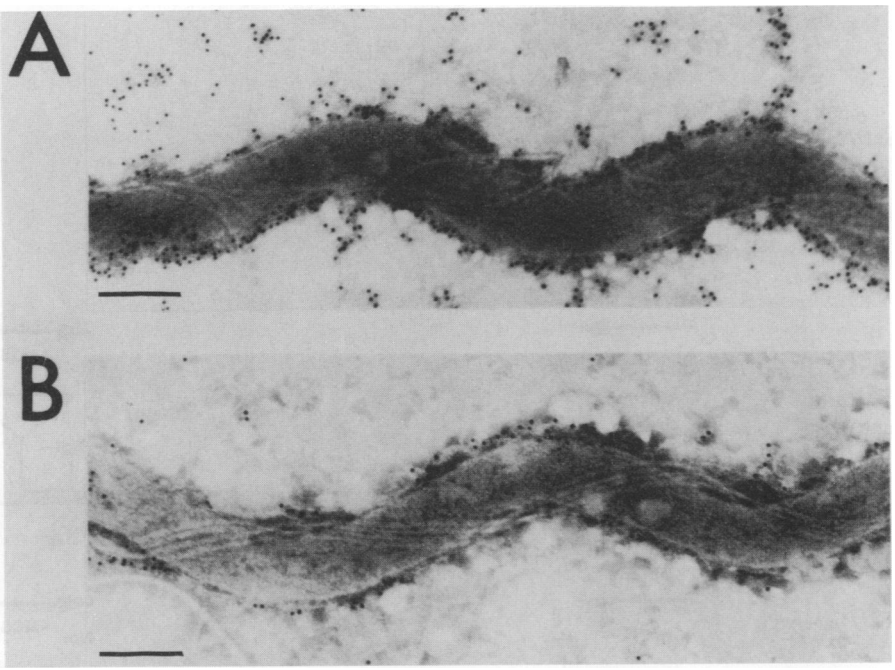

FIG. 5. Immunogold labelling of *T. denticola* with whole-cell antiserum (A) and anti-53-kDa-protein antibody (B). Bars, 200 nm.

extraction. They assumed this protein to be a breakdown product of a large, heat-modifiable polypeptide. It is probable that the 53-kDa protein that we describe here is present in high-molecular-mass aggregates in the intact cell. Indeed, we found that electrophoresis of unheated NP-40 extracts yielded a number of high-molecular-mass proteins which dissociated into a 53-kDa protein upon heating. Although the 53-kDa protein was the predominant component in all of the complexes, the 300-kDa complex was different from the 130- to 200-kDa complexes with respect to the minor components (Fig. 2, lanes E and G). High salt concentrations did not dissociate the aggregates, nor did repeated cycles (45 times) of freezing and thawing (data not shown).

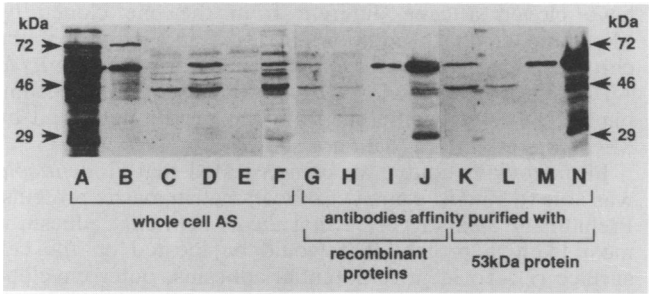

FIG. 6. Identification of recombinant proteins by Western blotting. Lanes A to F, protein incubated with whole-cell antiserum raised against *T. denticola* ATCC 35405; lanes G to J, protein incubated with antibodies affinity purified with a lysate from λ ZAP II recombinant clone 1; lanes K to N, protein incubated with antibodies affinity purified with pure 53-kDa protein from *T. denticola* ATCC 35405. Lanes A, J, and N, *T. denticola* ATCC 35405 whole-cell lysate; lanes B, I, and M, semipurified 53-kDa protein from *T. denticola* ATCC 35405; lanes C, H, and L, whole-cell lysate from *E. coli* XL-1 Blue/pKX102; lanes D, G, and K, whole-cell lysate from *E. coli* XL-1 Blue/pKX101; lane E, whole-cell lysate from *E. coli* XL-1 Blue/pKX10; lane F, lysate from λ ZAP II recombinant clone 1.

The high-molecular-mass aggregates were highly resistant to hydrolysis by proteases, whereas the 53-kDa monomeric form was readily degraded. This is interesting in light of our earlier observation, that proteinase K had no effect on the attachment of *T. denticola* to a number of matrix proteins (9). Our results may indicate, therefore, that the in situ conformation of *T. denticola* surface proteins protects them against proteolytic enzymes produced by the bacterial cells themselves as well as by the host or other bacteria.

Umemoto et al. (31) showed that a 53-kDa protein was a major antigen of the cell envelope of *T. denticola* ATCC 33520. In their study, anti-53-kDa-protein antibodies showed no cross-reactivity with other *T. denticola* strains, including strains ATCC 35405 and ATCC 35404. Our experiments indicate that the 53-kDa proteins of these three strains share at least some common antigenic determinants, since the antibody against the 53-kDa protein of *T. denticola* ATCC 35404 cross-reacted strongly with the 53-kDa band of strain ATCC 35405 and a clear, although weaker, reaction was observed also with the 53-kDa protein of strain ATCC 33520. Our results, obtained by using polyacrylamide electrophoresis, Western blotting, and immunoelectron microscopy, suggest that the 53-kDa protein described here is similar to the proteins studied by Umemoto et al. (31) and Cockayne et al. (5).

With whole-cell and anti-53-kDa-protein antiserum, we were able to identify a recombinant *E. coli* clone that expressed the gene for the 53-kDa major surface protein of *T. denticola* ATCC 35405. The gene requires the *lacZ* promoter of the vector for expression. Miyamoto et al. (15) recently reported the cloning of an antigen gene from *T. denticola* Johnson producing a recombinant protein in *E. coli* with a molecular mass of 53 kDa. The authors concluded that their protein was different from those reported by Cockayne et al. (5) and Umemoto et al. (31). Our results strongly

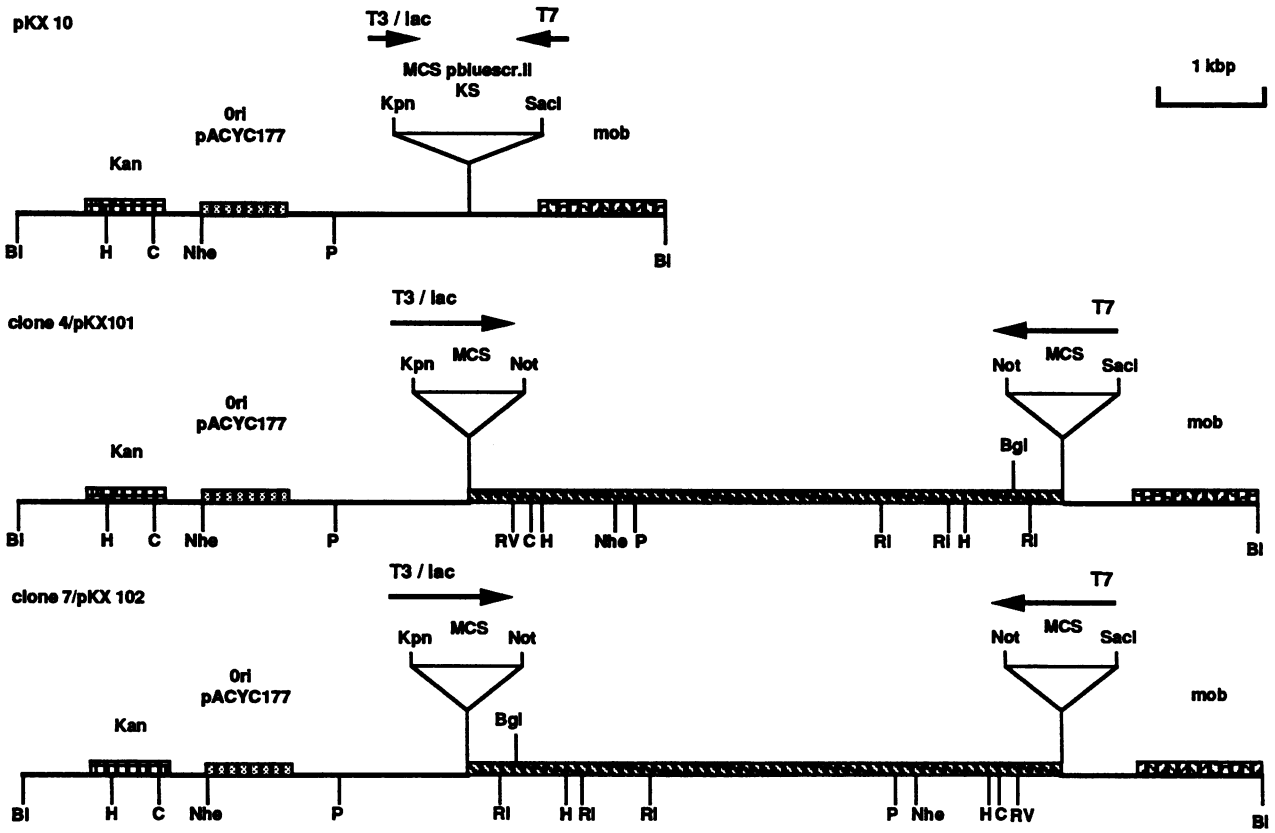

FIG. 7. Restriction maps of recombinant clones containing the gene for the 53-kDa protein. pKX10 was derived from pSUP401 (27) by introducing the *Pvu*II fragment containing the *lacZ*, T7, and T3 promoters and the multiple cloning site from pBluescript II KS(−). The plasmids pKX101 and pKX102 are derived from pKX10 by cloning the 5.5-kbp *Not*I recombinant fragment from λ ZAP II recombinant clone 1 into its *Not*I site. The 53-kDa recombinant protein is expressed from pKX101 but not from pKX102, which contains the insert in the opposite orientation. Abbreviations: BI, *Bam*HI; Bgl, *Bgl*II; C, *Cla*I; H, *Hin*dIII; Kpn, *Kpn*I; Nhe, *Nhe*I; Not, *Not*I; P, *Pst*I; RI, *Eco*RI; RV, *Eco*RV; SacI, *Sac*I.

indicate that the *T. denticola* gene that we have cloned codes for the same protein studied by Umemoto et al. (31). In both studies, the 53-kDa protein was the major component of the cell surface as shown by SDS-PAGE and immunoelectron microscopy as well as the major antigen when whole cells were used for immunization of rabbits. Data from immunoblots or protein electrophoresis do not allow comparison between the present study and the one by Miyamoto et al. However, the observations noted above indicate that we have cloned a gene different from the one cloned by Miyamoto et al. In addition, the size and pattern of the chromosomal *Eco*RV and *Pst*I fragments from *T. denticola* ATCC 35405 and ATCC 33520 hybridizing with our recombinant DNA were different from the results published by Miyamoto et al. (15) (data not shown).

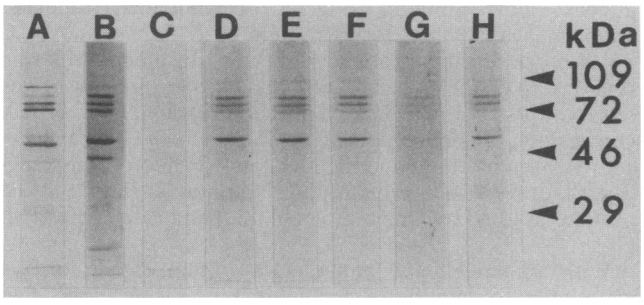

FIG. 8. Binding of various proteins to an NP-40 extract of *T. denticola* ATCC 35405 bound to nitrocellulose membranes. NP-40 extracts of *T. denticola* ATCC 35405 were electrophoresed, and the proteins were transferred to nitrocellulose and incubated with a number of mammalian proteins. The detection of the bound proteins was described in Materials and Methods. Lanes: A, fibronectin; B, fibrinogen; C, gelatin; D, laminin; E, laminin after gelatin blocking; F, laminin after BSA blocking; G, laminin after fibrinogen blocking; H, laminin after treatment of the membrane with oxidized glutathione.

In a previous study, we demonstrated that *T. denticola* was able to bind to a number of mammalian matrix proteins. Preliminary data suggested that the *T. denticola* adhesin is most likely a protein that would be located on the cell surface (9). To identify potential adhesins, outer envelope preparations were separated by SDS-PAGE and transferred to nitrocellulose, where they were exposed to a number of mammalian proteins. Binding of these proteins was then determined immunologically. Laminin, fibrinogen, and fibronectin bound to a number of *T. denticola* proteins, whereas BSA and gelatin did not bind to any protein. The multiple binding profiles raise the question of significance and the relative importance of each binding protein in the adhesive interaction of the whole cell. We believe that the 53-kDa protein may be an adhesin, because of its ability to bind fibronectin, fibrinogen, and laminin and its location on the *T. denticola* cell surface. This finding is supported by the

observation that the high-molecular-mass forms of the protein also bind to eukaryotic proteins (data not shown) and that this form of the molecule is resistant to proteases, as is the binding reaction.

The 45-kDa band was detected by SDS-PAGE only occasionally. When present, it was detected with the anti-53-kDa-protein antibody in Western blot analysis and it bound to fibronectin, laminin, and fibrinogen. This suggests that the 45-kDa protein is a degradation product of the 53-kDa protein. The anti-53-kDa-protein antibodies reacted with a number of low-molecular-mass polypeptides in the *T. denticola* whole-cell lysate. *T. denticola* produces potent proteases, and our results show that the 53-kDa protein is susceptible to proteolytic degradation. The bands smaller than 53 kDa reacting with the anti-53-kDa-protein antibody are most likely degradation products of the same protein. Four other *T. denticola* proteins ranging from 75 to 95 kDa that also bound to substrate proteins could not be detected with the anti-53-kDa-protein antiserum or antiserum prepared against whole *T. denticola* cells. It appears that they are not exposed to the surface of *T. denticola*, and their importance in host protein binding is questionable.

In conclusion, we have cloned a *T. denticola* gene coding for a 53-kDa major surface protein which is also the major antigen of studied *T. denticola* strains. The 53-kDa protein readily forms protein aggregates that are highly resistant to proteases. Our results also suggest that the 53-kDa protein may play a role in the attachment of *T. denticola* to host tissues.

ACKNOWLEDGMENTS

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