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 Ca²⁺ 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 mg/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 [³²P]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 *ClaI* and *PstI*. After electrophoresis on a 1.0% agarose gel, the separated DNA fragments were transferred to nitrocellulose membranes by
using the PosiBlot pressure-blotting system (Stratagene, La Jolla, Calif.) and hybridized with biotin-labeled DNA probe. The 1.9-kb Clal-HindIII 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 Clal-HindIII 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 14C-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 collagenses 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 P2-PLGPA (4-phenylazobenzyl-oxycarboxybenzyl-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-1 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 Clal and PStI and probed with a 1.9-kb Clal-HindIII 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 polycistrionic 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 polycistrionic 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
n. 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.

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 Ca²⁺ but is strongly inhibited by Zn²⁺ and Fe³⁺ (Table 2). Neither Mg²⁺ nor Mn²⁺ 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. 2. Southern blot analysis of Clal-PstI-digested chromosomal DNA of P. gingivalis with the biotin-labeled Clal-HindIII 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.

FIG. 1. Nucleotide sequence of the prc gene and the deduced amino acid sequence of the collagenase. The locations of the restriction enzyme digestion sites are indicated along the sequence.
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.

**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 fibrilar 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

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

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Total activity (U/mg)</th>
<th>Sp act (U/mg)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sonic extract</td>
<td>660</td>
<td>488.4</td>
<td>100</td>
<td>0.74</td>
</tr>
<tr>
<td>Mono Q</td>
<td>107</td>
<td>1350.4</td>
<td>267</td>
<td>12.2</td>
</tr>
<tr>
<td>TSK-G3000SW</td>
<td>1.6</td>
<td>64.2</td>
<td>13</td>
<td>40.1</td>
</tr>
<tr>
<td>TSK-G3000SW</td>
<td>1.1</td>
<td>48.7</td>
<td>10</td>
<td>44.3</td>
</tr>
</tbody>
</table>

* Units of enzyme activity per milligram of protein in the pooled fraction from each step. Collagenase activity was determined by the [3H]-labeled type I collagen assay system.
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TABLE 3. Effects of chemical agents on collagenase activity

<table>
<thead>
<tr>
<th>Chemical agent*</th>
<th>Relative collagenase activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>PCMB (1.0 mM)</td>
<td>41</td>
</tr>
<tr>
<td>2-ME (1.0 mM)</td>
<td>77</td>
</tr>
<tr>
<td>DTT (1.0 mM)</td>
<td>84</td>
</tr>
<tr>
<td>EDTA (5.0 mM)</td>
<td>56</td>
</tr>
<tr>
<td>PMSF (1.0 mM)</td>
<td>91</td>
</tr>
<tr>
<td>TLCK (1.0 mM)</td>
<td>105</td>
</tr>
<tr>
<td>TPCCK (1.0 mM)</td>
<td>96</td>
</tr>
<tr>
<td>Cystatin SA-I</td>
<td>134</td>
</tr>
<tr>
<td>(0.7 μM)</td>
<td></td>
</tr>
<tr>
<td>Histain 5</td>
<td></td>
</tr>
<tr>
<td>(1.0 μM)</td>
<td>73</td>
</tr>
<tr>
<td>(2.0 μM)</td>
<td>67</td>
</tr>
</tbody>
</table>

* Abbreviations: PCMB, p-chloromercurobenzoic acid; 2-ME, 2-mercaptoethanol; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; TLCK, N-a-o-tosyl-l-lysine chloromethyl ketone; TPCCK, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

**Collagenase activity was determined by the 3H-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 33281.

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

![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 3H-labeled type I collagen assay system. No collagenase activity was detected in fractions 4 through 14.](http://jb.asm.org/download)
proposed for \textit{P. gingivalis} (15), including proteases, hemagglutinins, and neutrophil-neutralizing factors. Our laboratory has demonstrated that the genes for superoxide dismutase (sod), a trypsinalike protease (prtT), and a hemagglutinin are present downstream from the \textit{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 \textit{prtC} gene and examine the regulation of this potential virulence-related mRNA. Experiments addressing this issue are currently in progress in our laboratory.

Since \textit{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 trypsinalike protease of \textit{P. gingivalis} could be inhibited by the salivary peptide histatin (25) and that this peptide also inhibited the \textit{C. histolyticum} collagenase. Likewise, this peptide produced moderate inhibition of the \textit{prtC} gene product (Table 3). Another salivary peptide, cystatin SA-I, has been demonstrated to inhibit thiol-dependent proteases (1). Since the \textit{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 \textit{P. gingivalis} trypsinalike protease displayed collagenase activity (40). Since this latter enzyme both is thiol dependent and appears to have a larger molecular size than the \textit{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.

\textit{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 \textit{prtC} gene.

The isolation of the \textit{prtC} gene will make it possible to construct \textit{P. gingivalis} mutants which are defective in this gene for testing in appropriate animal model systems. The recent development of a \textit{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 \textit{prtC} in the virulence of these periodontopathogenic organisms.

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