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Inducible Expression of a *Porphyromonas gingivalis* W83 Membrane-Associated Protease

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The Tpr protease of *Porphyromonas gingivalis* W83 is a membrane-associated enzyme capable of hydrolyzing a chromogenic bacterial collagenase substrate. An isogenic mutant lacking a functional *tpr* gene had a greatly reduced ability to hydrolyze the collagenase substrate. Activity was restored to the *tpr* mutant by introducing a shuttle plasmid containing the *tpr* gene. Expression of the gene is induced by nutrient limitation, as shown by enzymatic and Northern analyses.

The numerous proteases of *Porphyromonas gingivalis* have been studied to determine their role in periodontitis (37, 38). These enzymes are able to hydrolyze a number of host proteins important in host defenses (10, 14, 35, 36, 44, 45) or in maintaining tissue integrity (3, 4, 16, 17, 39, 40, 42, 43, 47, 48). They are also involved in binding *P. gingivalis* to host tissues and to other oral microorganisms (7, 18, 26, 27). In recent years, several research groups have reported the cloning of *P. gingivalis* protease genes and the isolation of isogenic mutants (1, 5, 8, 20, 24, 28, 31, 33, 46). In an earlier study (30) we reported on the isolation of an isogenic mutant created by insertional mutagenesis. The mutant was unable to synthesize the 80-kDa Tpr protease. In its native, enzymatically active form the Tpr protease has a molecular mass of approximately 80 kDa; when boiled under reducing conditions, a peptide of 53 kDa is formed. The recombinant enzyme has the same properties.

Regulation of gene expression can be important in the expression of virulence factors and for adaptation of pathogens to the host environment. It has been suggested that expression of *P. gingivalis* proteases is regulated by the growth environment (6, 22, 23, 34). Robertson et al. (34) reported that the collagenolytic activity of *P. gingivalis* was enhanced in a peptide-depleted growth medium; others have found that in hemin excess trypsin-like activity increased 3.5-fold, whereas collagenolytic activity was increased when hemin was limiting (22).

In this study, we were able to show by enzymatic assay and Northern analysis that the expression of the *tpr* gene in *P. gingivalis* W83 was dependent on the growth medium. Tpr is a membrane-associated enzyme. A *tpr*-negative mutant was complemented with a shuttle vector containing the *tpr* gene.

(Part of the present study was reported at the 94th General Meeting of the American Society for Microbiology in 1994.)

P. gingivalis strains were grown in Bacto Brain Heart Infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) as described elsewhere (31). In some studies, *P. gingivalis* was grown in TYE broth (10) or 0.5TYE, TYE broth in which the Trypticase peptone content was reduced from 1.7 to 0.5%.

Pz-peptidase activities of *P. gingivalis* W83 and W83/PM. *P. gingivalis* has a number of different cell-associated proteolytic activities, making it difficult to assess the role of a specific enzyme. To circumvent this problem, we compared enzyme activity of an isogenic mutant with that of the parent. Zymography showed that Tpr was much more active against gelatin than against bovine serum albumin, suggesting that the enzyme was recognizing an amino acid sequence common to collagen. The bacterial collagenase substrate *p*-phenylazo-benzyloxycarbonyl-L-prolyl-L-leucyl-glycyl-L-prolyl-D-arginine (Pz-peptide; Sigma Canada, Mississauga, Ontario, Canada) was chosen as the substrate. The Pz-peptidase assay was carried out as described previously (29). Trypsin-like protease activity was measured with *N*-benzoyl-L-arginine-*p*-nitroanilide as the substrate as described by Grenier and McBride (11). Whole-cell extract, soluble cell extract, and crude membrane fractions of *P. gingivalis* strains were prepared by French pressure cell disruption followed by differential centrifugation as described elsewhere (29). Protein concentration was determined by the method of Bradford with a protein assay solution (Bio-Rad Laboratories, Hercules, Calif.). As shown in Table 1, the Pz-peptidase activity of the whole-cell extract and the membrane fraction from the mutant (W83/PM) was significantly reduced compared with that of the parent strain (W83). This shows that Tpr is a membrane-associated enzyme and is the major *P. gingivalis* protease capable of hydrolyzing the Pz-peptide. Some level of Pz-peptide-hydrolyzing activity of W83/PM may be due to other *P. gingivalis* proteases capable of hydrolyzing collagen (3, 4, 17, 39, 47) or due to nonspecific reaction by the other proteases. A comparable level of the activity could be detected when substrate was incubated with purified trypsin (Type XI; Sigma Chemical Co., St. Louis, Mo.).

Pz-peptidase activity in *P. gingivalis* has been reported by several research groups (13, 25). Ng and Fung (25) suggested that the Pz-peptidase activity of *P. gingivalis* is associated with the cell membrane, which is consistent with the result of the present study. Hino et al. (12) showed high levels of Pz-peptidase activity in inflamed gingivae. Recently, Sojar et al. (41) reported purification of a membrane-associated protease from *P. gingivalis* ATCC 33277 capable of hydrolyzing Pz-peptide as well as salt-solubilized collagen. This enzyme is believed to be another *P. gingivalis* protease, since its N-terminal sequence is different from the deduced N-terminal sequence of Tpr.

Although our study indicates that Tpr does not hydrolyze native collagen (29), it is very active in hydrolyzing denatured collagen (gelatin) and the Pz-peptide. Since *P. gingivalis* produces proteases capable of hydrolyzing native collagen, it is possible that these enzymes open up the collagen molecule and expose the sequences that can be recognized by Tpr. In this sense, Tpr may be involved in the late stages of degradation of

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TABLE 1. Pz-peptidase activities and trypsin-like protease activities of *P. gingivalis* W83 and W83/PM grown in TYE broth

Extract	Strain	Pz-peptidase activity (ΔA_{320}/h/mg of protein)[a]	Trypsin-like protease activity (Δμmol of *p*-nitroanilide/min/mg of protein)[b]
Whole cell	W83	1.6	1.3
	W83/PM	0.3	1.5
Soluble cell	W83	0.5	0.7
	W83/PM	0.3	0.8
Membrane	W83	1.8	1.8
	W83/PM	0.4	2.1

[a] Mean values obtained from four different Pz-peptidase assays.
[b] Mean values obtained from two different *N*-benzoyl-L-arginine-*p*-nitroanilide assays.

collagen or proteins containing a Pz-peptide-like sequence, in order to produce small peptides and/or amino acids which can be used as an energy source for the growth of *P. gingivalis*. The location of Tpr on the cell membrane accentuates its potential role in bacterial nutrition.

Identification of the Tpr protein. Identification of the Tpr protein in *P. gingivalis* was done by comparing parent and mutant in two-dimensional polyacrylamide gel electrophoresis (PAGE). Cells were grown in 0.5TYE and fractionated as described previously (29), and proteins were separated by two-dimensional PAGE (19). As shown in Fig. 1, the membrane fraction of *P. gingivalis* W83 contains a protein of approximately 55 kDa that is not present in the soluble cell extract (data not shown); nor is it present in extracts of W83/PM. This 55-kDa protein is believed to be the *tpr* gene product. The molecular mass of the protein was similar to that of the recombinant gene product (30, 31). An additional protein with an approximate molecular mass of 27 kDa was seen in the crude membrane of the parent strain (Fig. 1). This may be a degradation product of Tpr or another protein whose expression depends on Tpr expression. Western immunoblot analysis using polyclonal antiserum to the recombinant Tpr confirmed the two-dimensional electrophoresis results (30). Membranes of the parent strain possessed an antigen reacting with anti-Tpr antibody; the antigen was absent in all fractions from the mutant. When gels were run under nondenaturing conditions, the antigen migrated with an apparent molecular mass of 80 kDa; when denatured, the mass was 53 kDa. This data is consistent with the known properties of the recombinant and native Tpr proteins. This also suggested that the active protease Tpr exists as a dimer of the 55-kDa *tpr* gene product.

Complementation studies. To complement the *tpr* isogenic mutant, a shuttle vector plasmid of *Bacteroides* spp., pNJR12 (21), was chosen as the base to construct the shuttle plasmid containing the *tpr* gene. The 2.4-kb *Bam*HI-*Hin*dIII fragment of pYS307-2(+) (31) was subcloned into pNJR12, and the recombinant plasmid was named pBY3. The shuttle plasmid pBY3 was transferred into the Tpr-deficient mutant *P. gingivalis* W83/PM by conjugation as described previously (30). Plasmids were isolated from the transconjugants, and restriction patterns of the plasmids were identical to that of pBY3 (data not shown). When used to transform *Escherichia coli*, all of the *E. coli* transformant colonies produced the typical clear zone of protein hydrolysis on skim-milk plates. These results showed that the transconjugants contained an intact autonomous plasmid, pBY3. One of the transconjugants was selected for further study. Western immunoblots and gelatin substrate zymography were performed to determine if pBY3 had restored the ability of W83/PM to express the Tpr protein. Sodium dodecyl sulfate-PAGE was done by the Laemmli method (15), in a Mini PROTEAN II cell (Bio-Rad Laboratories). Protein samples were obtained by solubilization in 4% sodium dodecyl sulfate buffer without 10% β-mercaptoethanol at 37°C for 30 min. Gelatin substrate zymography and Western immunoblot analysis were done as described previously (30). The results are shown in Fig. 2. The complemented strain produced a proteolytic band in zymography and a reactive band in a Western immunoblot at 80 kDa identical to those produced by the parent strain, W83. *P. gingivalis* W83/PM or W83/PM mobilized with the original shuttle plasmid pNJR12 lacking the *tpr* gene did not produce the 80-kDa proteolytic band in a gelatin zymogram, nor was there a reactive band in a Western immunoblot. On the Western blot, additional bands with molecular masses of >110 kDa could be seen. Since the proteins were run under nondenaturing conditions, they could represent Tpr associated with other cell components. The Pz-peptidase activity of the whole-cell extract of the complemented strain, *P. gingivalis* W83/PM(pBY3), was approximately two times higher than that of the parent strain W83 (data not shown). This higher Pz-peptidase activity may due to multiple copies of the shuttle plasmid. Mobilization of pNJR12 alone into W83/PM did not result in an increase in Pz-peptidase activity in the mutant. Although foreign plasmid DNA can exist as an autonomous replicating form in the organism, the transconjugants

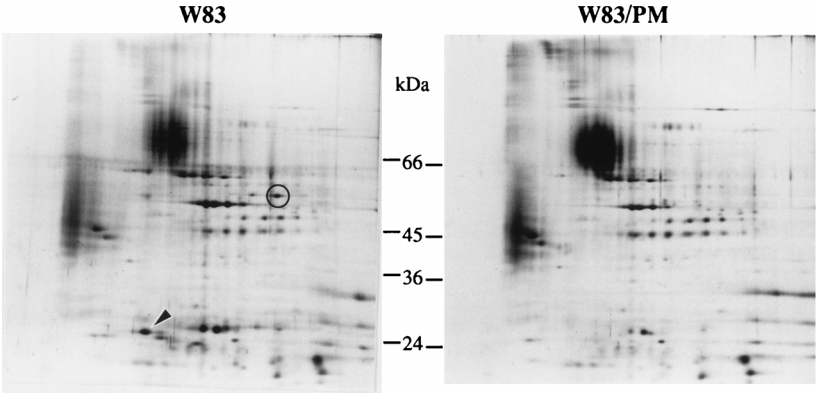

FIG. 1. Two-dimensional PAGE analysis of the crude membranes of *P. gingivalis* W83 and W83/PM. Cells were cultivated in 0.5TYE. Twelve micrograms of proteins was used for each gel. The circle marks the 55-kDa protein believed to be the *tpr* gene product, and the arrowhead marks an additional 28-kDa protein.

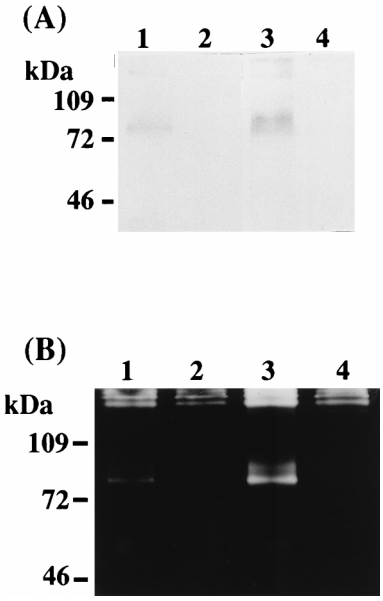

FIG. 2. Western immunoblot (A) and gelatin substrate zymogram (B) of the complemented strain, *P. gingivalis* W83/PM(pBY3). Lanes: 1, *P. gingivalis* W83; 2, *P. gingivalis* W83/PM; 3, *P. gingivalis* W83/PM(pBY3); 4, *P. gingivalis* W83/PM(pNJR12). Cell extracts were prepared by French press and differential centrifugation and used in all lanes. For Western immunoblot and zymography, 10 μg and 150 ng of proteins, respectively, were used. All samples are nonreduced-nondenatured. In Western immunoblot analysis, anti-rTpr was the primary antibody.

were unstable, as seen in their inability to grow in selective media after a number of subcultures.

Induction of Tpr expression. The effect of growth media on the level of Pz-peptidase activity was studied in the membrane fraction of cells grown in BHI broth, TYE broth, and 0.5TYE. As seen in Fig. 3, there was five times as much activity in cells grown in 0.5TYE as there was in cells grown in BHI broth and twice as much activity as in cells grown in full-strength TYE broth. To determine if the differences in activity were reflected in differences in levels of transcription, expression of the *tpr*

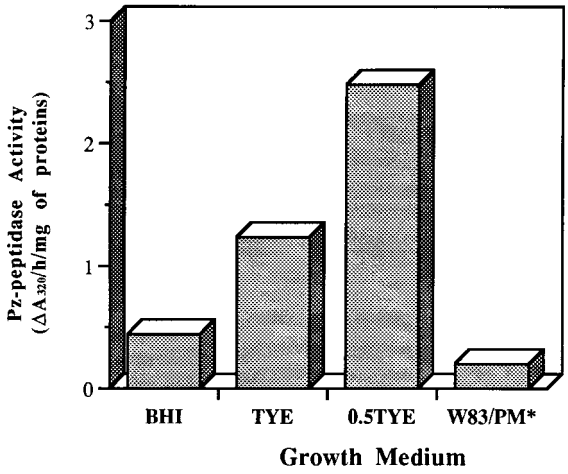

FIG. 3. Pz-peptidase activities of W83 and W83/PM. Crude membrane fractions of W83 grown in BHI broth, TYE broth, and 0.5TYE and W83/PM grown in TYE broth were analyzed for Pz-peptidase activity. Pz-peptide was used as the substrate.

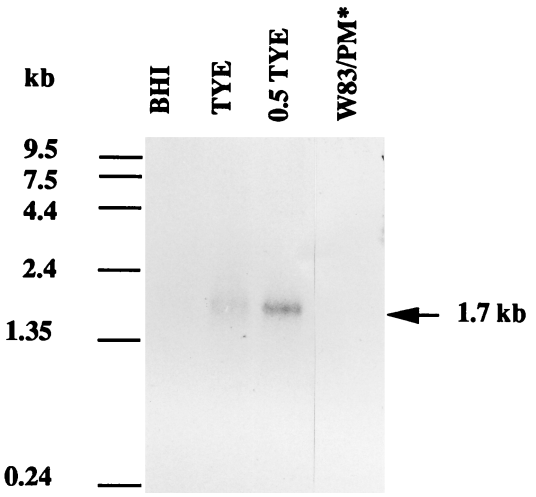

FIG. 4. Northern blot analysis of *tpr* mRNA from W83 grown in BHI broth, TYE broth, and 0.5TYE and W83/PM grown in TYE broth. Twenty micrograms of total RNA was loaded on each lane. A 0.7-kb *Kpn*I-*Pst*I internal fragment of the *tpr* gene was used as the probe. kb, RNA size markers in kilobases.

gene was examined by Northern hybridization. Total RNA was isolated from *P. gingivalis* W83 in logarithmic growth phase with TRIzol Reagent (Gibco BRL, Gaithersburg, Md.) according to the manufacturer's protocol. Northern blot analysis was performed as described by Ausubel et al. (2). Equal amounts of total RNA (20 μg) per lane were loaded and confirmed by equal intensity of rRNA bands by ethidium bromide staining. A 0.7-kb *Kpn*I-*Pst*I internal fragment of the *tpr* gene was labeled with biotin by following the nick translation protocols described by the manufacturer (BluGENE Nonradioactive Nucleic Detection System; Gibco BRL) and used as the probe to detect *tpr* mRNA. The results as seen in Fig. 4 paralleled the enzymatic activity studies. Cells grown in 0.5TYE had the highest level of *tpr* transcript, there was less in cells grown in TYE broth, and the transcript was below the level of detection in cells grown in BHI broth. No hybridized band was detected with total RNA from *tpr* mutant W83/PM, since the 0.7-kb *Kpn*I-*Pst*I fragment of the *tpr* gene had been replaced with the Em^r fragment (30). The size of the *tpr* mRNA was approximately 1,700 nucleotides and comparable with the open reading frame of the gene (approximately 1,500 nucleotides) (5), suggesting that the *tpr* gene is transcribed monocistronically.

To our knowledge, this is the first report of inducible expression of a protease gene in *P. gingivalis*. Several studies (6, 22, 23, 34) have shown that the general proteolytic activities of *P. gingivalis* are increased by culturing cells in certain growth environments and suggested the regulation of gene expression of those enzymes. This finding may provide an explanation for why Potempa et al. (32) and Gharbia et al. (9) were unable to see expression of *tpr*.

In conclusion, we observed inducible expression of the Tpr protease and by Northern blot analysis determined that this regulation occurred at the transcriptional level. However, it still remains to be determined whether regulation of the *tpr* gene expression is due to growth limitation or due to depletion of certain nutrients, such as peptides or amino acids. Furthermore, it would be interesting to know how this regulation is achieved at the genetic level. We are currently investigating the regulation of the *tpr* expression in different growth environments and the potential regulatory factors that are involved in gene expression.

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