

## Revised Sequence of the *Porphyromonas gingivalis* PrtT Cysteine Protease/Hemagglutinin Gene: Homology with Streptococcal Pyrogenic Exotoxin B/Streptococcal Proteinase

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The *prtT* gene from *Porphyromonas gingivalis* ATCC 53977 was previously isolated from an *Escherichia coli* clone possessing trypsinlike protease activity upstream of a region encoding hemagglutinin activity (J. Otogoto and H. Kuramitsu, *Infect. Immun.* 61:117–123, 1993). Subsequent molecular analysis of this gene has revealed that the PrtT protein is larger than originally reported, encompassing the hemagglutination region. Results of primer extension experiments indicate that the translation start site was originally misidentified. An alternate open reading frame of nearly 2.7 kb, which encodes a protein in the size range of 96 to 99 kDa, was identified. *In vitro* transcription-translation experiments confirm this size, and Northern (RNA) blot experiments indicate that the protease is translated from a 3.3-kb mRNA. Searching the EMBL protein database revealed that the amino acid sequence of the revised PrtT is similar to sequences of two related proteins from *Streptococcus pyogenes*. PrtT is 31% identical and 73% similar over 401 amino acids to streptococcal pyrogenic exotoxin B. In addition, it is 36% identical and 74% similar over 244 amino acids with streptococcal proteinase, which is closely related to streptococcal pyrogenic exotoxin B. The similarity is particularly high at the putative active site of streptococcal proteinase, which is similar to the active sites of the family of cysteine proteases. Thus, we conclude that PrtT is a 96- to 99-kDa cysteine protease and hemagglutinin with significant similarity to streptococcal enzymes.

*Porphyromonas gingivalis* has been shown to be a primary etiologic agent in periodontitis (24, 28, 54). Two potentially important virulence factors of *P. gingivalis* are the production of proteases and agglutination of erythrocytes (hemagglutination) (37). Proteases are able to degrade serum proteins involved in immunity which normally eradicate invading microorganisms (50, 60). *P. gingivalis* proteases also degrade connective tissue proteins directly and indirectly by activating latent human fibroblast procollagenase and neutrophil interstitial procollagenases (59).

Hemagglutination is thought to reflect the ability of a periodontal microorganism to adhere to and colonize oral tissues (55, 56), and in *P. gingivalis*, it is mediated by fimbrial and nonfimbrial molecules (6, 39, 68). Some of the hemagglutinins possess proteolytic activity, and others are found complexed with proteases (22, 41, 45), suggesting that hemagglutination may be involved in processes other than colonization.

We report the further characterization of the *P. gingivalis* trypsinlike protease gene, *prtT*, which was previously identified downstream of collagenase (*prtC*) and superoxide dismutase (*sod*) genes on a 5.9-kb *P. gingivalis* DNA fragment. Hemagglutination activity expressed from this fragment was localized to the 3' end of the clone and believed to be encoded by a separate gene downstream of *prtT* (42). However, we demonstrated that the *prtT* gene is larger than originally reported and that it encompasses the region encoding hemagglutination.

PrtT is translated from a different open reading frame than was originally reported. The deduced amino acid sequence of the 96- to 99-kDa protein translated from the revised reading

frame has significant homology to two streptococcal proteins. We present the corrected nucleotide sequence of *prtT* and accompanying sequence analysis.

### MATERIALS AND METHODS

**Bacterial strains, plasmid vectors, and culture conditions.** *Escherichia coli* HB101 and JM109 were used as host strains for pUC18 and pUC19. JM109 was used as the host strain for pBluescript KS+. *E. coli* clones were grown on Luria agar plates. Supplements included ampicillin (100 µg/ml), X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 50 mg/ml), and 50 mM IPTG (isopropyl-β-D-thiogalactopyranoside; Sigma Chemical Co., St. Louis, Mo.). *P. gingivalis* ATCC 53977, originally designated A7A1-28, was isolated from the subgingival dental plaque of a patient with periodontitis and non-insulin-dependent diabetes from the Pima Indian Reservation in Arizona (35). Strain ATCC 53977 (A7A1-28) was grown in PY broth (23) supplemented (per liter) with 5.0 mg of hemin, 1.0 mg of vitamin K, 0.42 g of NaHCO<sub>3</sub>, and 1.0 g of soluble starch and on prerduced paromomycin-vancomycin laked blood agar (Carr Scarborough Microbiologicals, Decatur, Ga.). Cultures were incubated at 37°C in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.) under an atmosphere of 5% CO<sub>2</sub>–10% H<sub>2</sub>–85% N<sub>2</sub>. Purity of the cultures was assessed by Gram stain and phase-contrast microscopy.

**Preparation of DNA.** *P. gingivalis* chromosomal DNA was prepared by using 1% cetyltrimethylammonium bromide in 0.7 M NaCl and a CsCl density gradient (1). Plasmid DNA was isolated by using Wizard or Magic Minipreps (Promega, Madison, Wis.) or a CsCl density gradient.

**DNA sequencing.** Double-stranded sequencing was carried out on both DNA strands (Fig. 1), using the dideoxy-chain termination method (48). Sequenase version 2.0 DNA polymerase, [<sup>35</sup>S]dATP, and Sequenase kit reagents were used as recommended by the manufacturer (United States Biochemical Corp., Cleveland, Ohio). Sequencing gels (8% polyacrylamide) were run by using a model S2 sequencing gel electrophoresis system (Bethesda Research Laboratory [BRL], Gaithersburg, Md.). Cloning vectors pUC18, pUC19, and pBluescript KS+ (Stratagene, La Jolla, Calif.) were used for subcloning. Compressions were resolved by the use of 7-deaza-dGTP. Automated sequencing was performed directly on the *Hae*III inverse PCR (IPCR) product, using a model 370A DNA sequencer (Applied Biosystems, Foster City, Calif.). The 2.9-kb *Tha*I IPCR product was cloned into the *Sma*I site of pBluescript KS+. Oligonucleotide primers were synthesized with a model 380A DNA synthesizer (Applied Biosystems).

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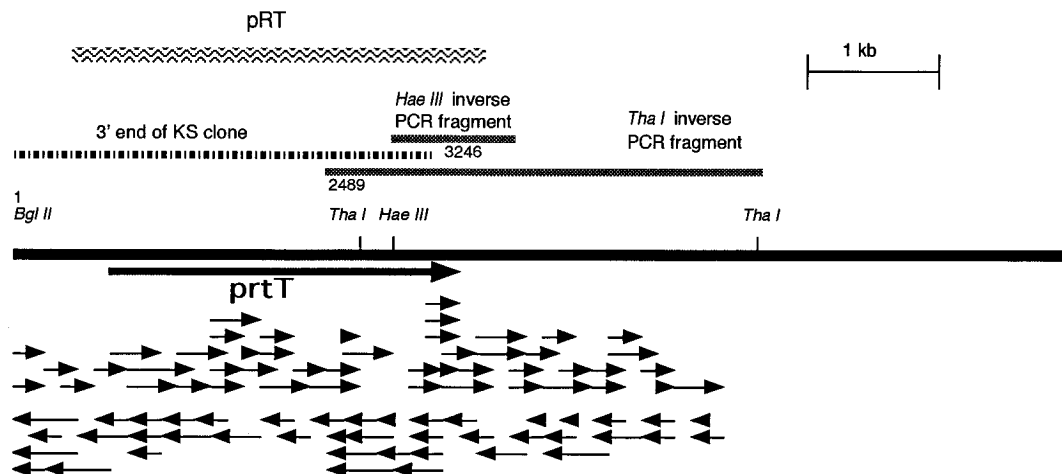


FIG. 1. Genetic map of the *prtT* locus and sequencing and cloning strategies. Nucleotide 1 occurs at the *Bgl*II site, as in the previous report (42). Sequence derived from the original pS1 clone and KS subclone extends to position 3246, and downstream sequence was derived from *Hae*III and *Tha*I PCR fragments. Individual arrows represent the approximate region sequenced by each reaction. A clone (pRT) containing the entire PCR-amplified *prtT* open reading frame is described in the text.

**IPCR.** Since the entire *prtT* open reading frame was not contained on the original pS1 clone, IPCR was used to isolate contiguous downstream chromosomal DNA fragments. Two samples of 1.2  $\mu$ g of strain 53977 chromosomal DNA were digested with the restriction endonucleases *Hae*III and *Tha*I (BRL) at 37 and 60°C, respectively. The fragments were precipitated, resuspended in a 350- $\mu$ l ligation reaction mixture, and incubated with 5  $\mu$ l of T4 ligase (BRL) at 15°C overnight; 10  $\mu$ l of this product was used for each PCR as previously described. Primer pairs (5'GTAATAGTCCCAACAAT3' and 5'TATGTCATGTAGCCATTCC3') successfully amplified an 800-bp fragment from the *Hae*III religation product and a 2.9-kb fragment from the *Tha*I religation product.

**PCR.** Following identification of the *prtT* open reading frame, a subclone (pRT) which contained the entire *prtT* open reading frame was constructed (Fig. 1). PCR was used to amplify a 3.7-kb DNA molecule from *P. gingivalis* ATCC 53977 chromosomal DNA. Twenty-five cycles were carried out, using a denaturing temperature of 95°C, annealing temperature of 55°C, and extension temperature of 72°C, in a model 60 Coy Tempcycler (Coy Laboratory Products, Ann Arbor, Mich.). Similar conditions were used for amplification of IPCR products. Approximately 0.5  $\mu$ g of DNA template was used in each reaction with buffer, deoxynucleoside triphosphates (dNTPs), and *Taq*I DNA polymerase (GeneAmp; Perkin-Elmer, Norwalk, Conn.) according to the manufacturer's recommendations.

**Isolation of *prtT* subclones.** Plasmid pS1, a 5.9-kb *P. gingivalis* clone with collagenase, benzoyl-L-arginine-*p*-nitroanilide (BAPNA) hydrolase, superoxide dismutase, and hemagglutination activities, was previously isolated (27). Subclones were constructed by *Kpn*I-*Cla*I double digestion of pS1 and ligation into the similarly digested pUC and pBluescript KS+ vectors. The pRT clone containing the entire *prtT* open reading frame was prepared by PCR with primers 5'GGGGTACCCCAATCCTATCAAGCC3' (*Kpn*I site plus bases 195 to 213) and 5'GCTCTAGAATTCAAGAGAGCTATGAAGGC3' (*Xba*I site plus bases 3760 to 3777) (see Fig. 2 for nucleotide position numbers). The PCR fragment was digested with *Kpn*I and *Xba*I and ligated into pUC18. Restriction digestion with *Sca*I, *Pst*I, and *Hind*III as well as partial DNA sequencing confirmed that pRT contained the *prtT* gene. Three sequencing reactions (primers 5'GGGC-CGATTGCTATATAGCTTTG3', 5'TGAGGAGTAGCAAACGGG3', and 5'GGGGATGTGCTGCAAGGCG3') which spanned bp 190 to 259 and 3673 to 3818 were completed to confirm that the clone contained the entire *prtT* open reading frame in the desired orientation.

**Sequence analysis.** DNA and amino acid sequence, amino acid composition, and Pustell DNA matrix analyses were performed, in part, with MacVector sequence analysis software (International Biotechnologies, New Haven, Conn.). Free energy of each potential stem-loop RNA structure was calculated by the method of Tinoco et al. (62). Protein homology was searched in the GenBank/EMBL sequence data library and the SwissProt and PIR protein sequence data libraries. FindPattern analysis was performed on a 12-nucleotide repeat occurring in the downstream region of the *prtT* gene (Fig. 2).

**In vitro transcription-translation.** Each reaction was performed with 2.5  $\mu$ g of CsCl-prepared plasmid DNA, using a prokaryotic DNA-directed translation kit (Amersham, Arlington Heights, Ill.). Samples were incubated for 45 min at 37°C with [<sup>35</sup>S]methionine as recommended by the manufacturer. Reaction products along with <sup>14</sup>C-labeled methylated molecular weight standard proteins (Amersham) were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The gels were fixed for 30 min in isopropanol-H<sub>2</sub>O-acetic acid

(25:65:10) followed by 15 min in Amplify fluorographic reagent (Amersham) and dried under vacuum. Dried gels were scanned for digital imaging by using a Phosphor screen and PhosphorImager together with ImageQuant version 3.2 software (Molecular Dynamics, Sunnyvale, Calif.) and printed with a LaserJet 4 printer (Hewlett-Packard, Cupertino, Calif.).

**Preparation of RNA.** A single-step method was used for isolation of RNA (11). Three-day cultures of *P. gingivalis* ATCC 53977 were processed with TRI (total RNA isolation) reagent (Molecular Research Center, Inc., Cincinnati, Ohio) or total RNA isolation reagent (BRL) according to the manufacturer's directions, with the following modifications. The pellet from a 500-ml culture was resuspended in 7 ml of TRI reagent and incubated 60 min at room temperature with 50  $\mu$ g of RNase-free DNase I, 100 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, and 10  $\mu$ l of RNasin at 37°C. The reaction was stopped by incubation at 60°C in 2.5 ml of 50 mM EDTA-1.5 M sodium acetate (pH 7.0)-1% SDS. Phenol-chloroform extraction and ethanol precipitation followed. Purified RNA samples were stored at -70°C.

**Primer extension.** Primer 5'GTGGCGGAGATTTTTTTTCG3' was end labeled with [ $\gamma$ -<sup>32</sup>P]ATP (New England Nuclear, Boston, Mass.); 10<sup>5</sup> cpm was added to 1.5  $\mu$ g of *P. gingivalis* RNA-10  $\mu$ M dithiothreitol-10 mM dNTPs-200 U of Superscript H<sup>-</sup> reverse transcriptase enzyme (BRL) and incubated at 42°C for 30 min. The Sequenase reaction was run simultaneously on an 8% polyacrylamide gel. Dried gels were scanned for digital imaging, using a PhosphorImager and ImageQuant version 3.2 software as described above.

**Northern (RNA) blotting.** RNA was electrophoretically separated in 1.2% agarose-formaldehyde, and the gel was washed in 10 $\times$  SSC (1.5 M NaCl, 0.15 M sodium citrate) to remove formaldehyde. Overnight capillary transfer of RNA onto a Hybond N filter (Amersham) was carried out. The *Kpn*I-*Hind*III restriction fragment of pRT containing the entire *prtT* gene was used as a probe. Probe preparation and hybridization were carried out by using ECL (enhanced chemiluminescence) direct nucleic acid labeling and detection system (Amersham) according to the manufacturer's directions. The prepared membranes were exposed to Hyperfilm ECL (Amersham).

**Nucleotide sequence accession number.** The *P. gingivalis* *prtT* gene sequence deposited in the GenBank database (accession number M83096) has been updated.

## RESULTS

**Identification of a transcriptional start site and potential promoter upstream of *prtT*.** The results of primer extension analysis (Fig. 3) demonstrated that transcription begins at the adenine at nucleotide position 256 (Fig. 2). This rules out the possibility that translation of *prtT* originates at nucleotide 239, as previously hypothesized (42). Putative -35 (TTCAGA) and -10 (TACCAT) promoter sequences with homology to the *E. coli* consensus sequence were identified upstream of the transcription start site (Fig. 2).

**Nucleotide sequence of the *prtT* gene.** The nucleotide sequence downstream of the *prtT* gene, including DNA frag-



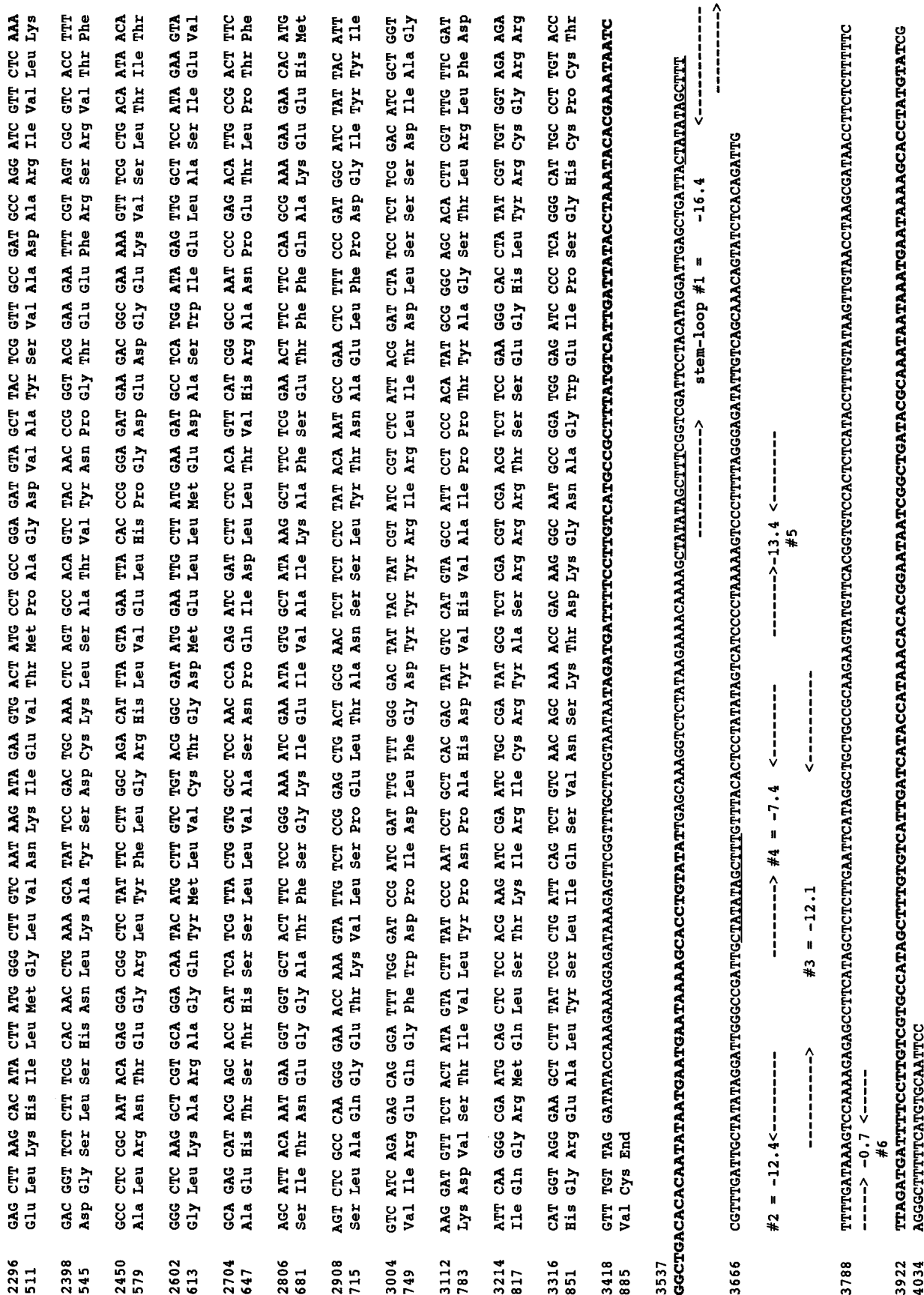


FIG. 2. Nucleotide and deduced amino acid sequences of the *P. gingivalis* *prtT* gene. Transcriptional start site, ribosome binding site (SD), and potential promoter are indicated. Potential translational start codons are underlined at nucleotide positions 766 and 820. Downstream of *prtT* are seven regions of dyad symmetry (arrows), three direct repeats of 12 nucleotides (underlined), and a pair of long direct repeats (boldface). A decanucleotide sequence also found downstream of papain is underlined. Free energies are indicated for the six potential stem-loop termination structures. Nucleotide 1 at the *Bg*II site is identical to that reported previously (42).



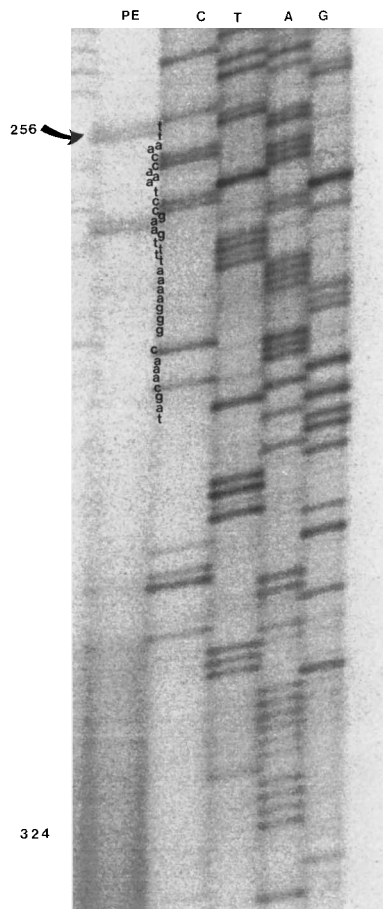


FIG. 3. Primer extension (PE) indicates the start of transcription at nucleotide 256.

ments encompassing the 3' end of the gene, was found to differ from the published sequence. Therefore, the entire gene was resequenced. Nine single-nucleotide corrections were made between the *Bgl*III site at position 1 and the *Bam*HI site at position 1280. In addition, the previously published sequence between the *Bam*HI sites at 1280 and 1740 was incorrect. Multiple overlapping sequence reactions involving several different subclones and PCR products yielded identical sequence results (Fig. 1). Hence, the corrected sequence is presented here with confidence. In the original cloned fragment (plasmid pS1), no stop codon for the *prtT* gene was identified. Therefore, sequence downstream of the cloned fragment was obtained by IPCR. The stop codon TAG was then located at position 3426. The G+C content of the *prtT* gene is 46.3%, which corresponds to that expected for *P. gingivalis* (52).

**The *prtT* gene product and identification of potential start translational.** Potential start codons for the *prtT* open reading frame were identified at positions 820 (ATG) and 766 (ATT). The former has no identifiable ribosome binding site. It does, however, encode a characteristic signal sequence, 5'MKRIF YTLG LLLLCLPMLQAGPV3' (65). The predicted size of the gene product translated from position 820 is 96.4 kDa.

On the other hand, translation may start at position 766, utilizing the alternate start codon ATT. Seven nucleotides upstream is the sequence AGAGGA, a possible Shine-Dalgarno sequence (20). However, the 5' amino acid sequence (MIASSASPASEQKQNAIM) does not appear to be a signal

TABLE 1. Amino acid composition of PrtT<sup>a</sup>

Amino acid	No. (%) of residues
<b>Nonpolar</b>	
Ala .....	70 (7.9)
Val .....	44 (5.1)
Leu .....	77 (8.9)
Ile .....	52 (5.9)
Pro .....	45 (5.1)
Met .....	25 (2.8)
Phe .....	37 (4.2)
Trp .....	12 (1.4)
<b>Polar</b>	
Gly .....	74 (8.5)
Ser .....	69 (7.8)
Thr .....	60 (6.9)
Cys .....	11 (1.3)
Tyr .....	41 (4.7)
Asn .....	33 (3.7)
Gln .....	22 (2.5)
<b>Acidic</b>	
Asp .....	48 (5.5)
Glu .....	57 (6.6)
<b>Basic</b>	
Lys .....	40 (4.5)
Arg .....	44 (5.1)
His .....	24 (2.8)

<sup>a</sup> Based on start of translation at nucleotide position 766.

sequence. If translation begins at this site, the primary translation product is 98.3 kDa. The amino acid composition of the *prtT* gene product translated from position 766 is shown in Table 1 and has a deduced pI of 5.48.

Proteins obtained from in vitro transcription-translation experiments support our conclusion about the size of the *prtT* gene product. The in vitro product made from the pRT plasmid template containing the entire *prtT* gene is in the size range of 96 to 99 kDa (Fig. 4b, lane 4). The in vitro product made from the KS plasmid template (Fig. 4b, lane 5, and 4a, lane 2) is approximately 108 kDa. Since KS does not contain a stop codon for the *prtT* open reading frame, the in vitro transcription-translation reads through to the first in-frame stop codon within the pUC vector, located 469 nucleotides past the 3' end of the insert. The predicted molecular size of this chimeric open reading frame is 107.7 kDa.

**Size of the *prtT* transcript.** Results of Northern blot analysis indicate that the transcript in *P. gingivalis* is approximately 3.3 kb (Fig. 5). This finding supports our hypothesis that transcription begins at position 256 and continues through the stop codon at position 3424. Transcription most likely ends within the downstream region of potential secondary structure described below.

**Homology results.** The PrtT deduced amino acid sequence was found to have 30.7% identity and 72.6% similarity over 401 amino acids with streptococcal pyrogenic exotoxin B (SPE B) and 36.1% identity and 74% similarity over 244 amino acids with streptococcal proteinase (Fig. 6). Amino acid identity of PrtT was particularly high in the putative active site of the streptococcal proteinase. When aligned with the active center of other cysteine proteases, striking conservation was found at the reactive cysteine, histidine, and glutamine residues (Fig. 7). Significant homology of PrtT with these other proteases was not found beyond amino acids of the active site cleft.

**Potential secondary structures.** Downstream from the stop codon for the *prtT* gene are six regions of overlapping dyad symmetry, which may be rho-dependent transcription termina-

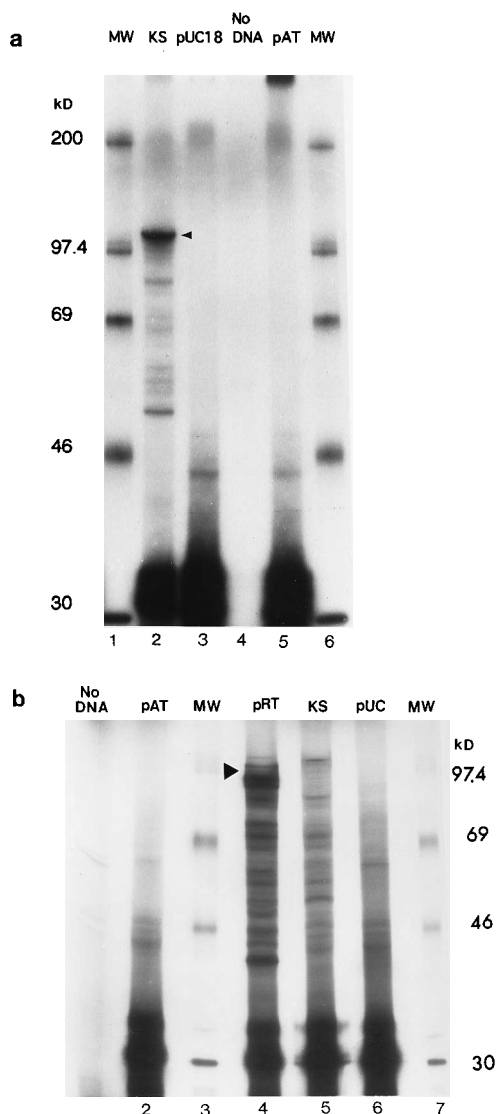


FIG. 4. (a) In vitro transcription-translation. Lanes: 1 and 6, molecular weight (MW) standards; 2 to 5, 108-kDa product obtained by using the KS template (lane 2) and control templates (lanes 3 to 5) (pAT is an Amersham kit control template). (b) In vitro transcription-translation. The product is approximately 96 to 99 kDa with use of the pRT template (lane 4); other lanes are as in panel a.

tors (33, 44). The calculated free energies (62) of the potential RNA hairpin loops formed by these sequences are  $-16.4$ ,  $-12.4$ ,  $-12.1$ ,  $-7.4$ ,  $-13.4$ , and  $-0.7$  kcal (1 kcal = 4.184 kJ), respectively, indicating that loops 1, 2, 3, 5, and possibly 4 form in vivo. Overlaps within the stem regions, however, would limit the number of hairpins to three at any given time (hairpins 1, 3, and 5 or 2, 4, and 5). Within this same region is a 12-nucleotide direct repeat, CTATATAGCTTT, which occurs three times within the overlapping stems of the potential RNA stem-loop secondary structure (underlined in Fig. 2). Results of a FindPattern analysis of this 12-nucleotide sequence revealed no significant similarity to other prokaryotic sequences. However, if this were a binding site for a regulatory protein, formation of hairpins 1, 2, 4, and possibly 3 would be blocked. This region is flanked by one pair of long direct repeats. Between the stop codon and stem loop 1 are 107 nucleotides nearly identical (92%) to 110 nucleotides repeated after dyad

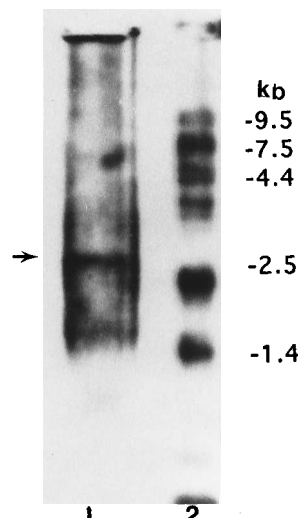


FIG. 5. Northern blot probed with a 3-kb fragment containing the entire *prtT* open reading frame. The transcript is approximately 3.3 kb in size (lane 1). Molecular weight markers are in lane 2.

symmetry 6 (Fig. 2, in boldface). Within the second repeat is the decanucleotide AAATAATAAAA at positions 4009 to 4018, which also appears downstream of the papain gene but has no known function (13).

## DISCUSSION

The gene *prtT*, shown previously to have trypsinlike protease and hemagglutinin activities, is nearly 2.7 kb and encodes a protein in the size range of 96 to 99 kDa. Its amino acid sequence is similar to those of *Streptococcus pyogenes* proteinase and SPE B, two closely related proteins. The sequence similarity spans the entirety of the streptococcal proteins, with the greatest conservation being at the putative active-site amino acids. Eleven cysteine residues are found in the deduced amino acid sequence of PrtT (Table 1), making possible the formation of extensive intramolecular disulfide bridges under nonreducing conditions. If the enzymatic mechanism of PrtT resembles that of the other cysteine proteinases, Cys-202 and His-345 are likely reactive amino acids. This conclusion is based on similarity of two regions of PrtT to the identified or putative active sites of papain, actinidin, cathepsins, streptococcal proteinase, and other cysteine proteases (Fig. 7) (7, 61).

Like PrtT, streptococcal proteinase, is similar at the amino acid level to other cysteine proteinases solely across their active sites. The putative active cysteine is the sole cysteine residue found in streptococcal proteinase. Thus, intramolecular disulfide bridges do not form. The mature protein is derived from a 40,314-Da, 398-amino-acid precursor. Cleavage of the 27-amino-acid signal sequence is followed by successive proteolysis to yield a stable 27.6-kDa protein (21). Streptococcal proteinase precursor and SPE B are thought to be identical proteins or variants of the same protein. Comparison of the presently deposited SPE B and streptococcal proteinase precursor sequences shows differences at 16 amino acid residues (Fig. 6) (21). Both are encoded by a single gene, *speB* (9, 17, 21). By insertional inactivation of *speB*, wild-type extracellular protease activity of strain NZ131 was significantly reduced (9).

Similarity between PrtT and SPE B and streptococcal proteinase suggests that PrtT may have significant biologic activity. SPE B is one of three pyrogenic exotoxins produced by group

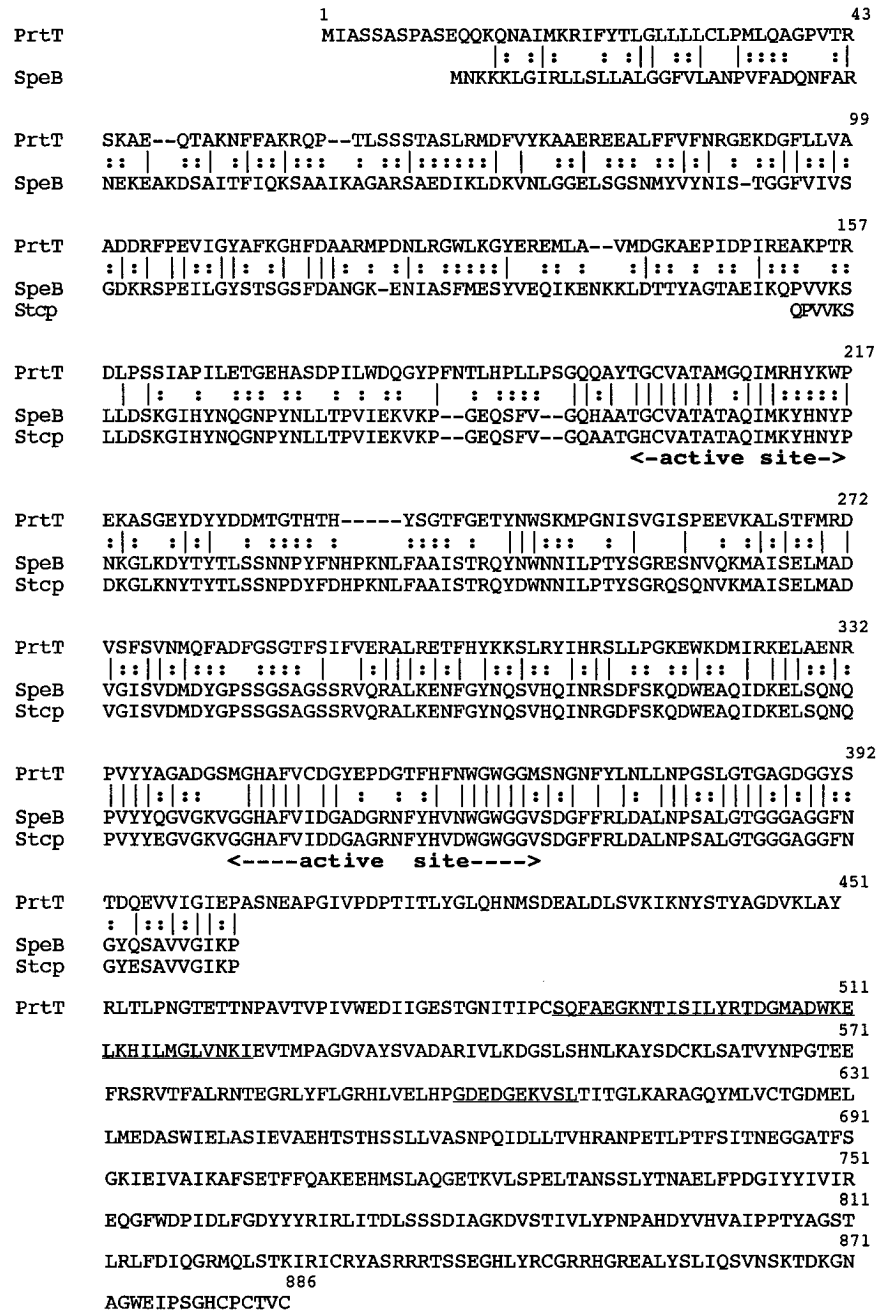


FIG. 6. Amino acid alignment of PrtT with SPE B and streptococcal proteinase (Step). Putative streptococcal active-site residues are indicated.

A beta-hemolytic streptococci (SPE A, B, and C). The SPE proteins are also known as scarlatinal, Dick, cardiac, erysipelas, and erythrogenic toxins (4, 32). The cause of scarlet fever and possibly toxic shock-like syndrome, they induce hypersensitive skin rash, fever, and T-lymphocyte mitogenesis. They enhance myocardial damage due to streptolysin O, potentiate endotoxic shock, and suppress immunoglobulin secretion (32). Preparations of SPE from strains NY5 and ATCC 19165 potentially augment natural killer cell activity of human peripheral blood mononuclear cells (47). In the presence of splenic macrophages, SPE preparations augment glucose consumption and superoxide anion production as well as inhibit phagocytosis by rabbit macrophages (40).

Sequence similarity between enzymes in dissimilar bacterial genera may have arisen by one of several mechanisms. Certainly, divergent evolution of an enzyme possessed by a common ancestor of *Streptococcus* and *Porphyromonas* species is a possible explanation of the origin of *prtT* in *P. gingivalis*. A second mechanism would be one of genetic exchange. Close proximity of the two bacteria at some point in their life cycles would enhance such opportunities for genetic exchange. Although beta-hemolytic streptococci are cultured from the periodontal lesions of 33% of patients with advanced periodontitis, isolates did not include group A (*S. pyogenes*) (18). However, group A streptococci and *P. gingivalis* (classified as *Bacteroides melaninogenicus* at that time) have been commonly isolated

QQAYTGCVATAMGQIM	MGHAFVCDGYEPDGTFFHFNWGWGMSNGNF	<i>P. gingivalis prtT</i>
QHAATGCVATATAQIM	GGHAFVIDGADGRNFYHVNWGWGVSDFGF	SpeB (21)
QAATGHCVATATAQIM	GGHAFVIDDAGRNPFYHVDWGWGVSDFGF	streptococcal proteinase (61)
QGSCGSCYAFSAVVTI	VDHAVAAVGYNPGYILIKNSWGTGWGE	papain (13)
QGSCGSCWAFSAVATV	VDHAVTAVGYGKSGGKGYILIKNSWGTAWD	Papaya cysteine proteinase omega (38; 67)
QGYCESCWAFSTVATV	VDHAVTAVGYGKSGGKGYILIKNSWGPWGW	Papaya cysteine proteinase IV (46)
QGECGGCWAFSAIATV	VDHAIIVVGYGTEGGVDYWIWKNWSDTTWG	kiwi actinidin (7)
QGSCGSCWAFGAVEAM	GGHAIRILGWGIENGVPYWLWVANSWNVDWG	cathepsin B (7)
QGACGSCWTFSTTGAL	VNHAVLAVGYGEQNGLLYWIWKNWGSNNG	cathepsin H (26)
QNPFCGACWAFGAIATV	LNHAVTAIGYNKAIEFGDGSKKARWG	stem bromelain (7)
QGIAGDCYMLAALSAL	AYHAYTVLGYTVSNGAYYLIIRNPWGVTEP	<i>P. gingivalis tpr</i> (5)
QGALGDCWLLAAIASL	KGHAVSVTGAKQVNYRQVVSILIRNRPWGW	human calpain(m large subunit (58)
QGALGDCWLLAVVASI	GSHAYSLTGVYVNYRGRTOQLMRLRNPWGW	Shistosoma mansoni calpain (12)
QQCGSCWAFSTI	LDHGVLVGYNDNSNPPYWIWKNW	<i>Trypanosoma brucei</i> cysteine protease (16)
QGACGSCWAFSTIATV	LDHAVTAVGYGTSDFGKNYIIIKNSWGPWGW	chymopain B (67)
QGGCGSCWAFSGVAAT	NYHAVNIVGYSNAQGVVDYWIWRNSWDTNNG	<i>Dermatophagoides pteronyssinus</i> cysteine protease (12)

FIG. 7. Comparison of active-site amino acid residues of various cysteine proteinases. Reactive cysteine and histidines are in boldface, as is contributory glutamine (7, 29, 34).

together from acutely infected tonsils (8). Both *Streptococcus* and *Bacteroides* species contain conjugal transposons, and *P. gingivalis* has been successfully transformed with elements from *Bacteroides* species (49, 53, 64). In addition, there does not appear to be a barrier to conjugal transfer between gram-negative and gram-positive bacteria. The *Streptococcus tetM* gene freely infects *Bacteroides* hosts, conferring high-level resistance to tetracycline and minocycline (14, 30, 57).

In determining the *prtT* start of translation, two possible sites were identified. The codon at nucleotide position 766 is ATT, is preceded by a ribosome binding site, but does not encode a signal sequence. In *P. gingivalis*, alternate (not ATG) start codons have been identified for the methylase (*pgiM*) and the fimbrial subunit (*fimA*) genes (2, 19). It is not known whether their use affects the rate of translation of these proteins. The ATG at position 820 encodes a signal sequence but is not preceded by a classic ribosome binding site (Fig. 2). A protein initiated at position 766 would be 96.4 kDa, and a protein initiated from position 820 would be 98.3 kDa. A size difference this small could not be resolved from the in vitro transcription-translation experiments carried out in this work.

The transcriptional start at position 256 is preceded by the putative promoter sequences TTCAGA (−35) and TACCAT (−10) (Fig. 2). The consensus prokaryotic Pribnow box is −10 T<sub>89</sub>A<sub>89</sub>T<sub>50</sub>A<sub>65</sub>A<sub>65</sub>T<sub>100</sub>, with subscripts indicating percent agreement of each nucleotide position with known promoters. Two cytosine residues of the putative *prtT* promoter occur at positions 3 and 4 (underlined in Fig. 2), which are the bases most likely to diverge from the consensus (33). Therefore, the 3.3-kb transcript (Fig. 5) begins at position 256 and includes 0.5 kb of 5' untranslated message, 2.6 kb of translated message, and approximately 0.14 kb of 3' untranslated message.

If translation begins at position 766, the amino terminus of PrtT does not have a signal sequence. It is not known if PrtT is secreted in vivo from *P. gingivalis* or if secretion requires a signal peptide. While extracellular proteases from gram-positive bacteria depend on signal peptides for secretion, proteases from gram-negative bacteria utilize a variety of mechanisms to reach the extracellular environment. Two metalloproteases which do so without signal peptides are the products of *prtB* and *prtC* of *Erwinia chrysanthemi* (66).

We show that PrtT is translated as a 96- to 99-kDa protein. Previous purification of recombinant chimeric PrtT expressed in *E. coli* yielded an active protein with an approximate mo-

lecular size of 51 to 53 kDa (42). As previously stated, *prtT* in the KS clone lacks the 3' end of the gene, and so the recombinant protein lacks its carboxyl terminus. In addition to the possibility that the signal peptide is removed from the protein, it apparently undergoes additional size reduction by processing in *E. coli*. Precedence for size reduction of bacterial cysteine proteases is seen with streptococcal proteinase as well as clostripain from *Clostridium histolyticum*. We have not yet determined the nature of this processing, nor do we know the size of the mature PrtT protein when expressed in *P. gingivalis*. Although not all gram-negative proteases require signal peptides for secretion, finding one in this work suggests that PrtT may use this mechanism to reach the extracellular environment (66).

PrtT appears to be unique among a number of *P. gingivalis* trypsinlike, cysteine proteases being characterized at the molecular level. Porphyain is a large, cell surface cysteine protease from strain W12 with arginyl and lysyl endopeptidase activities. The porphyain open reading frame is 4.6 kb, encoding 1,830 amino acids, and has no homology to *prtT* (31). It is, however, related to a hemagglutinin gene, *hagA*. The significance of this relatedness is not clear; however, similar binding epitopes may be used by the hemagglutinins and the proteases of *P. gingivalis* (45).

Currently, the biochemical and molecular characterization of similarly acting arginine- and lysine-specific cysteine proteases is under way in several laboratories. It remains unclear whether these proteins are related to each other. Gingipain is a calcium-stabilized cysteine protease purified from strain H66, which specifically cleaves peptides at the carboxyl-terminal side of arginine residues (43). The amino-terminal sequence is not homologous with any region of *prtT* (10). The gene for gingipain appears to have a much larger open reading frame than would be predicted from the 50-kDa molecular size of the purified enzyme (63), suggesting posttranslational modification of the enzyme. Further studies have indicated that there are two forms of gingipain with different molecular weights and activities (43). Two of these subunits have been termed arg-gingipain and lys-gingipain because they cleave specifically at the carboxyl-terminal side of arginine and lysine residues, respectively. Arg-gingipain is 50 kDa, and lys-gingipain is 60 kDa, and both form noncovalent bonds with a 44-kDa protein and with 30-, 27-, and 17-kDa proteins thought to be subunits of the 44-kDa protein. These associating proteins are believed to be



responsible for the hemagglutination of high-molecular-weight gingipain complexes (43).

A distinct 64-kDa thiol protease has been cloned and sequenced from strain W83. The *tpr* gene was isolated by screening recombinant *E. coli* clones for general protease activity on agar plates containing skim milk. It also has activity against bovine serum albumin, azocoll, denatured collagen, casein, and fibrinogen but lacks activity against BAPNA and native collagen (5). The *tpr* gene encodes a 482-amino-acid protein which has homology across a 24-amino-acid region with eukaryotic and prokaryotic cysteine proteases, encompassing their active sites (Fig. 7). The deduced amino acid sequence of *tpr* does not have other homology with that of *prtT*.

A 97- to 110-kDa protease, PrtH, which cleaves human complement component C3 has recently been cloned from *P. gingivalis* W83. By direct comparison of the *prtT* sequence, we have found that this protease is expressed from a different gene (36). Mutant strain HF18, produced by allelic exchange, has diminished proteolytic activity against C3 and is noninvasive and avirulent in the mouse model. HF18 accumulates activated C3b opsonins on its surface to a level 12-fold higher than that of the wild-type strain (49). This finding suggests that *prtH* encodes a protease that may enable *P. gingivalis* to evade complement-mediated killing during the immune response.

Others have characterized an enzyme from *P. gingivalis* ATCC 33277 and named it lys-gingivain (51). It is a 68- to 70-kDa thiol lysyl-amidase with potent activity against high-molecular-weight kininogen and fibrinogen. This dual activity against kininogen and fibrinogen mimics the host enzymes trypsin, plasmin, factor XIa, and neutrophil elastase.

Three additional *P. gingivalis* proteases are under study, but sequence information is not yet available for comparison with known genes. A 150-kDa thiol gelatinase/hemagglutinin which stimulates collagen degradation by rat oral mucosal keratinocytes by activation of tissue matrix metalloproteinases has been purified (15). A 45-kDa trypsinlike enzyme increases interstitial collagenase production 10-fold by cultured human gingival fibroblasts but with no effect on tissue inhibitor of metalloproteinase (25). A 55-kDa arginine-specific cysteine protease with a pH optimum of 8.0 has been purified from the culture supernatant of *P. gingivalis* 381 and is able to degrade collagens, C3,  $\alpha$ 1 and  $\beta$  chains of fibrinogen, fibronectin,  $\alpha$ 1-antitrypsin,  $\alpha$ 2-macroglobulin, apotransferrin, albumin, and the  $\beta$  chain of insulin (3).

Further molecular characterization of this group of enzymes will add clarity in understanding the relationship among multiple *P. gingivalis* proteases and hemagglutinins beyond that achieved solely from biochemical purification and characterization. In summary, *prtT* encodes a cysteine protease with hemagglutinin activity. Its sequence similarity with streptococcal proteinase and the active site of the family of cysteine proteases supports experimental findings that it is a cysteine protease. Because of its similarity with SPE B, the possibility exists that it also has toxin activity. However, additional experiments with the purified enzyme are necessary to confirm this hypothesis.

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