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Sequence Analysis and Characterization of the *Porphyromonas gingivalis prtC* Gene, Which Expresses a Novel Collagenase Activity

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In order to examine the potential role of bacterial collagenases in periodontal tissue destruction, we recently isolated a gene, *prtC*, from *Porphyromonas gingivalis* ATCC 53977, which expressed collagenase activity (N. Takahashi, T. Kato, and H. K. Kuramitsu, FEMS Microbiol. Lett. 84:135–138, 1991). The nucleotide sequence of the gene has been determined, and the deduced amino acid sequence corresponds to a basic protein of 37.8 kDa. In addition, Southern blot analysis indicated that the *prtC* gene is conserved among the three major serotypes of *P. gingivalis*. The enzyme has been purified to near homogeneity from *Escherichia coli* clone NTS1 following Mono Q anion exchange and sequential gel filtration chromatography. The molecular mass of the purified enzyme was estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to be ca. 35 kDa, and the active enzyme behaved as a dimer following gel filtration chromatography. The collagenase degraded soluble and reconstituted fibrillar type I collagen, heat-denatured type I collagen, and azocoll but not gelatin or the synthetic collagenase substrate 4-phenylazobenzyloxycarbonyl-Pro-Leu-Gly-Pro-D-Arg. Enzyme activity was enhanced by Ca^{2+} and inhibited by EDTA, sulfhydryl-blocking agents, and the salivary peptide histatin. Preliminary evidence for the existence of a second collagenase expressed by strain 53977 was also obtained.

Porphyromonas gingivalis, a gram-negative anaerobic rod-shaped bacterium, has been isolated from the lesions of advanced adult periodontitis (35, 45) and has been implicated as a periodontal pathogen (20, 38, 47). These organisms exhibit a number of potential virulence traits, including high proteolytic activity (15). The proteases from *P. gingivalis* may degrade periodontal tissues (36, 40, 46) as well as inactivate host defense mechanisms (6, 31, 43). Since type I collagen serves as a major supporting structure for the teeth, it may be particularly relevant that these organisms exhibit collagenase activity (10, 46). This activity appears to be localized primarily in the membrane fraction of these organisms (28), but the enzymes have not yet been purified to homogeneity and extensively characterized. However, since the collagenase activity detected in the gingival fluid from diseased sites appears to be primarily of human origin (12, 15), there is no direct evidence that bacterial collagenases play a role in periodontal diseases.

In order to investigate the potential role of *P. gingivalis* collagenases in periodontitis, we have recently isolated the *prtC* gene which expressed collagenase activity from *P. gingivalis* ATCC 53977 (44). Although extensive biochemical characterization of the collagenase activity from *Clostridium histolyticum* has been carried out (32), little molecular genetic characterization of the bacterial enzymes has been reported. In contrast, both the isolation and sequence characterization of a number of different eucaryotic collagenase genes has been described (11). In addition, except for some partial sequence data for a collagenase isolated from *Vibrio alginolyticus* (9), no information is presently available regarding the nucleotide sequences of the bacterial enzymes.

In the present communication, we report the sequence analysis and further characterization of a purified collagenase expressed from the *prtC* gene of *P. gingivalis*. To our knowledge, this information represents the first report of the complete sequence of a bacterial collagenase gene. These results are discussed in light of the potential role of the collagenase in *P. gingivalis* virulence.

MATERIALS AND METHODS

Bacteria and growth conditions. *Escherichia coli* NTS1 containing plasmid pS1 was grown and maintained in LB broth supplemented with ampicillin (50 μg/ml) at 30°C as previously described (44). *P. gingivalis* strains were maintained anaerobically on blood agar plates containing mycoplasma broth (BBL Microbiology Systems, Cockeysville, Md.) supplemented with 1.5% agar, 10% sheep blood, hemin (5 μg/ml), and menadione (1 μg/ml). *P. gingivalis* strains were grown anaerobically in mycoplasma broth supplemented with hemin (5 μg/ml) and menadione (1 μg/ml).

Nucleotide sequencing of the *prtC* gene. For nucleotide sequence determination, overlapping DNA fragments from plasmid pS1 were subcloned into M13 mp18 and M13 mp19. Single-stranded template DNA was isolated by the procedure of Messing (23). To sequence the promoter region of the *prtC* gene, double-stranded pS1 DNA was used together with synthetic oligonucleotide primers. Nucleotide sequences were determined for the M13 DNA inserts by the dideoxy-chain termination method (30) with Sequenase version 2.0 (United States Biochemical Corp., Cleveland, Ohio) and standard M13 primers using [^{35}S]dATP. Sequence analysis was performed with the IBI Pustell sequence analysis program (International Biotechnologies, Inc., New Haven, Conn.).

Southern blot analysis. For Southern blot analyses, chromosomal DNA was digested with *Cla*I and *Pst*I. After electrophoresis on a 1.0% agarose gel, the separated DNA fragments were transferred to nitrocellulose membranes by

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using the PosiBlot pressure-blotting system (Stratagene, La Jolla, Calif.) and hybridized with biotin-labeled DNA probe. The 1.9-kb *Cla*I-*Hin*dIII biotin-labeled DNA probe was constructed by using the random primer DNA-labeling method (3).

Northern (RNA) blot analysis. Total RNA from *P. gingivalis* ATCC 53977 was extracted with guanidium thiocyanate and then subjected to ultracentrifugation in a cesium chloride solution (29). The isolated RNA was separated by electrophoresis on a 2.2 M formaldehyde–1.0% agarose gel and transferred to a nitrocellulose membrane by capillary elution. The ECL direct nucleic acid labeling and detection system (Amersham International plc, Amersham, United Kingdom) was used to identify the RNA fragments. The *Cla*I-*Hin*dIII 1.9-kb DNA fragment from plasmid pS1 was used as a probe. The blots were exposed on blue-light-sensitive autoradiography film (Hyperfilm-ECL; Amersham) for 3 or 4 h.

Purification of the *prtC* gene product. *E. coli* NTS1 was grown overnight in 1.0 liter of LB broth containing ampicillin (50 μg/ml) at 30°C. The harvested cells were washed with 50 mM Tris HCl buffer (pH 7.8) and suspended in 6 ml of the same buffer. The bacterial cells were disrupted by sonication and centrifuged to remove the cellular debris (44), and the resultant crude extract was filtered through a Millipore filter (pore size, 0.22 μm). The filtrate was injected into a Mono Q anion exchange chromatography column for separation with a fast-protein liquid chromatography system (Pharmacia-LKB Biotechnology, Inc., Piscataway, N.J.). Elution with a gradient of 0 to 1 M NaCl in 50 mM Tris-HCl buffer (pH 7.8) was utilized to purify the collagenase. The active fractions were then pooled and concentrated through a Centricon-10 ultrafilter (Amicon Corp., Danvers, Mass.). The enzyme sample from the Mono Q column was next applied onto a TSK-G3000SW gel filtration column. Collagenase-containing fractions were pooled and concentrated by ultrafiltration. The enzyme was reapplied to a TSK-G3000SW column, and the active fractions were again pooled and used as the purified enzyme.

Enzyme assays. Collagenase activity was determined by an assay system using ^{3}H-labeled reconstituted fibrillar type I collagen (specific activity, 0.020 GBq/mg; New England Nuclear, Boston, Mass.) as described by the supplier. Incubation was performed at 22°C for 3 h. In this system, collagenolytic activity is monitored by quantitating the production of soluble radioactive collagen fragments, which are readily separated from undigested collagen fibrils by centrifugation. Units of enzyme were defined by using the collagenase from *C. histolyticum* (315 U/mg; Sigma, St. Louis, Mo.) as a standard. In addition, collagenase activity was assayed as previously described (44) by incubating the enzymes with type I collagen (Sigma) at 30°C for 24 h. Reaction mixtures were then analyzed by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (19, 44). The ability to hydrolyze azocoll and the synthetic collagenase substrate PZ-PLGPA (4-phenylazobenzyl-oxycarbonyl-Pro-Leu-Gly-Pro-D-Arg) were examined by utilizing standard assay procedures (4, 48). Gelatinase activity was determined by the X-ray film method (26).

Purified cystatin SA-I was obtained from M. J. Levine (State University of New York, Buffalo), and histatin 5 was obtained from Y. Kuboki (Hokkaido University, Sapporo, Japan).

Nucleotide sequence accession number. The nucleotide sequence of the *prtC* gene has been deposited in the GenBank data base and given the accession number M60404.

RESULTS

Sequence analysis of the *prtC* gene. Recently, the *prtC* gene coding for collagenase activity was isolated on a 5.9-kb DNA fragment from *P. gingivalis* ATCC 53977 cloned into plasmid vector pPL-lambda (44). Since deletion analysis and subcloning indicated that the gene was located at one end of the insert, overlapping DNA fragments from this region were utilized to determine the nucleotide sequences of both DNA strands. A single long open reading frame was identified in this region (Fig. 1) which contained 1,002 base pairs (initiating at base position 85) coding for a putative protein with 333 amino acids (with a calculated molecular mass of 37.8 kDa). Alternatively, initiation at base position 94 would yield a slightly smaller protein with 330 amino acids. Either protein would be relatively basic, with an estimated pI of 9.7 for the larger protein.

A potential ribosome-binding site (34), AGGA, was identified starting at base position 80, but the actual sequences for *P. gingivalis* binding sites have yet to be determined. No sequences corresponding to *E. coli* promoter regions could be identified upstream from the potential initiation codons for the collagenase. However, fewer than 100 base pairs have been sequenced upstream from the start of the *prtC* gene, since this gene is located at the extreme end of the *P. gingivalis* insert in plasmid pS1 (see Fig. 3). A comparison of the amino acid sequence of the *P. gingivalis* collagenase with other proteins in the National Biomedical Research Foundation data base did not reveal significant similarities with any other protein sequence, including those of several eucaryotic collagenases.

The presence of the *prtC* gene in other *P. gingivalis* strains. In order to determine whether or not the *prtC* gene is unique to strain 53977 or whether it is present in other strains of *P. gingivalis*, Southern blot analyses of several other representative strains were carried out utilizing a *prtC* probe. Chromosomal DNA from strains of the three major serotypes of *P. gingivalis* (7) (381 [serotype a], W50 [serotype b], and 53977 [serotype c]) were cleaved with *Cla*I and *Pst*I and probed with a 1.9-kb *Cla*I-*Hin*dIII fragment containing most of the *prtC* gene and some downstream sequences (Fig. 2 and 3). The results demonstrated identical patterns for all three strains. In addition, an avirulent mutant of strain W50, W50/BE1 (14, 15), displayed the same two positive bands. These results suggest that the *prtC* gene and the 3′-flanking sequence are present in all three serotypes of *P. gingivalis* and that DNA rearrangement of the *prtC* gene in mutant W50/BE1 is apparently not responsible for its avirulent phenotype.

Identification of the *prtC* transcript. In order to determine whether the *prtC* gene was transcribed as part of a polycistronic mRNA or whether it was transcribed independently, Northern blot analysis of mRNA extracted from strain 53977 was carried out with the 1.9-kb DNA fragment from plasmid pS1 as a probe. The results (Fig. 3) indicated that the single mRNA containing the *prtC* transcript was approximately 4.4 kb in length. Since the *prtC* gene is approximately 1.0 kb in length, this gene appears to be transcribed as part of a polycistronic mRNA. It was not possible to determine the start site of this transcript, since only a small portion of the sequence upstream from *prtC* gene is presently available.

Purification and characterization of the collagenase expressed by *E. coli* NTS1. The collagenase activity present in the cytoplasmic fluids from *E. coli* NTS1 was purified to near homogeneity (Table 1). After passage of the crude enzyme through a Mono Q anion exchange column, there was an

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          10          20          30          40          50
          *           *           *           *           *
GAT CTG TAC AAA ATA GCA GAG ATT TGC AGA GAT AAA GGC GTA AAG AGC TAT TTA ACG

  60          70          80          90          100         ClaI
  *           *           *           *           *
GTG AAT ACC GTC ATA TAC GAC GAG GAC ATG ACA CTC ATG CGC TCC GTC ATC GAT GCG
                            Met Thr Leu Met Arg Ser Val Ile Asp Ala

     120         130         140         150         160         170
      *           *           *           *           *           *
GCA CAA AAG GCA CAA ATA TCT GCC ATT ATA GCT TCC GAC GTG GCT GCG ATG ACG TAT
Ala Gln Lys Ala Gln Ile Ser Ala Ile Ile Ala Ser Asp Val Ala Ala Met Thr Tyr

         180         190         200         210         220
          *           *           *           *           *
GCC AAC GAG ATC GGA GTA GAA GTG CAT CTG TCC ACT CAG CTC AAT ATC AGC AAT GCG
Ala Asn Glu Ile Gly Val Glu Val His Leu Ser Thr Gln Leu Asn Ile Ser Asn Ala

230         240         250         260         270         280
 *           *           *           *           *           *
GAA GCC CTA CGC TTT ATC GCG CTT GCC ATG TGG TCG TAT TGG CAG AGA GCT GAA TAT
Glu Ala Leu Arg Phe Ile Ala Leu Ala Met Trp Ser Tyr Trp Gln Arg Ala Glu Tyr

     290         300         310         320         330         340
      *           *           *           *           *           *
GGA TCA GGT ACG TAC AAT CCA CGA GAC CAT CGT CAG GGC CAT ATC TGT GGA CCT AAA
Gly Ser Gly Thr Tyr Asn Pro Arg Asp His Arg Gln Gly His Ile Cys Gly Pro Lys

        350         360         370         380         390
         *           *           *           *           *
GGC CAT CCC GTA CGT ATA GAG ATG TTC GCT CAC GGC GCT CTG TGT ATG GCC GTT TCG
Gly His Pro Val Arg Ile Glu Met Phe Ala His Gly Ala Leu Cys Met Ala Val Ser

400         410         420         430         440         450
 *           *           *           *           *           *
GGC AAG TGC TAT CTA AGC CTG CAC GAA CAC AAC ACG TCC GCC AAC AGA GGA GCC TGT
Gly Lys Cys Tyr Leu Ser Leu His Glu His Asn Thr Ser Ala Asn Arg Gly Ala Cys

      BglII PstI            480         490         500         510
                             *           *           *           *
GCG CAG ATC TGC AGG AGG GGC TAC ACC GTC AAG GAT AGC GGT CTG GAA CTG GAC ATT
Ala Gln Ile Cys Arg Arg Gly Tyr Thr Val Lys Asp Ser Gly Leu Glu Leu Asp Ile

       520         530                 BglII   550         560         570
        *           *                   *       *           *           *
GAG AAC CAA TAC ATC ATG TCG CCG AAA GAT CTG AAG ACT ATT CAT TTC ATC AAT AAG
Glu Asn Gln Tyr Ile Met Ser Pro Lys Asp Leu Lys Thr Ile His Phe Ile Asn Lys

           580         590         600         610         620
            *           *           *           *           *
ATG ATG GAT GCC GGC GTA CGA GTA TTC AAG ATA GAA GGA AGG GCA CGT GGC CCC GAA
Met Met Asp Ala Gly Val Arg Val Phe Lys Ile Glu Gly Arg Ala Arg Gly Pro Glu

 630         640         650         660         670         680
  *           *           *           *           *           *
TAC GTC TAT ACG GTC TGC CGC TGC TAC AAA GAA GCG ATC GAA GCC TAC TGC AAC GGC
Tyr Val Tyr Thr Val Cys Arg Cys Tyr Lys Glu Ala Ile Glu Ala Tyr Cys Asn Gly

     690         700         710         720         730         740
      *           *           *           *           *           *
ACC TAT GAT GAA GAG TCC ATA GGC CGG TGG GAC GAA CAA TTG GCT ACG GTA TTC AAC
Thr Tyr Asp Glu Glu Ser Ile Gly Arg Trp Asp Glu Gln Leu Ala Thr Val Phe Asn

         750         760         770         780         790
          *           *           *           *           *
CGA GGC TTT TGG GAT GGC TAC TAC CTC GGA CAA CGG CTC GGC GAA TGG ACA CAT CGT
Arg Gly Phe Trp Asp Gly Tyr Tyr Leu Gly Gln Arg Leu Gly Glu Trp Thr His Arg

800         810         820         830         840         850
 *           *           *           *           *           *
TAC GGC TCA GGA CGT ACG CGA CAG AAA ACA TAT GTA GGC AAG GGA ATC AAA TAC TTC
Tyr Gly Ser Gly Arg Thr Arg Gln Lys Thr Tyr Val Gly Lys Gly Ile Lys Tyr Phe

     860         870      EcoRI              890         900         910
      *           *                          *           *           *
AGC CGT CTC GGT GTG GCT GAA TTC GAG ATA GAG TCC GGC GAA CTG CAT ATA GGC GAT
Ser Arg Leu Gly Val Ala Glu Phe Glu Ile Glu Ser Gly Glu Leu His Ile Gly Asp

         920         930         940         950         960
          *           *           *           *           *
GAG ATT GTG ATC ACC GGC CCT ACT ACA GGT GTG ATC ATC CAA AAG GTG GAA GAG ATC
Glu Ile Val Ile Thr Gly Pro Thr Thr Gly Val Ile Ile Gln Lys Val Glu Glu Ile

970         980         990         1000        1010        1020
 *           *           *           *           *           *
CGA TAC GAA CTG CAA ACC GTG GAA AAG GCG ACA AAG GGA CAA CGC ATT TCC ATT CCG
Arg Tyr Glu Leu Gln Thr Val Glu Lys Ala Thr Lys Gly Gln Arg Ile Ser Ile Pro

   1030        1040        1050        1060        1070        1080
    *           *           *           *           *           *
GTA AAG GAG AAA GTG CGT CCG TCG GAC AAG CTC TAC CGG TTC GAC AAA AGA GAA GAA
Val Lys Glu Lys Val Arg Pro Ser Asp Lys Leu Tyr Arg Phe Asp Lys Arg Glu Glu

       1090        1100        1110        1120        1130        1140
        *           *           *           *           *           *
TAA GAT GTA GGG ACC GAG AGC GAA TTA TCA CCT CTC AAA CCC GAC AAA ACC CAA TGA
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          1150        1160        1170        1180        1190
           *           *           *           *           *
TAT GAA AGA GAA AGA ATA CCT GTT CTC CCT CGA CAT GAA GGT GAG AGA CTA TGA ATG
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FIG. 1. Nucleotide sequence of the *prtC* gene and the deduced amino acid sequence of the collagenase. The locations of the restriction enzyme digestion sites are indicated along the sequence.

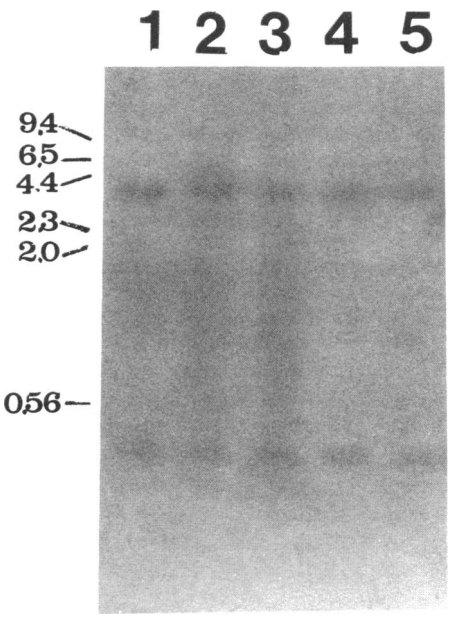

FIG. 2. Southern blot analysis of *Cla*I-*Pst*I-digested chromosomal DNA of *P. gingivalis* with the biotin-labeled *Cla*I-*Hin*dIII 1.9-kb fragment from plasmid pS1 as a probe. Lanes: 1, pS1; 2, *P. gingivalis* ATCC 53977 (serotype c); 3, *P. gingivalis* 381 (serotype a); 4, *P. gingivalis* W50 (serotype b); 5, *P. gingivalis* W50/BE1 (beige or nonpigmented colonial variant of W50). Numbers at the left indicate molecular sizes in kilobase pairs.

apparent enhancement of activity. This may represent the removal of an inhibitor of the collagenase activity which is present in *E. coli* extracts, but this has not yet been documented. As predicted from the apparent pI of the enzyme deduced from the amino acid sequence, the enzyme did not bind to the Mono Q column and was present in the flowthrough fractions. Sequential gel filtration of the concentrated enzyme on TSK-G3000SW columns resulted in an enzyme fraction displaying only a single protein band of approximately 35 kDa following SDS-PAGE (Fig. 4). This size corresponds to the predicted size of 37.8 kDa determined following nucleotide sequencing. In addition, the collagenase activity displayed an apparent molecular size of 70 kDa on the gel filtration column (data not shown). Therefore, it appears that the enzyme behaves as a dimer in its native form.

The purified enzyme degraded soluble (Fig. 5) as well as fibrillar reconstituted type I collagen (Table 1), azocoll, and heat-denatured (boiled for 5 or 10 min) type I collagen. However, the enzyme did not hydrolyze gelatin or the synthetic bacterial collagenase substrate PZ-PLGPA (data not shown). Therefore, the *P. gingivalis* collagenase appears to display properties which are distinct from those of other bacterial collagenases (18, 41). In addition, unlike eucaryotic collagenases (32), the *P. gingivalis* collagenase did not yield distinct degradation products when it was analyzed at different time periods (Fig. 5). It is likely that the purified enzyme degrades type I collagen to small peptide fragments which migrate off of the SDS-PAGE gel.

The purified collagenase is slightly stimulated in the presence of Ca^{2+} but is strongly inhibited by Zn^{2+} and Fe^{3+} (Table 2). Neither Mg^{2+} nor Mn^{2+} significantly affected enzyme activity. The enzyme appears to contain essential sulfhydryl groups, since *p*-chloromercuribenzoic acid is a strong inhibitor of the collagenase activity (Table 3). However, reducing agents such as 2-mercaptoethanol and dithiothreitol did not enhance enzyme activity, as has been observed for many enzymes from anaerobic bacteria (8, 14, 27, 39, 49). A metal cofactor appears to be required for activity, since the chelator EDTA inhibited collagenase activity. Several general protease inhibitors (phenylmethylsulfonyl

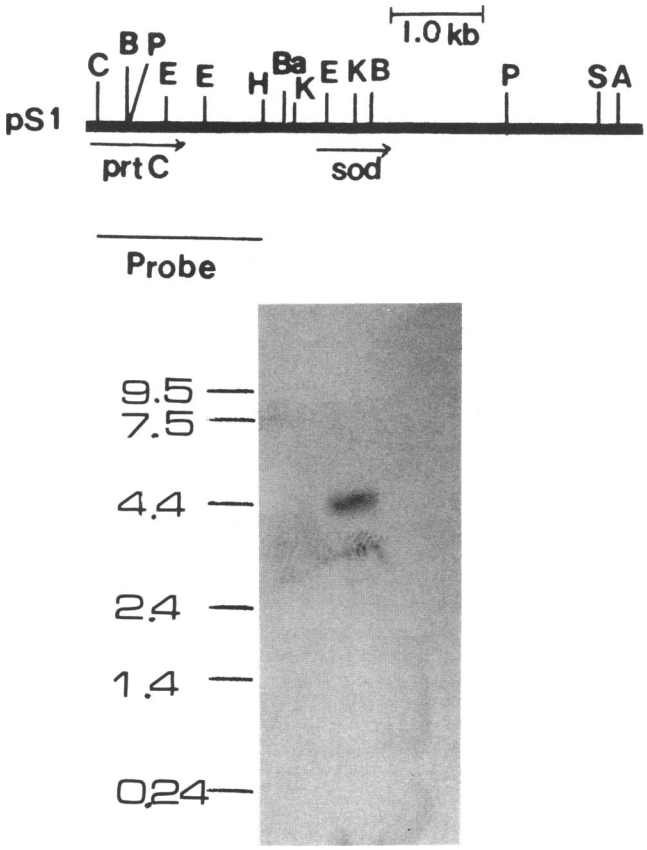

FIG. 3. Northern blot analysis. Hybridization of the blotted total RNA from *P. gingivalis* ATCC 53977 was performed with the labeled *Cla*I-*Hin*dIII fragment from plasmid pS1 (at top) as a probe with the ECL direct nucleic acid-labeling and detection system. Numbers at the left indicate molecular sizes in kilobases. Restriction sites: B, *Bgl*II; Ba, *Bal*I; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sma*I; A, *Ava*I. sod, superoxide dismutase gene.

fluoride [PMSF], *N*-α-D-tosyl-L-lysine chloromethyl ketone [TLCK], and L-1-tosylamido-2-phenylethyl chloromethyl ketone [TPCK]) had little effect on the *prtC* gene product. It was also of interest that two salivary peptides, cystatin and histatin, had opposite effects on the collagenase activity. The cysteine-rich peptide cystatin (2) enhanced enzyme activity, while histatin (25) acted as an inhibitor.

Evidence for two collagenases expressed by strain 53977. When crude extracts of *P. gingivalis* ATCC 53977 were subjected to anion exchange chromatography on Mono Q columns (Fig. 6), two peaks of collagenase activity could be detected. The active fractions not retained by the column contained a protein of approximately 35 kDa as detected following SDS-PAGE (data not shown) and therefore appears to correspond to the product of the *prtC* gene. However, a second collagenase activity was retained by the column and was eluted with the salt gradient. This enzyme fraction did not display a 35-kDa protein when subjected to SDS-PAGE. Therefore, these results are consistent with the presence of at least two collagenase enzymes produced by strain 53977.

TABLE 1. Purification of collagenase activity from *E. coli* NTS1

Purification step	Total protein (mg)	Total activity		Sp act (U/mg)[a]	Purification (fold)
		U	%		
Sonic extract	660	488.4	100	0.74	1
Mono Q	107	1350.4	267	12.2	16.5
TSK-G3000SW	1.6	64.2	13	40.1	54.2
TSK-G3000SW	1.1	48.7	10	44.3	59.9

[a] Units of enzyme activity per milligram of protein in the pooled fraction from each step. Collagenase activity was determined by the ^{3}H-labeled type I collagen assay system.

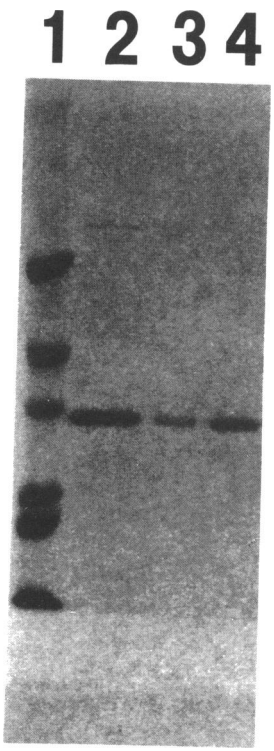

FIG. 4. SDS-PAGE analysis of the purified collagenase. Lanes: 1, molecular mass standards (from top to bottom, 66, 45, 36, 29, 24, and 20.1 kDa); 2, partially purified enzyme from the initial TSK-G3000SW column (5 μg); 3, final enzyme preparation (1 μg); 4, final enzyme preparation (5 μg).

DISCUSSION

The recently isolated *prtC* gene was shown to express an enzyme which hydrolyzes soluble type I collagen (44). The present investigation revealed that the collagenase was also capable of degrading reconstituted fibrillar collagen similar to the enzyme extensively characterized from *C. histolyticum* (32). However, unlike the latter enzyme, the *P. gingivalis* collagenase did not hydrolyze the synthetic collagenase substrate PZ-PLGPA. Likewise, unlike most collagenases (17, 18, 32, 41), the *prtC* gene product did not degrade gelatin but could hydrolyze the more extensively denatured type I collagen. The reasons for this difference in substrate specificity for the collagenase is presently unknown. Additional investigation will be required to determine the cleavage specificity of the *P. gingivalis* enzyme. In addition, the *prtC* gene product exhibited no structural similarity with eucaryotic collagenases and did not contain the HELGH peptide consensus sequence found in these enzymes (11). Likewise, degradation of type I collagen by the *P. gingivalis* enzyme

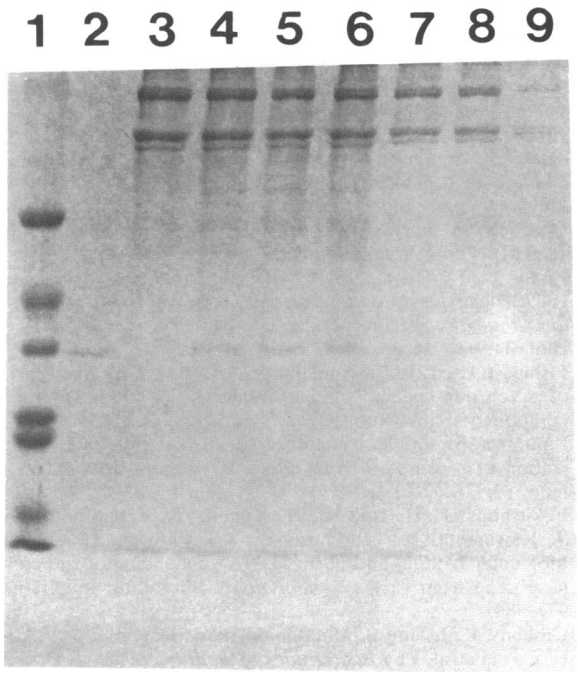

FIG. 5. Identification of the type I collagen degradation products following digestion with the purified collagenase. Collagenase activity of the purified enzyme was analyzed by SDS-PAGE on 10% polyacrylamide gels. In all assays, 20 μg of soluble type I collagen in 50 mM Tris HCl–5 mM $CaCl_2$ buffer (pH 7.8) was used as a substrate. Lanes: 1, molecular mass standards (from top to bottom, 66, 45, 36, 29, 24, and 20.1 kDa); 2, purified enzyme (0.7 μg); 3, native type I collagen incubated with buffer as a control; 4 through 9, native type I collagen incubated with purified enzyme for 4 (lane 4), 8 (lane 5), 10 (lane 6), 14 (lane 7), 18 (lane 8), and 24 (lane 9) h at 30°C.

resulted in multiple cleavage products (Fig. 5) rather than the two distinct peptides generated by the action of mammalian collagenases (24, 32). Therefore, the properties of the *prtC* gene product suggest that the encoded collagenase is a unique enzyme. However, since no other bacterial collagenase has yet been completely sequenced, the *P. gingivalis* enzyme could be related to some of these enzymes (18, 32, 41).

Sequence analysis of the *prtC* gene revealed two potential ORFs which differed by only three amino acids in length. Hydrophobicity analysis revealed that the amino-terminal sequences of both putative proteins were relatively hydrophobic (data not shown). Therefore, it may be possible that these sequences represent a signal peptide-like structure and

TABLE 2. Effects of metal ions on collagenase activity

Metal ion (concn [mM])	Relative collagenase activity (%)[a]
None	100
Ca^{2+} (5.0)	135
Mg^{2+} (5.0)	96
Mn^{2+} (5.0)	93
Zn^{2+} (5.0)	2
Fe^{3+} (5.0)	39

[a] Collagenase activity was determined by the ^{3}H-labeled type I collagen assay system.

TABLE 3. Effects of chemical agents on collagenase activity

Chemical agent[a] (concn)	Relative collagenase activity (%)[b]
None	100
PCMB (1.0 mM)	41
2-ME (1.0 mM)	77
DTT (1.0 mM)	84
EDTA (5.0 mM)	56
PMSF (1.0 mM)	91
TLCK (1.0 mM)	105
TPCK (1.0 mM)	96
Cystatin SA-I (0.7 μM)	134
(1.4 μM)	145
Histatin 5 (1.0 μM)	73
(2.0 μM)	67

[a] Abbreviations: PCMB, *p*-chloromercuribenzoic acid; 2-ME, 2-mercaptoethanol; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; TLCK, *N*-α-D-tosyl-L-lysine chloromethyl ketone; TPCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

[b] Collagenase activity was determined by the ^{3}H-labeled type I collagen assay system.

the collagenase is located external to the cytoplasmic membrane in *P. gingivalis*. In support of this idea, collagenase activity could be detected in the outer membrane fraction of one strain of these organisms (28), and activity characteristic of the *prtC* gene product was observed in both the cytoplasmic and membrane fractions of strain 53977 (data not shown). However, since this investigation has also suggested the presence of two collagenases in strain 53977, additional approaches will be required to clearly demonstrate the cellular localization of the *prtC* gene product.

The molecular size of the *prtC* gene product predicted from the amino acid sequence (37.8 kDa) and that estimated from gel filtration chromatography (70 kDa) suggest that the enzyme behaves as a dimer in its native state. It is of interest that Sorsa et al. (42) identified a collagenase of the same molecular size following gel filtration chromatography of extracts of *P. gingivalis* ATCC 33277.

The present demonstration that the *prtC* gene is transcribed as part of a polycistronic mRNA is of great interest, since a number of different virulence factors have been

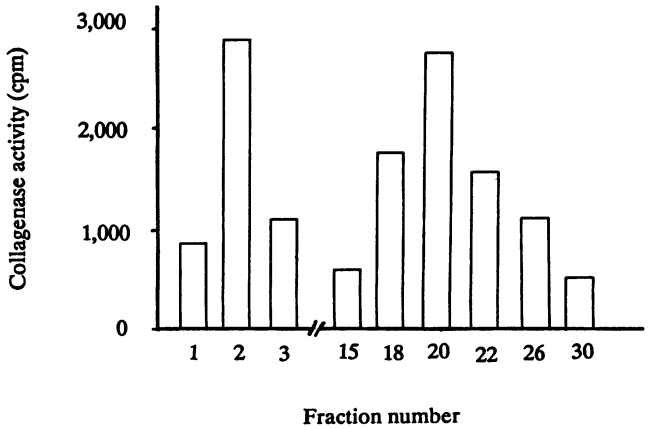

FIG. 6. Collagenase activity from the contents of a Mono Q anion exchange column of *P. gingivalis* ATCC 53977 sonic extracts. Collagenase activity was determined by the ^{3}H-labeled type I collagen assay system. No collagenase activity was detected in fractions 4 through 14.

proposed for *P. gingivalis* (15), including proteases, hemagglutinins, and neutrophil-neutralizing factors. Our laboratory has demonstrated that the genes for superoxide dismutase (*sod*), a trypsinlike protease (*prtT*), and a hemagglutinin are present downstream from the *prtC* gene on the strain 53977 chromosome (5, 27). Therefore, it will be of great interest to identify the genes which are cotranscribed with the *prtC* gene and examine the regulation of this potential virulence-related mRNA. Experiments addressing this issue are currently in progress in our laboratory.

Since *P. gingivalis* is present in the human oral cavity, it was of interest to examine the effects of potential salivary inhibitors on its collagenase activity. It has been reported that the trypsinlike protease of *P. gingivalis* could be inhibited by the salivary peptide histatin (25) and that this peptide also inhibited the *C. histolyticum* collagenase. Likewise, this peptide produced moderate inhibition of the *prtC* gene product (Table 3). Another salivary peptide, cystatin SA-I, has been demonstrated to inhibit thiol-dependent proteases (1). Since the *prtC* gene product is not dependent upon reducing agents (Table 3), it was not surprising that cystatin did not act as an inhibitor of collagenase activity. In contrast, moderate stimulation of activity was demonstrated. This effect does not appear to be dependent upon the high cysteine content of cystatin, since both 2-mercaptoethanol and dithiothreitol did not stimulate activity.

The results of this study have also suggested the presence of two distinct collagenases produced by strain 53977. A recent report has suggested that a *P. gingivalis* trypsinlike protease displayed collagenase activity (40). Since this latter enzyme both is thiol dependent and appears to have a larger molecular size than the *prtC* gene product, it may be possible that this enzyme is responsible for the collagenase activity which is retained by Mono Q columns. However, this latter enzyme has not been extensively characterized either genetically or enzymatically.

P. gingivalis strains have been utilized in experimental animal models to demonstrate virulence (13, 16, 21, 37). A spontaneous mutant of strain W50 was isolated and demonstrated to be less virulent in a rodent model system (22). This mutant, W50/BE1, was shown to be defective in several potential virulence characteristics, including collagenase activity (33). However, the present investigation suggests that the loss of virulence in the mutant does not result from extensive rearrangement of the *prtC* gene.

The isolation of the *prtC* gene will make it possible to construct *P. gingivalis* mutants which are defective in this gene for testing in appropriate animal model systems. The recent development of a *P. gingivalis* gene transfer system for inactivating cloned genes in these organisms on the basis of conjugation (5a) will make it possible to construct such mutants. In this manner, it should be possible to define the potential role of the *prtC* in the virulence of these periodontopathogenic organisms.

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