

# Human serum antibodies recognize *Treponema denticola* Msp and PrtP protease complex proteins

R. Capone<sup>1</sup>, H. T. Wang<sup>1</sup>, Y. Ning<sup>1</sup>,  
D. G. Sweier<sup>1,2</sup>, D. E. Lopatin<sup>1</sup>,  
J. C. Fenno<sup>1</sup>

Departments of <sup>1</sup>Biologic and Materials Sciences and <sup>2</sup>Cariology, Restorative Sciences and Endodontics, University of Michigan, Ann Arbor, MI, USA

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**Background/aims:** *Treponema denticola* outer membrane proteins are postulated to have key roles in microbe–host interactions in periodontitis. Because there are no reports of *in vivo* expression of these putative virulence factors, we examined several *T. denticola* strains to determine whether sera from human subjects recognized specific *T. denticola* outer membrane proteins.

**Methods:** Soluble extracts were prepared from exponential phase cultures of *T. denticola* strains representing three serotypes, from defined *T. denticola* mutants defective in Msp (major surface protein) or PrtP lipoprotein protease complex (CTLP; dentilisin), and *Escherichia coli* strains expressing distinctly different *T. denticola* Msp. Extracts were subjected to Western immunoassays using archived human serum samples.

**Results:** Human serum antibodies (immunoglobulin G class) recognized multiple protein bands in *T. denticola* strains. In the parent strain ATCC 35405, these included bands at 72-, 53-, 40-, and 30-kDa. Bands corresponding to Msp and the PrtP protease complex proteins were absent in isogenic *msp* and protease complex mutants, respectively. Individual human sera showed specificity for one or more Msp types.

**Conclusions:** This is the first definitive report of human serum antibody responses to specific *T. denticola* antigens. *T. denticola* Msp and the proteins comprising the PrtP lipoprotein protease complex are expressed *in vivo* and are immunogenic in humans. Human antibody recognition of Msp exhibits strain specificity and is consistent with strain serotyping. These results demonstrate the utility of *T. denticola* isogenic mutants in characterizing host immune responses to periodontal pathogens.

Key words: PrtP lipoprotein protease complex (CTLP); dentilisin; immunoglobulins; major surface protein (Msp); protease

J. Christopher Fenno, Biologic and Materials Sciences, School of Dentistry, University of Michigan, Michigan, 48109-1078, USA  
Tel.: 734-763-3331;  
fax: 734-647-2110;  
e-mail: fenno@umich.edu

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Periodontal disease is the primary cause of tooth loss after the age of 35 years, and up to half of the adult population is in at least the early stages of disease (19). The periodontal pathogen *Treponema denticola* is highly associated with severe periodontal disease (26), and many studies have noted serum antibody responses to *T. denticola*; reviewed in ref. (9). While *T. denticola* is only one of more

than 50 oral *Treponema* species that have been detected in periodontal lesions (8), it is the most readily isolated and thus the most extensively studied oral spirochete. *T. denticola* expresses several outer membrane components with key roles in microbe–host interactions, including the pore-forming major surface protein (Msp) (11, 14) and an acylated protease complex (CTLP; dentilisin) (16, 27). The activities

of these protein complexes have been studied *in vitro* and in cell challenge studies (10, 21, 28). Studies in mice suggest that expression of the protease complex has a role in tissue destruction (15).

Evidence of *in vivo* expression is crucial for the identification and characterization of putative virulence determinants. Direct evidence of immunogenicity of specific

*T. denticola* antigens is practically absent from the literature. To date only TdpA, an otherwise uncharacterized putative lipoprotein, has been reported to be reactive in serum from periodontitis subjects. However this report was based on a single uncontrolled experiment using a recombinant TdpA protein (22). No studies have reported expression of Msp or the protease complex in human subjects. For this reason, we chose to focus on the identification of specific *T. denticola* protein antigens recognized by serum antibodies from human subjects.

The role of humoral immune responses to *T. denticola* in progression or resolution of disease is not well understood. The results of several studies of serum responses to oral spirochetes in periodontal disease [reviewed in (9)] suggest that significant exposure to oral treponemes may result in a non-productive or suppressed immune response. More recently, Kesavalu et al. reported that humoral immune responses, as manifested by antibody levels, isotype, and antigenic specificity, were not capable of resolving *T. denticola* infection in a mouse infection model (17). This pattern is not unlike what is seen in other spirochete pathogens, including *Treponema pallidum* and *Borrelia burgdorferi*, both of which cause chronic infection mediated by one or more mechanisms of immune evasion or suppression. In the case of both of these organisms, there has been considerable controversy as to the identity of cell surface proteins expressed during infection and their roles in disease (7, 25).

Our laboratory has developed a series of defined isogenic *T. denticola* mutants defective in one or more components of Msp and the PrtP protease complex (4, 12, 18). To characterize the immunogenicity of these outer membrane components, we used human serum samples to probe *T. denticola* ATCC 35405 parent and isogenic mutant strains as well as *T. denticola* and *Escherichia coli* strains expressing different Msp types. Here we present definitive evidence that Msp and the proteins comprising the PrtP complex are expressed in the human host. Furthermore, we demonstrate that anti-Msp human antibodies can show strain specificity consistent with serogrouping of *T. denticola* strains.

## Materials and methods

### Human serum samples

Archived adult serum samples were used in this study. Initial characterization of

these samples has been described in previous studies (2, 20). Subjects whose sera were used were classified as having moderate to severe gingivitis, but exhibited little evidence of destructive periodontal disease. Use of these samples was conducted under protocols approved by the University of Michigan Health Sciences Institutional Review Board.

### Bacterial growth and sample preparation

The *T. denticola* strains used in this study (listed in Table 1) were grown anaerobically in new oral spirochete (NOS) medium, with erythromycin (40 µg/ml) added as appropriate (12). Three-day exponential phase cultures were harvested by centrifugation (1200 g, 10 min, 4°C). All further manipulations were carried out at 4°C. Cell pellets were washed twice with phosphate-buffered saline (PBS) containing 1 mM phenylmethylsulfonyl fluoride (PMSF) to inactivate proteases. To ensure equivalent loading of samples, pellets were resuspended in 300–500 µl lysis buffer (1% nonidet P-40, 20 mM HEPES pH 7.4, 250 mM NaCl, 10 mM NaF, 1 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 3 mM ethylenediaminetetraacetic acid, 1 mM PMSF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 2 mM dithiothreitol, 0.27 M sucrose, 1 mM pNPP, and Roche complete protease inhibitor cocktail in 10 ml) based on optical density of the culture, and mixed until translucent. Extracts consisting of total cell lysates were clarified by centrifugation (16,000 g, 20 min). Soluble supernatants containing proteins from equivalent numbers of cells per µl were collected, heated at 100°C for 5 min, divided into aliquots and stored at –80°C until use.

*E. coli* strains were grown in Luria-Bertani (LB) broth or agar with ampicillin (50 µg/ml), carbenicillin (50 µg/ml) or chloramphenicol (34 µg/ml), as appropriate. DNA encoding the mature Msp peptides of *T. denticola* 35405, 33520 and OTK (13) was amplified from

genomic DNA by polymerase chain reaction and cloned into *E. coli* plasmid vector pET17b (Novagen, Madison, WI), as described previously (11). For expression of recombinant Msp, the resulting plasmids (or empty vector) were introduced into *E. coli* Rosetta™ (DE3)pLysS (Novagen). Expression of Msp was induced as described previously (11), and cultures were harvested after 3 h. Cell pellets were rinsed once in PBS, recentrifuged, and resuspended in PBS to a standardized optical density and lysed in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) sample buffer. After heating at 100°C for 5 min, sample aliquots were stored at –80°C until use.

### SDS–PAGE and Western blots

Protein extract aliquots were thawed on ice, mixed with sample buffer, boiled for 7 min and subjected to 4–20% SDS–PAGE. Proteins in gels were stained with Coomassie brilliant blue or transferred to nitrocellulose membranes at 25 V for 1 h. Membranes were stained with Ponceau-S to verify protein transfer, blocked for at least 1 h (5% non-fat milk, 0.2% Tween-20 in Tris-buffered saline), and probed with human sera (dilution 1 : 500) or anti-T•7Tag monoclonal antibody (Novagen, #69522; dilution 1 : 10,000) in blocking solution for a minimum of 2 h. Membranes were rinsed three times with TBS–0.2% Tween-20 for 10 min at room temperature and incubated with peroxidase-conjugated goat anti-human immunoglobulin G (IgG; Jackson ImmunoResearch, West Grove, PA, #109-035-088) or peroxidase-conjugated goat anti-mouse IgG (Pierce, Rockford, IL, #31430), as appropriate. Western blots were developed using a chemiluminescent substrate. The secondary antibodies did not recognize *T. denticola* antigens (data not shown).

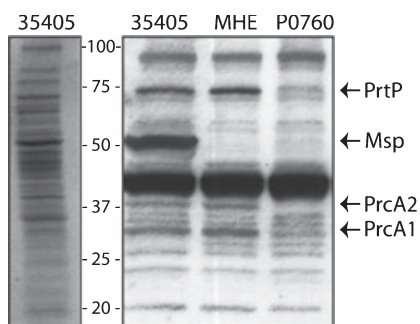
Table 1. *Treponema denticola* strains used in this study

Strain	Characteristics	Phenotype	Source
OTK	Serotype b-like	CTLP (dentilisin) positive 64-kDa Msp	(13)
33520	Serotype c	CTLP (dentilisin) positive 53-kDa Msp	ATCC
35405	Serotype a	CTLP (dentilisin) positive 53-kDa Msp	ATCC
MHE	Isogenic <i>msp</i> mutant of 35405	CTLP (dentilisin) positive Msp negative	(12)
P0760	Isogenic TDE0760 mutant of 35405	CTLP (dentilisin) negative Greatly reduced Msp	(4)

## Results

### Human serum antibodies recognize Msp, PrcA, and PrtP

Archived human sera from a previous study (20) were used to probe the *T. denticola* 35405 parent and isogenic mutant strains. Fig. 1 shows a representative Western blot in which human IgGs recognized multiple bands in *T. denticola* 35405. As in other spirochete pathogens, the prominent antigenic band at approximately 43-kDa was most likely flagellin (23, 29). Comparison with isogenic *T. denticola* mutants demonstrated that the 53-kDa band was Msp (11), and that the 72-, 40-, and 30-kDa bands were PrtP, PrcA2, and PrcA1, respectively (18). The 53-kDa Msp band was absent in the *msp* mutant MHE. In protease locus mutant P0760, bands corresponding to PrtP, PrcA1, and PrcA2 were absent. Consistent with our previous characterization of this and other protease locus mutants, Msp was also absent from P0760 (4, 12, 18). This definitive evidence that Msp and the protease complex proteins were expressed and were immunogenic in humans supports their identification as likely virulence determinants. Mechanism(s) involved in inter-related expression of Msp and the protease complex are currently under investigation in our laboratory.



**Fig. 1.** Human serum antibodies (IgG) recognize Msp, PrtP, PrcA1, and PrcA2. *Treponema denticola* whole cell extracts were separated on 4–20% SDS–PAGE gels and stained with Coomassie brilliant blue (left panel, strain 35405) or transferred to a nitrocellulose membrane and probed with human serum sample 293 (right panel). Positions of molecular size standards are shown in kDa. Msp and protease complex proteins are expressed in parent strain 35405. Isogenic mutants MHE and P0760 carry mutations in the *msp* gene and the first gene of the protease locus, respectively. MHE lacks Msp, while P0760 lacks both the protease complex proteins and Msp. The strongly reactive band at 43-kDa is likely to represent a flagellar protein.

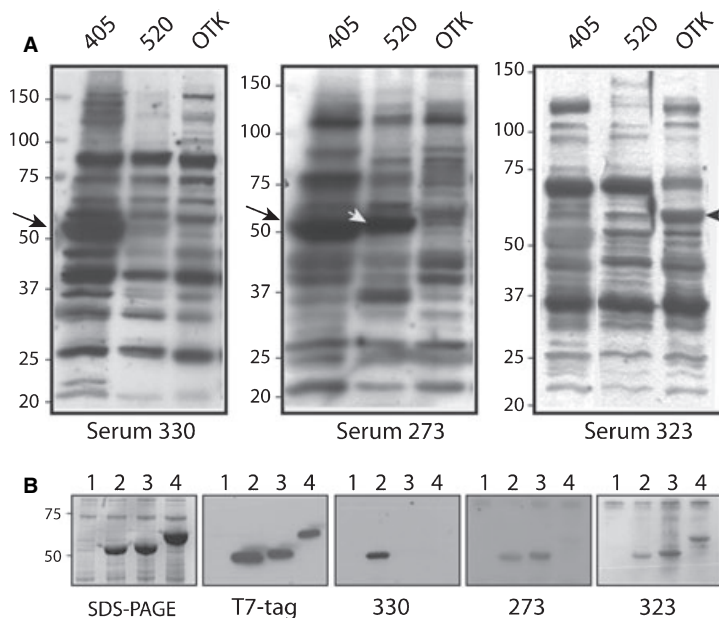
### Human serum antibodies recognize specific Msp types

*T. denticola* strains contain a single *msp* gene, and Msp polypeptides fall into one of three broad groups as defined by DNA sequence and predicted antigenic domains (13). Strains 35405, 33520, and OTK represent three distinct *T. denticola* serotypes (5, 13). As shown in Fig. 2A, human IgGs recognized prominent bands in strains OTK, 33520, and 35405 that were consistent with the distinctly different Msp of each strain. There was considerable variability between serum samples in the level of Msp recognition, and Msp recognition appeared to be strain-specific. In Fig. 2A, Msp was a major immunoreactive protein in at least one strain probed with each of the three sera shown. Serum 330 strongly recognized the 53-kDa Msp of 35405 (serotype a) but did not recognize either the 53-kDa Msp of 33520 (serotype c) or the 64-kDa Msp of OTK (serotype b-like). Serum 273 recognized the Msp of both 35405 and 33520 but not that of strain OTK. Serum 323 strongly recognized a band consistent with the 64-kDa OTK Msp, while reactivity with 35405 and 33520 Msp appeared, at best, weak. To confirm the identity of the putative

Msp bands in Fig. 2A, we used the same sera to probe extracts of *E. coli* strains expressing recombinant Msp fused to a vector-encoded T7Tag (Novagen). As shown in Fig. 2B, the sera showed the same pattern of specificity for individual Msp, with the exception that serum 323 reacted less strongly with recombinant OTK T7-Msp than with the native protein. These results are in agreement with our previous finding that Msp sequences from numerous isolates have considerable amino acid differences in predicted surface-exposed domains ((13) and data not shown). Taken together, these data show that Msp is strongly immunogenic in humans, and that *T. denticola* serotypes are at least partially defined by their Msp type.

## Discussion

Previous studies reported that conspicuous differences in anti-*T. denticola* IgG titers exist between periodontally diseased and healthy subjects [reviewed in (9)]. Serum antibody titers to periodontal pathogens were reduced during the maintenance phase following periodontal therapy (1). However, other studies reported that, despite successful periodontal therapy,



**Fig. 2.** Human serum antibodies (IgG) discriminate between different *T. denticola* Msp. (A) Western immunoblots of *Treponema denticola* strains ATCC 35405 (405), ATCC 33520 (520), and OTK probed with individual human serum samples. Arrows indicate the positions of immunoreactive bands corresponding to the 53-kDa Msp of 35405, the 53-kDa Msp of 33520, and the 64-kDa Msp of OTK. Positions of molecular size standards are shown in kDa. (B) From left to right: Coomassie-stained SDS–PAGE gel and four Western immunoblots of *Escherichia coli* strains expressing recombinant Msp. Lane 1, empty vector control; lane 2, 35405 Msp; lane 3, 33520 Msp; lane 4, OTK Msp. Individual blots were probed with human serum samples as in (A) or with anti-T7Tag monoclonal antibody, as indicated below each blot.

anti-*T. denticola* titers remained elevated over a 30-month period, suggesting that serology may mark the history of past periodontal infection but that it is not indicative of current colonization (24). Some of the sera used in the present study were from subjects classified as having at most low levels of *T. denticola*, as measured by reactivity of a subgingival plaque sample with rabbit antibodies raised against *T. denticola* ATCC 35404 (20). Interestingly, some of these sera reacted strongly with Msp of one or more *T. denticola* strains. It cannot be determined from the available reagents whether these subjects had a previous *T. denticola* infection, or whether the immunological reagents used to detect colonization in the previous study were overly strain-specific (20). Some serum samples did not react with Msp of the three strains tested (data not shown). Because human serum recognition of Msp can be both strong and strain-specific, previously reported colonization data may be somewhat less than reliable. Our data strongly suggest that the utility of immunological methods to detect or quantify *T. denticola* and total spirochetes in clinical samples is limited not only by the diversity of oral *Treponema* species, but also by major antigenic differences between *T. denticola* strains.

Our data demonstrate that Msp is one of the predominant *T. denticola* antigens recognized by human serum antibodies. Interstrain differences in Msp appear to be a defining element of strain serotype because each wild-type strain tested has a distinct Msp and represents a different serotype. This is consistent with our previous report that rabbit polyclonal antibodies raised against the Msp of strain 35405 did not recognize the Msp of 33520 and OTK (13). Barron et al. reported that *T. denticola* is the first spirochete to colonize the oral cavity and that *T. denticola* comprises a major proportion of all spirochetes at all ages tested (3). Furthermore, that study found at least two distinct serotypes of *T. denticola* in plaque from most children tested. Our results are consistent with this previous finding, with some subjects having serum reactivity to only one *T. denticola* Msp type, while others recognize two or more Msp types. It should be noted that our study does not address colonization by or immune responses to oral *Treponema* species other than *T. denticola*, which probably further complicates the overall picture. The question as to whether anti-Msp antibodies are protective against subsequent infection remains to be addressed.

Isogenic mutants of *T. denticola* are becoming important tools for characterizing host immune responses in periodontal disease. We provide here the first definitive evidence that Msp is a major immunoreactive protein and that the immunogenicity is often strain specific. We also demonstrate that the proteins comprising the PrtP protease complex are immunogenic. This protease complex (CTLp, dentilisin) is present in the three *T. denticola* serotypes tested as well as in several other species of oral spirochetes (6). While we cannot rule out the possibility that antibody responses to these proteins result from cross-reactivity with other bacterial or human proteins, this is highly unlikely because Msp-like proteins are found only in spirochetes. While PrtP contains conserved peptidase domains, the polypeptides of the protease complex are otherwise unique to oral *Treponema* species. As shown by their ability to elicit humoral immune responses in humans, our data provide the first evidence that these putative virulence determinants are in fact expressed *in vivo*. Validation of this key prerequisite for definition as virulence determinants lends strong support to work underway in several laboratories characterizing the role of these surface proteins in the interactions between *T. denticola* and host immune response pathways.

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