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Sequence Analysis, Expression, and Binding Activity of Recombinant Major Outer Sheath Protein (Msp) of *Treponema denticola*

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The gene encoding the major outer sheath protein (Msp) of the oral spirochete *Treponema denticola* ATCC 35405 was cloned, sequenced, and expressed in *Escherichia coli*. Preliminary sequence analysis showed that the 5′ end of the *msp* gene was not present on the 5.5-kb cloned fragment described in a recent study (M. Haapasalo, K. H. Müller, V. J. Uitto, W. K. Leung, and B. C. McBride, Infect. Immun. 60:2058–2065, 1992). The 5′ end of *msp* was obtained by PCR amplification from a *T. denticola* genomic library, and an open reading frame of 1,629 bp was identified as the coding region for Msp by combining overlapping sequences. The deduced peptide consisted of 543 amino acids and had a molecular mass of 58,233 Da. The peptide had a typical prokaryotic signal sequence with a potential cleavage site for signal peptidase I. Northern (RNA) blot analysis showing the *msp* transcript to be approximately 1.7 kb was consistent with the identification of a promoter consensus sequence located optimally upstream of *msp* and a transcription termination signal found downstream of the stop codon. The entire *msp* sequence was amplified from *T. denticola* genomic DNA and cloned in *E. coli* by using a tightly regulated T7 RNA polymerase vector system. Expression of Msp was toxic to *E. coli* when the entire *msp* gene was present. High levels of Msp were produced as inclusion bodies when the putative signal peptide sequence was deleted and replaced by a vector-encoded T7 peptide sequence. Recombinant Msp purified to homogeneity from a clone containing the full-length *msp* gene adhered to immobilized laminin and fibronectin but not to bovine serum albumin. Attachment of recombinant Msp was decreased in the presence of soluble substrate. Attachment of *T. denticola* to immobilized laminin and fibronectin was increased by pretreatment of the substrate with recombinant Msp. These studies lend further support to the hypothesis that Msp mediates the extracellular matrix binding activity of *T. denticola*.

Periodontal diseases are characterized by mixed bacterial infections associated with progressive destruction of the tissue surrounding and supporting the teeth (37, 41, 46, 54, 55). Oral spirochetes, including *Treponema denticola*, are associated with both acute and chronic periodontal diseases and are frequently found in high numbers at diseased sites (3, 38, 40, 60). The interaction of periodontal organisms with host tissues is complex, involving mechanisms of adhesion, proteolysis, and evasion and modulation of the immune system (37, 39, 46, 54, 55). *T. denticola* binds to a number of extracellular matrix (ECM) components and other host factors that may mediate binding to gingival tissue (11, 18, 23). Several studies have demonstrated that *T. denticola* adheres to and damages a number of different host cell types (5, 31, 48, 52, 66, 72). *T. denticola* is able to interfere with host defenses (57) and induce tissue damage (5, 16, 52, 62). It has been shown to interact with other organisms populating the oral cavity in what may be an important step in colonization (19, 32).

A variety of *T. denticola* surface antigens with molecular masses in the range of 50 to 65 kDa have been identified (9, 22, 45, 47, 61, 69, 70, 73, 75). Several of these surface antigens have been proposed as adhesins directed toward host cell ECM components (22, 68). The major protein component of the outer sheath of the oral spirochete *T. denticola* ATCC 35405 is a 53-kDa protein (Msp). Msp is a highly immunogenic protein found on the cell as a protease-resistant, heat-modifiable high-molecular-weight complex (22). Our previous studies showed that Msp is organized as a regular hexagonal array in the outer sheath of *T. denticola*. Transmission electron microscopy of whole cells and isolated outer sheaths showed dark-staining hexagonal patterns with center-to-center distance of approximately 20 nm (12). ECM components including fibronectin, fibrinogen, and laminin, but not bovine serum albumin (BSA) or gelatin, bound to immobilized Msp in a modified Western blot (immunoblot) (22). *T. denticola* whole cells were found to similarly bind ECM components in an enzyme-linked immunosorbent assay (ELISA) (23).

In addition to its apparent role as an adhesin targeted to abundant host cell surface molecules, Msp has properties of a bacterial outer membrane porin. In this respect, it may be functionally similar to the major outer membrane porins of *Chlamydia trachomatis* (63) and *Legionella pneumophila* (6), which have been reported to have adhesin functions. The Msp protein can be extracted from *T. denticola* in the form of sodium dodecyl sulfate (SDS)-stable oligomers and has a slightly acidic pI (22). In single channel conductance studies, Msp formed extremely large, nonselective ion channels in a black lipid bilayer model (12). The hexagonal array ultrastructure of Msp in the outer sheath is consistent with observations of outer membrane porins of several other bacteria (29).

In a recent study, we reported the initial cloning of the *msp* gene. DNA encoding an antigenic Msp peptide was identified on a 5.5-kb fragment in a genomic library of *T. denticola*. A recombinant protein recognized by anti-Msp sera was expressed at low levels in *Escherichia coli* (22). Preliminary sequence analysis showed that the 5′ end of the *msp* gene was not present on this clone and that the immunoreactive peptide was a fusion with the NH_2 terminus of the vector-encoded LacZ. The present study was undertaken to identify the 5′ end of *msp*

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TABLE 1. PCR primers used in this study

Primer	Nucleotide sequence[a]	Location[b]
KX01	5′ dCCT GTT CCT GCA AGG TCA TCG CTT 3′	612–589
KX02	5′ dTTC GTA TCT CCT GAA GCT GCT GAT 3′	1302–1279
KX11	5′ dGTC TGC ATC TAT TCA TTT CTT CGG 3′	424–447
KX14	5′ dGCT TGA CAA GTG AAT TTG GCT GTG 3′	1–24
53L1	5′ dGGG GTA GAT CTG CAG GTT ACC CAA CTT ACA CCT CAA GTT ACA GCA AAG 3′	128–154
53L0	5′ dGGG GGG GGG CAT ATG AAA AAA ATT CTG GCG ATT TTG ATG 3′	68–94
53R	5′ dGGG GGG GTG ACC CTC GAG TTA GTA GAT AAC TTT AAC ACC GAT TAC 3′	1699–1673

[a] Single-stranded oligonucleotide primers were synthesized by the Oligonucleotide Synthesis Laboratory at the University of British Columbia.
[b] Position in the nucleotide sequence shown in Fig. 1.

and to obtain recombinant Msp for use in studies of the interaction of *T. denticola* with oral epithelial cells.

MATERIALS AND METHODS

Bacterial strains and plasmids. *T. denticola* ATCC 35405 was grown anaerobically in NOS broth medium as described previously (22, 23). Three-day 10-ml cultures of *T. denticola* were harvested by centrifugation at 10,000 × *g* (10 min, 4°C), washed twice in phosphate-buffered saline (PBS; 10 mM Na_2HPO_4, 150 mM NaCl, 2.5 mM KCl, 1.5 mM KH_2PO4 [pH 7.2]), and resuspended in PBS to an optical density at 600 nm (OD_{600}) of 0.2 (5×10^9 cells per ml).

E. coli XL1-Blue (Stratagene Cloning Systems, La Jolla, Calif.) was used for plasmid preparations. Plasmid vector pBluescript IIKS(−) (Stratagene Cloning Systems) was used for routine subcloning and preparation of sequencing templates. *E. coli* plasmid pKX101 carries a 5.5-kb fragment of *T. denticola* 35405 DNA in the multiple cloning site. A truncated recombinant Msp peptide is expressed from this plasmid under control of the vector-encoded *lac* promoter (22). *E. coli* XL1-Blue was grown in terrific broth (56), LB broth, or LB agar media supplemented with ampicillin (50 μg/ml) or kanamycin (50 μg/ml) as appropriate.

E. coli BL21(DE3)/pLysS and plasmid vector pET17b (Novagen) were used for expression studies. *E. coli* BL21(DE3)/pLysS cultures freshly transformed with pET17b-based plasmids were inoculated into LB broth containing carbenicillin (50 μg/ml) and chloramphenicol (34 μg/ml) to an OD_{600} of 0.05. Cultures were incubated at 30°C with shaking until reaching an OD_{600} of 0.4. Isopropylthiogalactopyranoside (IPTG) from a 100 mM stock solution was added to a final concentration of 0.4 mM, and the cultures were incubated at 37°C for a further 1 to 3 h.

Chemicals. All chemicals were purchased at the highest available purity from Sigma Chemical Co. (St. Louis, Mo.) or Fisher Scientific (Ottawa, Ontario, Canada).

Preparation of chromosomal DNA from *T. denticola*. DNA from a 1,000-ml culture of *T. denticola* ATCC 35405 was prepared as described by Silhavy et al. (59), 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 further purified by cesium chloride density gradient ultracentrifugation.

Recombinant DNA methods. Unless stated otherwise, we followed methods described in reference 4 or 56. DNA fragments were purified from agarose gels by using either a Gene Clean kit (Bio 101, La Jolla, Calif.) or a Qiaex kit (Qiagen Inc., Chatsworth, Calif.).

Introduction of nested deletions into the *msp* region in recombinant plasmids. A series of nested deletions in pKX101 extending through the *msp* region was obtained either by limited DNase I digestion as described by Lin et al. (35) or with an Erase-a-Base kit (Promega Corporation, Madison, Wis.).

PCR amplification of *T. denticola* library DNA. PCR amplification of DNA was performed on a Twin Block system (EriComp, Inc., San Diego, Calif.). The reaction mixture (80 μl) contained 20 pmol of each primer, 62.5 mM each of the four deoxynucleoside triphosphates, 50 mM KCl, 10 mM Tris-HCl (pH 8.0), 1.5 mM $MgCl_2$, 0.01% gelatin, and 2.5 U of *Taq* DNA polymerase (Promega). The following reaction conditions were used: annealing, depending on primers and template, 50 to 60°C for 1 min; extension, 72 to 74°C for 75/kb; and denaturation, 95°C for 1 min. The cycles were repeated 30 times.

Cloning of PCR fragments. PCR fragments were purified either from agarose gels or by passage through Qiagen columns as recommended by the manufacturer. To obtain blunt ends, the purified fragments were treated with the Klenow fragment of DNA polymerase I or T4 DNA polymerase in the presence of all four deoxynucleotides. To obtain cohesive ends, purified PCR products were digested in the appropriate enzyme and repurified from agarose gels. PCR fragments were ligated to vectors by using T4 DNA ligase at 25°C for blunt ends or 16°C for cohesive ends.

DNA sequence analysis. Plasmid templates for DNA sequence analysis were purified either by polyethylene glycol precipitation or with Qiagen columns. Sequencing reactions were performed on plasmid templates by using a *Taq* cycle sequencing kit with either fluorescence-labeled primers or fluorescence-labeled dideoxynucleoside triphosphates (Applied Biosystems Inc., Foster City, Calif.) as instructed by the manufacturer. DNA sequence analysis was performed on an Applied Biosystems model 373A automated DNA sequencer. Computer analysis of the DNA sequence data was performed with DNA Strider (Service de Biochimie, Département de Biologie, Institut de Recherche Fondamentale Commissariat a l'Energie Atomique, Paris, France) or GeneWorks (IntelliGenetics Inc., Mountain View, Calif.). The nonredundant SWISS-PROT, PIR, EMBL, and GenBank databases were searched for homologous amino acid and nucleotide sequences, using the BLAST (1) network service at the National Center for Biotechnology Information, National Institutes of Health, Bethesda, Md.

Prediction of secondary structure of Msp. The membrane criterion value (H + <u>) (29), a linear combination of hydrophobicity (H) and hydrophobic moment (<u>) values (13, 33), was used to predict the transmembrane regions of the deduced Msp peptide.

Northern (RNA) blot analysis. Total RNA of *T. denticola* was isolated by using a monophasic solution of phenol and guanidine isothiocyanate (TRIzol; Life Technologies, Inc.). Two hundred milliliters of NOS broth was inoculated with 20 ml of 3-day-old *T. denticola* and grown for an additional 2 days. Cells were harvested at 10,000 × *g*, resuspended in TRIzol reagent at a density of 10^9/ml, and lysed by repetitive pipetting. Homogenized cells were incubated for 5 min at room temperature and extracted once with 0.2 volume $CHCl_3$. RNA was precipitated from the aqueous phase with 0.5 ml of isopropanol per ml of TRIzol. The RNA pellet was washed once with 1 ml of 75% ethanol per ml of TRIzol, air dried, and redissolved in RNase-free H_2O. The yield of RNA was determined by absorbance spectrophotometry at 260 nm. Twenty micrograms of RNA per lane was electrophoresed in 1% agarose gel made in 2.2 M formaldehyde–40 mM 3-(*N*-morpholino)propanesulfonic acid (pH 7.0)–10 mM sodium acetate–1 mM EDTA. The gel was soaked in several changes of RNase-free H_2O, after which the RNA was transferred to a nitrocellulose membrane by capillary transfer in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate) and immobilized by drying under vacuum. An *msp* probe constructed by using PCR primers KX02 and KX11 (Table 1) and labeled with biotin-dATP by random oligonucleotide-primed DNA synthesis (New England BioLabs) (15) was hybridized to the membrane. The probe hybridizing to the *msp* transcript was visualized by using a streptavidin-alkaline phosphatase detection system.

PCR amplification of *msp* from *T. denticola* genomic DNA. The method described by Feavers et al. (14) was used to amplify DNA encoding the mature Msp peptide from *T. denticola* genomic DNA. The primers used were 53L1 and 53R (Table 1). To amplify the entire *msp* gene, primer 53L0 was substituted for 53L1.

Preparation of *T. denticola* sonicated extract. *T. denticola* cells were harvested, washed, and suspended in PBS to an OD_{600} of 0.2 as described above. Cell suspensions were subjected to mild sonication (six pulses of 15 s spaced 10 s apart at an output of 2 with a Sonifier Cell Disrupter 350 [Branson Sonic Power Co., Danbury, Conn.]), which primarily releases outer sheath material of the treponemes. 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 and suspended in PBS.

Protein assay. Protein concentrations of samples were measured by using a Bio-Rad protein assay (Bio-Rad, Richmond, Calif.) in either the standard or detergent-compatible format as appropriate, using BSA as a reference.

Gel electrophoresis and immunoblotting. SDS-polyacrylamide gel electrophoresis (PAGE) was done by the method of Laemmli (34), using a Mini Protean II electrophoresis apparatus (Bio-Rad). Proteins or peptides solubilized in sample buffer were electrophoresed at 200 V and detected by silver staining or by Coomassie blue staining. For immunoblotting, proteins were transferred electrophoretically to nitrocellulose membrane as described by Towbin et al. (64). Following transfer, the membrane was blocked with 3% BSA in Tris-buffered saline (20 mM Tris, 0.5 M NaCl [pH 7.5]) and then incubated with primary antibodies followed by alkaline phosphatase-conjugated secondary antibodies at appropriate dilutions. The membranes were developed with 5-bromo-4-chloro-3-indolylphosphate and nitroblue tetrazolium (Sigma).

FPLC purification of T7-Msp fusion protein. The T7-Msp fusion protein was purified by using a variation of the method of Qi et al. (51). Briefly, a 25-ml culture was induced with IPTG at an OD_{600} of 0.4, incubated for 3 h at 37°C, harvested at 10,000 × *g* for 10 min, and resuspended in 3 ml of TEN buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 100 mM NaCl) per g (wet weight) of cells. To this culture were added 5 μl of phenylmethylsulfonyl fluoride stock solution (100 mM in isopropanol) and 25 μl of lysozyme stock solution (50 mg/ml in water) per g (wet weight) of cells. These mixtures were stirred at room temperature for 20 min. Deoxycholate (4 mg/g [wet weight] of cells) was added while the mixtures were being stirred, and the mixtures were transferred to 37°C with continued stirring until the cells were lysed. DNase I stock solution (1 mg/ml) was added to 20 μl/g (wet weight) of cells, and the mixture was incubated at room temperature until no longer viscous. The insoluble pellet was recovered by centrifugation at 15,000 rpm in a JA-20 rotor for 20 min at 4°C and washed twice with TEN buffer. The pellets were resuspended in TEN buffer containing 0.1 mM phenylmethylsulfonyl fluoride and 8 M urea and then sonicated with a microtip (Heat Systems, Inc., Farmingdale, N.Y.) probe at maximum setting. Protein concentration was adjusted to less than 10 mg/ml in TEN-urea buffer, as measured by using a Bio-Rad protein assay. The samples were then diluted 1:1 with 10% Zwittergen 3,14 (Calbiochem, La Jolla, Calif.), sonicated, and fractionated by fast protein liquid chromatography (FPLC; Pharmacia, Uppsala, Sweden) on a Superose 12 column previously equilibrated in TEN buffer containing 0.05% Zwittergen 3,14 and 0.02% azide. The column flow rate was adjusted to 0.15 ml/min, and 1.0-ml fractions were collected and analyzed by SDS-PAGE and Western immunoassay in anti-*T. denticola* serum.

Preparation of antisera. A New Zealand White rabbit was immunized with approximately 1 mg of purified T7-Msp fusion protein in complete Freund's adjuvant. Subsequent intramuscular injections without adjuvant were performed after 1, 2, 3, 5, and 7 weeks. The rabbit was bled 1 week after the last injection. The specificity of the antiserum was determined by ELISA, using alkaline phosphatase-conjugated goat anti-rabbit antibody (1:5,000; Bethesda Research Laboratories, Gaithersburg, Md.). Antiserum to the 95-kDa chymotrypsin-like protease of *T. denticola* was prepared as described previously (21) and did not recognize Msp in a Western immunoblot assay. For adhesion assays, immunoglobulins were purified from immune serum by using protein A-Sepharose (25).

Immunoaffinity purification of Msp. Recombinant Msp (rMsp) was purified by immunoaffinity chromatography. Immunoglobulins raised against the T7-Msp fusion protein were bound to protein A-Sepharose (Sigma) in the presence of 0.1M borate (pH 9.0) and covalently coupled by using dimethyl suberimidate-2HCl (Pierce Chemical Co., Rockford, Ill.) as described by Harlow and Lane (25). A 5-ml column of the affinity matrix was washed and equilibrated with TSA buffer (10 mM Tris-Cl [pH 8.0 at 4°C], 140 mM NaCl, 0.025% NaN_3) as described by Ausubel et al. (4). Six-liter cultures were induced with IPTG for 1 h, lysed, and suspended in approximately 50 ml of TSA buffer containing 0.5% Triton X-100, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and 5 mM iodoacetate and passed over the column. After being washed at low pH (50 mM glycine [pH 2.5], 140 mM NaCl, 0.1% Triton X-100), purified rMsp was eluted from the column at high pH (50 mM triethanolamine [pH 11.5], 140 mM NaCl, 0.1% Triton X-100) and neutralized with 0.2 volume of 1 M Tris-HCl (pH 6.7). Eluate fractions were analyzed by duplicate SDS-PAGE and Western immunoblotting in anti-T7-Msp serum. For use in attachment studies, rMsp was passed over a buffer exchange column (Bio-Rad Econo-Pac 10DG) equilibrated with PBS containing 0.3% Triton X-100.

Determination of the N-terminal amino acid sequence. Purified proteins were subjected to SDS-PAGE and transferred electrophoretically to Immobilon-P membranes (Millipore Co., Bedford, Mass.) in 25 mM Tris-Cl (pH 8.3)–192 mM glycine–10% methanol. The transferred protein was analyzed on a gas-phase sequenator (model 475A; Applied Biosystems) by the Nucleic Acids and Protein Sequencing Unit at the University of British Columbia.

Attachment of *T. denticola* proteins to ECM components. BSA, human fibronectin, and murine laminin were prepared at 0.1 mg/ml in 50 mM carbonate buffer (pH 9.6). Ninety-six-well microtiter plates (Immulon; Corning Laboratory Sciences) were coated with 0.1 ml of protein solution for 18 h at 4°C. Since BSA was used to block nonspecific adhesion of *T. denticola* proteins to plastic, measurements of adhesion to BSA-coated wells were reported for initial adhesion experiments. After washing (all washes were done twice with 0.05% Tween 20 in PBS and once with PBS), the wells were blocked with 0.2 ml of BSA (1 mg/ml) for 1 h, washed, and incubated for 2 h at room temperature with 0.1 ml of *T. denticola* whole cells in PBS (5×10^9/ml), *T. denticola* sonic extract (30 μg/ml), or rMsp (10 μg/ml). The wells were washed, probed with antibodies to T7-Msp, washed, probed with alkaline phosphatase-conjugated goat anti-rabbit antibodies (1:5,000 in PBS containing 1% BSA, 1 h; Life Technologies), and washed again. Color formation of a chromogenic substrate for alkaline phosphatase (*p*-nitrophenylphosphate; Sigma) was monitored at 405 nm. Control wells containing each substrate were incubated with PBS or *T. denticola* cells in PBS and then incubated with either the primary or the secondary antibodies followed by chromogenic alkaline phosphate substrate. No significant alkaline phosphatase activity was observed in these control wells. For experiments comparing levels of rMsp binding to soluble and insoluble ECM components, fibronectin or laminin (50 μg/ml, final concentration) was added to the rMsp solution before addition to ECM-coated wells. For experiments measuring inhibition of attachment of *T. denticola* to ECM components by rMsp, *T. denticola* cells were added to ECM-coated wells which had been washed following 1 h of incubation with BSA (1 mg/ml) and 1 h of incubation with rMsp (30 μg/ml) or PBS. *T. denticola* cells were removed after 5, 15, and 60 min. Attachment of *T. denticola* was detected with antibodies to the *T. denticola* 95-kDa chymotrypsin-like surface protease (21). Results show mean values from experiments performed at least twice, using triplicate samples. Standard deviations among samples within each experiment were less than 0.05.

Nucleotide sequence accession number. The nucleotide sequence reported here has been submitted to GenBank and assigned accession number U29399.

RESULTS

Nucleotide sequence of the *msp* gene. DNA encoding Msp was identified on a 5.5-kb fragment of *T. denticola* ATCC 35405 DNA in the multiple cloning site of pKX101. Analysis of pKX101 showed that *msp* was located at one end of the cloned fragment. Preliminary sequence analysis showed that the 5′ end of the *msp* gene was not present on pKX101 and that it produced a truncated β-galactosidase–Msp fusion protein. This finding was confirmed by introducing a frameshift mutation into the *lacZ* portion of the fusion, thereby abolishing expression of the recombinant protein (data not shown). The missing 5′ end of *msp* was obtained by PCR amplification from the genomic library, using a primer (KX01; Table 1) near the 5′ end of the original clone and a vector-based M13 primer, yielding a 0.5-kb product. The sequence of the amplified product was combined with the original sequence to yield the DNA sequence shown in Fig. 1. To confirm the identity of the amplified sequence as part of *msp*, *T. denticola* DNA was amplified by using primer KX14 (corresponding to the 5′ end of the DNA sequence shown) and several oligonucleotide primers internal to or 3′ to *msp*, yielding amplification products of the expected sizes (not shown). All attempts to clone PCR products generated by using primer KX14 in *E. coli* plasmid vectors yielded either no products or rearranged plasmids containing deletions in the KX14 region of the sequence (data not shown).

An open reading frame of 1,629 bp was identified as the coding region for Msp. The G+C content of *msp* is 42%, which is slightly higher than the *T. denticola* overall G+C content of 37 to 38%. Codon usage in the *msp* gene was similar to that of the other *T. denticola* sequences in the database (2, 26–28, 42, 45). A promoter-like sequence (underlined in Fig. 1; bases 3 to 31) was identified upstream of *msp*, and a potential ribosome binding site was located 8 bp upstream of the ATG start codon. The composition and spacing of the putative promoter and ribosome binding site are in very close agreement with published consensus sequences (36, 53). The sequence beginning 19 bp downstream of the TAA stop codon at base 1719 (underlined in Fig. 1) represents a typical transcription terminator which could form a GC-rich stem-loop structure.

Analysis of the Msp peptide. We found no significant homology between Msp and any other published sequences at either the DNA or amino acid level. The deduced Msp peptide contained 543 amino acids and had a molecular mass of 58,233 Da. The protein displays a typical prokaryotic signal sequence, with two positively charged amino acids after the first methionine followed by a stretch of hydrophobic amino acids and a turn-promoting amino acid preceding the putative signal peptidase I cleavage site following residue 20 (10, 50). The Msp peptide contains no cysteine residues. The mature protein contains 523 amino acids and has a molecular mass of 56,151 Da. Analysis of the deduced Msp peptide revealed secondary structure features consistent with identification of Msp as a pore-forming outer membrane protein. As in other porins, the signal sequence is the only strongly hydrophobic region of Msp. Calculations of membrane criterion values (H + <u>), as illustrated in Fig. 2, demonstrated the potential for a number of

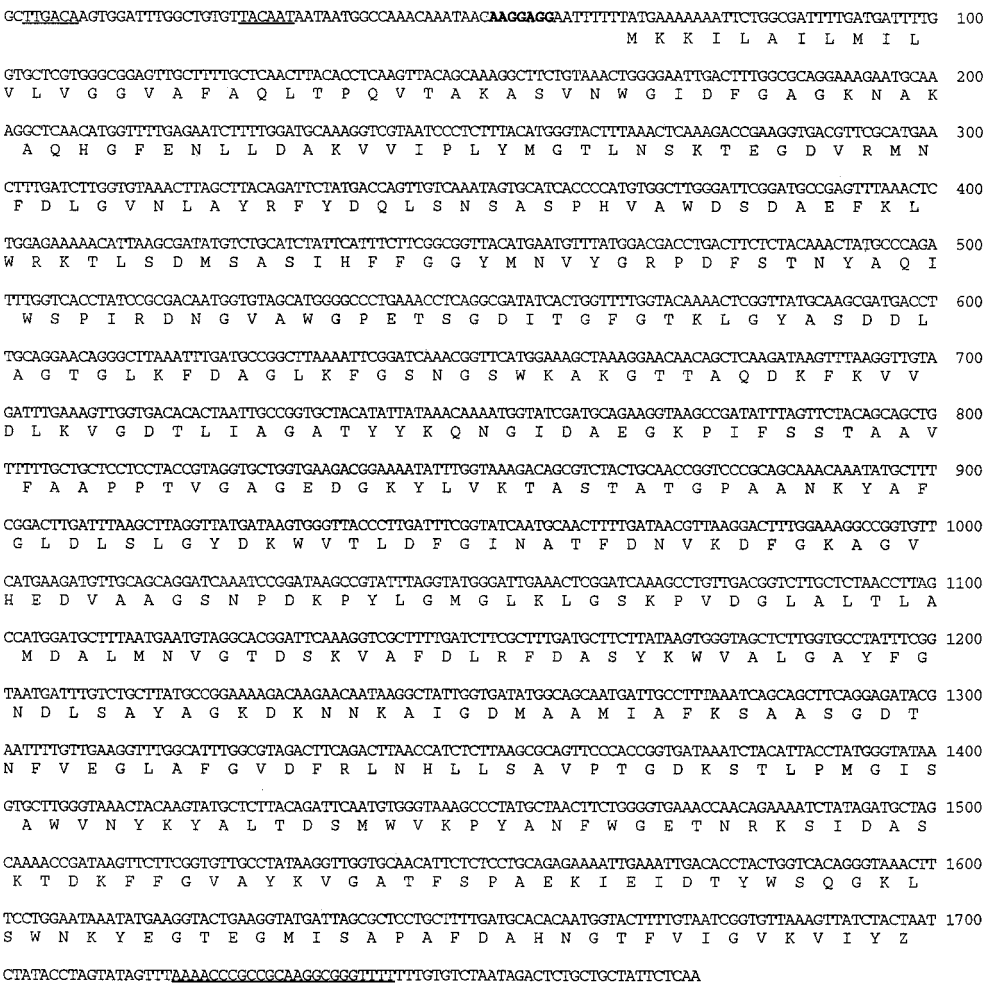

FIG. 1. Nucleotide sequence of the *T. denticola msp* locus and deduced amino acid sequence of Msp. The locations of the putative −35 and −10 promoter regions and ribosome binding site are underlined and boldfaced, respectively. The potential transcription termination sequence downstream of *msp* is underlined.

amphipathic β sheets that could form a hydrophilic pore across the outer membrane of *T. denticola* in the form of a β barrel (29). Neither the high content of clustered hydroxyamino acids nor the frequent Asn-X-Ser/Thr N-glycosylation sites commonly found in S-layer proteins (43, 44, 71) were found in the deduced Msp sequence. This observation was consistent with identification of Msp as a porin-like molecule rather than as an S-layer protein.

Northern blot analysis of *msp*. To determine the size of the *msp* mRNA, Northern blot analysis was performed with *T. denticola* RNA. Total RNA (20 μg) of *T. denticola* ATCC 35405 was separated on a 1% agarose–formaldehyde gel and transferred to nitrocellulose. As shown in Fig. 3, a biotin-labeled *msp* probe hybridized with a single transcript of approximately 1.7 kb, which includes the 1,629-bp *msp* coding region. This size closely corresponds with the *msp* transcript size predicted from the DNA sequence and is suggestive that *msp* is transcribed monocistronically from the putative promoter sequence.

Plasmid construction for expression of *msp* in *E. coli*. Our inability to isolate a recombinant clone that contained the entire *msp* gene from the *T. denticola* library suggested that expression of the Msp protein might be toxic to *E. coli*. Furthermore, attempts to clone PCR products encoding the putative *msp* promoter and signal peptide region were unsuccessful. To circumvent this problem, we used oligonucleotide primers to amplify *msp* directly from *T. denticola* genomic DNA and cloned the resulting PCR products directly downstream of a tightly controlled T7 RNA polymerase promoter in plasmid vector pET17b. The gene encoding Msp was amplified from *T. denticola* genomic DNA by using primer pairs 53L0-53R and 53L1-53R (Table 1), derived from the *msp* DNA sequence. Primer 53L0 creates an *Nde*I site at the ATG start codon of *msp*. Primer 53L1 contains a *Bam*HI site located such that ligation of *Bam*HI-digested vector and PCR product produces an in-frame fusion of the vector-encoded T7 phage capsid protein with the mature Msp peptide. Primer 53R contains an *Xho*I site followed by DNA complementary to the stop codon and 3′ end of *msp*.

The 53L0-53R PCR product contains the entire *msp* coding sequence. The PCR product was digested with *Nde*I and *Xho*I and ligated to the plasmid vector pET17b, which had been similarly digested. Sequence analysis of the resulting plasmid, pET53L0, confirmed that the *msp* start codon was located optimally downstream of the vector-encoded T7 RNA polymerase promoter and ribosome binding site (data not shown).

Studies of other cloned porins suggested that expression levels might be increased by preventing the targeting of Msp to

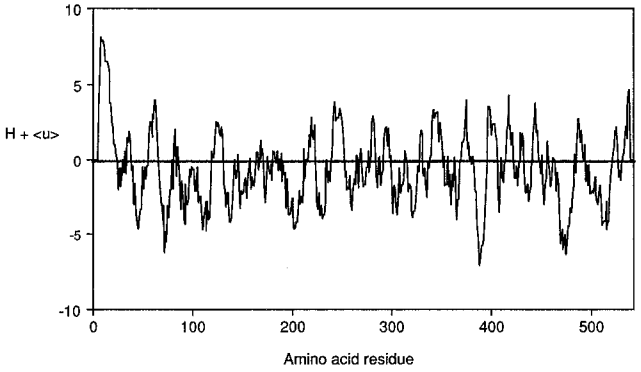

FIG. 2. Predicted secondary structure of the Msp peptide. The horizontal axis shows residue numbers of the complete deduced amino acid sequence of Msp. The vertical axis represents membrane criterion values (H + <u>) calculated over a window of nine amino acids. Potential transmembrane β strands show positive peaks corresponding to high transmembrane probability. The putative signal peptide shows an extremely strong hydrophobic peak.

the *E. coli* membrane. To express Msp without its signal peptide, the 53L1-53R PCR product was cloned in pET17b as a *Bam*HI-*Xho*I fragment. In this construct, pET53L1, the putative Msp signal peptide was replaced by a T7 phage peptide sequence of similar length, and expression was under control of T7 RNA polymerase.

Expression and purification of T7-Msp fusion protein and recombinant Msp. Both pET53L1 and pET53L0 were stable in *E. coli* XL1-Blue, and growth rates were similar to that of a strain carrying the vector alone. As shown in Fig. 4, lane 4, the T7-Msp fusion protein was produced in very high quantity when broth cultures of *E. coli* BL21(DE3)/pLysS transformed with pET53L1 were induced with IPTG during log phase and grown for an additional 3 h. The T7-Msp fusion protein present in cytoplasmic inclusion bodies was purified from induced cultures by FPLC, using a method which has been shown to yield recombinant bacterial porin that retains the ability to form native oligomers (51). Fractions containing the T7-Msp fusion protein were collected and analyzed by SDS-PAGE.

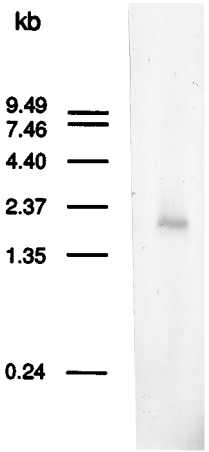

FIG. 3. Northern blot analysis of *msp* mRNA. Hybridization of total RNA from *T. denticola* ATCC 35405 was performed with a biotin-labeled PCR probe made with primers KX11 and KX02. The probe hybridized with a single transcript of approximately 1.7 kb, as calculated from the positions of the RNA molecular weight standards shown on the left. This corresponds with the *msp* transcript size predicted from the DNA sequence.

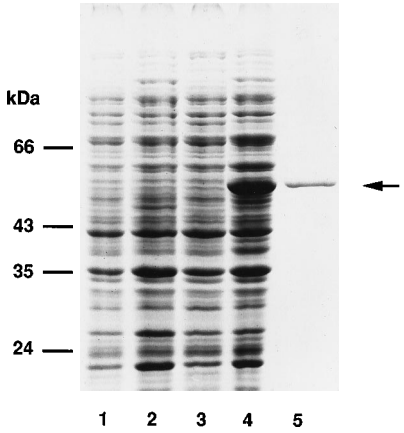

FIG. 4. Purification of T7-Msp fusion protein overexpressed in *E. coli*. Shown is an SDS-polyacrylamide gel stained with Coomassie blue. Cultures were harvested 3 h after reaching an OD_{600} of 0.4. Lanes: 1 and 2, *E. coli* BL21(DE3)/pLysS transformed with vector pET17b; 3 and 4, *E. coli* BL21(DE3)/pLysS transformed with pET53L1; 2 and 4, T7 RNA polymerase induced with IPTG at an OD_{600} of 0.4; 5, T7-Msp fusion protein purified from the culture in lane 4.

The purified T7-Msp fusion protein migrated in SDS-PAGE as a single band of approximately 55 kDa (Fig. 4, lane 5), which corresponds with the expected size of the unprocessed fusion protein. SDS-PAGE of heated and unheated samples of T7-Msp did not show oligomer formation (data not shown). Antiserum to the T7-Msp fusion protein was raised in rabbits and used in subsequent experiments to detect and purify recombinant Msp.

When pET53L0 was present in expression host *E. coli* BL21(DE3)/pLysS, growth in the presence of IPTG for more than 1 h resulted in extensive lysis, as monitored by a sharp drop in OD_{600}. Growth of uninduced cultures was normal. To detect rMsp, *E. coli* cultures were harvested no more than 1 h after IPTG induction, and equal amounts of protein from whole cell lysates of induced and uninduced cultures were analyzed. In contrast to the high-level expression of T7-Msp, the rMsp gene product expressed from pET53L0 in induced cultures was not visible in a Coomassie blue-stained gel and could be detected only by Western blot immunoassay (Fig. 4, lane 5; Fig. 5A, lanes 1 and 2). Antiserum to the T7-Msp fusion protein recognized rMsp as a doublet migrating at approximately 53 to 55 kDa (Fig. 5B, lanes 2 and 3). The lower band of the doublet had the same relative molecular weight as native Msp from *T. denticola* and is assumed to be the result of cleavage of the Msp signal peptide. Antiserum to the T7-Msp fusion protein recognized both the oligomeric complex (150- to 200-kDa) and monomeric (53-kDa) forms of native Msp present in *T. denticola* extracts (Fig. 5B, lanes 4 and 5).

rMsp was purified by immunoaffinity chromatography from induced cultures carrying pET53L0. Purified rMsp migrated as a doublet of approximately 53 to 55 kDa in SDS-PAGE (Fig. 5A and B, lanes 3; for purposes of visualization, lane 3 in Fig. 5A contains 10-fold more protein than lane 3 in Fig. 5B). N-terminal sequencing of the first 15 residues of the lower band of rMsp showed that the processed peptide sequence begins Q-L-T-P-Q-V-T-A-K-A-S-V-N-W-G, in agreement with the predicted cleavage of Msp by signal peptidase following the alanine residue at position 20 of the deduced Msp peptide sequence (Fig. 1). Because of apparent N-terminal blockage, we were unable to determine the N-terminal sequence of native Msp purified from *T. denticola.*

rMsp binds to ECM components. To determine the role of

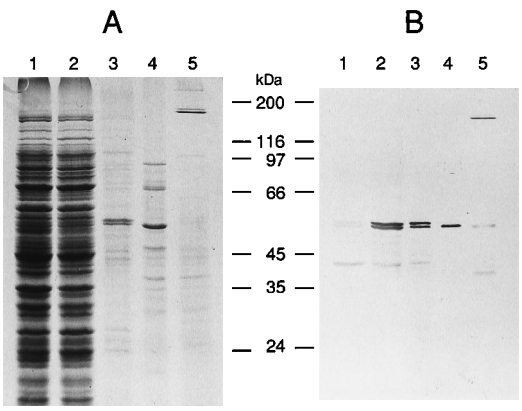

FIG. 5. Purification of recombinant Msp and comparison with native Msp. (A) Coomassie blue-stained SDS-polyacrylamide gel; (B) Western blot probed with antiserum to T7-Msp. Lanes: 1 and 2, *E. coli* BL21(DE3)/pLysS transformed with pET53L0; 2, T7 RNA polymerase induced with IPTG at an OD_{600} of 0.4; 3, recombinant Msp purified from the culture in lane 2; 4, *T. denticola* sonic extract, heated; 5, *T. denticola* sonic extract, unheated. *E. coli* cultures were harvested 1 h after reaching an OD_{600} of 0.4.

Msp in binding to epithelial cell surface molecules, adhesion of *T. denticola* preparations and rMsp to the ECM components laminin and fibronectin was measured in an ELISA. As shown in Fig. 6, rMsp expressed from pET53L0 bound specifically to immobilized laminin and fibronectin. The pattern of binding to ECM components was the same as that observed both for *T. denticola* whole cells and for sonicated outer membrane preparations of *T. denticola*. Attachment to BSA was negligible, as expected. The T7-Msp fusion protein did not show specific binding to ECM components (data not shown), suggesting that binding of rMsp to ECM components required correct folding of the peptide. Fibronectin in solution inhibited attachment of rMsp to immobilized fibronectin by 49.5% (±2%) and 41.7% (±4%) in two separate assays. Attachment of rMsp to immobilized laminin was inhibited by laminin in solution to a lesser degree (25% ± 3%). Recombinant Msp did not inhibit attachment of *T. denticola* to laminin or fibronectin. *T. denticola* cells attached very rapidly to ECM components, reaching over 60% of the 1-h value for attachment to fibronectin within 5 min (data not shown). Preincubation of immobilized fibronectin with rMsp had no significant effect on attachment of *T. denticola* to fibronectin. Interestingly, preincubation of immobilized laminin with rMsp enhanced the attachment of *T. denticola*. After 5 min, attachment of *T. denticola* to rMsp-treated laminin was nearly two times higher than attachment to laminin alone. After 60 min, attachment to rMsp-treated laminin was approximately 1.5 times higher than attachment to laminin alone.

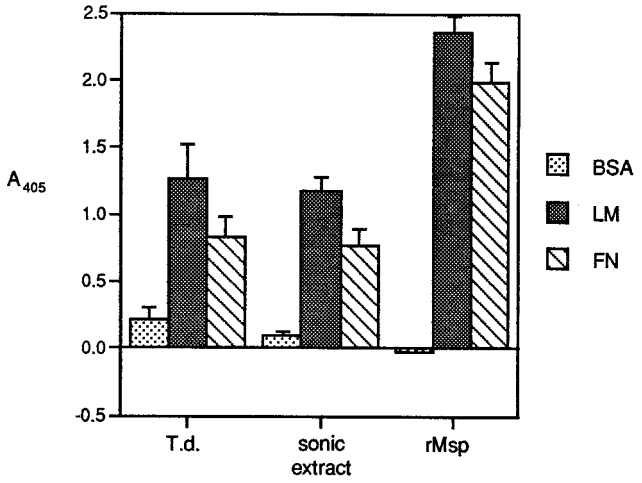

FIG. 6. Attachment of recombinant Msp to ECM components. ELISA plate wells were coated with laminin (LM), fibronectin (FN), or BSA and blocked with BSA. *T. denticola* whole cells (T.d.), *T. denticola* outer sheaths (sonic extract), and rMsp were allowed to attach to the proteins for 2 h. Attachment was measured by ELISA with antiserum to T7-Msp. The vertical axis shows A_{405} values in alkaline phosphatase substrate. Insert bars show standard deviations of triplicate samples in two representative experiments.

DISCUSSION

This study reports the complete sequence and expression in *E. coli* of the *T. denticola* gene encoding Msp, a surface protein involved in the interaction of the bacterium with host cell components. Of the *T. denticola* DNA sequences reported to date, only that of *tdpA* has been shown to encode an antigen reactive with sera from patients with periodontitis. In contrast to the highly expressed Msp protein, the TdpA protein was reported to be expressed at very low levels in *T. denticola* (45). On the basis of the apparent secretion of the cloned gene products by *E. coli*, several other recently isolated *T. denticola* genes have been suggested as encoding secreted or surface-expressed proteins. *E. coli* clones expressing PrtB (2), a 30-kDa chymotrypsin-like protease of *T. denticola* with a substrate hydrolysis pattern distinct from that of the surface-expressed 95-kDa chymotrypsin-like protease of *T. denticola* (65), were identified on skim milk agar. Genes encoding two *T. denticola* proteins that express hemolytic activity in *E. coli* have also been reported (8, 30). Analysis of the deduced amino acid sequence a neutral phosphatase (PhoN) of *T. denticola* suggested a possible membrane localization (28). However, the cellular locations of these proteins in *T. denticola* have not yet been reported.

A recent study identified several antigenically related outer membrane proteins of 50 to 65 kDa in oral treponemes (47). The same study also reported two 50- to 65-kDa antigens which appeared to be specific to particular *T. denticola* strains. Haapasalo et al. (22) showed that Msp was present in at least three different *T. denticola* strains (ATCC 35404, ATCC 35405, and ATCC 3520), while Weinberg and Holt (73) reported that some *T. denticola* strains did not contain the 64-kDa major outer sheath protein, which appeared to be antigenically unrelated to Msp. No homology was seen between the deduced Msp peptide and any of the cyanogen bromide cleavage fragments of the 64-kDa outer sheath protein of *T. denticola* GM-1 reported by Weinberg and Holt (73), nor was homology evident between *msp* and *tdpA*, a gene encoding a 53-kDa protein of *T. denticola* Johnson (45). Taken together, these findings suggest that there may be at least two classes of major outer sheath proteins found among *T. denticola* strains. We are currently examining Msp-like proteins in several strains of oral treponemes in order to clarify this issue.

We had considerable difficulty in cloning the entire *msp* gene. We found that the 5′ end of gene including the putative promoter was not stable in *E. coli* and that the complete coding region was not tolerated by *E. coli* except when placed under stringent transcriptional control. Purification of sufficient rMsp for use in adhesion assays required carefully timed growth and induction of expression of large batch cultures so that cells could be harvested while most of the cells were still intact. Attempts to purify rMsp from culture supernatants were un-

successful, possibly because of release of endogenous proteases in lysing cells. Similar cloning and expression problems have been reported for other bacterial porins as well as other membrane proteins (7, 17, 24, 58). There are several possible explanations for this. First, Msp forms the largest pore of any bacterial porin described to date (12). If the recombinant protein is assembled as a porin in the outer membrane of *E. coli*, it may create pores of a size detrimental to *E. coli*. Second, processing of the Msp signal peptide and secretion of the protein may interfere with the general secretory apparatus of *E. coli* (50). This interpretation is supported by the ability of *E. coli* to express large amounts of a T7-Msp fusion protein that lacks the signal peptide of Msp. This hybrid protein accumulated in the form of cytoplasmic inclusion bodies and was the most abundant cellular protein. In contrast, induction of expression of the intact *msp* gene carried on pET53L0 was toxic in growing *E. coli* cultures, and very small amounts of the Msp protein were produced in this recombinant system. Third, the putative *msp* promoter might direct extremely high levels of Msp expression in *E. coli*. The composition and spacing of the proposed −35 and −10 regions show almost perfect homology with the *E. coli* promoter consensus sequence. In a recent compilation of 300 *E. coli* promoter sequences, only 4 showed the same degree of homology with the consensus sequence as does the putative *msp* promoter, and none showed greater homology (36). We have been unable to construct a stable *E. coli* plasmid containing the putative *msp* promoter.

Msp is produced in large amounts in *T. denticola* and is a major protein component of the outer sheath. Northern blot data presented here showed that the *msp* transcript was very abundant in *T. denticola* and was detectable by a relatively insensitive method. Using the same method, we were unable to detect a transcript of another gene that was expressed at lower levels (data not shown). The size of the *msp* transcript relative to the coding region and the high degree of homology to the *E. coli* consensus promoter sequence lend support to our preliminary identification of the *msp* promoter. The instability of *msp* clones containing the putative *msp* promoter suggests that the promoter sequence is recognized by *E. coli* RNA polymerase and that there may be functional homology between the RNA polymerases of *E. coli* and *T. denticola*. Studies are in progress to identify the 5′ end of the *msp* transcript.

The ribosome binding site proposed for the *msp* gene is highly complementary to published sequences of the 3′ ends of the 16S RNA of *E. coli* and several spirochetes, with the exception of *T. denticola* ATCC 33520 (42, 49). However, the 16S RNA sequence reported for *T. denticola* ATCC 33520 is 60 to 70 bp shorter than those reported for other spirochetes, suggesting that this sequence may be incomplete. The apparent functional similarity of treponemal and *E. coli* transcriptional and translational apparatus could facilitate further genetic studies of *T. denticola.*

Our previous studies showed that native Msp exists in the outer sheath of *T. denticola* as an oligomeric structure with pore-forming capabilities (12, 22). Secondary structure analysis of the amino acid sequence predicted a structure similar to those of other bacterial porins, in which a number of membrane-spanning amphipathic β sheets could be arranged in a pore-forming β barrel (29). Although the Msp protein is associated with the outer sheath of *T. denticola*, no strongly hydrophobic regions other than the signal sequence were predicted by the Kyte-Doolittle algorithm (33). This result is similar to that found in other porins. The structures and conformations of recombinant Msp in *E. coli* and native Msp in *T. denticola* are being investigated.

The demonstration that rMsp and whole *T. denticola* cells bind similarly to ECM components is an important step in understanding the initial interaction between the bacterium and host tissue. Outer membrane-associated components of *T. denticola* have been previously associated with attachment to host ECM (11, 67, 68), but characterization of the role of specific surface components of *T. denticola* has been problematical. It is difficult to obtain outer membrane proteins that retain biological activity after the relatively harsh purification procedures necessary to completely dissociate them from other surface-associated components that have been suggested as having roles in adherence and cytopathic effects of *T. denticola*, including a lipopolysaccharide-like material and peptidoglycan (20) and the 95-kDa chymotrypsin-like surface protease (65). This protease has been shown to be involved in migration of *T. denticola* through the basement membrane (21) and is implicated in cytopathic effects of *T. denticola* on epithelial cells (66).

The observation that rMsp attachment was partially inhibited by soluble ECM components is compatible with its identification as an adhesin directed to host cell ECM. While some bacterial ECM-binding proteins do not interact with soluble ECM components, other important pathogenesis determinants such as the mycobacterial fibronectin-binding proteins bind both the soluble and insoluble forms (reviewed in reference 74). The nature of the interaction between Msp and fibronectin remains to be further characterized in terms of the relevant binding domains of both molecules.

The inability of rMsp to block adherence of *T. denticola* to ECM components might suggest that adhesins other than Msp are involved. It is important to note, however, that rMsp did not form the oligomeric structures characteristic of the native molecule. The rapid binding of *T. denticola* to fibronectin and laminin suggests a very high affinity for these substrates. The affinity of *T. denticola* for ECM may be greater than that of monomeric rMsp because of this difference in macromolecular structure of native Msp. The enhancement of *T. denticola* attachment to laminin resulting from pretreatment of immobilized ECM components with rMsp is interesting in light of the fact that this spirochete continually releases outer membrane fragments and vesicles when grown in liquid culture. Membrane fragments containing Msp might serve to enhance attachment if the Msp molecules present in these fragments were exposed differently than on an intact cell. In support of this speculation, the oligomeric structure of native Msp implies that it has strong affinity for itself under certain topological conditions. Further characterization of the affinity of Msp for host cell surface components and other *T. denticola* surface components are required to resolve this issue.

In this study, we have shown that Msp, a major outer sheath protein of *T. denticola*, can be expressed and purified from *E. coli* in a biologically active form. Msp, in addition to having pore-forming ability, has an adhesin function that strongly implicates it in the pathogenic interaction of *T. denticola* with the ECM of subgingival epithelial tissues. We confirmed and expanded upon earlier studies that suggested a role for Msp in adhesion to ECM components of subgingival epithelial tissues. We are continuing to investigate the functional properties of the Msp molecule and its structural role in the outer sheath architecture of this important treponeme.

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