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Cloning and Characterization of a New Protease Gene (*prtH*) from *Porphyromonas gingivalis*

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***Porphyromonas gingivalis* has been implicated as a contributing etiological agent of adult periodontitis and generalized forms of early-onset periodontitis. Proteases of *P. gingivalis* may contribute to its pathogenicity by destroying connective tissue as well as inactivating key plasma proteins that might mediate protective host functions. In order to explore this problem, antiserum raised against membrane vesicles of *P. gingivalis* W83 was used to screen a genomic library of strain W83 constructed by using the λ DASH vector system. A recombinant phage (λ34) expressing a *P. gingivalis* protease from the library was identified and characterized. Casein substrate zymography of λ34 lysates revealed a protease with an apparent molecular mass of 97 kDa. The gene encoding this protease was designated *prtH*. It was localized to a 3.7-kb *Hin*dIII-*Bam*HI fragment and specified an enzyme which hydrolyzed the human C3 complement protein under defined conditions. The nucleotide sequence of this 3.7-kb fragment was determined, and one 2.9-kb open reading frame (992 amino acids) corresponding to a 110-kDa protein was detected, suggesting it might be a precursor of the 97-kDa active protease. *prtH* is not similar to any previously cloned protease gene from *P. gingivalis*.**

Porphyromonas gingivalis, a black-pigmented, gram-negative anaerobe, has been implicated as an important etiological agent in the pathogenesis of periodontal disease. Several putative virulence factors may contribute to the pathogenicity of this organism. Such candidate virulence factors have been reviewed (23, 39) and include a capsule (antiphagocytosis), lipopolysaccharide (bone resorption), fimbriae (adhesin), hydrolytic enzymes (specific and generalized tissue destruction), and a variety of toxic by-products (e.g., ammonia).

P. gingivalis produces several extracellular proteases of differing molecular masses (reviewed in reference 15). Specific proteases of *P. gingivalis* are able to hydrolyze type I and type IV collagen (20, 37), complement (12, 32, 41), and immunoglobulin A (IgA) and IgG (11, 16). Thus, the proteolytic capacity of this organism may be an important virulence factor. Proteases could destroy tissue components as well as key plasma proteins that could mediate protective host functions. The complement system plays an important role as a possible means of protection in periodontal diseases (10, 33). In addition to this protective role which includes bacterial opsonization and killing, activation of complement can initiate a number of other events including release of inflammatory agents and modulation of other immune reactions (10). Although some of these responses are directed at defending the host against the invading organism, they may also cause local tissue damage typical of periodontal disease (10).

Several proteases produced by *P. gingivalis* are present in membrane vesicles which bud off from the outer surface of the cell (reviewed in reference 35). Although the exact function of these vesicles is unclear, biological activities previously reported have suggested that their production could be important in enhancing the virulence of *P. gingivalis* (35).

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Several groups have reported cloning and expression of *P. gingivalis* protease genes in *Escherichia coli* (1, 5, 27, 28, 37). Kuramitsu et al. (17, 27) have determined the nucleotide sequences of genes expressing collagenase activity (*prtC*) and trypsin-like activity (*prtT*). Bourgeau et al. (5) have determined the nucleotide sequence of a protease gene (*tpr*) expressing a 64-kDa protein. We now report the cloning of a *P. gingivalis* gene in *E. coli* that specifies an enzyme which hydrolyzed the human C3 complement protein under defined conditions. The nucleotide sequence of a 3.7-kb *Hin*dIII-*Bam*HI fragment encodes a gene we have designated *prtH*. *prtH* consists of a 2.9-kb open reading frame corresponding to a 110-kDa protein. The deduced protein product of the gene is not similar to other characterized proteases from *P. gingivalis*.

MATERIALS AND METHODS

Bacterial strains. *P. gingivalis* W83 was grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with hemin (5 μg/ml), vitamin K (0.5 μg/ml), and cysteine (1%). *E. coli* strains were grown in Luria-Bertani broth (30). Unless otherwise stated, all cultures were incubated at 37°C. *P. gingivalis* was maintained in an anaerobic chamber (Coy Manufacturing, Ann Arbor, Mich.) in 10% H_2–10% CO_2–80% N_2.

DNA isolation and analysis. Chromosomal DNA from *P. gingivalis* was prepared by the method of Marmur (22). Plasmid DNA extraction followed the alkaline lysis procedure of Birnboim and Doly (3). Plasmids were purified by cesium chloride-ethidium bromide centrifugation as described by Sambrook et al. (30). λ DNA was prepared as described by Sambrook et al. (30). DNA was digested with restriction enzymes as specified by the manufacturer (GIBCO/BRL, Gaithersburg, Md.). Southern blot transfer followed the method of Smith and Summers (36). DNA hybridization was done at 42°C with 50% formamide to allow a stringency of 80% (30). The blot was washed with 0.1% SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]) and 0.1% sodium dodecyl

sulfate (SDS). DNA labelling and autoradiography were done as reported previously (21).

Construction of a *P. gingivalis* genomic library. Chromosomal DNA was partially digested with *Sau*3A enzyme and separated by velocity centrifugation through a 10 to 30% sucrose density gradient (30). Fragments of 15 to 23 kb were isolated and combined with λ DASH II-*Bam*HI arms (Stratagene, La Jolla, Calif.), ligated at 4°C overnight, and then packaged in vitro by using the Gigapack II gold extract according to the manufacturer's instruction (Stratagene). Phage banks were adsorbed onto maltose-grown *E. coli* SRB(P2) cells for 15 min at 37°C and then mixed with soft L agar (0.7% agar) (30) and overlaid onto an L-agar (1.5% agar) base. The plates were incubated overnight at 37°C. This procedure yielded a λ DASH library of cloned chromosomal DNA with a titer of 1.1×10^6 PFU/ml.

Preparation of membrane vesicles and antiserum. Membrane vesicles from *P. gingivalis* cells were prepared by the method of Grenier and Mayrand (14). Briefly, a 4-liter culture of *P. gingivalis* was grown for 4 days in tryptic soy broth enriched with hemin (1 μg/ml), dithiothreitol (0.2 μg/ml), and menadione (1 μg/ml). The cells were removed by centrifugation, and the culture supernatant fluid was concentrated to 600 ml, using a Pellicon ultrafiltration system (Millipore Corporation, Bedford, Mass.), and then centrifuged at 120,000 × *g* for 60 min at 4°C. The sediments containing the membrane vesicles were suspended in 10 ml of distilled water, lyophilized, and stored at −70°C. Membrane vesicles suspended in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4 [pH 7.4]) were mixed with Freund's complete adjuvant (Difco) and used to immunize rabbits. The animals were boosted after 4 weeks with membrane vesicles in Freund's incomplete adjuvant and were exsanguinated at the end of 8 weeks. Preparation of antiserum was completed under an approved protocol authorized by the Virginia Commonwealth University Institutional Animal Care and Use Committee (40).

Screening of recombinant phage clones. Phage plaques obtained from infection of *E. coli* SRB(P2) with λ DASH were screened by following the methods of the Bio-Rad Immuno-Blot assay kit (Richmond, Calif.). Immunoreactive proteins of *P. gingivalis* produced by the phage plaques were detected by incubation of the filters with rabbit antiserum raised against membrane vesicles, followed by incubation with goat anti-rabbit IgG conjugated to alkaline phosphatase (Bio-Rad). Color development was with the reagent containing 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium (Bio-Rad Immuno-Blot assay kit).

Enzyme assay. The presence of trypsin-like activity was determined by using *N*-α-benzoyl-DL-arginine *p*-nitroanilide (BAPNA) as a substrate (Sigma Chemical Co., St. Louis, Mo.) in 50 mM Tris (pH 7.5). The incubation period was 5 h. Each assay was done at least twice. Buffer alone and a λ clone not recognized by antivesicle antibodies were used as controls.

Effect of inhibitors. To test the effect of various agents on proteolytic activity, a mixture of λ34 lysate (50 μl) and 50 mM Tris-HCl (pH 7.5; 400 μl) containing potential inhibitors was incubated at 37°C for 30 min before the addition of 50 μl of 100 mM BAPNA. The mixtures then were incubated at 37°C for 5 h. Following incubation, the mixtures were centrifuged and the A_{410} was read. Buffer alone and a λ clone not recognized by antivesicle antibodies were used as controls. The following concentrations of potential inhibitors were tested: 5 mM phenylmethylsulfonyl fluoride (PMSF), 5 mM EDTA, 5 mM *p*-chloromercuribenzoate (PCMB), 5 mM iodoacetic acid, 5 mM Nα-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), 5 mM tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK), 100 μg of leupeptin per ml, 100 μg of α-1-antitrypsin per ml, 5 mM dithiothreitol (DTT), 5 mM cysteine, 5 mM β-mercaptoethanol, 5 mM $CaCl_2$, 5 mM $MgCl_2$, and 5 mM $MnCl_2$.

SDS-PAGE and Western blot analysis. SDS-polyacrylamide gel electrophoresis (PAGE) was performed with a 12% separating gel by the method of Laemmli (19). The samples were solubilized in 2% SDS buffer at 100°C for 3 min and electrophoresed at a constant current of 15 mA. The protein bands were visualized by Coomassie blue staining. The separated proteins were electrophoretically transferred (38) to a nitrocellulose membrane at a constant voltage of 15 V for 2 h. Blots were probed with rabbit antiserum against the membrane vesicles of *P. gingivalis*. Immunoreactive proteins were detected by the procedure described in the Bio-Rad Immuno-Blot (GAR-AP) assay kit.

Preparation of protease from λ lysate. Approximately 500 ml of liquid lysate of λ34 absorbed onto maltose-grown *E. coli* SG21155 (obtained from S. Gottesman, Laboratory of Molecular Biology, National Institutes of Health) was harvested at 20 h and centrifuged at 8,000 × *g* for 20 min. The supernatant fraction was collected and gradually mixed by a gentle stirring with 2 volumes of acetone chilled at −20°C. After standing for 20 min at 4°C, the suspension was centrifuged at 8,000 × *g* for 30 min at 4°C. The resulting precipitate was dissolved in 50 mM Tris-HCl buffer (pH 7.5) and dialyzed overnight against the same buffer.

Casein substrate zymography. SDS-polyacrylamide was mixed with casein-polyacrylamide conjugate (18) to give a concentration of 200 μg of protein per ml of gel. Samples were solubilized in SDS buffer (4% SDS, 20% glycerol, 0.124 M Tris [pH 6.8]) for 30 min at 37°C. Electrophoresis was conducted with a SE 250-Mighty Small II slab gel unit (0.075 cm thick) (Bio-Rad) at room temperature with a constant current of 15 mA. Development of proteolytic bands followed the method of Greiner et al. (13). Briefly, after electrophoresis, the gel was shaken gently for 30 min in 100 mM Tris-hydrochloride buffer (pH 7.0) containing 2% Triton X-100, rinsed twice in distilled water, and then shaken for a further 30 min in 100 mM Tris-hydrochloride buffer (pH 7.0). The gel was transferred into development buffer consisting of 100 mM Tris-hydrochloride (pH 7.0) containing 2.5 mM $CaCl_2$ and 50 mM cysteine. The gel was incubated overnight (16 h) and stained with Coomassie blue. After the gel was destained, proteolytic activity was visualized as a clear band against a blue background.

DNA sequencing. DNA fragments of the 3.7-kb *Hin*dIII-*Bam*HI fragment from λ 34 (see Fig. 3) was subcloned into pUC19. Nucleotide sequences were determined by the dideoxy chain termination method (31) with the Sequenase version 2.0 DNA sequencing kit, using M13 (−40) and M13 reverse (−48) primers (United States Biochemical Corporation, Cleveland, Ohio) and [α-^{35}S]dATP (Amersham, Arlington Heights, Ill.). The Sequenase kit was used according to the manufacturer's recommendations (United States Biochemical). Additional overlapping oligonucleotide primers used were synthesized by Oligos Etc. Inc. (Wilsonville, Oreg.). Confirmation of some nucleotide sequences was performed with the *Taq* DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems, Foster City, Calif.) and analyzed on an Applied Biosystems model 373A DNA sequencing system (Foster City, Calif.). Nucleotide sequences were analyzed with the Genetics Computer Group sequence analysis software package (8) and the MacVector sequence analysis software package for the Macintosh (International Biotechnologies, Inc., New Haven, Conn.).

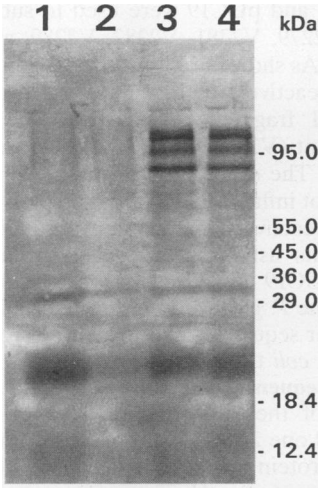

FIG. 1. Western immunoblot analysis of the proteins in lysate of the λ recombinant clones. Lane 1, λ1, a negative control (a recombinant not recognized by antivesicle antibodies); lanes 2 to 4, λ3, λ24, and λ34, respectively. All lanes contain 10 μg of protein from acetone-precipitated lysates. Samples were solubilized at 100°C in sample buffer with β-mercaptoethanol. The filter was reacted with antiserum raised in rabbits against the membrane vesicles of *P. gingivalis* W83. Secondary antibody was goat anti-rabbit IgG alkaline phosphate conjugate (Bio-Rad).

Nucleotide sequence accession number. The nucleotide sequence reported here was submitted to GenBank and assigned accession number L27483.

RESULTS

Screening of recombinant clones and biochemical assay of immunoreactive phage plaques. Antibodies raised against the membrane vesicles of *P. gingivalis* W83 were used to screen the λ DASH recombinant phage bank for the presence of immunoreactive clones. Eighty-three of 1.4×10^4 phage clone plaques (0.55%) reacted with the antivesicle serum.

The immunoreactive phage plaques were amplified and absorbed onto maltose-grown *E. coli* SG21155 (Δ*lon* Δ*lac* Δ*gal*) cells. Liquid lysates from each of the phage were assayed for proteolytic activity by using BAPNA as a substrate. Three recombinants (λ3, λ24, and λ34) expressed proteolytic activity above the host cell background. The proteolytic activity expressed by these three clones was enhanced 8 to 21% by the presence of 5 mM cysteine. This slight stimulation of activity was in contrast to observations made with other cloned proteases from *P. gingivalis* (1, 5, 28). The protease activities of λ3, λ24, and λ34 did not appear to require reducing conditions.

Analysis of recombinant λ clones. The identity of the cloned *P. gingivalis* protein was determined by immunoblotting with the antiserum raised to the *P. gingivalis* W83 membrane vesicles. SDS-PAGE-separated proteins from each of the three λ recombinant clones were transferred to nitrocellulose membranes by Western blotting (immunoblotting) (38). Three major immunoreactive components with apparent molecular masses of 116, 97, and 88 kDa were detected in λ24 and λ34 lysates (Fig. 1). No immunoreactive component differing from the control signals was detected in λ3. In addition, no immunoreactive band was detected in any of the λ clones when preimmune serum was used as the probe (data not shown).

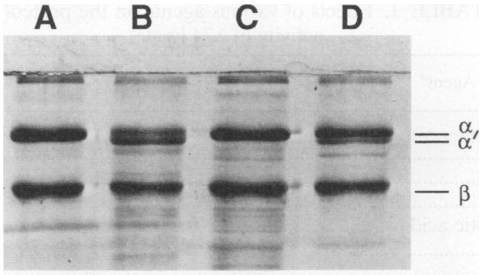

FIG. 2. Cleavage of human C3 by λ34. C3 (50 μg/ml) was incubated for 2.5 h at 37°C with membrane vesicles from *P. gingivalis* W83 and protein from an acetone precipitate of lysate of λ34. The reaction mixture was subjected to SDS–10% PAGE. Lane A, untreated C3; lane B, C3 incubated with membrane vesicles from W83; lane C, C3 incubated with λ1, a nonimmunoreactive clone (negative control); lane D, C3 incubated with λ34. All lanes contained 10 μg of C3. Lane B shows appearance of a new fragment corresponding to α′, a nicked α-chain. A product corresponding to a similarly cleaved fragment is seen in lane D (treated with λ34 lysate).

This indicated that the cloned *P. gingivalis* DNA was the source of immunoreactive material and that λ24 and λ34 were similar.

*Bam*HI cleavage of the DNA purified from λ3, λ24, and λ34 revealed that the phage clones had different restriction fragment patterns. However, λ24 and λ34 contained similar 6.0-, 2.4-, and 0.6-kb fragments. In addition, there was a 4.4-kb fragment present in λ24 that was missing in λ34 (data not shown). Further, λ34 contained a 3.4- and a 0.6-kb fragment that was missing in λ24 (data not shown). Taken together, the similar fragments and immunoreactive material of λ24 and λ34 indicated a common chromosomal origin and suggested that both these phage clones were expressing a common gene(s). Furthermore, these data indicated that the clones were independent isolates and not siblings from a single cloning event. λ34 was chosen for further study.

Expression of the protease activity was demonstrated by casein substrate zymography. A proteolytic band of approximately 97 kDa was detected in the λ34 extract. A component of similar size was seen in the Western blot of λ34 probed with antiserum raised against the membrane vesicles of *P. gingivalis* W83. No proteolytic band was detected in the negative control, a λ clone that was not recognized by antivesicle antibodies.

The ability of the cloned protease to hydrolyze the C3 complement protein under defined conditions was examined. Depending on the concentration of the protease, a 30-min to 2-h incubation of purified C3 with membrane vesicles from strain W83 resulted in the cleavage of the α chain of C3 to produce an α′ fragment of molecular mass identical to that of C3b (32). As seen in Fig. 2, incubation for 2 h of purified C3 with λ34 resulted in cleavage of the α chain of C3. This hydrolysis was identical to that seen when C3 was incubated with the membrane vesicles from W83. No hydrolysis of C3 was detected with phage lysate from the negative control. These results suggested that the C3 protein is one substrate for the cloned protease. Furthermore, the similar patterns of hydrolysis for this protease and the protease(s) contained in the membrane vesicles imply a common origin.

Effects of inhibitors and metal ions. The effects of various agents on proteolytic activity of λ34 are summarized in Table 1. Proteolytic activity was sensitive to TLCK and leupeptin, was slightly affected by PMSF, but was unaffected in the presence of EDTA. Significant activation was not seen in the presence of cysteine, DTT, β-mercaptoethanol, Mg^{2+}, and Mn^{2+}.

TABLE 1. Effects of various agents on the proteolytic activity of λ34 lysate

Agent[a]	% Protease activity[b]
None	100
PMSF	94
EDTA	100
PCMB	100
Iodoacetic acid	93
TLCK	2
TPCK	97
Leupeptin	23
α-1-Antitrypsin	94
DTT	104
Cysteine	115
β-Mercaptoethanol	108
$CaCl_2$	99
$MgCl_2$	107
$MnCl_2$	104

[a] Standard reaction conditions; see Materials and Methods for concentrations and agents used.
[b] Absolute optical density at 410 nm for the control is 1.16.

Subcloning and characterization of recombinant plasmid. The recombinant λ34 contained an 18-kb DNA insert. The origin of the cloned DNA fragment was confirmed by Southern hybridization, using λ34 as a probe. Chromosomal DNA of W83 digested with *Eco*RI possessed a 20-kb fragment which hybridized with the probe. A restriction endonuclease site map of the λ34 insert is shown in Fig. 3.

Subcloning was used to determine the location of the protease gene on the cloned DNA fragment of λ34. Since antibodies raised against the membrane vesicles recognized a 97-kDa protein that was similar in molecular mass to the proteolytic component, the antiserum was used in Western blots to analyze a crude lysate of the subclones. The plasmid vectors pUC18 and pUC19 were used to subclone fragments (designated V2279, V2281, V2282, V2289, and V2290) from the λ34 insert. As shown in Fig. 3, the sequences encoding the three immunoreactive protein bands were localized to a 3.7-kb *Hin*dIII-*Bam*HI fragment. A subclone of a 2.9-kb *Sma*I-*Bam*HI fragment (V2290) did not encode the three immunoreactive bands. The orientation of the 3.7-kb *Hin*dIII-*Bam*HI fragment did not influence its expression. As seen in Fig. 3, the three immunoreactive components were evident in Western blot experiments using the insert cloned in either pUC18 (V2279) or pUC19 (V2289). These results suggested that expression of the *P. gingivalis prtH* gene was initiated from its native promoter sequences and that these signals were recognized by the *E. coli* transcription machinery.

Nucleotide sequence determination of the protease gene. Both strands of the 3.7-kb *Hin*dIII-*Bam*HI fragment were sequenced, and one 2.9-kb open reading frame corresponding to a 110-kDa protein was detected (Fig. 4). This open reading frame was designated *prtH*. There was a start codon at base position 385. Alternatively, initiation at base position 394 would yield a protein that would be three amino acids smaller. The initiation codon at the alternative site is 11 bases downstream from an ACAG sequence that is complementary to the sequence UGUC found at the 3′ end of the 16S rRNA of *P. gingivalis* (29). As shown in Fig. 5, this sequence is conserved in several *P. gingivalis* genes and is located −26 to −5 upstream of the initiation start codon. There were no detected sequences resembling the consensus procaryotic −10 and −35 promoter regions. The calculated G+C content for the *prtH* gene was 48%, which corresponded well to the 46 to 48% previously reported for genomic *P. gingivalis* DNA (34). A comparison of the amino acid sequence of this gene with the National Center for Biotechnology Information genetic sequence data bank revealed a 13-amino-acid consensus sequence (amino acids 599 to 611) observed in cysteine proteases from both procaryotic and eukaryotic species (Fig. 6). No significant amino acid

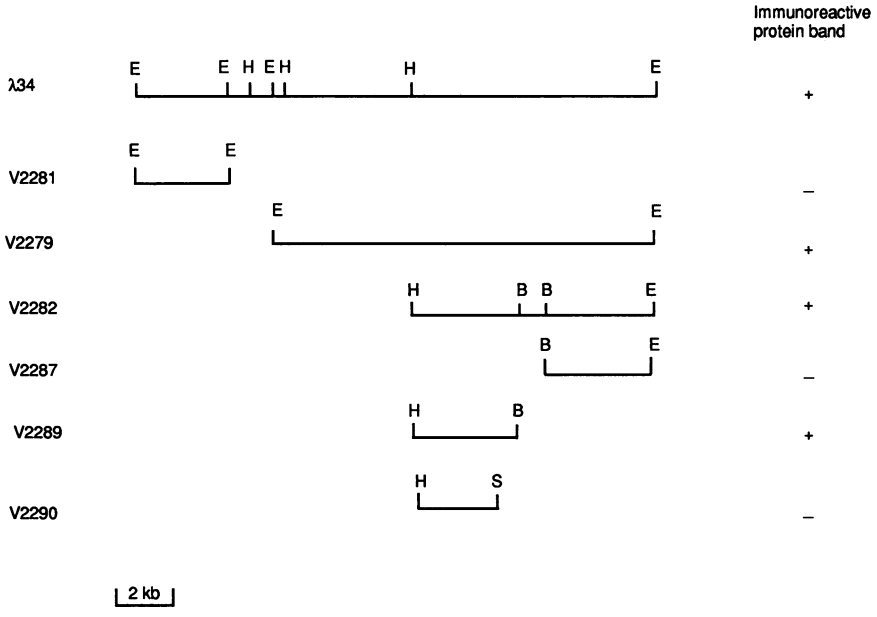

FIG. 3. Partial endonuclease restriction map of λ34 and its subclones. V2279 and V2281 contained fragments subcloned on pUC18. V2282, V2287, and V2289 contained fragments subcloned into pUC19. + and −, the presence or absence of the three protein species recognized by antiserum raised against membrane vesicles of W83 (Fig. 1). Cloning vector sequences are not shown in the map. Abbreviations are as follows: E, *Eco*RI; H, *Hin*dIII; B, *Bam*HI. The cloned protease gene is contained on a 3.7-kb *Hin*dIII-*Bam*HI fragment.

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                                                                      60
GATCCAATGAATTTGCTCCTGTACAGAACCTGACCGGTAGTGCAGTCGGCCAGAAAGTAA
                                                                     120
CGCTTAAGTGGGATGCACCTAATGGTACCCCGAATCCAAATCCAAATCCGAATCCAAATC
                                                                     180
CGAATCCCGGAACAACTACACTTTCCGAATCATTCGAAAATGGTATTCCTGCCTCATGGA
                                                                     240
AGACGATCGATGCAGACGGTGACGGGCATGGCTGGAAGCCTGGAAATGCTCCCGGAATCG
                                                                     300
CTGGCTACAATAGCAATGGTTGTGTATATTCAGAGTCATTCGGTCTTGGTGGTATAGGAG
                                                                     360
TTCTTACCCCTGACAACTATCTGATAACACCGGCATTGGATTTGCCTAACGGAGGTAAGT
                                                                     420
TGACTTTCTGGGTATGCGCACAGGATGCTAATTATGCATCCGAGCACTATGCGGTGTATG
                SD          M  H  P  S  T  M  R  C  M
                                                                     480
CATCTTCGACCGGTAACGATGCATCCAACTTCACGAATGCTTTGTTGGAAGAGACGATTA
H  L  R  P  V  T  M  H  P  T  S  R  M  L  C  W  K  R  R  L
                                                                     540
CGGCCCAAAGGTGTTCGCTCGCCGGAAGCTATTCGTGGTCGTATACAGGGTACTTGGCGC
R  P  K  G  V  R  S  P  E  A  I  R  G  R  I  Q  G  T  W  R
                                                                     600
CAGAAGACGGTAGACCTTCCCGCAGGTACCGAAATATGTTGCTTTCCGTCACTTCCCAAA
Q  K  T  V  D  L  P  A  G  T  E  I  C  C  F  P  S  L  P  K
                                                                     660
GCACCGATATGTTCTACATCGACCTTGATGAGGTTGAGATCCAAGACCAATGCCAAGCGC
A  P  I  C  S  T  S  T  L  M  R  L  R  S  K  T  N  A  K  R
                                                                     720
GCAGACTTCACGGAAACGTTCGAGTCTTCTACTCATGGAGAGGCACCAGCGGAATGGACT
A  D  F  T  E  T  F  E  S  S  T  H  G  E  A  P  A  E  W  T
                                                                     780
ACTATCGATGCCGATGGCGATGGTCAGGGTTGGCTCTGTCTGTCTTCCGGACAATTGGAC
T  I  D  A  D  G  D  G  Q  G  W  L  C  L  S  S  G  Q  L  D
                                                                     840
TGGCTGACAGCTCATGGCGGCACCAACGTAGTAAGCTCTTTCTCATGGAATGGAATGGCT
W  L  T  A  H  G  G  T  N  V  V  S  S  F  S  W  N  G  M  A
                                                                     900
TTGAATCCTGATAACTATCTCATCTCAAAGGATGTTACAGGCGCAACGAAGGTAAAGTAC
L  N  P  D  N  Y  L  I  S  K  D  V  T  G  A  T  K  V  K  Y
                                                                     960
TACTATGCAGTCAACGACGGTTTTCCCGGGGATCACTATGCGGTGATGATCTCCAAGACG
Y  Y  A  V  N  D  G  F  P  G  D  H  Y  A  V  M  I  S  K  T
                                                                    1020
GGCACGAACGCCGGAGACTTCACGGTTGTTTTCGAAGAAACGCCTAACGGAATAAATAAG
G  T  N  A  G  D  F  T  V  V  F  E  E  T  P  N  G  I  N  K
                                                                    1080
GGCGGAGCAAGATTCGGTCTTTCCACGGAAGCCGATGGCGCCAAACCTCAAAGTGTATGG
G  G  A  R  F  G  L  S  T  E  A  D  G  A  K  P  Q  S  V  W
                                                                    1140
ATCGAGCGTACGGTAGATTTGCCTGCGGGCACGAAGTATGTTGCTTTCCGTCACTACAAT
I  E  R  T  V  D  L  P  A  G  T  K  Y  V  A  F  R  H  Y  N
                                                                    1200
TGCTCGGATTTGAACTACATTCTTTTGGATGATATTCAGTTCACCATGGGTGGCAGCCCC
C  S  D  L  N  Y  I  L  L  D  D  I  Q  F  T  M  G  G  S  P
                                                                    1260
ACCCCGACCGATTATACCTACACGGTGTATCGTGATGGTACGAAGATCAAGGAAGGTTTG
T  P  T  D  Y  T  Y  T  V  Y  R  D  G  T  K  I  K  E  G  L
                                                                    1320
ACCGAAACGACCTTCGAAGAAGACGGCGTAGCTACGGGCAATCATGAGTATTGCGTGGAA
T  E  T  T  F  E  E  D  G  V  A  T  G  N  H  E  Y  C  V  E
                                                                    1380
GTGAAGTACACAGCCGGCGTATCTCCGAAGAAATGTGTAAACGTAACTGTTAATTCGACA
V  K  Y  T  A  G  V  S  P  K  K  C  V  N  V  T  V  N  S  T
                                                                    1440
CAGTTCAATCCTGTAAAGAACCTGAAGGCACAACCGGATGGCGGCGACGTGGTTCTCAAG
Q  F  N  P  V  K  N  L  K  A  Q  P  D  G  G  D  V  V  L  K
                                                                    1500
TGGGAAGCCCCGAGCGCAAAGAAGACAGAAGGTTCTCGTGAAGTAAAACGGATCGGAGAC
W  E  A  P  S  A  K  K  T  E  G  S  R  E  V  K  R  I  G  D
                                                                    1560
GGTCTTTTCGTTACGATCGAACCTGCAAACGATGTACGTGCCAACGAAGCCAAGGTTGTG
G  L  F  V  T  I  E  P  A  N  D  V  R  A  N  E  A  K  V  V
                                                                    1620
CTCGCAGCAGACAACGTATGGGGAGACAATACGGGTTACCAGTTCTTGTTGGATGCCGAT
L  A  A  D  N  V  W  G  D  N  T  G  Y  Q  F  L  L  D  A  D
                                                                    1680
CACAATACATTCGGAAGTGTCATTCCGGCAACCGGTCCTCTCTTTACCGGAACAGCTTCT
H  N  T  F  G  S  V  I  P  A  T  G  P  L  F  T  G  T  A  S
                                                                    1740
TCCGATCTTTACAGTGCGAACTTCGAGTATTTGATCCCGGCCAATGCCGATCCTGTTGTT
S  D  L  Y  S  A  N  F  E  Y  L  I  P  A  N  A  D  P  V  V
                                                                    1800
ACTACACAGAATATTATCGTTACAGGACAGGGTGAAGTTGTAATCCCCGGTGGTGTTTAC
T  T  Q  N  I  I  V  T  G  Q  G  E  V  V  I  P  G  G  V  Y
                                                                    1860
GACTATTGCATTACGAACCCGGAACCTGCATCCGGAAAGATGTGGATCGCAGGAGATGGA
D  Y  C  I  T  N  P  E  P  A  S  G  K  M  W  I  A  G  D  G
                                                                    1920
GGCAACCAGCCTGCACGTTATGACGATTTCACATTCGAAGCAGGCAAGAAGTACACCTTC
G  N  Q  P  A  R  Y  D  D  F  T  F  E  A  G  K  K  Y  T  F
                                                                    1980
ACGATGCGTCGCGCCGGAATGGGAGATGGAACTGATATGGAAGTCGAAGACGATTCACCT
T  M  R  R  A  G  M  G  D  G  T  D  M  E  V  E  D  D  S  P
                                                                    2040
GCAAGCTATACCTATACAGTCTATCGTGACGGCACGAAGATCAAGGAAGGTCTGACCGAA
A  S  Y  T  Y  T  V  Y  R  D  G  T  K  I  K  E  G  L  T  E
                                                                    2100
ACGACCTACCGCGACGCAGGACTGAGTGCACAATCTCATGAGTATTGCGTGGAAGTTAAG
T  T  Y  R  D  A  G  L  S  A  Q  S  H  E  Y  C  V  E  V  K
                                                                    2160
TACACAGCCGGTGTTTCTCCGAAGGTTTGTGTGGATTATATTCCTGACGGGGTGGAAGAC
Y  T  A  G  V  S  P  K  V  C  V  D  Y  I  P  D  G  V  E  D
                                                                    2220
GTTACGGTTCAGAAGCCTCACACGCTGACAGTTGTCGGAAAGACGATCACGGTATCTTGG
V  T  V  Q  K  P  H  T  L  T  V  V  G  K  T  I  T  V  S  W
```

```
                                                                    2280
CAGGGGGAAGCTATGATCTACGACATGAACGGTCGTCGTCTGGCAGCCGGTCGCAACACG
Q  G  E  A  M  I  Y  D  M  N  G  R  R  L  A  A  G  R  N  T
                                                                    2340
GTTGTTTACACGGCTCAGGGCGGCTACTATGCAGTTATGGTTGTCGTTGACGGCAAGTCT
V  V  Y  T  A  Q  G  G  Y  Y  A  V  M  V  V  V  D  G  K  S
                                                                    2400
TACGTAGAAGAAACTCGCTATCAAGTAAATCTGTCTTGGACTCGGAGACTTTGTGCAGAC
Y  V  E  E  T  R  Y  Q  V  N  L  S  W  T  R  R  L  C  A  D
                                                                    2460
ACTTCTAAGCATAGGTCTGTAATTGTCTCAGAGTATGAATCGGTCGCCCGACCTCCTTTT
T  S  K  H  R  S  V  I  V  S  E  Y  E  S  V  A  R  P  P  F
                                                                    2520
AAGGAAGTCGGGCGACTTCGTTTTTATGCCTATTATTCCAATATACTTCTGAAACAATTT
K  E  V  G  R  L  R  F  Y  A  Y  Y  S  N  I  L  L  K  Q  F
                                                                    2580
GTTCCAAAAAGTTGCATGAAAAGATTATCTTACTATCTTCCGCACTGCAAAAAGGGGAGT
V  P  K  S  C  M  K  R  L  S  Y  Y  L  P  H  C  K  K  G  S
                                                                    2640
TTCCTAAGGTTTCCCCGGAGTAGTACGGTAATCACGGTGTGCCAGTTCAGCTGGTTAGAA
F  L  R  F  P  R  S  S  T  V  I  T  V  C  Q  F  S  W  L  E
                                                                    2700
TACCTGCCTGTCACGCAGGGGGTCGCGGGTTCGAGTCCCGTCCATACCGCTAAAATAAGG
Y  L  P  V  T  Q  G  V  A  G  S  S  P  V  H  T  A  K  I  R
                                                                    2760
AGTTGTGTTGAAATAGTTTTTCGGCACAGCTCCATTTTTGTATGTTATCGCAGCACCGGA
S  C  V  E  I  V  F  R  H  S  S  I  F  V  C  Y  R  S  T  G
                                                                    2820
AAGTATAATTACCGGATGAGATTATTCAATATGCTCGGGAAGAATTTCCTTAGAACGAAG
K  Y  N  Y  R  M  R  L  F  N  M  L  G  K  N  F  L  R  T  K
                                                                    2880
CAGAAGTGTTTGTCTTTATTACGATCTGCTTGGGACATAGGGATTAAATTAGTATTATTC
Q  K  C  L  S  L  L  R  S  A  W  D  I  G  I  K  L  V  L  F
                                                                    2940
CAGGAGGGACGGTACATGGAGTCGCCCGGCCAATCAGATGAAGAAAGAAGAACTACGATT
Q  E  G  R  Y  M  E  S  P  G  Q  S  D  E  E  R  R  T  T  I
                                                                    3000
GATTTTTATGGGAACGGGCGATATTTGCTGTTCCGGCACTCCGAGCTTTTGGTCGAAAAC
D  F  Y  G  N  G  R  Y  L  L  F  R  H  S  E  L  L  V  E  N
                                                                    3060
GGATACCAAGTAAAAGCTGTGGTCACTATGCTCGGAACAAAACCTATGGGTCGAGGACAT
G  Y  Q  V  K  A  V  V  T  M  L  G  T  K  P  M  G  R  G  H
                                                                    3120
AAGGTAAGTCCCAGTATGGTCAAACTATACGCACAGGAATTGGGTCTGCCTATTCTCCAG
K  V  S  P  S  M  V  K  L  Y  A  Q  E  L  G  L  P  I  L  Q
                                                                    3180
CCGGACAATCTGAACGAGGAATCTTTTCTCGATGAACTACGGACTTATCAGCCGCACTTG
P  D  N  L  N  E  E  S  F  L  D  E  L  R  T  Y  Q  P  H  L
                                                                    3240
CAAATCGTAGTGGCTTTCCGTATGCTTCCTCGCTCCGTATGGCAAATGCCCCCCATGGGA
Q  I  V  V  A  F  R  M  L  P  R  S  V  W  Q  M  P  P  M  G
                                                                    3300
ACAATCAATCTGCATGGCTCTCTGCTGCCCATGTATCGAGGAGCAGCCCCCTATCCAACCA
T  I  N  L  H  G  S  L  L  P  M  Y  R  G  A  A  P  I  Q  P
                                                                    3360
CGCGATACGCCATGGCGATACGGGAAACGGGAGTTACCACCTTCCGCCTCCGGCATGAGA
R  D  T  P  W  R  Y  G  K  R  E  L  P  P  S  A  S  G  M  R
                                                                    3420
TAGATACGGGTGAAGTACTGCTGCAAGAGAAGTTGCCTATAGGACATGAAGAGACTTTCG
*
                                                                    3480
GCGAATTGTACGAACGTATGGCTACTCTCGGTGCATCCGTATTGGTGCACACAGTGGACT
                                                                    3540
TGTTTCTCGAAGGAGAACCCGTCTCCATACCTCAGGAGCAACTTCCGGGCTATGTTGATG
                                                                    3600
CTCGACCGGCTCCGAAGATTTTCAAAGACGACTGCCGTATCGATTGGGACAAACCGGCTG
                                                                    3658
AAGAGGTACACAATTTCATCCGCAGCATATCGCCTTGTCCTGCAGCTTGGACCTACCT
```

FIG. 4. Nucleotide sequence of the cloned protease gene from *P. gingivalis* W83. The sequence is derived from the *Bam*HI-*Hin*dIII fragment of λ34. The deduced amino acid sequence of the open reading frame is indicated below the nucleotide sequences. A possible Shine-Dalgarno (SD) sequence is underlined. The stop codon (TAG) is indicated by an asterisk.

```
prtH        GGGTATGCGCACAGGATGCTAATTATG
FimA      TTACAGAAGGTAATGCCACCATCAGCGTG
sod         TTAATTACAATAAAATCAGACGTTATG
Sod         TTACAATAAAAATCAGACGTTATG
pgiIM       CAAATAGAAGAGGTATCTTTGTG
prtC      CGGTGAATACCGTCATATACGACGAGGACATG
tpr     GTCACAGTTTTAATCAATCTAAAATTTTTCAATTATG
```

FIG. 5. Comparison of published sequences of *P. gingivalis* with an ACAG sequence that is complementary to the sequence UGUC. The sequence UGUC is found at the 3′ end of the 16S rRNA of *P. gingivalis*. Sequences of *prtH* (this study), *FimA* (9), *sod* (6), *Sod* (25), *pgiIM* (2), *prtC* (17), and *tpr* (5) are shown. The 3′ end of the *P. gingivalis* (ATCC 33277) 16S rRNA is as follows: 3′ UGUCACCUC UCAAACUAGGA 5′ (29).

homology was seen between *prtH* and other protease genes from *P. gingivalis* (5, 17, 27).

DISCUSSION

A genomic library of *P. gingivalis* W83 was constructed by using the λ DASH *E. coli* vector. Assuming that the genome of this organism is similar to those of other gram-negative bacteria such as *E. coli*, a representative genomic library with fragment sizes of 15 to 23 kb would be achieved with as few as 1.3×10^3 recombinants (7). The procedure used in these experiments yielded 1.1×10^6 independent recombinants, suggesting the construction of a complete library.

Assay of lysates of the immunoreactive clones with a synthetic peptide substrate revealed that three of the clones encoded a protease that degraded BAPNA at levels above the host background. In contrast with other reports (1, 5, 28), reducing agents appeared not to enhance significantly proteolytic activity of our recombinant λ clones. Proteolytic activity was inhibited by TLCK and leupeptin but not by any of the other agents tested. We cannot explain this atypical profile, but work is in progress to purify this protease in order to perform a definitive biochemical characterization. The identity of the cloned protease gene as determined by Western immunoblotting of lysates revealed three components of molecular masses of 116, 97, and 88 kDa present in both λ24 and λ34. This indicated that both clones were expressing the same gene and further, as confirmed by restriction endonuclease analysis, contained similar fragments, indicating a common chromosomal origin. Although λ3 had proteolytic activity, there was

```
1.  H T L T V V G K T I T V S W Q G E A M I Y D M N
2.  H A Y T V L G Y T V S N G A Y Y L I I R N P W G
3.  H A V T A V G Y G K S G G K G Y I L I K N S W G
4.  H A V L A V G Y G E Q N G L L Y W I V K N S W G
5.  H A V N I V G Y S N A Q G V D Y W I V R N S W D
6.  H C V T A V G Y G S N S N G K Y W I V K N S W
7.  H G V L L V G Y N D N S N P P Y W I V K N S W
8.  H G L L L V G Y N D S A A V P Y W I I K N S W
9.  H A I V I V G Y G T E G G V D Y W I V K N S W D
10. H A I R I L G W G V E N G T P Y W L V A N S W N
11. H A V A A V G Y - - N P G - - Y I L I K N S W G
```

FIG. 6. Comparison of conserved amino acid sequences of the C3 protease gene of *P. gingivalis* W83 and other cysteine proteases of procaryotic and eukaryotic origin (adopted from reference 5). Boxes surround conserved amino acids and/or amino acids with conservative replacements according to the following groupings: ILVM, C, PAGST, HKR, and QNED. Row 1, *P. gingivalis* W83 C3 protease gene; row 2, *P. gingivalis* protease (*tpr*); row 3, *Carica papaya*; row 4, rat cathepsin; row 5, *Dermatophagoides pteronyssinus*; row 6, *Entamoeba histolytica*; row 7, *Trypanosoma brucei*; row 8, *Trypanosoma cruzi*; row 9, Chinese gooseberry actinidin; row 10, human cathepsin B; row 11, papaya papain.

no evidence of any immunoreactive band that was different from the control. We cannot explain the phenotype of this recombinant. It is possible that the acetone precipitation of the λ3 lysate resulted in loss or degradation of the putative immunoreactive protein which enabled us to originally detect this phage. We are investigating this further.

The active proteolytic component as determined by zymography was a 97-kDa protein. If we assume the standard relationship of genetic coding capacity in procaryotes (1 kb of DNA = a 35-kDa polypeptide), approximately 8.6 kb of DNA would be needed to specify the three protein products. However, the 3.7-kb *Hin*dIII-*Bam*HI fragment from λ34 encoded three immunoreactive bands of similar sizes. Moreover, the nucleotide sequence of the 3.7-kb *Hin*dIII-*Bam*HI fragment revealed one 2.9-kb open reading frame corresponding to a 110-kDa protein. Collectively, these data are consistent with the notion that the three immunoreactive bands are different forms of the same product and that the 97-kDa protein represents the active protease. It is possible that the three immunoreactive bands seen could represent a precursor, active form, and a truncated, inactive form of the same gene product. Processing or autodegradation of proteases in *P. gingivalis* has been demonstrated (28).

The *prtH* gene product can hydrolyze substrates including casein and the C3 protein of the complement system. The hydrolysis of the C3 protein had a pattern similar to that of the protease(s) contained in membrane vesicles, suggesting a common origin. Several other proteases in *P. gingivalis* that degrade similar substrates have already been described by other groups (reviewed in reference 15), and a proteolytic band with a molecular mass of 97 kDa has been demonstrated by Grenier et al. (13). We do not know the relationship of the *prtH* gene product to this previously reported protease, as it was neither cloned nor biochemically characterized. Although the *prtH* gene product can hydrolyze casein and C3 complement protein, its ability to degrade other substrates is the subject of an ongoing investigation.

A putative Shine-Dalgarno region for the *prtH* gene was detected 11 bases upstream of the AUG start codon. This deduction was based on sequence complementarity to the 3′ end of the 16S rRNA of *P. gingivalis* (29). Further, a survey of other published data from *P. gingivalis* revealed that this sequence is highly conserved and is located −25 to −5 upstream of the initiation start codon (Fig. 5). Although this distance upstream from the initiation codon is in contrast to those in *E. coli* (i.e., −12 to −5 upstream from the AUG start codon) and other procaryotic systems, there are reports of potential ribosomal binding sequences further upstream (up to 100 bp) from the AUG initiation triplet (4).

The translation product deduced from the nucleotide sequence of the *prtH* gene had a calculated molecular mass of 110 kDa, which is comparable to the 116-kDa species observed in Western blot analysis. The gene coding for the protease had an opening frame of 2,976 nucleotides that terminated with a TGA stop codon. There was, however, a lack of obvious procaryotic transcription termination sequences downstream of the stop codon. It is possible that *prtH* is part of an operon. A collagenase (*prtC*) from *P. gingivalis* ATCC 53977 has been shown to be transcribed as part of a polycistronic mRNA (17). Further, Choi et al. (6) have demonstrated that the genes for superoxide dismutase (*sod*), a trypsin-like protease (*prtT*), and a hemagglutinin are present downstream from the *prtC* gene on the chromosome. However, their cotranscription with the *prtC* gene has not been demonstrated. Experiments addressing the notion that the *prtH* gene is part of an operon are under way.

A comparison of the amino acid sequence of the cloned protease with the National Center for Biotechnology Information genetic sequence data bank revealed a consensus sequence (amino acids 599 to 611) with other known cysteine proteases. This consensus region, which begins with a histidine residue, is part of the active site in papain, a well-characterized cysteine protease (24, 26), and may also represent part of the active site of the protease described in this study. The protease described in this study was unique on the basis of nucleotide and amino acid sequence comparison with other genes from *P. gingivalis*.

The cloning and sequencing of a 97-kDa protease gene (*prtH*) represents the initial step in elucidating the precise role of complement-degrading proteases in the pathogenicity of *P. gingivalis*. Among other things, this work now makes it possible to construct mutants of *P. gingivalis* defective in *prtH* for use in in vivo virulence testing.

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