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Cloning, Expression, and Sequencing of a Protease Gene (*tpr*) from *Porphyromonas gingivalis* W83 in *Escherichia coli*

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***Porphyromonas gingivalis* is a highly proteolytic organism which metabolizes small peptides and amino acids. Indirect evidence suggests that the proteases produced by this microorganism constitute an important virulence factor. In this study, a gene bank of *P. gingivalis* W83 DNA was constructed by cloning 0.5- to 20-kb *Hin*dIII-cut DNA fragments into *Escherichia coli* DH5α by using the plasmid vector pUC19. A clone expressing a protease from *P. gingivalis* was isolated on LB agar containing 1% skim milk. The clone contained a 3.0-kb insert that coded for a protease with an apparent molecular mass of 64 kDa. Sequencing part of the 3.0-kb DNA fragment revealed an open reading frame encoding a protein of 482 amino acids with a molecular mass of 62.5 kDa. Putative promoter and termination elements flanking the open reading frame were identified. The activity expressed in *E. coli* was extensively characterized by using various substrates and protease inhibitors, and the results suggest that it is possibly a thiol protease.**

Porphyromonas gingivalis is frequently isolated from cases of advanced adult periodontitis in humans (38, 40, 44, 47). Holt et al. (18) recently demonstrated the progression of periodontal lesions in nonhuman primates with the implantation of *P. gingivalis* alone. This microorganism is capable of causing tissue breakdown and possesses surface adhesins that may play a significant role in adherence of the cells to oral tissue surfaces and other bacteria (25). *P. gingivalis* also possesses several proteolytic enzymes, including immunoglobulin A- and immunoglobulin G-degrading enzymes (15, 17, 21, 42), a collagenase (14, 24), and a trypsinlike protease (45). Recent studies have reported the purification and characterization of numerous proteases from *P. gingivalis* (for a review, see reference 26), but the specific role that they play remains to be determined. However, evidence obtained until now points to four possible roles. Because *P. gingivalis* is an asaccharolytic organism whose metabolism is dependent on the uptake of small peptides and amino acids, the role of proteases in the degradation of proteins represents an important metabolic requirement for its growth (5, 6, 9, 17). A second role for the proteases produced by *P. gingivalis* involves attachment to surfaces in the oral cavity. In some cases, proteases may adversely influence attachment but may, at other times, favor bacterial adherence either through exposure of hidden binding sites or through direct binding by using the active site of the enzyme (7, 13, 28, 29). A third role for the proteases produced by *P. gingivalis* is the degradation of various macromolecules and tissue components (4, 22, 33, 43). Finally, proteases produced by *P. gingivalis* can perturb various host defense mechanisms (21, 36, 41, 42).

Molecular cloning should provide the means for the production and purification of substantial quantities of rare or minor proteins or surface proteins that cannot be adequately purified by biochemical means. The cloning of genes coding for putative virulence factors will also allow the construction of well-defined mutants which lack various factors and which could be compared with the parent strain for their capacity to cause infections in various rodent models. This strategy will be helpful in determining the role of each factor in the pathogenesis of *P. gingivalis*. Very little information is available on the cloning of putative virulence factors from *P. gingivalis* (46). Recent studies indicate that at least one hemagglutinin gene from *P. gingivalis* (30), an outer membrane protein gene (1), a gene coding for a superoxide dismutase (8), and the gene encoding the fimbrial subunit protein of *P. gingivalis* (11) have been successfully cloned and characterized. To our knowledge, only the fimbrillin (11) and *sod* genes (8) have been sequenced.

In the work presented here, recombinant DNA technology was used to characterize a protease produced by *P. gingivalis*. *P. gingivalis* W83 was chosen for our study because this particular strain is capable of infecting guinea pigs when it is injected in pure culture (16). A clone (hereafter referred to as clone 46) that expresses a protease from *P. gingivalis* in *Escherichia coli* was isolated, and the activity was characterized. This protease was able to degrade bovine serum albumin (BSA), azocoll, and heat-treated collagen. The gene, called *tpr*, encoding the protease produced in *E. coli* has been sequenced, and the presence of putative promoter and transcription termination elements flanking the gene have been indicated.

MATERIALS AND METHODS

Bacterial strains, plasmid vectors, and culture conditions. *P. gingivalis* W83 was maintained on Todd-Hewitt (Difco Laboratories, Detroit, Mich.) agar plates supplemented with laked human blood (50 ml/liter), hemin (10 μg/ml), and vitamin K (1 μg/ml). Cells were transferred to fresh plates at 7-day intervals. Growth was at 37°C in an anaerobic chamber (COY Laboratory Products, Ann Arbor, Mich.) under an atmosphere of 80% nitrogen, 10% hydrogen, and 10% carbon dioxide. Growth of *P. gingivalis* in liquid medium was carried out by inoculating a single colony into 10 ml of a Trypticase (17 g/liter) (BBL Microbiology Systems, Cockeysville, Md.)-yeast extract (3 g/liter) medium containing hemin (10 μg/ml) and vitamin K (1 μg/ml). A 2-day preculture was used to inoculate larger volumes of Trypticase-

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yeast extract medium. Culture purity was assessed by Gram stain, phase-contrast microscopy, gas-liquid chromatographic analysis of culture supernatants, and anaerobic and aerobic culture on Todd-Hewitt blood agar plates.

E. coli DH5α was used as the host strain for pUC19 (Pharmacia) and pBluescript II KS M13(−) (Stratagene 212208) plasmid subclones. *E. coli* strains were grown at 37°C in Luria broth (LB) or on LB agar. Transformed cells were grown in LB or on LB agar supplemented with 100 μg of sodium ampicillin (Sigma Chemical Co., St. Louis, Mo.) per ml.

Isolation of chromosomal DNA. DNA was isolated from *P. gingivalis* W83 by a modification of the method of Hull et al. (19). Cells from a 2-day culture (300 ml) were harvested and washed twice with 50 mM Tris buffer (pH 8.0) containing 0.4 M EDTA. Proteinase K was then added to a final concentration of 800 μg/ml, and the preparation was gently mixed. Sarkosyl was added to a final concentration of 0.5% and mixed well by rotation, and the preparation was incubated for 60 min on ice. The suspension was heated for 20 min at 70°C to destroy the strong nuclease activity of strain W83. The suspension was then incubated overnight at 50°C. The DNA was centrifuged on a CsCl density gradient containing phenylmethylsulfonyl fluoride, a protease inhibitor (100 μl of a 30-mg/ml solution), in a VTi-50 rotor for 20 to 24 h at 49,000 rpm. The DNA was dialyzed against three changes of 10 mM Tris buffer (pH 7.5) containing 50 mM NaCl.

Cloning in pUC19. *P. gingivalis* W83 DNA was partially digested with *Hin*dIII to yield a maximum recovery of 0.5- to 20-kb fragments. The pUC19 plasmid was digested with *Hin*dIII, dephosphorylated, and ligated with the 0.5- to 20-kb W83 *Hin*dIII fragments. DH5α-competent cells (BRL 8258SA) were transformed by using this ligation mixture and spread on LB plates containing 50 to 100 μg of sodium ampicillin per ml, 50 mg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (Sigma) per ml, 50 mM IPTG (isopropyl-β-D-thiogalactopyranoside) (Sigma), and 1% skim milk. Recombinant clones produced white colonies. The plates were either kept at 37°C in the presence of oxygen for 4 to 5 days or, after 24 h at 37°C in the presence of oxygen, transferred to an anaerobic chamber (COY) and incubated at 37°C for a further 3 to 4 days to detect the expression of proteolytic activity by a protease that may be inactive or have reduced activity in the presence of oxygen, as is the case for several proteases produced by *P. gingivalis* (15). Proteolytic activity was detected by the presence of clear zones around the colonies. Positive clones were reinoculated onto LB–ampicillin–X-Gal–IPTG–skim milk plates and treated in the same manner as described above to verify the presence of proteolytic activity.

DNA sequencing. A protease-positive clone (clone 46) was characterized by restriction mapping by using the general method described by Maniatis et al. (23). Subclones were then constructed in the plasmid pBluescript (Stratagene) and tested for protease activity. The plasmid-strain W83 fragment DNA from each subclone was isolated by the method of Berger and Kimmel (3) and denatured with NaOH. The DNA was sequenced by using the dideoxynucleotide chain termination method (34) with 17-mer T3 and T7 universal primers (Stratagene) and [α-^{35}S]dATP (Dupont Canada Inc.). The T7 sequencing kit was used according to the manufacturer's recommendations (Pharmacia). Additional oligonucleotides used as primers (5′-CTA TCG CTA CTT CAT AGG G-3′, 5′-CGG TAG AGC GGG CAG CCT C-3′) were synthesized by using a Cyclone (Milligen/Biosearch) DNA synthesizer and purified by using a Sep-pak cartridge (Waters). The sequencing gels (4.5%) were run by using a Bio-Rad Sequi-Gen Sequencing Cell, and the sequences were analyzed by using the Genetics Computer Group Sequence Analysis Software Package (10).

Preparation of bacterial cells for the characterization of proteolytic activity. The protease-producing clone was inoculated onto LB agar plates containing 50 μg of sodium ampicillin per ml and 1% skim milk by using sterile, cotton-tipped applicators. The plates were incubated at 37°C in the presence of oxygen to ensure good growth and then transferred to an anaerobic chamber at 37°C for an additional 3 to 4 days. Activity was detected by the clearing of the opaque agar medium. Bacterial cells were harvested by scraping with a sterile bent glass rod and resuspended in 1 ml of 100 mM Tris-HCl (pH 7.2) for each agar plate. The bacterial suspensions were either used as is or adjusted to a concentration appropriate for the testing of certain substances that either stimulated or inhibited protease activity. All tests were carried out a minimum of three times, with many of the tests being repeated up to 10 times.

Effect of temperature. Undiluted bacterial suspensions (equivalent to approximately 5 mg of protein per ml) were tested for activity as a function of temperature. The test consisted of mixing 100 μl of bacterial suspension with 650 μl of 100 mM Tris (pH 7.2), 250 μl of distilled water, 10 mM sodium dodecyl sulfate (SDS) (Sigma), and 20 mg of azocoll (Sigma). The cell lysates were incubated at various temperatures (between 20 and 90°C [in 5-degree increments]) for 2 h. The lysates were then sonicated to disrupt released DNA and thus to facilitate pelleting and centrifuged in an Eppendorf Microfuge for 2 min to pellet cellular debris and undigested azocoll, and the optical density at 520 nm (OD_{520}) of the supernatant was read. A suspension of *E. coli* DH5α containing pUC19 was always used as a control to compensate for any inherent proteolytic activity. An increase in OD indicated the presence of proteolytic activity.

Effect of pH. Bacterial cells scraped from the LB agar plates were suspended directly in the appropriate buffer (sodium citrate-citric acid [pH 3.5 to 6.0], Na_2HPO_4-NaH_2PO_4 [pH 6.5 to 7.5], Tris [pH 8.0 to 9.0], sodium carbonate-sodium bicarbonate [pH 9.5 to 10.0], Na_2HPO_4-NaOH [pH 11.0 to 11.5], or KCl-NaOH [pH 12.0]). Increments of 0.5 pH unit were used. A 100-μl suspension of bacterial cells was added to 650 μl of the appropriate buffer with 250 μl of distilled water, 10 mM SDS, and 20 mg of azocoll. The cell lysates were incubated at 37°C for 4 h and vigorously shaken every 30 min to redisperse the azocoll. After 4 h, the cell lysates were sonicated to disrupt released DNA and centrifuged, and the OD_{520} was read by using DH5α(pUC19) as the control.

Effect of divalent and monovalent cations. To test the effect of divalent and monovalent cations, a mixture of 100 μl of undiluted cell suspension, 650 μl of 100 mM Tris (pH 7.2), 10 mM SDS, and suitable volumes of distilled water and cation solution were incubated for 30 min at room temperature, before the addition of 20 mg of azocoll. The suspensions were then incubated at 37°C for 4 h with periodic shaking. The suspensions were sonicated and centrifuged, and the OD_{520}s were read by using DH5α(pUC19) as the control.

Effect of inhibitors. A series of potential inhibitors of proteolytic activity were also tested: 1 to 100 mM EDTA, 1 to 100 mM 2-mercaptoethanol, 1 to 100 mM cysteine, 1 to 100 mM dithiothreitol, 1 to 10 mM phenylmethylsulfonyl fluoride, 1 to 10 mM sodium iodoacetate, 1 to 100 mM SDS, 1 to 10 μg of amastatin per ml, 1 to 10 μg of leupeptin per ml, 1 to 10 μg of pepstatin per ml, 1 to 10 μg of bestatin per ml,

1 to 10 mM PCMB (*p*-chloromercuribenzoate), 1 to 10 mM TLCK (Nα-*p*-tosyl-L-lysine chloromethyl ketone), 1 to 10 mM TPCK (tolyl sulfonyl phenylalanyl chloromethyl ketone), 1 to 20 mM ascorbic acid, 1 to 10 mM *N*-ethylmaleimide, 1 to 10 mM dithiobisnitrobenzoic acid, 1 to 10 mM E64, 500 μmol of phosphoramidon per ml, 200 mM 2,3-dichloroisocoumarin, 10 to 100 μg of α-1-antitrypsin per ml, and 1 to 10 mM benzamidine. To test the effect of various inhibitors on proteolytic activity, an appropriate bacterial concentration was first determined. Undiluted cell suspensions usually resulted in a maximum OD reading after 2 to 3 h of incubation. The cell suspensions were therefore diluted to achieve a concentration that had half-maximal activity after 4 h (typically a 1:4 or 1:8 dilution). A volume of 100 μl of diluted bacterial cells was mixed with 650 μl of 100 mM Tris (pH 7.2), 10 mM SDS, a suitable volume of inhibitor, and enough distilled water to give a final volume of 1 ml. This mixture was incubated at room temperature for 30 min before adding 20 mg of azocoll. The suspensions were incubated at 37°C for 4 h with periodic shaking. They were then sonicated and centrifuged, and the OD_{520} was read.

Determination of the molecular weight on SDS-polyacrylamide-BSA gels. The molecular weight of the protease was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by using a 4.5% stacking gel and a 12% resolving gel (0.75 mm) containing polyacrylamide-conjugated BSA (20). Bacterial cells were recovered from LB agar plates in a dissociating buffer containing 125 μl of 0.5 M Tris-HCl (pH 6.8), 300 μl of 50% glycerol, and 10 μl of 0.5 M EDTA (pH 8.0). When the cells were well resuspended, 10 μl of a 1% bromophenol blue solution was added along with 10 μl of 2-mercaptoethanol, 300 μl of distilled water, and 150 μl of 10% SDS. After being mixed on a Vortex mixer, the cell lysates were incubated at 37°C and sonicated after 15 min and at the end of a 30-min incubation period (Heat Systems Ultrasonics Inc., model W150; power setting 5, microprobe). They were then centrifuged in an Eppendorf Microfuge for 2 min, and a sample volume (30 μg per well) of the supernatant was deposited on the gel (6 by 10 cm; by using a Bio-Rad mini-gel system). After a 40-min migration at 200 V by using a buffer containing 0.3% Tris-HCl, 1.5% glycine, and 0.1% SDS, the gels were washed for 30 min in a 10 mM Tris-HCl buffer (pH 8.0) containing 1% Triton X-100, then for 30 min in a 10 mM Tris-HCl buffer (pH 8.0), and finally for 2 to 4 h in a 50 mM Tris-HCl buffer (pH 8.0) in the presence of 10 mM dithiothreitol or in an anaerobic chamber to activate the protease. The gels were then incubated for 15 min in the following series of solutions: (i) 40% methanol–10% acetic acid; (ii) 0.25% Coomassie blue G-250–50% methanol–12.5% trichloroacetic acid (TCA); (iii) 5% TCA; (iv) 40% methanol–10% acetic acid; (v) 10% ethanol–5% acetic acid; (vi) 5% acetic acid. The gels were then either stored in Ziploc bags with a few drops of 5% acetic acid or dried between two cellulose membranes by using the Biodesign (Carmel, N.Y.) system for drying gels. Proteolytic activity appears as a clear zone against a dark blue background. Protein low-molecular-weight standards (Bio-Rad) remained visible on the stained gels.

Activity against other protease substrates. Various protease substrates other than azocoll were used to test the extent of the proteolytic activity of clone 46. These substrates included azoalbumin (Sigma A-2382), azocasein (Sigma A-2765), hide powder azure (Calbiochem 37716), BSA (Sigma A-7906), benzoyl-arginine-*p*-nitroaniline (BAPNA) (Sigma B-4875), casein yellow (Calbiochem 218681), glycyl-L-proline-*p*-nitroanilide (Gly-Pro-NA) (Sigma G-0513), acid-soluble collagen (Sigma C-3511), autoclaved collagen, and gelatin (Bio-Rad).

Activity against azoalbumin was determined by mixing 100 μl of cell suspension (3 mg of protein) with 500 μl of an azoalbumin solution (25 mg/ml in 100 mM Tris [pH 8.5]). After 4 h of incubation at 37°C, 500 μl of 20% TCA was added to the mixture, which was then centrifuged. The supernatant was mixed with 105 μl of 6 N NaOH, and the OD_{440} was determined.

Activity against hide powder azure was determined by mixing 500 μl of cell suspension (15 mg of protein) with 500 μl of 100 mM Tris (pH 7.2) and adding 20 mg of hide powder azure. After 4 h of incubation at 37°C, the suspension was sonicated to disrupt released *p*-nitroaniline and centrifuged. The OD_{595} of the supernatant was determined.

Activity against BSA, native collagen, autoclaved collagen, fibrinogen, gelatin, and casein was determined by mixing 500 μl of cell suspension (15 mg of protein) with 500 μl of a 1% solution of the substrate (in 100 mM Tris [pH 8.5]). After a 4-h incubation at 37°C, 500 μl of 10% TCA was added, and after 30 min, the mixture was centrifuged. The OD_{280} of the supernatant was determined.

Activity against BAPNA and Gly-Pro-NA was determined by mixing 100 μl of cell suspension (3 mg of protein) with 100 μl of chromogenic substrate (2 mM) and 300 μl of 75 mM Tris (pH 8.0). After a 4-h incubation period, 500 μl of 40% TCA and 150 μl of 0.1% sodium nitrate were added. After a 5-min incubation period, 150 μl of 0.5% ammonium sulfamate was added. After another 5-min incubation period, 150 μl of 0.1% *n*-1-naphthylethylenediamine dihydrochloride was added, and the OD_{545} was determined.

Activity against casein yellow was determined by mixing 500 μl of cell suspension (15 mg of protein) with 500 μl of a solution of casein yellow (25 mg/ml in 100 mM Tris [pH 8.0]). After a 4-h incubation period at 37°C, 4 volumes of 500 mM perchloric acid was added. After 30 min at room temperature, the mixture was centrifuged. The supernatant was mixed with an equal volume of 300 mM NaCl, and the OD_{285} was determined.

Nucleotide sequence accession number. The nucleotide sequence reported here has been submitted to GenBank and assigned accession number M84471.

RESULTS AND DISCUSSION

Cloning of a *P. gingivalis* protease. A gene coding for a protease from *P. gingivalis* W83 has been cloned in *E. coli* DH5α by using the plasmid vector pUC19. Approximately 60,000 clones were examined. Initially, the LB-skim milk plates were examined after growth for 24 h at 37°C and then discarded. The final series of plates containing approximately 5,000 clones was incubated for a further 4 days in an anaerobic chamber (COY) at 37°C. The one positive clone (clone 46) was detected after a 3-day incubation period in the anaerobic chamber. While subsequent testing of the clone showed the presence of a small clear zone after growth for 1 week on LB-skim milk agar in the presence of oxygen, a marked and clearly visible zone could be seen after 3 to 4 days in the absence of oxygen (Fig. 1). While activity does develop under aerobic conditions, the presence of oxygen delays its appearance by up to 1 week. The appearance of increased production of the protease in the absence of oxygen may be due simply to an increase in activity resulting in cellular disruption and release of the protease into the surrounding medium. Plate-grown cells kept in an anaerobic chamber invariably lysed, releasing their DNA onto the plate

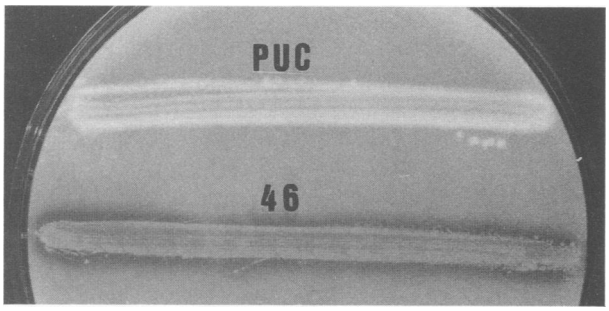

FIG. 1. Enzymatic activity of clone 46 on LB-skim milk agar as compared with *E. coli* containing only the plasmid vector (pUC19).

surface and giving a large clear zone as the protease diffused into the agar medium. Growth scraped from the agar surface was thus extremely viscous.

The molecular weight of 64,000, determined by SDS-PAGE (Fig. 2), is very similar to the molecular weight of the protease described by Fujimura and Nakamura (12) and to that of the trypsinlike enzyme described by Smalley and Birss (39). While these three enzymes share several common properties, such as being inhibited by *N*-ethylmaleimide and stimulated by dithiothreitol, that indicate that they are all thiol proteases, the protease described here is not trypsinlike because it does not degrade the artificial substrate BAPNA to any great extent nor is it inhibited by α-1-antitrypsin (100 μg/ml).

Restriction mapping and sequencing. The positive clone was shown to contain a 3.0-kb DNA fragment. This fragment was characterized by restriction endonuclease mapping (Fig. 3). The various fragments were subcloned into *E. coli* DH5α by using pBluescript and analyzed for the presence of protease activity (Fig. 3). Only the 1.9-kb *Hin*dIII-*Hin*cII fragment retained protease activity. Figure 3 also shows the various fragments used in the sequencing strategy. Six fragments were sequenced to obtain the nucleotide sequence shown in Fig. 4. The translation product deduced from the *P. gingivalis tpr* sequence presented in Fig. 4 has a calculated molecular weight of 62,510, which is comparable to the molecular weight of 64,000 deduced from polyacrylamide-BSA gel analysis. The gene coding for the protease has an open reading frame of 1,445 nucleotides (nt) that ends with a TGA stop codon at nt 1642. The sequence beginning 32 nt upstream from the start codon and the sequence 60 nt upstream could correspond to the −10 and −35 transcription sequences, respectively, of *E. coli*. These sequences are very similar to those already reported for a *P. gingivalis* superoxide dismutase gene (8) and a *Bacteroides fragilis* β-lactamase gene (32). There are 21 nt separating the two consensus regions as compared to an average of 20 nt in the case of *E. coli*. A potential Shine-Dalgarno site (ATT) is located 10 nt upstream from the start codon (37). A clone containing the *tpr* gene in the reverse orientation (as determined by restriction mapping) also expressed the proteolytic activity, indicating that the gene is controlled by its own promoter. There is a sequence resembling a rho-independent transcription termination sequence from nt 1674 to nt 1707 (delta G_0 = −9.9) that contains an inverted repeated sequence with the potential to form a hairpin structure with a 6-nt loop and a 14-nt stem including five pairs of CGs (Fig. 4). A stretch rich in T residues much like typical *E. coli* terminators and another stop codon (TAA) immediately follow this structure.

A comparison of the amino acid sequence with sequences listed in the GenEMBL Sequence Data Library, the GenNBRF Protein Sequence Data Library, and the SwissProt Protein Sequence Data Library revealed the presence of a 23-amino-acid consensus sequence (amino acids 406 to 430) near the C terminus that had a degree of homology of 60 to 65% with thiol or cysteine proteases from a wide range of species (Fig. 5). This region, which begins with a histidine

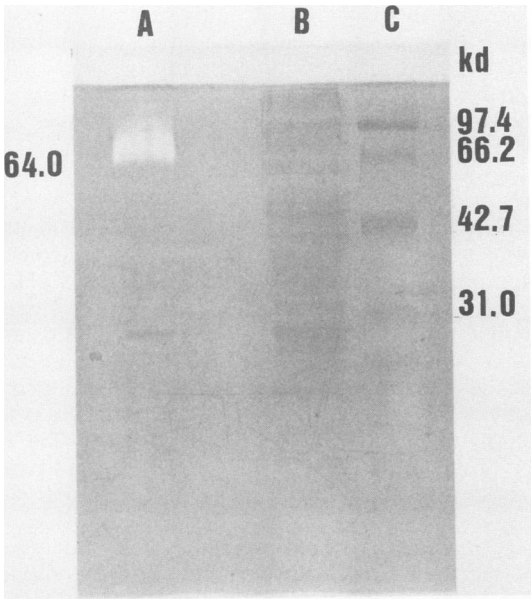

FIG. 2. Molecular weight determination of the protease. The molecular weight of the protease was determined by SDS-PAGE on a gel containing polyacrylamide-conjugated BSA. The molecular weight calculated from the leading edge of the clear zone was approximately 64,000. Lanes: A, clone 46; B, *E. coli*(pUC19); C, molecular weight markers. Thirty micrograms of protein was deposited in both lanes A and B.

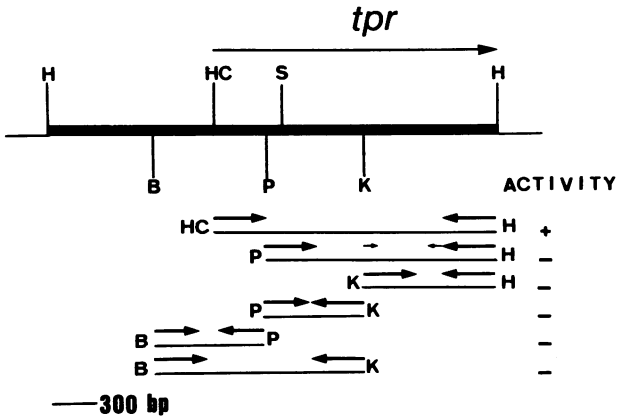

FIG. 3. Restriction endonuclease and functional map of the protease gene *tpr* of *P. gingivalis*. Abbreviations: H, *Hin*dIII; B, *Bam*HI; HC, *Hin*cII; P, *Pst*I; S, *Sph*I; K, *Kpn*I; + or −, presence or absence, respectively, of protease activity in *E. coli* subclones harboring the respective DNA fragments. Also indicated are the restriction fragments of clone 46 subcloned for sequence analysis. The *Hin*cII-*Hin*dIII fragment is 1.9 kb in length, and the *Hin*dIII-*Hin*dIII fragment is 3.0 kb in length.

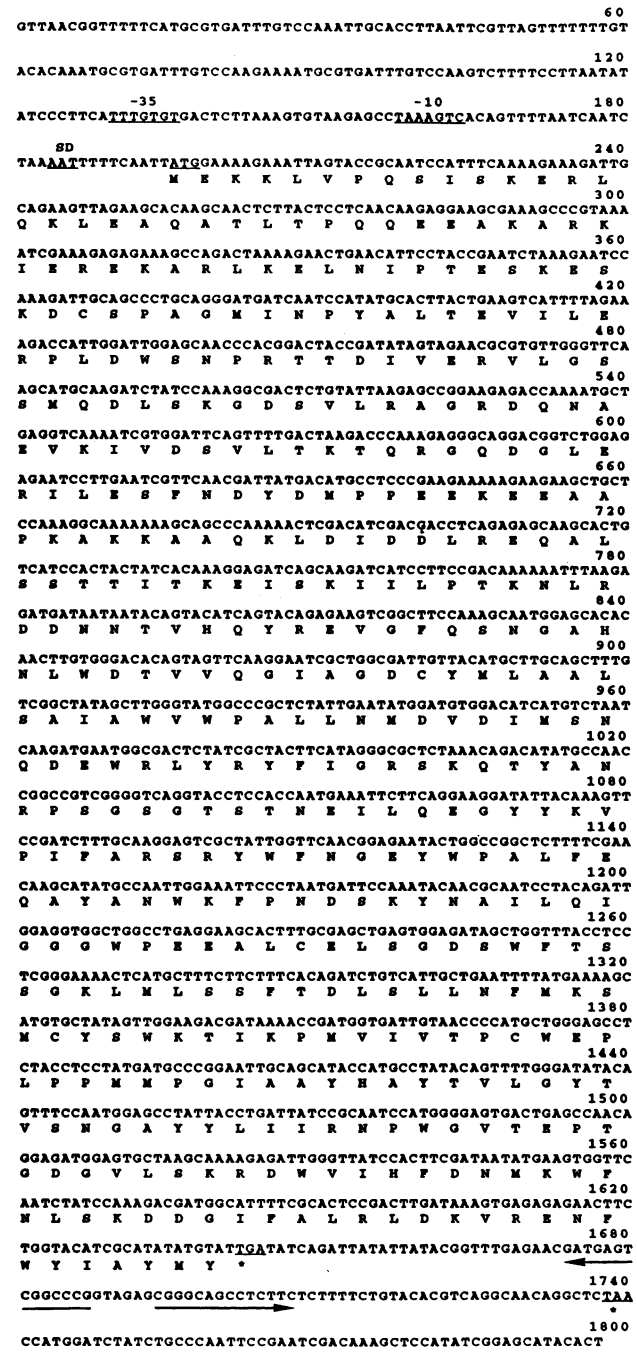

FIG. 4. Nucleotide sequence of the cloned *tpr* gene from *P. gingivalis* W83. The sequence is derived from the *Bam*HI-*Hin*dIII fragment of the insert. The deduced amino acid sequence of the open reading frame is indicated below the nucleotide sequence. Two stop codons are indicated by asterisks. Putative −35 and −10 promoter elements as well as a possible Shine-Dalgarno (SD) sequence are indicated. The possible rho-independent dual terminator is indicated by divergent arrows.

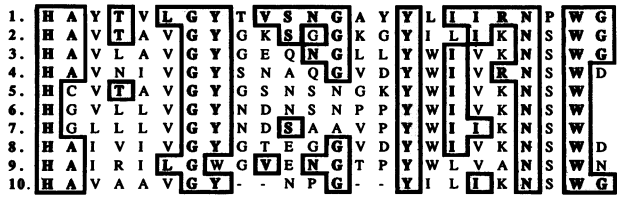

FIG. 5. Amino acid homologies between the deduced *P. gingivalis tpr* gene and amino acid sequences deduced from published sequences of other cysteine proteases. The numbers in parentheses below refer to the EMBL/GenBank accession number or a published sequence. Rows: 1, *P. gingivalis*, protease (*tpr*) (M84471); 2, *Carica papaya*, cysteine protease [65.2% homology, 47.8% identity] (X03970, M24252); 3, rat cathepsin, cysteine protease [60.9% homology, 43.5% identity] (Y00708); 4, *Dermatophagoides pteronyssinus*, cysteine protease [60.9% homology, 43.5% identity] (Y00641); 5, *Entamoeba histolytica*, cysteine protease [56.6% homology, 34.8% identity] (M27037); 6, *Trypanosoma brucei*, cysteine protease [60.9% homology, 30.4% identity] (M27306); 7, *Trypanosoma cruzi*, cysteine protease [65.2% homology, 39.1% identity] (M27305); 8, Chinese gooseberry actinidin [62.5% homology, 37.5% identity] [reference 2]; 9, human cathepsin B [62.5% homology, 41.7% identity] [reference 35]; 10, papaya papain [58.3% homology, 41.7% identity] [reference 31].

residue, corresponds to the active site of papain (27) and, most likely, also represents part of the active site of the thiol protease described here. Although histidine and aspartic acid residues are generally associated with the active site of cysteine proteases, e.g., papain, it is the thiol group of a cysteine residue that is acylated by the substrate. The aspartic acid and histidine residues stabilize the reaction of the cysteine residue with the substrate but are not modified during the reaction. Three-dimensional structures of homologous proteins tend to be more highly conserved in a wide range of species, while there appears to be little homology elsewhere between the amino acid sequences of the proteins. A three-dimensional model of our protease would be required to determine the active-site cysteine residue. No other regions with high degrees of homology were detected.

Characterization of the protease. The protease was inactive at and below 20°C when azocoll was used as the test substrate. Activity increased fairly rapidly above 30°C (OD_{520} = 0.4) and reached a maximum at approximately 45°C (OD_{520} = 1.0). Maximum activity was retained up to 70°C and was lost completely at 90°C. This temperature stability may be surprising, but there are examples of other proteases, such as proteinase K, that are active at 80°C. The protease was inactive below pH 6.0, reached maximum activity at pH 6.5, and retained its activity up to pH 12.0. Since *P. gingivalis* is an asaccharolytic organism that ferments peptides and amino acids, it might be expected to produce proteases that are functional over this pH range. Both NaCl and KCl exhibited no inhibitory or stimulatory effect up to a concentration of 100 mM. Divalent cations, on the other hand, had a strong inhibitory effect since 5 mM cobalt, nickel, mercury, or zinc completely inhibited protease activity (Table 1).

Proteolytic activity was sensitive to TLCK, TPCK, and EDTA (Table 2), which inhibit both serine and cysteine proteases. It was also inhibited by PCMB and divalent cations, including Hg^{2+}, which suggests that thiol groups may be involved in a configurational or catalytic role. Furthermore, reducing agents such as cysteine, dithiothreitol, and 2-mercaptoethanol stimulated activity. Other protease inhibitors such as amastatin, bestatin, pepstatin A, and aprotonin had no effect on protease activity (up to 50 μg/ml) (Table 2). Leupeptin, a thiol protease inhibitor, had no effect on protease activity. This may be due to the fact that it is a small peptide and may be degraded quickly enough by the protease to avoid any inhibitory effect.

TABLE 1. Effect of divalent and monovalent cations on the proteolytic activity[a] of clone 46

Ion	Inhibitory concn (mM)[b]
Manganese	5–10
Calcium	15–20
Magnesium	15–20
Copper	<5
Iron	<5
Cobalt	<5
Nickel	<5
Mercury	<5
Zinc	<5

[a] Proteolytic activity was determined by using azocoll as the substrate and 100 μl of cell suspension (3 mg of total protein).
[b] Concentration needed to completely inhibit the activity.

As was previously mentioned, clone 46 was detected when LB plates containing 1% skim milk were used. Initial testing with azocoll (without SDS) confirmed the proteolytic activity. SDS (10 mM) was found to stimulate activity, perhaps by causing bacterial cells to break open and release the proteolytic activity present into the cytoplasm. It is for this reason that 10 mM SDS was subsequently added to the test mixtures.

Finally, the activity of the clone against other natural and synthetic protease substrates was tested (Table 3). The clone 46 enzyme was particularly active against general protease substrates such as azocoll, casein, and BSA but had no effect on native collagen (Sigma type III, acid soluble) or synthetic peptides such as BAPNA and Gly-Pro-NA, indicating that it is not a collagenase nor a trypsinlike protease. It was, however, extremely active against autoclaved collagen, indicating that the heat treatment had exposed recognition sites.

In summary, we have cloned, sequenced, and characterized a protease from *P. gingivalis* that contains 482 amino acids and that shares a relatively high degree of homology between amino acids 406 and 430 with a number of cysteine proteases from a wide range of eucaryotes and procaryotes.

TABLE 2. Effect of various inhibitory substances on the proteolytic activity of clone 46

Inhibitory substance	Concn	% Inhibition[a]
Serine proteases		
Phenylmethylsulfonyl fluoride	5 mM	0
Benzamidine	5 mM	0
3,4-Dichloroisocoumarin	200 μg/ml	0
Aprotonin	100 μg/ml	0
Leupeptin	100 μg/ml	0
TLCK	0.25 mM	100
TPCK	0.25 mM	100
Metalloproteases		
Phosphoramidon	500 μg/ml	0
Ascorbic acid	10 mM	0
EDTA	5 mM	100
Trypsinlike proteases		
TLCK	0.25 mM	100
α-1-Antitrypsin	100 μg/ml	0
Leupeptin	100 μg/ml	0
Thiol proteases		
TLCK	0.25 mM	100
TPCK	0.25 mM	100
PCMB	0.25 mM	100
N-Ethylmaleimide	1 mM	85
Dithiobisnitrobenzoic acid	1 mM	80
E64	10 mM	85
Iodoacetic acid	0.25 mM	100
Leupeptin	100 μg/ml	0
Others		
Amastatin	50 μg/ml	0
Bestatin	50 μg/ml	0
Pepstatin A (aspartic)	50 μg/ml	0

[a] Inhibition of proteolytic activity was determined by using azocoll as the substrate and 100 μl of cell suspension containing 375 to 750 μg of total protein.

TABLE 3. Activity of clone 46 against various protein substrates[a]

Substrate	Substrate concn[b]	OD	
		Clone 46[c]	Strain W83[d]
Azocoll	20	1.70	0.65
Azoalbumin	20	0.13	0.10
Azocasein	20	0.06	0.045
Hide powder azure	20	0.76	0.50
BSA	5	0.54	0.29
BAPNA	0.4 mM	0	0.78
Gly-Pro-NA	0.4 mM	0	0.31
Autoclaved collagen	20	0.65	0.11
Native collagen	1	0	0.33
Fibrinogen	20	0.62	0.40
Gelatin	20	0.82	0.71
Casein	20	0.74	0.35
Casein yellow	12.5	0.58	0.21

[a] The tests using BSA, gelatin, casein, collagen (native and denatured), azocoll, casein yellow, and hide powder azure all contained 15 mg of cell extract per ml while the tests for activity against BAPNA, Gly-Pro-NA, azoalbumin, and azocasein all contained 3 mg of cell extract per ml. In all cases, reaction mixtures contained 5 mM SDS. Activities were measured after a 4-h incubation period at 37°C with vigorous shaking every 30 min. After 4 h, the mixtures were sonicated to disrupt released DNA. Activities against the various substrates were determined as described in Materials and Methods.
[b] Expressed in milligrams per milliliter except where otherwise indicated.
[c] *E. coli* DH5α containing pUC19 was not active against any of these substrates.
[d] For comparative purposes, cells from the original strain were tested in these assays.

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