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Cloning and Sequence Analysis of a Chymotrypsinlike Protease from *Treponema denticola*

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A clone expressing a *Treponema denticola* chymotrypsinlike protease from recombinant plasmid pSA2 was identified in a genomic library of *T. denticola* ATCC 35405. Nucleotide sequencing of the insert identified an open reading frame, designated the *prtB* gene, which codes for the protease. Two potential inverted repeat sequences are present both upstream and downstream from the *prtB* gene. The *prtB* gene would code for a putative protein of 273 amino acids with a calculated molecular mass of 30.4 kDa and an estimated pI of 7.0. The G+C content of the gene is 40.3%. The results of maxicell analysis are consistent with the expression of a 30-kDa protease from the *prtB* gene. Preliminary characterization of the protease indicated that it was inhibited by the protease inhibitors phenylmethylsulfonyl fluoride, diisopropylfluorophosphate, and *N*-tosyl-L-phenylalanine chloromethyl ketone but not by *Nα-p*-tosyl-L-lysine chloromethyl ketone. Purification of the protease was accomplished with the PinPoint protein purification system following construction of site-directed mutagenized plasmid pXa-3:2. The purified protease degraded human and bovine serum albumins as well as casein. Furthermore, hemolysis of sheep erythrocytes by the protease was observed. Northern (RNA) blot analysis of mRNA extracted from strain 35405 indicated a single 1.9-kb mRNA species containing the *prtB* transcript. In addition, the results of primer extension analysis indicated that transcription was initiated primarily at a T residue. However, no corresponding −10 and −35 sequences related to *Escherichia coli* promoter sequences were identified. The availability of the purified protein and its gene will aid in evaluating the potential role of the protease in the physiology and virulence of *T. denticola* since proteases may play a key role in oral treponemal pathogenicity.

Gram-negative oral bacteria, including spirochetes, have been implicated in the etiology of human periodontal diseases (4, 6, 26, 32, 48, 50, 52, 55). Histological investigations have revealed the presence of spirochetes at the leading edge of subgingival plaque in rapidly progressive periodontitis (43), in the periodontal tissues of acute necrotizing ulcerative gingivitis (14), and in the junctional epithelium of experimentally induced periodontal lesions (29). In addition, several reports have also demonstrated that elevated numbers of spirochetes are present in dental plaque associated with various types of periodontal diseases (16, 17, 20). *Treponema denticola*, which is the most frequently isolated oral spirochete, has been positively correlated with the presence of severe periodontal lesions (33). Successful treatment of periodontal pockets leads to both a reduction in spirochetes in the subgingival microbiota and a reduction in specific pocket proteolytic activities associated with *T. denticola* (15, 19, 37).

T. denticola expresses a number of potential virulence properties, including the inhibition of superoxide production by human polymorphonuclear leukocytes (2), the suppression of lymphocyte blastogenesis by mitogens (18), the inhibition of fibroblast proliferation (3), the induction of detachment of human gingival fibroblasts (1), cytotoxicity to epithelial cells (42), and the ability to attach to host tissues (10, 36) and invade periodontal tissue (7, 30, 43). *T. denticola* strains have also been demonstrated to be highly proteolytic (24, 34) and keratinolytic (27). Therefore, proteases are also potential virulence factors in these organisms (18, 36) and may be of importance in providing essential amino acids for growth, neutralizing host defense mechanisms, and playing a key role in the invasion of and subsequent multiplication in host cells. Previous studies have demonstrated that *T. denticola* possesses a number of enzymes capable of degrading synthetic peptides (23, 24, 28, 35), as well as type IV collagen, gelatin, elastin, and fibronectin (53). Recently, Uitto et al. (54) isolated a chymotrypsinlike enzyme from *T. denticola*. Furthermore, Greiner et al. (9) demonstrated that this enzyme is associated with the cell envelope of *T. denticola* and might play an important role in the invasion and destruction of the basement membrane. In addition, several genes coding for *T. denticola* proteases have been previously isolated (22, 41). In this report, we present data on the isolation and characterization of a novel chymotrypsinlike protease gene, *prtB*, from *T. denticola* ATCC 35405.

MATERIALS AND METHODS

Bacterial strains and cultivation. *T. denticola* ATCC 35405, which was previously isolated from a human periodontal pocket (3), was maintained and grown at 37°C under an atmosphere of 85% N_2, 10% H_2, and 5% CO_2 in an anaerobic chamber (Coy Laboratory Products Inc., Ann Arbor, Mich.) in TYGVS broth medium (35).

Escherichia coli HB101 was used as a host strain for the PinPoint pXa-3 vector (Promega Corp. Madison, Wis.). *E. coli* CJ236 (*dut ung*) and MV1190 (*ung*$^+$) were employed for site-directed mutagenesis. *E. coli* JM109 was the host strain for pBluescript II KS +/− phagemid. *E. coli* strains were routinely grown at 30°C in Luria broth (LB) (44) or on LB agar plates. Transformed cells were grown on LB agar plates or in LB broth supplemented with ampicillin (100 μg/ml). For maxicell analysis, *E. coli* CSR603 (*uvrA phr*) (46) was used. 2XY

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medium (6 g of tryptone, 10 g of yeast extract, 5 g of NaCl, 1 liter of distilled water) and M-9 medium (44) were also used where indicated.

Isolation of chromosomal DNA and mRNA. Chromosomal DNA from *T. denticola* was isolated by the method of Marmur (25). mRNA was isolated from *T. denticola* by utilizing the TRIzol Reagent (GIBCO Bethesda Research Laboratories, Gaithersburg, Md.). Briefly, *T. denticola* 35405 was cultured overnight in 1 liter of TYGVS medium, and the cells were harvested by centrifugation and homogenized following mixing with 6 ml of TRIzol solution. The homogenized samples were next incubated for 5 min at room temperature. Chloroform (1.2 ml) was then added, and the combination was mixed vigorously and then incubated at room temperature for 3 min. Following centrifugation, a colorless aqueous phase was isolated and transferred to a fresh tube. The RNA was dissolved in diethyl pyrocarbonate (Sigma, St. Louis, Mo.)-treated distilled water following precipitation with isopropanol.

Construction of a genomic library and screening of clones. *T. denticola* ATCC 35405 chromosomal DNA was isolated, size fractionated (3 to 6 kb) by use of a sucrose gradient following partial *Sau*3AI digestion, and ligated to plasmid vector pUC118, which was digested with *Bam*HI and dephosphorylated. The ligation mixtures were transformed into *E. coli* HB101, and duplicate plates were screened for protease activity on LB-ampicillin agar plates containing 1.0% skim milk. Protease-positive colonies were detected following anaerobic incubation of the plates for 7 days. Clone extracts were prepared by sonication and assayed for hydrolysis of the synthetic chymotrypsin substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (SAAPNA; Sigma) at 37°C for 1 h (54).

Southern blot analysis. For Southern blot analysis, chromosomal DNA was digested with *Hin*dIII, separated on 1.0% agarose gels, transferred to GeneScreen hybridization transfer membranes (NEN Research Products, Boston, Mass.), and fixed by exposure to UV radiation in a UV Stratalinker (Stratagene, La Jolla, Calif.). Filters were prehybridized in buffer (0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin [BSA], 0.2% Ficoll, 0.05 M Tris-HCl [pH 7.5], 1 M NaCl, 0.1% sodium PP_i, 0.1% sodium dodecyl sulfate [SDS], 10% dextran sulfate, denatured salmon sperm DNA) for 2 h at 42°C. The probe (pSA2) was added following labeling with biotin by use of the random primer DNA labeling kit (Bio-Rad, Richmond, Calif.), and hybridization was performed at 42°C overnight. Posthybridization was carried out with a streptavidin-alkaline phosphatase-conjugated solution utilizing the BluGENE nonradioactive nucleic acid detection system (GIBCO Bethesda Research Laboratories) following washing.

Northern (RNA) blot analysis. Total RNA isolated from *T. denticola* was separated by electrophoresis on a 2.2 M formaldehyde–1.0% agarose gel and transferred to Hybond-N+ nylon membranes (Amersham International plc., Amersham, United Kingdom) by capillary elution. The ECL direct nucleic acid labeling and detection systems (Amersham) were used to identify the RNA fragments. The 0.95-kb *Nco*I-*Hin*dIII DNA fragment from plasmid *Hin*dIII-*Nco*I-*Eco*RI-pTrc99A was used as a probe. The blots were exposed on blue-light-sensitive autoradiography film (Hyperfilm-ECL; Amersham) for 2 h.

Nucleotide sequencing of the *prtB* gene. The nucleotide sequence of the *prtB* gene was determined from both DNA strands by use of the dideoxynucleotide sequencing strategy (47). Overlapping DNA fragments from pSA2 were subcloned into pUC118, pUC119, or pBluescript II KS + or KS − (Stratagene), and deletion mutants were constructed by restriction enzyme digestion. Single-stranded template DNA was isolated by the method of Yanish-Perron et al. (57) by utilizing M13K07 helper phage (Bio-Rad Laboratories, Hercules, Calif.). Sequencing was carried out with Sequenase, version 2.0 (United States Biochemical Corp., Cleveland, Ohio) and standard M13, KS, T3, and T7 primers by using ^{35}S-dATP. Sequence analysis was performed with the IBI Pustell sequence analysis program (International Biotechnologies, Inc., New Haven, Conn.) and the HIBIO DNASIS program (Hitachi Software Engineering Co., Ltd., Japan).

Primer extension analysis. A 21-mer oligonucleotide primer (5′-AAT ACC GCC GTT CTT TGT CAT-3′) synthesized by the oligonucleotide synthesis facility at the Department of Biochemical Pharmacology, State University of New York at Buffalo, was labeled with [γ-^{32}P]ATP (Dupont NEN Research Products, Wilmington, Del.) and used as a primer. This oligonucleotide anneals to nucleotides 118 to 138 (see Fig. 4). Reverse transcriptase (GIBCO Bethesda Research Laboratories) was used to extend this primer to produce cDNA complementary to *T. denticola* mRNA following annealing. After RNase A treatment, the resulting end-labeled cDNA was electrophoresed on a 6% polyacrylamide gel (Long Ranger; AT Biochem Inc., Malvern, Pa.) under denaturing conditions. Dideoxy sequencing reactions with the same oligonucleotide as a primer were also electrophoresed for reference. Dried gels were exposed to Kodak XAR-2 (X-Omat AR) film.

Expression of the *ptrB* gene in maxicells. Plasmids were introduced into maxicell strain *E. coli* CSR603 following transformation. Proteins encoded by the resident plasmid in each cell were then labeled with [^{35}S]methionine by the method of Sancar et al. (45). These cells were collected by centrifugation and suspended in sample buffer (2% SDS, 5% mercaptoethanol, 10% glycerol, 0.0005% bromophenol blue, 0.0625% Tris-HCl [pH 6.8]) and heated for 2 min at 100°C. The samples were next loaded onto an SDS–8.5% polyacrylamide gel, electrophoresed, dried, and subjected to autoradiography.

Purification of the *prtB* gene product. The PinPoint Xa System (Promega) was used for purification of the recombinant protein. For utilization of this system, plasmid pXa-3:2 (see Fig. 1) was constructed by site-directed mutagenesis. Briefly, the *Hin*dIII-*Eco*RI fragment from pSA2 was ligated to *Hin*dIII-*Eco*RI-digested pUC119. For construction of the *Nco*I site, the ATG site of *prtB* in pSA2 was altered by site-directed mutagenesis from AA ATG A to CC ATG G. A mutagenic 28-mer oligonucleotide (5′-GCC GTT CTT TGC CAT GGT AAG ACT CCT T-3′) was synthesized by V. Tryon (University of Texas Health Science Center, San Antonio). Site-directed mutagenesis of the DNA fragment was carried out by the method of Kunkel et al. (12). Subsequently, the fragment was ligated to vector pTrc99A to produce *Nco*I-*Eco*RI-pTrc99A. At the *Pvu*II site (upstream of the *Nco*I site), a *Hin*dIII site was constructed by addition of a *Hin*dIII linker, producing *Hin*dIII-*Nco*I-*Eco*RI-pTrc99A. The *Hin*dIII fragment containing the *prtB* gene was then ligated to vector PinPoint pXa-3, yielding plasmid pXa-3:1. Plasmid pXa-3:1 was then digested with *Nco*I, and the truncated fragment was filled in with the Klenow enzyme. After digestion with *Nru*I, the fragment was self-ligated and plasmid pXa-3:2 was obtained.

E. coli subclone Xa-3:2 was grown for 24 h on 80 LB agar plates (15 by 100 mm) containing ampicillin (100 μg/ml) at 30°C. The harvested cells were washed with 50 mM Tris-HCl (pH 7.5)–50 mM NaCl–5% glycerol and suspended in 10 volumes of the same buffer (milliliters per gram of cell paste) at 4°C. The bacterial cells were disrupted by sonication and centrifuged to remove the cellular debris, the resultant crude extract was applied to an equilibrated SoftLink Resin (Promega) column, and detection of the recombinant fusion pro-

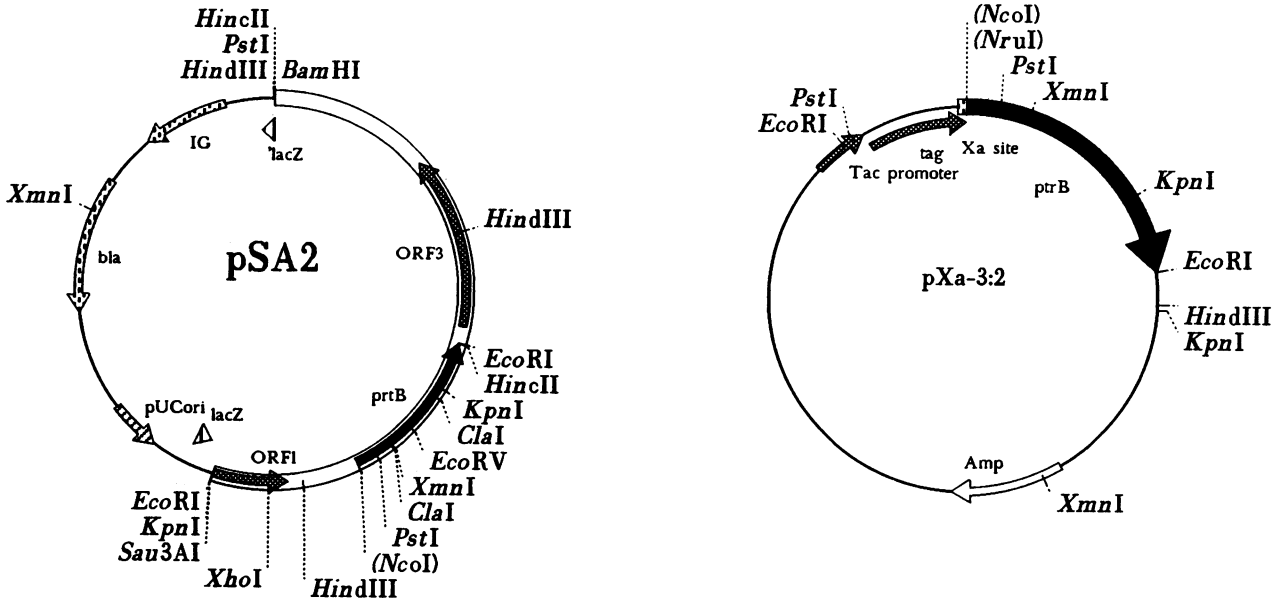

FIG. 1. Structures of plasmids pSA2 and pXa-3:2. To construct plasmid pSA2, partially *Sau*3AI-digested *T. denticola* chromosomal DNA was ligated to plasmid vector pUC118. To construct pXa-3:2, the site-directed mutagenized *Hin*dIII-*Hin*dIII fragment was ligated to the PinPoint pXa-3 vector and self-ligated following *Nru*I and *Nco*I cleavage and filling in of the *Nco*I site.

tein was carried out in accordance with the manufacturer's instructions. Briefly, the recombinant protein was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, Mass.). The biotinylated proteins were detected with streptavidin-alkaline phosphatase and nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate toluidinium (salt) reagent for alkaline phosphatase activity. The purified protease was also treated with factor Xa to cleave the recombinant protease from the Tag (12.5-kDa subunit of the transcarboxylase complex) protein.

Determination of substrate specificity. Degradation of BSA, human serum albumin, collagen types I and IV, fibrinogen, gelatin, fibronectin, immunoglobulin G, and transferrin was analyzed by incubating 1.5 μg of the purified enzyme with 25 μg of substrates in a final volume of 40 μl overnight under both aerobic and anaerobic conditions at 37°C, except that the incubation with collagen was carried out at 30°C. The reaction buffer contained 10 mM Tris-HCl (pH 7.5), 15 mM L-cysteine, and 1 mM $CaCl_2$. Analysis of the reaction products was performed by SDS-PAGE (13), and staining of the proteins was done with Coomassie brilliant blue.

Hemolysin activity. Hemolysin activity of the protease was examined by two methods. The purified enzyme, the factor Xa-treated enzyme, and a lysate of *E. coli* harboring only plasmid pXa-3 (10 μg) were added to wells cut into a 10% sheep blood agar plate. The plate was then incubated aerobically at 37°C for 18 h and examined for zones of hemolysis. For more quantitative assays, mixtures of 150 μl of each enzyme and 5% sheep blood were incubated at room temperature for 90 min. The supernatant fluids were isolated (120 μl), and the optical density at 570 nm was measured following centrifugation at 1,000 × *g* for 5 min at 4°C to quantitate the release of hemoglobin from the sheep erythrocytes (RBCs) (8).

Nucleotide sequence accession number. The nucleotide sequence reported here has been submitted to the GenBank database and assigned accession number L25603.

RESULTS

Cloning of a chymotrypsinlike protease gene. A clone bank of *T. denticola* DNA fragments was constructed in plasmid pUC118 and screened for protease-positive clones on skim milk agar plates. Protease-positive colonies capable of hydrolyzing skim milk were identified following 1 week of aerobic incubation. However, the zones of hydrolysis were intensified following anaerobic incubation of duplicate plates. Among a total of 1,500 colonies, 5 protease-positive colonies were observed. Two clones, SA2 (Fig. 1) and SA7, appeared to express a chymotrypsinlike protease activity since they hydrolyzed the synthetic chymotrypsin substrate SAAPNA. Since clones SA2 and SA7 appeared to contain similar plasmids, SA2 was selected for further characterization.

Southern blot analysis. To confirm that the protease gene detected in plasmid pSA2 was derived from *T. denticola* ATCC 35405 chromosomal DNA, Southern blot analysis was carried out. Plasmid pSA2 was labeled with biotin and utilized as a probe with chromosomal DNA digested with *Hin*dIII (Fig. 2). The results demonstrated that the probe did not react with *E. coli* DNA but did exhibit three positive bands with *T. denticola*. Three positive bands were anticipated upon the basis of the restriction map of the *T. denticola* insert present in plasmid pSA2. In addition, the internal 1.9-kb *Hin*dIII fragment was present in both the chromosomal DNA and plasmid patterns. These results suggested that the insert DNA of plasmid pSA2 was derived from *T. denticola* chromosomal DNA and the internal *Hin*dIII fragment of plasmid pSA2 was exclusively derived from *T. denticola*.

Proteolytic activity of deletion mutants. To localize the protease gene on the *T. denticola* insert in plasmid pSA2, DNA fragments were subcloned into pUC118 or pUC119. Crude extracts of subclones containing the 1.9-kb *Hin*dIII fragment in either orientation relative to the vector degraded SAAPNA (Fig. 3). In addition, the *Eco*RI fragment overlapping most of the *Hin*dIII fragment also coded for protease activity in both

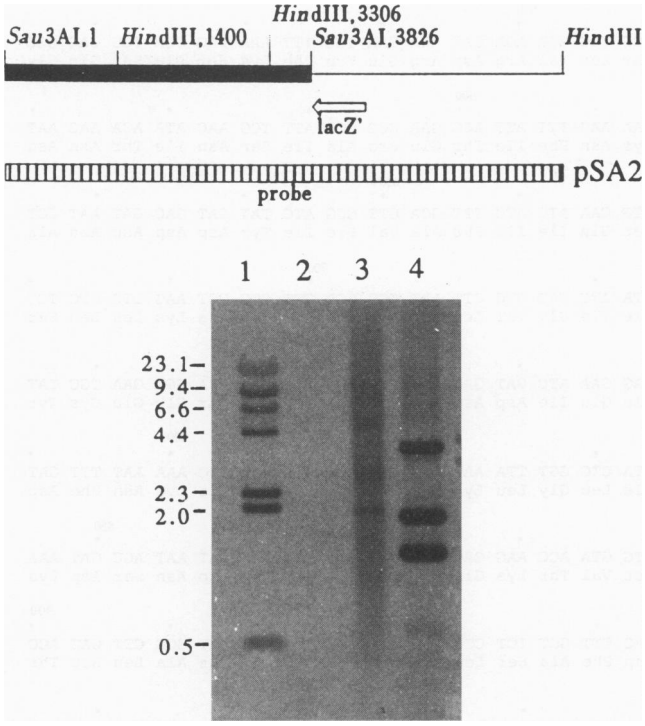

FIG. 2. Southern blot analysis of *Hin*dIII-digested chromosomal DNAs from *E. coli* and *T. denticola* with biotin-labeled plasmid pSA2 as a probe. Lanes: 1, molecular size markers; 2, *E. coli* HB101; 3, *T. denticola* ATCC 35405; 4, plasmid pSA2. The numbers at the left indicate molecular sizes in kilobase pairs.

orientations. These results suggested that the protease gene was located within the larger *Eco*RI-*Hin*dIII fragment of the insert. Since isopropyl-β-D-thiogalactopyranoside (IPTG) had no effect on the expression of protease activity in pSA2 and protease activity was expressed similarly from the 1.9-kb *Hin*dIII fragment in both fragment orientations, it is likely that a promoter present on the *T. denticola* insert initiates transcription of the protease gene.

Sequence analysis of the pSA2 gene. To characterize the protease gene, sequencing of both insert DNA strands of plasmid pSA2 was carried out. The sequence data identified three open reading frames (ORFs) on the insert contained in plasmid pSA2 (Fig. 1), and only one of these was located within the largest *Eco*RI-*Hin*dIII fragment of the insert. This ORF was designated the *prtB* gene (Fig. 4). The presumed initiation codon of the gene is preceded by a sequence which is highly homologous to the *E. coli* Shine-Dalgarno consensus sequence. In addition, a potential inverted repeat sequence is present just upstream of the ribosomal binding site and could play a role in regulating the expression of the *prtB* gene. Another potential stem-loop structure was identified downstream from the *prtB* gene and could act as a transcription terminator. The ORF corresponding to this gene, which contains 822 bp, would code for a putative protein of 273 amino acids with a calculated molecular mass of 30.4 kDa and an estimated pI of 7.0. The G+C content of the gene, 40.3%, is slightly higher than that estimated for chromosomal DNA from *T. denticola* strains (51). A comparison of the amino acid sequence of the protease with those of other proteins in the National Biomedical Research Foundation protein database revealed no other homologous proteins.

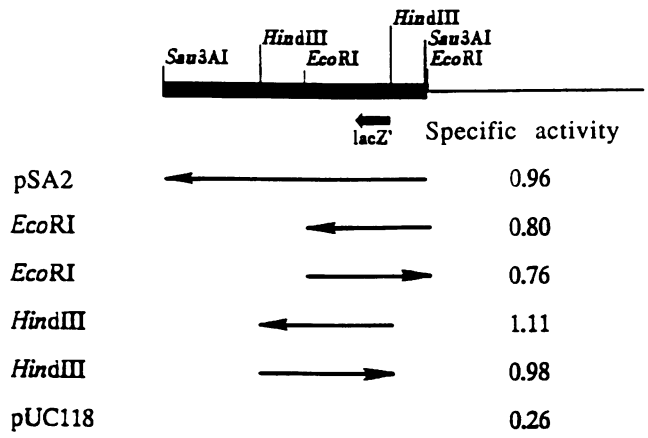

FIG. 3. Proteolytic activity of deletion mutants. Crude extracts from each *E. coli* subclone were assayed for SAAPNA-degrading activity.

Maxicell analysis of the proteins expressed from plasmid pSA2. To confirm that the ORF corresponding to the *prtB* gene codes for a protein of 30 kDa, maxicell analysis of pSA2 was carried out. As indicated in Fig. 5, a strongly labeled protein band near 30 kDa was expressed by pSA2 but not by vector pUC118 alone. These results are consistent with the expression of a 30-kDa protease from the *prtB* gene.

Purification and substrate specificity of the protease. The PinPoint protein purification system was used for purification of the protease following construction of site-directed mutagenized plasmid pXa-3:2 (Fig. 1). The recombinant fusion protein, detected following SDS-PAGE analysis of crude extracts transferred to polyvinylidene difluoride membranes and probed with streptavidin (Fig. 6), was expressed from plasmid pXa-3:2 following induction with IPTG. *E. coli* strains normally synthesize a 22.5-kDa biotinylated protein, which could be readily detected on the blots. In addition, the 12.5-kDa subunit of the transcarboxylase complex (Tag protein) was also detected in transformants containing only plasmid vector pXa-3. However, in the extracts containing plasmid pXa-3:2 expressing the recombinant fusion protein, several biotin-containing proteins as large as 42 kDa were detected. This size is consistent with that predicted for a fusion of the Tag protein (12.5 kDa) with the *prtB* protease (30 kDa). The lower-molecular-weight bands detected likely represent degradation products of the 42-kDa fusion protein. Expression of the fusion protein in *lon* mutant *E. coli* hosts did not prevent degradation of the fusion protein (data not shown).

Crude extracts containing the *prtB* protease fused to the Tag protein displayed similar SAAPNA-hydrolyzing activities in the presence or absence of Factor Xa (data not shown). This indicated that the fusion protein expressed the same activity as did the *prtB* protease. When the fusion protein was purified on SoftLink resin columns, the specific activity of the enzyme increased 18-fold. This likely represents a minimum purification value, since some of the lower-molecular-weight species may be enzymatically inactive. Coomassie blue staining of the purified enzyme following SDS-PAGE revealed several minor protein bands in addition to the predominant proteins, which corresponded to streptavidin-positive bands (data not shown).

The substrate specificity of the protease was examined by incubating the enzyme with several proteins, followed by analysis of the reaction products by SDS-PAGE (Fig. 7). The

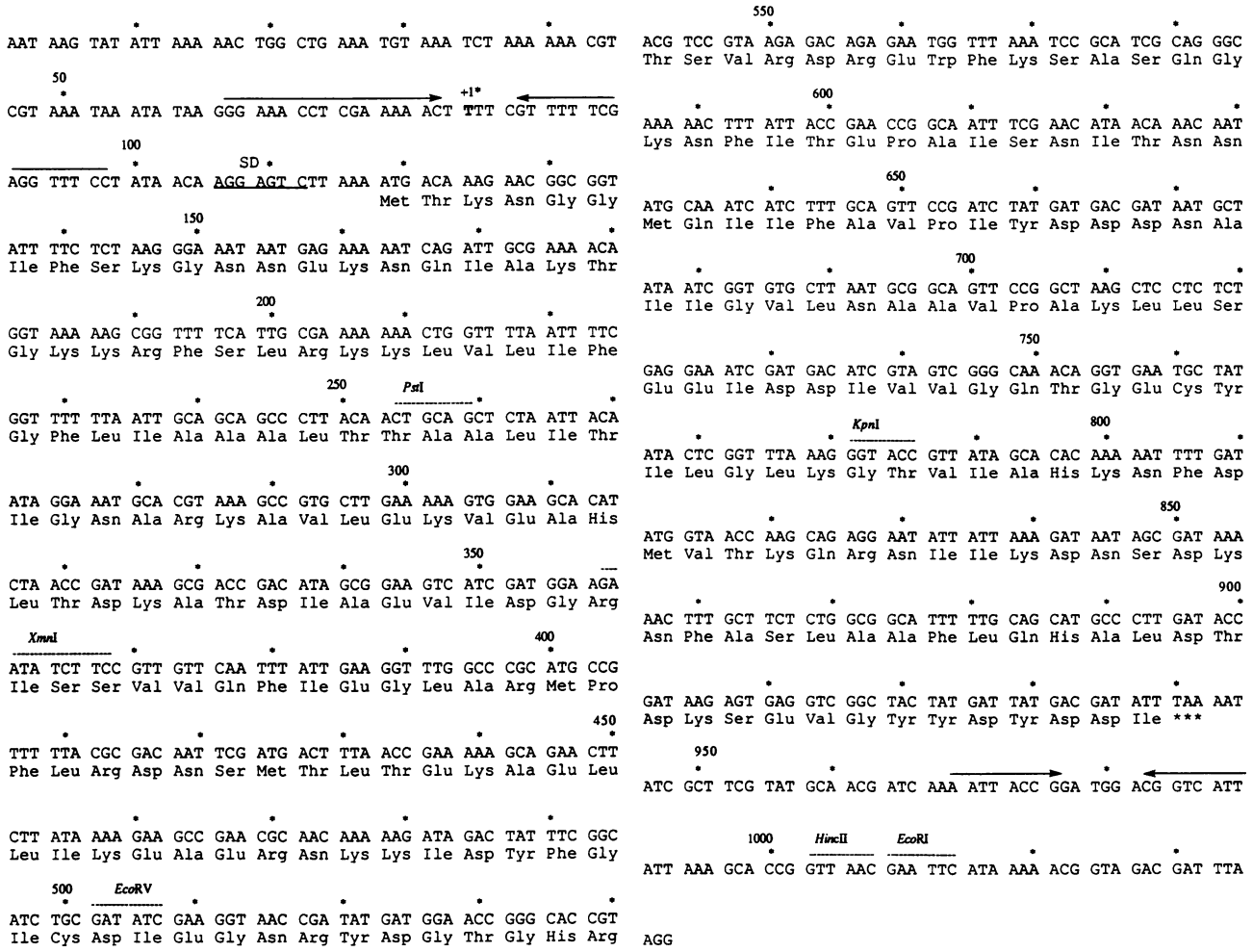

FIG. 4. Nucleotide sequence of the *T. denticola prtB* gene and deduced amino acid sequence of the chymotrypsinlike protease. The locations of the restriction enzyme digestion sites are indicated above the sequences. Potential stem-loop structures are depicted by the arrows.

protease degraded BSA and human serum albumin, as well as casein. Degradation of other proteins, collagen types I and IV, fibrinogen, fibronectin, gelatin, immunoglobulin G, and transferrin, was not detected. Furthermore, hemolysis of RBCs was detected with the intact or fusion protease on blood agar plates (Fig. 8). In addition, hemolysis of sheep RBCs was demonstrated by quantitating the release of hemoglobin from RBCs following incubation with the protease (data not shown). These results further suggest that the purified protease possesses hemolysin activity.

Characterization of the protease. Maximum protease activity of the enzyme was observed near pH 7.5 (data not shown) in the presence of 15 mM cysteine. The effects of protease inhibitors on the protease indicated that the two synthetic protease inhibitors phenylmethylsulfonyl fluoride and diisopropylfluorophosphate produced strong inhibition of enzyme activity (Table 1). In addition, the synthetic chymotrypsin inhibitor *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), but not the trypsin inhibitor *Nα-p*-tosyl-L-lysine chloromethyl ketone (TLCK), inhibited the enzyme. The *prtB* gene product also was inhibited by EDTA, suggesting a role for a metal cation in enzyme activity. Protease activity was also strongly inhibited by zinc and somewhat stimulated by calcium. The enzyme was only moderately activated by reducing agents such as cysteine and mercaptoethanol but not by dithiothreitol. The biochemical characterization of the enzyme is preliminary and requires further investigation.

Identification of the *prtB* transcript. Since plasmid pSA2 contains three identifiable ORFs, it was of interest to determine the size of the *prtB* transcript. Northern (RNA) blot analysis of mRNA extracted from strain ATCC 35405 was carried out with the 0.95-kb *Nco*I-*Hin*dIII DNA fragment from plasmid *Hin*dIII-*Nco*I-*Eco*RI-pTrc99A as a probe (Fig. 9). The results indicated that the single mRNA species containing the *prtB* transcript was approximately 1.9 kb long. Since the *prtB* gene is approximately 900 bp long, it is possible that this gene is transcribed as part of a polycistronic mRNA.

To identify the precise transcriptional start site for the *prtB* gene, primer extension analysis of mRNA extracted from *T. denticola* was carried out with a 21-mer oligonucleotide as a primer. The results (Fig. 10) indicated that transcription was initiated primarily at a T residue corresponding to position 79 (Fig. 4). Two other minor initiation sites (positions 73 and 86) were also detected. If transcription was initiated at the T residue at position 79, no corresponding −10 and −35 sequences related to *E. coli* promoter sequences could be identified.

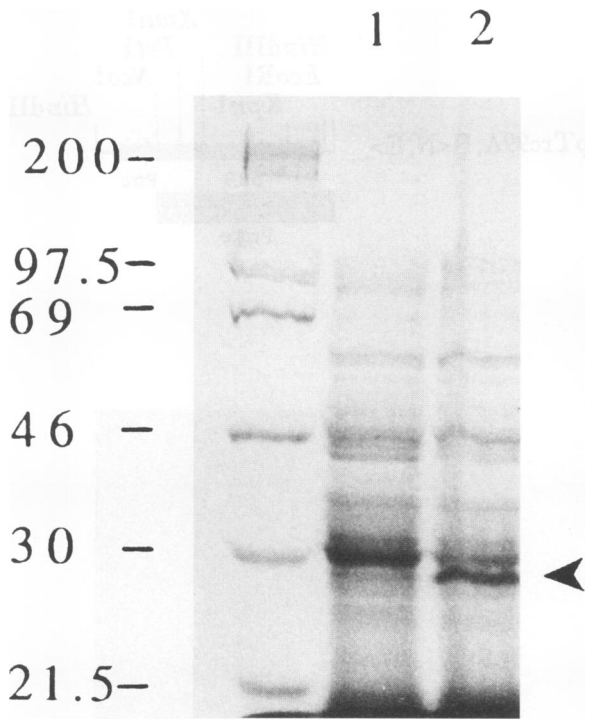

FIG. 5. Maxicell analysis of *E. coli* transformants. Lanes: 1, *E. coli* containing vector pUC118; 2, *E. coli* with pSA2. Molecular size standards in kilodaltons are indicated on the left. The arrowhead indicates the *prtB* gene product.

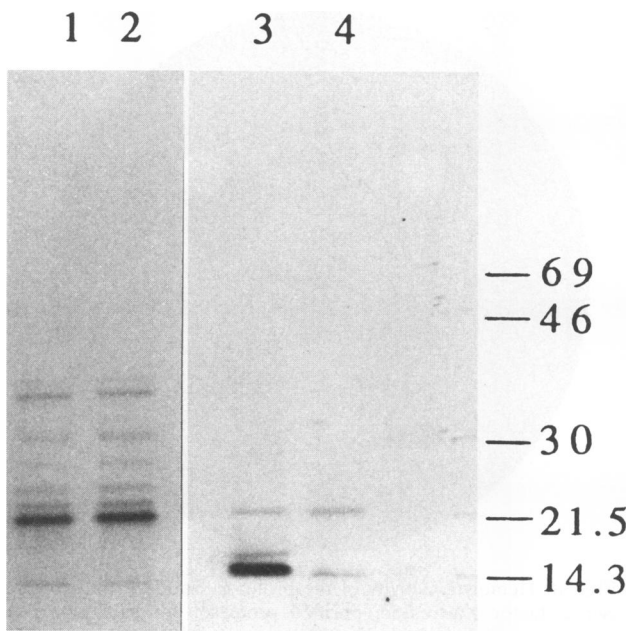

FIG. 6. Detection of expression of fusion constructs. Lanes: 1, *E. coli* subclone Xa-3:2 following 8 h of culture; 2, 22 h of culture; 3, *E. coli* with vector pXa-3; 4, *E. coli* with pUC119. The numbers at the right indicate molecular sizes in kilodaltons.

DISCUSSION

The present communication describes the cloning, characterization, and sequence analysis of a chymotrypsinlike protease gene, *prtB*, derived from *T. denticola* ATCC 35405. The gene was detected in an *E. coli* clone forming a zone of hydrolysis on skim milk agar plates. This is consistent with the subsequent observation that the purified protease hydrolyzed casein. The zones of hydrolysis on skim milk agar plates were intensified following anaerobic incubation. This is similar to the properties of *E. coli* clones expressing a protease from another anaerobe, *Porphyromonas gingivalis* (40). Apparently, maximum activity of the *prtB* protease requires anaerobic conditions.

Deletion analysis revealed the approximate location of the *prtB* gene on the plasmid pSA2 insert (Fig. 3). Nucleotide sequencing revealed the presence of an ORF in this region which codes for the protease. In addition, two other ORFs were identified upstream and downstream of the *prtB* gene, and they are under investigation in this laboratory. The presumed initiation codon of the *prtB* gene is preceded by a sequence which is highly homologous to the *E. coli* Shine-Dalgarno sequence. In addition, a potential inverted repeat sequence with a calculated ΔG of -28.3 kcal (1 cal = 4.184 J)/mol (residues 62 to 77 and 83 to 98) is present just upstream of the ribosomal binding site and could play a role in regulating the expression of the *prtB* gene in *T. denticola*.

Primer extension analysis indicated that transcription was initiated primarily at a T residue corresponding to position 79 (Fig. 4) in *T. denticola*. Since this site resides within the potential inverted repeat, it is likely that transcription of the *prtB* gene is regulated in the anaerobe. Accordingly, an inducer or repressor protein might bind to this inverted repeat region (38, 39). Furthermore, the results of the primer extension analysis revealed another initiation site (an A residue at base position 25) upstream from the stem-loop structure with corresponding -10 and -35 sequences which resemble the *E. coli* promoter consensus sequences. It is likely that transcription of the *prtB* gene is initiated at the latter position in the *E. coli* clone. This is consistent with the results suggesting that protease expression from plasmid pSA2 was initiated from a *T. denticola* sequence. Furthermore, the relatively weak expression of the *prtB* protease in *E. coli* clones might result from the presence of the inverted-repeat structure.

Koopman et al. (11) reported that sequences similar to the consensus ribosomal binding site and putative *E. coli* -10 and -35 promoter sequences could be identified upstream from

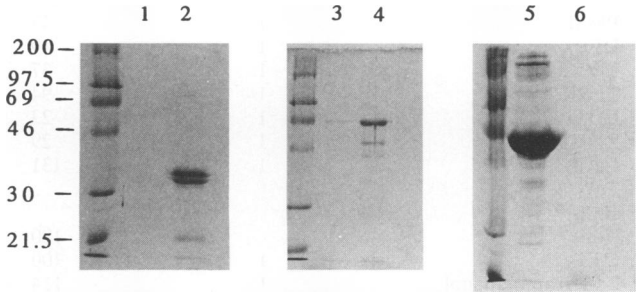

FIG. 7. SDS-PAGE analysis of protein degradation by the purified protease. Lanes: 1, 3, and 6, purified protease incubated with substrates; 2, 4, and 5, substrates incubated with *E. coli* lysates harboring parental plasmid pXa-3. Substrates: 1 and 2, casein; 3 and 4, BSA; 5 and 6, human serum albumin. Molecular size standards (kilodaltons) are indicated on the left. The gels were stained with Coomassie blue.

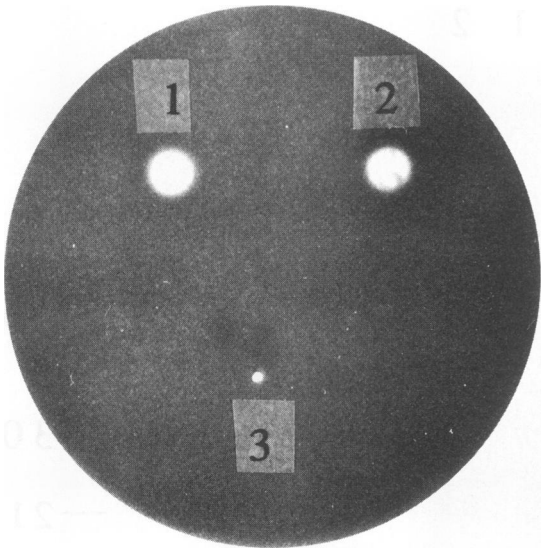

FIG. 8. Hemolytic activity of the protease. Spots: 1, purified protease; 2, factor Xa-treated, purified protease; 3, lysate of *E. coli* harboring parental plasmid pXa-3. The proteins (10 μg) were added to wells in a 10% sheep blood agar plate. The plate was photographed following aerobic incubation at 37°C for 18 h.

the *flaA* gene from the spirochete *Serpulina* (*Treponema*) *hyodysenteriae* but the actual transcription start site was not identified. However, Wallich et al. (56) identified a potential ribosomal binding site and a transcription start site but no *E. coli*-like −10 and −35 sequences for the *Borrelia burgdorferi* flagellum-associated antigen gene. Likewise, Miyamoto et al. (31) have reported the sequence of the *T. denticola* antigenic *tdpA* gene but could not detect an *E. coli*-like promoter sequence upstream of this gene. These observations, together with those from the present investigation indicating no *E. coli*-like promoter sequences upstream from the primary *prtB* transcription start site, suggest that the promoter sequences for the oral spirochete *T. denticola* could be distinct from those of *E. coli*. Further investigation is required to confirm this hypothesis.

TABLE 1. Effects of enzyme inhibitors and reducing agents[a]

Effector[b]	Concn (mM)	Residual activity (%)
Expt 1		
None		100
PMSF	1	47
DFP	1	19
TPCK	1	37
TLCK	1	94
EDTA	1	23
$ZnCl_2$	1	29
$CaCl_2$	1	131
Expt 2		
None		100
DTT	1	100
2-Mercaptoethanol	1	114
L-Cysteine	10	158

[a] Crude protease was assayed for SAAPNA-degrading activity in the presence of the indicated effectors. In experiment 1, but not experiment 2, all samples were incubated in the presence of 15 mM cysteine. In addition, the inhibitors were preincubated with the enzyme before addition of the substrates.

[b] PMSF, phenylmethylsulfonyl fluoride; DFP, diisopropylfluorophosphate; DTT, dithiothreitol.

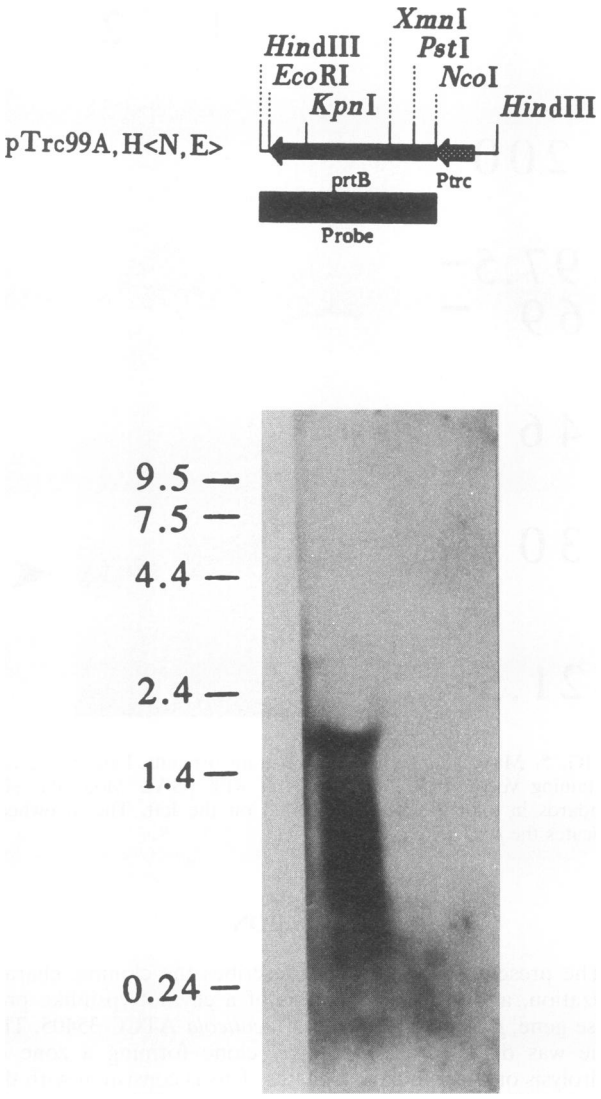

FIG. 9. Northern blot analysis of *prtB* mRNA. Hybridization of total RNA from *T. denticola* ATCC 35405 was performed with the labeled *Nco*I-*Hin*dIII fragment from plasmid *Hin*dIII-*Nco*I-*Eco*RI-pTrc99A (at the top) as a probe with the ECL direct nucleic acid labeling and detection systems. The numbers at the left indicate molecular sizes in kilobases. Ptrc, promoter of vector pTrc99A.

The results of Northern blot analysis suggested that the *prtB* gene may be transcribed as part of a polycistronic mRNA. In this regard, it will be of interest to characterize the two ORFs flanking this gene. Preliminary data suggest that the ORF downstream from the *prtB* gene, ORF3, may be of sufficient size to be cotranscribed with the protease gene.

On the basis of the *prtB* gene sequence data, the calculated molecular mass of the protease would be 30.4 kDa. This is consistent with the results of maxicell analysis (Fig. 5) and Western blots (immunoblots) of the biotinylated fusion product derived from subclone Xa-3:2 (Fig. 6). A chymotrypsinlike protease was recently purified from *T. denticola* 35405 and characterized (9, 54). The estimated molecular size of this

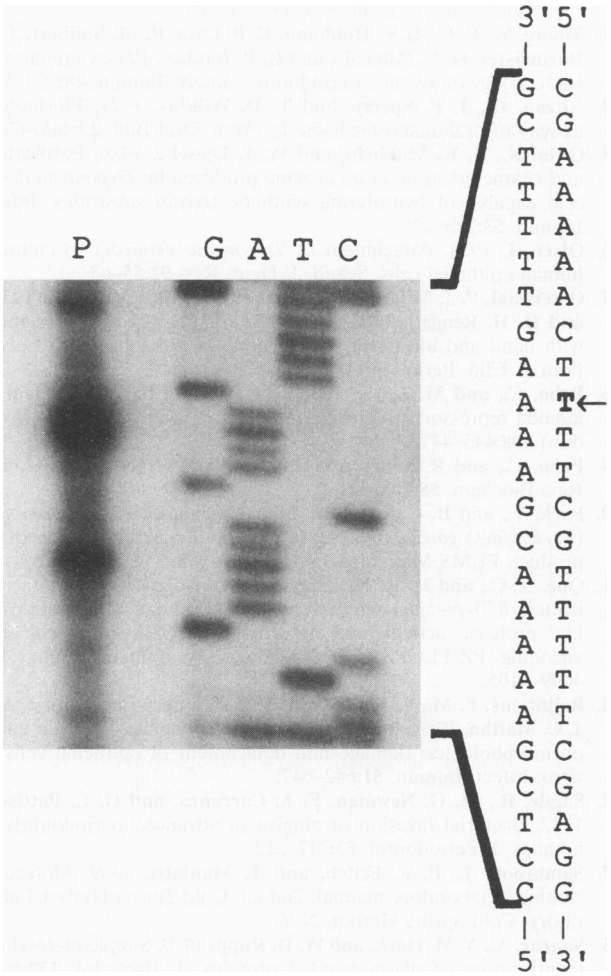

FIG. 10. Primer extension analysis of the *prtB* gene transcript. The size of the extension product (lane P) is 60 bp. The DNA sequence corresponding to this region was analyzed by using the same oligonucleotide primer. The arrow indicates the position of the primary transcriptional initiation site.

enzyme was 95 kDa following SDS-PAGE. This report further suggested that the possibility that there is more than one chymotrypsinlike enzyme in *T. denticola*. Although the 95-kDa enzyme hydrolyzed transferrin, fibrinogen, α_1-antitrypsin, immunoglobulin A, immunoglobulin G, gelatin, BSA, and a synthetic peptide containing phenylalanine, the protease expressed from the *prtB* gene degraded casein, BSA, and human serum albumin but not transferrin, fibrinogen, immunoglobulin A, and gelatin. Therefore, the protease encoded by the *prtB* gene appears to be distinct from the 95-kDa protease. Another chymotrypsinlike protease gene from *T. denticola* 35405 was also isolated in this laboratory (41). However, this enzyme has many properties in common with the 95-kDa protease and is also clearly distinct from the *prtB* gene product.

The effects of protease inhibitors on the *prtB*-encoded protease are consistent with the idea that the enzyme acts as a chymotrypsinlike protease (Table 1). However, unlike the 95-kDa protease, the *prtB*-encoded protease is inhibited by EDTA, suggesting a role for a metal ion in catalysis. The moderate stimulation of activity in the presence of cysteine is consistent with the enhanced anaerobic degradation of skim milk by *E. coli* clones expressing this enzyme.

Hemolytic activity for *T. denticola* has been reported previously by Greiner (8), who indicated that RBCs from humans, rabbits, and guinea pigs, but not sheep RBCs, were hemolyzed following incubation with a suspension of *T. denticola* at 37°C, while additional incubation at 4°C increased hemolysis. Furthermore, since this activity was not affected by the presence of either TLCK or phenylmethylsulfonyl fluoride, the proteases of *T. denticola* appeared not to be involved. In the present study, hemolysis of sheep RBCs was detected following incubation with the *prtB*-encoded protease. However, it is not clear if this enzyme plays a role in the observed hemolytic properties of *T. denticola* (5, 8).

The presence of more than one chymotrypsinlike protease in a single strain of *T. denticola* is not surprising, given the highly proteolytic nature of these organisms (18, 20, 24, 34, 36). Besides this activity, a trypsinlike activity has also been identified in these organisms (21, 49). Therefore, like another periodontopathic microorganism, *P. gingivalis* (26), it is likely that each *T. denticola* strain can express multiple proteases, some of which may contribute to the virulence of these organisms.

It is not possible to assign a physiological role for the *T. denticola prtB* gene product. The enzyme does not hydrolyze proteins which the organism would normally encounter in the periodontal pocket (fibronectin, type IV collagen, laminin, etc.). In addition, the cellular location of this enzyme in *T. denticola* remains to be determined. Nevertheless, the availability of the purified protein and its gene will aid in evaluating the potential role of the protease in the physiology and virulence of the organism.

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