Virulence of a *Porphyromonas gingivalis* W83 Mutant Defective in the *prtH* Gene

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In a previous study we cloned and determined the nucleotide sequence of the *prtH* gene from *Porphyromonas gingivalis* W83. This gene specifies a 97-kDa protease which is normally found in the membrane vesicles produced by *P. gingivalis* and which cleaves the C3 complement protein under defined conditions. We developed a novel *ermF-ermAM* antibiotic resistance gene cassette, which was used with the cloned *prtH* gene to prepare an insertionally inactivated allele of this gene. This genetic construct was introduced by electroporation into *P. gingivalis* W83 in order to create a protease-deficient mutant by recombinational allelic exchange. The mutant strain, designated V2296, was compared with the parent strain W83 for proteolytic activity and virulence. Extracellular protein preparations from V2296 showed decreased proteolytic activity compared with preparations from W83. Casein substrate zymography revealed that the 97-kDa proteolytic component as well as a 45-kDa protease was missing in the mutant. In in vivo experiments using a mouse model, V2296 was dramatically reduced in virulence compared with the wild-type W83 strain. A molecular survey of several clinical isolates of *P. gingivalis* using the *prtH* gene as a probe suggested that *prtH* gene sequences were conserved and that they may have been present in multiple copies. Two of 10 isolates did not hybridize with the *prtH* gene probe. These strains, like the V2296 mutant, also displayed decreased virulence in the mouse model. Taken together, these results suggest an important role for *P. gingivalis* proteases in soft tissue infections and specifically indicate that the *prtH* gene product is a virulence factor.

Porphyromonas gingivalis, a black-pigmented, gram-negative anaerobe, is widely implicated as an important etiological agent in the pathogenesis of periodontal disease. This organism possesses an array of potential virulence factors that can directly affect the periodontium or act in concert with other factors to elicit host functions that cause destruction typical of advanced periodontitis (reviewed in references 8 and 36). Despite an increased understanding of host factors in periodontal disease, little is known of the specific roles, interactions, or relative importance of the potential virulence factors of *P. gingivalis*. Unmasking the specific role played by each factor in pathogenesis requires precise inactivation of each contributing factor followed by biochemical evaluation and in vivo virulence testing.

The proteolytic capacity of *P. gingivalis* may contribute to virulence, since enzymatic activity may destroy proteinaceous tissue components as well as key plasma proteins that would otherwise mediate protective host functions (23, 47). A number of substrates (e.g., collagen, complement, and immunoglobulins A and G) important for host defenses and tissue integrity are hydrolyzed by *P. gingivalis* proteases (12, 18, 34). Although several groups have cloned and characterized protease genes from *P. gingivalis*, the specific role played by each of those protease genes in pathogenesis has not yet been fully investigated (1, 3, 16, 28, 29, 45).

In experimental animal models, heterogeneity in virulence between various strains of *P. gingivalis* has been observed (27, 46). Shah and coworkers have demonstrated that an avirulent, nonpigmented strain of *P. gingivalis* lacked ability to hemagglutinate and possessed low proteolytic activity (38). Other workers have shown that protease deficiency in *P. gingivalis* is correlated with virulence (17, 26, 46). In these early studies, chemical mutagenesis was used to isolate protease-deficient variants, but given the possibility of multiple mutations resulting from such treatment, interpretation of these results is difficult. Specifically, confidence in the predicted isogenicity of these strains is debatable. Park and McBride (30) recently have used allelic exchange mutagenesis to isolate a *P. gingivalis* W83 mutant deficient in a 90-kDa protease, but the in vivo contribution of this protease to virulence was not determined.

In a previous communication we reported the cloning and nucleotide sequence determination of the *prtH* gene involved in the cleavage of the C3 complement protein (7). The objective of this study was to evaluate the virulence of a *P. gingivalis* W83 mutant that was defective in the *prtH* gene encoding a 97-kDa protease. We report here that a *P. gingivalis* W83 mutant strain prepared by allelic exchange showed reduced proteolytic activity when the *prtH* gene was inactivated. In addition this mutant was dramatically less virulent than the wild-type strain in a mouse model of bacterial invasiveness. A limited survey of clinical isolates performed by using the *prtH* gene as a molecular probe suggested that this gene was conserved in several of the isolates and that related sequences were present in multiple copies in some strains. In addition, certain clinical isolates did not contain sequences which hybridized with the *prtH* gene probe. These strains were less virulent than naturally occurring isolates which carried *prtH* sequences.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Bacterial strains used in this study are described in Table 1. *P. gingivalis* was grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) supplemented with hemin (5 µg/ml),

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TABLE 1. Bacterial strains and plasmids used in this study

Species and strain	Plasmid	Description[a]	Source or reference
P. gingivalis			
W83			
ATCC 33277			
V2296		Em[r], protease-defective mutant	This study
V2298		Clinical isolate	D82F-5[b]
V2299		Clinical isolate	D172B-12[b]
V2300		Clinical isolate	D173A-2B[b]
V2301		Clinical isolate	D13B-11[b]
V2302		Clinical isolate	D207B-21[b]
V2303		Clinical isolate	D173A-20[b]
V2305		Clinical isolate	D67D-9[b]
V2306		Clinical isolate	D55D-13[b]
V2307		Clinical isolate	97A-18[b]
V2308		Clinical isolate	D40C-4[b]
E. coli			
V2049	pFD283	Sp[r]; *E. coli-Bacteroides* shuttle vector that can be mobilized by RK231	C. J. Smith
V831	pVA831	Carries *ermF*	24
V838	pVA838	Carries *ermAM*	39
V2198	pVA2198	pFD283 that carries the *ermF-ermAM* cassette	This study
V2289	pVA2289	Carries the 3.7-kb *Bam*HI-*Hin*dIII fragment that contains the *prtH* gene	7
V2290	pVA2290	Carries the 2.9-kb *Sma*I-*Hin*dIII fragment of pVA2289	This study
V2295	pVA2295	pVA2290 with the *ermF-ermAM* cassette inserted at the *Hin*cII site	This study

[a] Em[r] (erythromycin resistant), growth on medium containing 0.5 μg/ml; Sp[r] (spectinomycin resistant), growth on medium containing 50 μg/ml.
[b] Clinical isolate from L. V. H. and W. E. C. Moore, Virginia Polytechnic Institute and State University, Blacksburg.

vitamin K (0.5 μg/ml), and cysteine (1%). *Escherichia coli* strains were grown in Luria-Bertani broth (33). Unless otherwise stated, all cultures were incubated at 37°C. *P. gingivalis* was grown in an anaerobic chamber (Coy Manufacturing, Ann Arbor, Mich.) in 10% H_2, 10% CO_2, and 80% N_2. Antibiotics were used at the following concentrations: clindamycin, 0.5 μg/ml; erythromycin, 300 μg/ml; carbenicillin, 50 μg/ml; geneticin, 400 μg/ml; nalidixic acid, 30 μg/ml; and spectinomycin, 50 μg/ml.

Preparation and analysis of DNA. Chromosomal DNA from *P. gingivalis* was prepared by the method of Marmur (22). Plasmid DNA extraction and isolation were performed by the alkaline lysis procedure of Birnboim and Doly (2). Plasmids were purified by cesium chloride-ethidium bromide centrifugation according to the method of Sambrook et al. (33). DNA was digested with restriction enzymes as specified by the manufacturer (GIBCO/BRL, Gaithersburg, Md.). DNA blot transfer and hybridization were performed as previously reported (6). DNA fragments were isolated by electrophoresis (0.7% agarose; TAE buffer [0.4 M Tris-acetate, 0.001 M EDTA, pH 8.0]). The fragments were purified by using Gene Clean (Bio 101 Inc., La Jolla, Calif.). DNA labeling and autoradiography were done as previously reported (19). The following probes were used in hybridization experiments: (i) pVA2290, which contained a 2.9-kb *Sma*I-*Hin*dIII fragment of the DNA that carried the *prtH* gene with a truncated amino-terminal end; (ii) pVA2289, which contained a 3.7-kb *Bam*HI-*Hin*dIII fragment carrying the entire *prtH* gene and a small amount of flanking DNA; and (iii) pVA2198, which carried the *ermF-ermAM* cassette.

Extracellular protease and enzyme assay. Preparation of vesicles from *P. gingivalis* was performed by the method of Grenier and Mayrand (11). Ammonium sulfate was added to 1 liter of culture supernatant to 55% saturation. Vesicles were stored in 1 ml of Tris buffer (50 mM; pH 7.5) and kept at −20°C. The presence of trypsin-like activity was determined according to the method of Smalley and Birss (41).

Construction of the *ermF-ermAM* cassette. The *ermF* and *ermAM* genes confer resistance to macrolides and lincosamides and are expressed in *Porphyromonas* and *Bacteroides* spp. and in *E. coli*, respectively (14, 21, 39). Because these genes are not expressed heterologously in these host strains we constructed a cassette containing *ermF* and *ermAM* sequences. DNA sequencing of the region 3′ of *ermF* carried on pVA831 (24) was performed by using the Sequenase kit from United States Biochemical Corporation (Cleveland, Ohio). The *ermF* left PCR primer (5′-CCGATAGCTTCCGCTATTGC-3′) (Fig. 1) was synthesized by Oligos Etc. Inc. (Wilsonville, Oreg.). The following PCR primers were synthesized at the Nucleic Acid Core Facility, Virginia Commonwealth University, Richmond: *ermF* right primer, 5′-GCAAATTGGCGATGGAGCGGAAAC-3′; *ermAM* left primer, 5′-GCGATGGAGCGGAAACGTAAAAGAAG-3′; and *ermAM* right primer, 5′-GGTATACTACGACAGCTTC-3′.

The *ermF-ermAM* cassette was constructed by the PCR-based overlap extension method described by Horton et al. (15). PCR amplification was performed with a Perkin-Elmer Cetus DNA Thermal Cycler (Perkin-Elmer Corporation, Norwalk, Conn.) as described by Sambrook et al. (33). The reaction mixture (100 μl) contained 700 ng of each primer, 70 ng of template DNA, and 1 μl of Vent

Polymerase (New England Biolabs, Beverly, Mass.). The first PCR consisted of 28 cycles with a temperature profile of 1 min at 94°C, 2 min at 48°C, and 3 min at 72°C. The templates in the first PCR consisted of pVA831 (24) for amplification of *ermF* and pVA838 (39) for amplification of *ermAM*. Each of the first

A) Partial Sequence of *ermF* and its Flanking DNA.

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CCGATAGCTT CCGCTATTGC TTTTTTGCTC ATCGGTATTT GCAACATCAT AGAAATTGCA
TACCTTTGTT CCTCGGTTAT ATGTTTGCTC ATCTGCAACT TTTTTTTCTT TGGACGGACA
ATTAAAGCAA AGATAGCAAA CTTTATCCAT TCAGAGTGAG AGAAAGGGGG ACATTGTCTC
TCTTTCCTCT CTGAAAAATA AATGTTTTTA TTGCTTATTA TCCGCACCCA AAAAGTTGCA
TTTATAAGTT GAACTCAAGA AGTATTCACC TGTAAGAAGT TACTAATGAC AAAAAAGAAA
                                                     Start
TTGCTCCTTC............ermF coding sequence............GTAGTTCAAA

GTCGGGTGGT TGTCAAGATGA TTTTTTTGG TTTGGTCGTC TTTTTTTAAG CTGCCGCATA
                       ------>          <------ 
AGGCTGGCAA ATTGGCGATGG Agcggaaac
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B) Partial Sequence of *ermAM* and its Flanking DNA.

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gcgatggaGCG GAAACGTAAA AGAAGTTATG GAAATAAGAC TTAGAAGCAA

ACTTAAGAGT GTGTTGATAG TGCAGTATCT TAAAATTTTG TATAATAGGA ATTGAAGTTA
AATTAGATGC TAAAAATTTG TAATTAAGAA GGAGTGATTA CATGAACAAA .....ermAM
                                                        Start
coding sequence........GAAATAAT TCTATGAGTC GCTTTTGTAA ATTTGGAAAG
                              Stop
TTACACGTTA CTAAAGGGAA TGTAGATAAA TTATTAGGTA TACTACTGAC AGCTTC
```
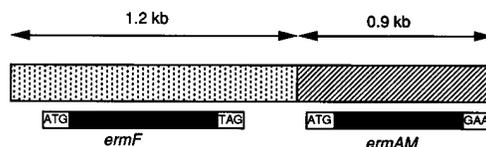
C)

FIG. 1. Partial sequences of the *ermF* (A) and *ermAM* (B) genes and their flanking DNA. The bold triplet codons represent the start and stop codons for the respective genes. The intervening DNA sequence for each gene is represented by the dotted lines. The arrows in panel A represent a possible stem and loop structure involved in transcription termination of *ermF*. The underlined sequences indicate primers used in PCR overlap extension reactions to join the two DNA sequences of the genes. Regions of overlapping sequences in the primers are represented as lowercase letters. Panel C shows a schematic representation of the *ermF-ermAM* cassette.
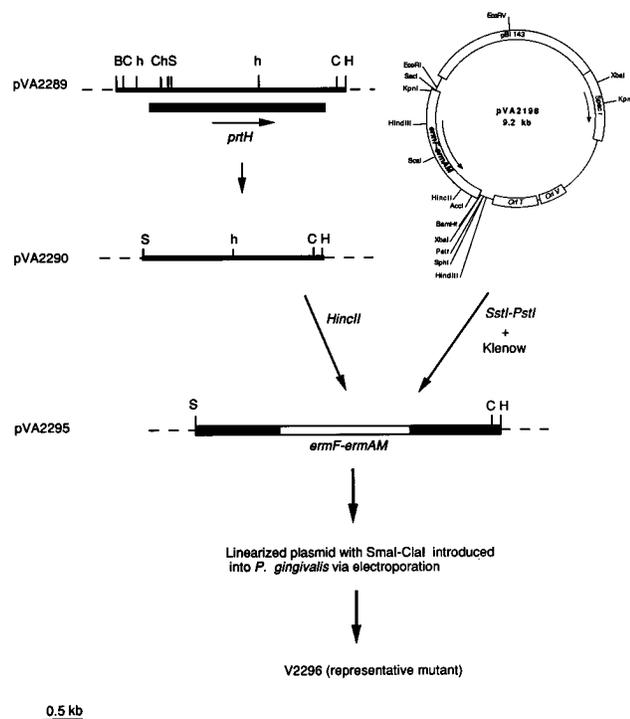
FIG. 2. Construction of site-specific mutant by allelic exchange. pVA2295 contained part of the *prtH* gene interrupted by an *ermF-ermAM* cassette (the cassette was constructed by PCR; *ermF* confers Em^r in *P. gingivalis*, and *ermAM* confers Em^r in *E. coli*). The plasmid was linearized with *Sma*I-*Cla*I and introduced into *P. gingivalis* W83 by electroporation. A reciprocal recombination event between areas of homology on the target cell's chromosome and regions flanking the Em^r cassette of pVA2295 replaced the protease gene with a fragment containing *ermF-ermAM*. *P. gingivalis* cells were incubated for about 12 h postelectroporation and plated on BHI with clindamycin (0.5 μg/ml) for 7 to 10 days at 37°C. Restriction sites shown are *Bam*HI (B), *Cla*I (C), *Hin*cII (h), *Hin*dIII (H), and *Sma*I (S). The pUC19 vector is represented by the broken lines.

reaction products was used as a template in the second PCR, which consisted of 28 cycles with a temperature profile of 1 min at 94°C, 2 min at 51°C, and 3 min at 72°C. The PCR-amplified DNA product was purified by phenol-chloroform extraction and ethanol precipitation and was identified by agarose gel electrophoresis. PCR fragments used in further manipulations were isolated from 0.7% agarose gels run in Tris-borate-EDTA buffer (33). Desired fragments were purified by a phenol freeze-thaw method followed by ethanol precipitation (33).

Cloning and expression of the *ermF-ermAM* cassette. The *ermF-ermAM* cassette constructed by the PCR-based overlap extension method (Horton et al. [15]) was cloned in the *E. coli-Bacteroides* shuttle vector pFD283 (obtained from C. J. Smith). The *ermF-ermAM* cassette was ligated into the *Hin*cII site of pFD283, resulting in pVA2198 (Fig. 2). *E. coli* DH5α was transformed with pVA2198 and spread on Luria-Bertani agar plates containing erythromycin and spectinomycin. To mobilize pVA2198 into *Bacteroides fragilis*, the plasmid RK231, which is a derivative of RK2 (40), was transferred by conjugation into the pVA2198 transformant by the filter mating technique (40). pVA2198 was mobilized into *B. fragilis* 638 by a modified method of Shoemaker et al. (40). Briefly, 1.8 ml of mid-log-phase *E. coli* donor cells was mixed with 0.2 ml of mid-log-phase *B. fragilis* recipient cells on a nitrocellulose filter. The filter was incubated on a Luria-Bertani agar plate at 37°C for 16 h under aerobic conditions. The filter containing the mating mixture then was placed in 1 ml of BHI broth and incubated at 37°C for 1 h under anaerobic conditions, allowing expression of *ermF*. *B. fragilis* transconjugants containing pVA2198 were selected on BHI agar plates containing geneticin, nalidixic acid, and clindamycin. The presence of pVA2198 in transconjugants was verified by electrophoretic analysis of restriction endonuclease-digested plasmid DNA. These experiments demonstrated the usefulness of pVA2198 as a shuttle in the *Bacteroides-Porphyromonas-E. coli* host system.

Casein substrate zymography. Sodium dodecyl sulfate (SDS)-polyacrylamide was mixed with casein-polyacrylamide conjugate (9) to obtain a concentration of 200 μg of protein per ml of gel. Samples were solubilized in SDS buffer (4% SDS, 20% glycerol, 0.124 M Tris [pH 6.8]) for 30 min at 37°C. Electrophoresis was conducted by using an SE 250-Mighty Small II slab gel unit (0.075 cm thick) (Bio-Rad Laboratories, Richmond, Calif.) at room temperature with a constant

current of 15 mA. Development of proteolytic zones was accomplished by the method of Grenier et al. (9). After electrophoresis the gel was shaken gently for 30 min in 100 mM Tris hydrochloride buffer (pH 7.0) containing 2% Triton X-100, rinsed twice in distilled water, and then shaken for a further 30 min in 100 mM Tris hydrochloride buffer (pH 7.0). The gel was transferred into development buffer, which was 100 mM Tris hydrochloride (pH 7.0) containing 2.5 mM CaCl$_2$ and 50 mM cysteine. The gel was incubated overnight (16 h) and stained for proteins with Coomassie blue. After the gel was destained, proteolytic activity was visualized as a clear band against a blue background.

Electroporation of *P. gingivalis*. Electroporation of cells was performed by a modified method of Smith et al. (43). Briefly, 1 ml of an actively growing culture of *P. gingivalis* was used to inoculate 10 ml of BHI broth supplemented with hemin and menadione, which then was incubated overnight at 37°C. Seventy milliliters of warmed medium (37°C) was then inoculated with 3 ml of the overnight culture and incubated an additional 4 h. The cells were harvested by centrifugation at 2,600 × *g* for 7 min at 4°C and washed in 70 ml of electroporation buffer (EP; 10% glycerol, 1 mM MgCl$_2$; filter sterilized; stored at 4°C), and the pellet was suspended in 0.5 ml of EP. A 100-μl sample of cells to which 1 μg of DNA was added was placed in a sterile electrode cuvette (0.2-cm gap). The cells were pulsed with a Bio-Rad gene pulser at 2,500 V and then incubated on ice for 3 min. The cell suspension then was added to 0.5 ml of BHI broth supplemented with hemin and menadione and incubated for approximately 16 h. Aliquots of cells (100 μl) were plated on solid medium containing clindamycin (5 μg/ml) and incubated anaerobically at 37°C for 7 to 10 days.

Virulence testing. *P. gingivalis* W83 and the mutant strain V2296 were tested for invasiveness in a mouse model as previously described by Neiders and coworkers (27). Both strains were grown for 18 h in tryptic soy broth supplemented with hemin (1 μg/ml), vitamin K (1 μg/ml), and dithiothreitol (0.5 μg/ml). The cells were centrifuged and washed in sterile phosphate-buffered saline (PBS; 0.147 M NaCl, 0.01 M sodium phosphate) under anaerobic conditions, counted in a Petroff-Hausser counting chamber, and adjusted to the desired concentration in PBS. All mice were challenged with subcutaneous injections of 0.1 ml of bacterial suspension at two sites on the dorsal surface. Mice were then examined daily to assess their general health status, as well as the presence and locations of lesions. Weights were determined for all surviving mice. These experiments were performed under the authorization of an institutionally approved animal use protocol (48).

RESULTS

Construction of an *ermF-ermAM* cassette. To facilitate genetic manipulations of *P. gingivalis*, we designed and constructed a cassette sequence with genes that could be expressed in *E. coli* or in *Porphyromonas* or *Bacteroides* spp. Such a cassette would permit selection of genetic constructs in either host, and its small size would facilitate its use to mark DNA sequences or inactivate genes. *ermF* and *ermAM* are expressed in *B. fragilis* and *P. gingivalis* and in *E. coli* hosts, respectively (14, 21, 39). The published sequence of *ermF* ends with a TGA stop codon and does not contain a cognate transcription termination region. Thus, in order to ensure the proper expression of *ermF* in the cassette, the DNA sequences downstream of *ermF* were determined. As shown in Fig. 1, inverted repeats that could form a stem-loop structure and possibly result in transcription termination were present downstream of the TAG stop codon. Primers for both *ermF* (32) and *ermAM* (4) were designed to include upstream regulatory regions and downstream termination regions for each gene (Fig. 1). The first PCR amplification created the individual genes as shown in Fig. 1A and B. Amplification products for each reaction were of the predicted size (1.2 kb for *ermF*; 0.9 kb for *ermAM*) (data not shown). These fragments were excised from the agarose gel and used as templates in the second PCR amplification step. The primers were designed to have 8-bp overlaps at the 3′ terminus of the *ermF* gene and the 5′ terminus of the *ermAM* gene as described by Horton et al. (15) (Fig. 1). The 8-bp overlaps allowed amplification of the entire DNA sequence when the *ermF* 5′ primer and the *ermAM* 3′ primer were used in the second PCR. The predicted 2.1 kb of amplified DNA containing both genes was observed when the DNA was analyzed on an agarose gel (data not shown). This DNA fragment contained the *ermF* and *ermAM* genes in tandem (Fig. 1C).
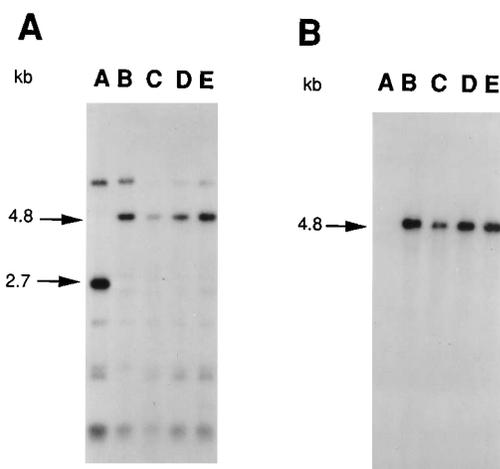
FIG. 3. Southern blot analysis of allelic exchange mutants of *P. gingivalis*. Total cellular DNA from *P. gingivalis* was cleaved with *Sma*I-*Cla*I, electrophoresed through 0.7% agarose, and bidirectionally transferred to nitrocellulose. (A) Lane A, W83; lanes B through E, allelic exchange mutants. The probe used was ^{32}P-labeled pVA2290 (Fig. 2). (B) Lanes A through E are the same as for panel A. The probe used was ^{32}P-labeled pVA2198 (Fig. 2). A 4.8-kb fragment was detected in all the strains which contained the protease gene inactivated with *ermF-ermAM*.

FIG. 4. Assay of proteolytic activity of membrane vesicles from *P. gingivalis*. Samples (0.2 µg/µl) were incubated for 10 min at 37°C in 50 mM Tris-HCl (pH 7.0)–1 mM *N*-α-benzoyl-DL-arginine *p*-nitroanilide in the presence or absence of 0.5 mM dithiothreitol (DTT). Controls contained buffer alone. Hydrolysis was monitored by the change of A_{410}.

Each gene in the construct contained upstream transcriptional control sequences to allow expression in the appropriate host.

Since *ermF* and *ermAM* are expressed in *B. fragilis* and *E. coli* hosts, respectively, pFD283 was chosen to verify the expression of both genes in their respective organisms. pFD283 contained an *E. coli* origin of replication, a spectinomycin resistance marker, an *oriT*, and a *Bacteroides* origin of replication, but it did not contain a *Bacteroides* resistance marker (42). The 2.1-kb *ermF-ermAM* cassette sequence was excised from an agarose gel and cloned into pFD283, resulting in pVA2198 (Fig. 2). pVA2198 conferred erythromycin (300 µg/ml) resistance in *E. coli* DH5α. When pVA2198 was mobilized into *B. fragilis* 638 it conferred clindamycin or erythromycin resistance at 5 µg/ml. The levels of macrolide resistance for each organism were consistent with those observed when the *erm* genes were cloned and tested individually.

Construction of a protease-deficient mutant of *P. gingivalis* W83. An isogenic *P. gingivalis* W83 mutant that was defective in the *prtH* gene was constructed by allelic exchange mutagenesis as depicted in Fig. 2. The 2.1-kb *ermF-ermAM* cassette was cloned in the *Hinc*II restriction site of pVA2290. The recombinant plasmid was linearized with *Eco*RI and electroporated into *P. gingivalis* W83. Since the plasmid was linear, clindamycin-resistant transformants could arise only as a result of two double-crossover events between the regions flanking the *erm* marker and the wild-type gene on the chromosome. This would result in the replacement of a segment of the wild-type gene with a fragment conferring clindamycin resistance.

We detected 11 Cc^r colonies following a 10-day incubation period. To confirm the presence of the *ermF-ermAM* cassette in the predicted location, chromosomal DNA from the transformants was probed with ^{32}P-labeled pVA2290 and pVA2198. Upon digestion of the DNA with *Sma*I and *Cla*I, a predicted 2.7-kb fragment should be seen in the wild-type cells. Since the *ermF-ermAM* cassette did not contain *Sma*I and *Cla*I sites, a 4.8-kb fragment should be seen in the Cc^r mutants. As shown in Fig. 3A, the predicted 2.7-kb fragment was seen in W83 cells (lane A). A 4.8-kb fragment was found to be present in the Cc^r mutants of W83 (lanes B through E) when they were probed
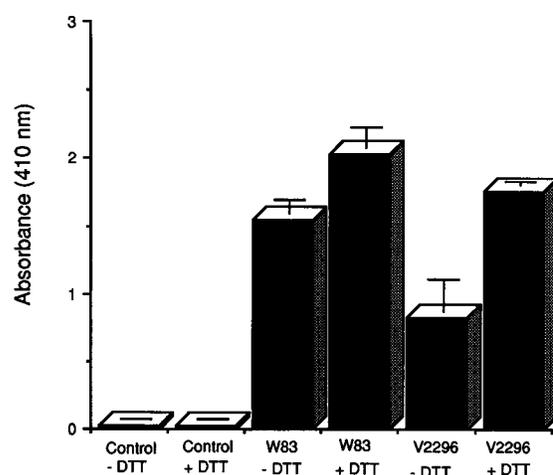
with ^{32}P-labeled pVA2290, which carried a truncated *prtH* gene. A similar blot probed with pVA2198, which carried the *ermF-ermAM* cassette, revealed an identical 4.8-kb hybridizing fragment present only in the Cc^r mutants (Fig. 3B, lanes B through E). No hybridizing sequences were seen in W83 cells (lane A). The results shown in Fig. 3A revealed a number of other signals of various intensities. Further analysis of these signals has revealed that a sequence present in *prtH* is repeated on the *P. gingivalis* genome (data not shown; see Discussion). pUC19 vector sequences did not hybridize with W83 or any of the Cc^r mutants (data not shown). These data indicated that the predicted recombination had occurred, resulting in the interruption of the wild-type protease gene by the antibiotic resistance gene cassette. One Cc^r mutant of *P. gingivalis* (V2296) was chosen for further study. The growth rate of V2296 was similar to that of W83; however, on prolonged incubation (greater than 1 week) in broth culture, autolysis of W83 occurred more readily than autolysis of the mutant (data not shown).

Proteolytic activity of *P. gingivalis* V2296. Extracellular protein preparations from W83 and V2296 were assayed for proteolytic activity by using *N*-α-benzoyl-DL-arginine *p*-nitroanilide. Wild-type W83 showed more proteolytic activity than V2296, and in addition the activity from both strains was enhanced in the presence of dithiothreitol (Fig. 4). A specific protease deficiency in V2296 was confirmed by casein-substrate zymography. As shown in Fig. 5, proteolytic activity in the range of 79 to 97 kDa was more intense for W83 than V2296. These data indicated that V2296 was missing the predicated proteolytic band of approximately 97 kDa. In addition, V2296 was also missing a 45-kDa proteolytic band that was present in the wild-type strain (lanes C and D).

Virulence testing of V2296. At a dose of 10^{10} bacteria per animal, strain W83 induced spreading, ulcerative lesions by 24 h in two of five animals (Table 2). These lesions were present on the abdomen and at the base of the tail; the mice did not display lesions at the dorsal surface site of injection. At 24 h the mice appeared cachectic and hunched with ruffled hair. By 48 h all animals challenged with this dose of W83 died. In contrast, all mice challenged with V2296 at a dose of 10^{10} bacteria per animal survived the 14-day observation period
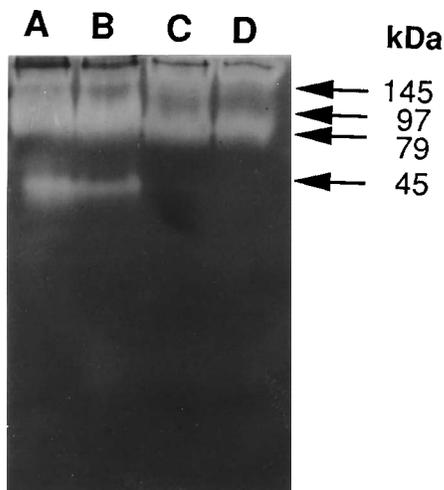
FIG. 5. Detection of proteolytic activity in casein-conjugated polyacrylamide. The gel was developed for 12 h at 37°C in 100 mM Tris-HCl (pH 7.0) containing 2.5 mM CaCl₂ and 50 mM cysteine. Lanes A and B, membrane vesicles from W83 (10 and 20 µg of protein, respectively); lanes C and D, membrane vesicles from V2296 (10 and 20 µg of protein, respectively). Samples were solubilized at 37°C for 30 min in buffer without β-mercaptoethanol prior to electrophoresis. Proteolytic bands of about 97 and 45 kDa were missing from the V2296 membrane vesicles.

(Table 2). The mice in this group displayed an unusual redness of the ears and tail by 48 h which resolved by day 5 or 6. No lesions of any sort appeared before day 6, and none of the animals in this group appeared cachectic. Secondary site lesions did appear in the axillary region beginning on day 6, but no ulcerative abdominal lesions or lesions at the base of the tail were observed. The secondary site lesions appeared to be confined to the skin, and all lesions were resolving or had substantially healed by the end of the 14 days.

When mice were challenged with 5×10^9 W83 bacteria, four of five developed secondary site lesions after 24 h. By 48 h, two of five mice in this group had died and by day 12 only one of five survived (Table 2). All had developed ulcerated abdominal lesions. The lesion in the surviving animal was resolving at day 14. The mean weights of surviving mice in this group declined substantially by day 6, and all mice displayed the cachectic appearance of those treated with the higher bacterial dose. In contrast, mice treated with the identical dose of V2296 appeared normal and healthy, continued to gain weight throughout the experiment, and failed to develop lesions of any sort at or distant from the injection site.

All mice in both groups survived challenge with 10^9 bacteria (Table 2). For the V2296 group that received 5×10^9 bacteria, no lesions were observed and no alterations in general health status were noted. In the W83 group, three of five animals developed secondary site lesions on the abdomen or at the base of the tail, and substantial weight loss was noted. Most of these lesions began healing by 6 days postchallenge.

Analysis of *P. gingivalis* clinical isolates for *prtH* sequences. Chromosomal DNA isolated from W83, 33277, and 10 clinical isolates of *P. gingivalis* (Table 1) was simultaneously digested with *Bam*HI and *Hin*dIII and probed with ³²P-labeled pVA2289 that contained the *prtH* gene with some flanking DNA sequences (Fig. 2). Since the *prtH* gene was cloned from W83 on a 3.7-kb *Hin*dIII-*Bam*HI fragment, the possibility of polymorphism could be determined by simultaneous digestion of chromosomal DNA with *Hin*dIII and *Bam*HI. As shown in Fig. 6, multiple bands including a 3.7-kb fragment were seen

for W83 (lane A), 33277 (lane B), and 7 of 10 clinical isolates (lanes D, E, and G through L). V2306 was missing a 3.7-kb band, but hybridizing fragments of 8.4, 6.5, 5.5, and 0.8 kb were seen (Fig. 6, lane J). DNA from strains V2298 (lane C) and V2301 (lane F) did not hybridize with the probe. A similar hybridization profile was seen for the isolates when an internal fragment of the *prtH* gene was used as the probe (data not shown). These two strains missing the *prtH* sequences had reduced proteolytic activity compared with W83 (data not shown). V2298 and V2301 were tested for invasiveness in a mouse model. At a challenge dose of 10^{10} bacteria per animal all animals survived the 14-day observation period (data not shown).

DISCUSSION

Proteases are known to contribute to virulence in some bacteria, including *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Clostridium* spp., and *Streptococcus* spp. (reviewed in references 13, 20, and 49). Molecular mechanisms for pathogenesis attributed to these proteases include enhancement of vascular permeability with edema formation, degradation of structural matrices (e.g., fibronectin and collagen), and destruction of proteins involved in host protective functions (reviewed in reference 20). For *P. gingivalis*, however, a possible role for proteases in virulence is not well defined. van Steenbergen et al. (46) and others (27) found no consistent association between proteolytic activity (measured by hydrolytic activity against synthetic substrates) of clinical isolates of *P. gingivalis* and their pathogenic potential. Grenier and Mayrand (10) separated *P. gingivalis* into two groups, but no correlation between protease activity and infectivity could be established. Pathogenic strains demonstrated high collagenolytic and proteolytic activities, whereas nonpathogenic strains showed lower collagenolytic but high proteolytic activity. In contrast to these reports, the work of McKee et al. (25) has suggested a correlation between the level of protease activity and virulence. In our study, we have demonstrated a correlation between protease activity and the pathogenesis of *P. gingivalis* in a mouse model. Although the infection in our model was extraoral, the data have provided evidence for the contribution of the proteolytic property to virulence of *P. gingivalis*. Inactivation of the protease gene that specifies an enzyme that degrades the C3 complement protein resulted in reduced proteolytic activity. Further, Schenkein et al. (37) have demonstrated that, compared with the wild-type strain, V2296 has a significantly reduced ability to degrade the C3 complement protein. This mutant strain displayed dramatically reduced virulence compared with wild-type W83. The involvement of the missing protease in tissue destruction was not determined and is the focus of further studies. Despite the strong potential of *P. gingivalis* proteinases for participating in tissue destruction typical of periodontal disease, there is no direct evidence or data proving such involvement.

The *prtH* gene that specified a 97-kDa protease involved in the cleavage of the C3 complement protein was previously cloned from *P. gingivalis* W83, and its nucleotide sequence was determined (7). Inactivation of this protease gene by allelic exchange has allowed us to evaluate its role in pathogenesis by using a soft tissue infection mouse model. The inactivation of the wild-type protease gene of W83 was made possible by the construction of an *ermF-ermAM* antibiotic resistance cassette. Since *ermAM* is expressed in *E. coli* (39) and *ermF* is expressed in *P. gingivalis* (14, 21, 31), selection for recombinant clones was possible in either host. The *ermF-ermAM* cassette will be an important tool for genetic manipulations in *P. gingivalis* and

TABLE 2. Virulence of *P. gingivalis* strains W83 and V2296 in mice

No. of days postchallenge	Strain	Inoculum (CFU/animal)	No. of animals surviving/total	No. of animals with primary lesion/total	No. of animals with secondary lesion/total	Wt of animal(s) (g)[a]
0	W83	1×10^{10}	6/6			ND[b]
	V2296		5/5			ND
	W83	5×10^{9}	5/5			18.8
	V2296		5/5			18.6
	W83	1×10^{9}	5/5			19.5
	V2296		6/6			18.8
1	W83	1×10^{10}	6/6	0/6	2/6	ND
	V2296		5/5	0/5	0/5	ND
	W83	5×10^{9}	5/5	0/5	4/5	17.5
	V2296		6/6	0/6	0/6	17.5
	W83	1×10^{9}	5/5	0/5	1/5	18.5
	V2296		5/5	0/5	0/5	18.6
2	W83	1×10^{10}	0/6			ND
	V2296		5/5	0/5	0/5	ND
	W83	5×10^{9}	3/5	0/3	3/3	16.5
	V2296		6/6	0/6	0/6	18.1
	W83	1×10^{9}	5/5	0/5	3/5	18.3
	V2296		5/5	0/5	0/5	19.0
4	W83	1×10^{10}	0/6			ND
	V2296		5/5	0/5	0/5	ND
	W83	5×10^{9}	2/5	1/2	2/2	15.1
	V2296		6/6	0/6	0/6	19.6
	W83	1×10^{9}	5/5	0/5	3/5	18.8
	V2296		5/5	0/5	0/5	19.7
6	W83	1×10^{10}	0/6			ND
	V2296		5/5	1/5	2/5	ND
	W83	5×10^{9}	2/5	1/2	2/2	15.1
	V2296		6/6	0/6	0/6	19.1
	W83	1×10^{9}	5/5	0/5	3/5	16.6
	V2296		5/5	0/5	0/5	19.4
8	W83	1×10^{10}	0/6			ND
	V2296		5/5	2/5	4/5	ND
	W83	5×10^{9}	2/5	1/2	2/2	15.6
	V2296		6/6	0/6	0/6	19.1
	W83	1×10^{9}	5/5	0/5	3/5	18.8
	V2296		5/5	0/5	0/5	19.3
10	W83	1×10^{10}	0/6			ND
	V2296		5/5	2/5	4/5	ND
	W83	5×10^{9}	2/5	1/2	2/2	16.1
	V2296		6/6	0/6	0/6	19.2
	W83	1×10^{9}	5/5	0/5	3/5	19.1
	V2296		5/5	0/5	0/5	19.5
12	W83	1×10^{10}	0/6			ND
	V2296		5/5	2/5	0/5	ND
	W83	5×10^{9}	1/5	0/1	1/1	18.9
	V2296		6/6	0/6	0/6	20.2
	W83	1×10^{9}	5/5	0/5	3/5	20.0
	V2296		5/5	5/5	5/5	20.0
14	W83	1×10^{10}	0/6			ND
	V2296		5/5	1/5	0/5	ND
	W83	5×10^{9}	1/5	0/1	1/1	19.2
	V2296		6/6	0/6	0/6	20.2
	W83	1×10^{9}	5/5	0/5	3/5	20.2
	V2296		5/5	0/5	0/5	20.4

[a] Data are averages except for cases in which only one animal was surviving.
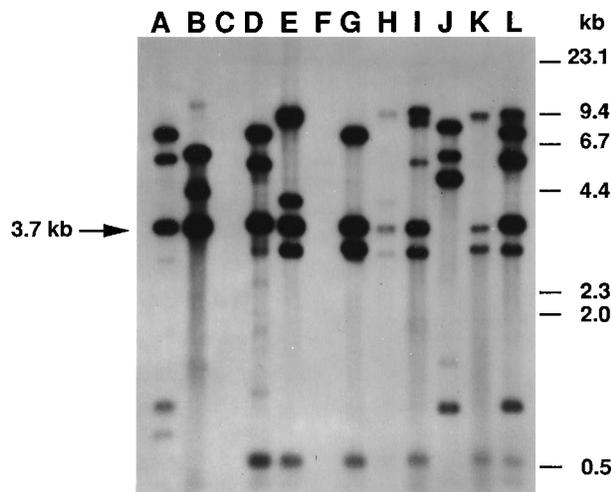[b] ND, not determined.

FIG. 6. Southern blot analysis of *P. gingivalis* strains performed by using a DNA probe internal to the 97-kDa protease gene. All chromosomal DNA was cleaved with *Bam*HI-*Hin*dIII. Lanes: A, W83; B, 33277; C, V2298; D, V2299; E, 2300; F, V2301; G, 2302; H, 2303; I, V2305; J, 2306; K, V2307; L, V2308. The probe used was the ^{32}P-labeled pVA2289 that carried the *prtH* gene.

other gram-negative anaerobes. With this cassette, genetic constructs can be easily created in *E. coli* and then moved into and evaluated in such anaerobic hosts.

A comparison of the proteolytic activities of the wild-type W83 and the isogenic mutant (V2296) showed that even in the presence of a reducing agent there was significantly less activity for the mutant (Fig. 4). V2296 was missing 97- and 45-kDa proteolytic bands that were present in W83. The missing 45-kDa proteolytic band in the mutant is noteworthy (Fig. 5). Several possibilities may explain this phenomenon. First, the gene that specifies this protease could be part of an operon, and it is downstream from the *prtH* gene. The allelic exchange event might have resulted in transcriptional polarity affecting the expression of this gene. Second, the 45-kDa protease could be a product that is processed from a larger precursor form by the 97-kDa protease. Third, expression of the 45-kDa protease could be regulated by a protein that is processed by the *prtH* gene product. These possibilities are being investigated. The wild-type strain and the mutant did not differ in growth rate; however, it was noted that V2296 persisted longer in liquid culture than W83, which suggests that the 97-kDa protease may be involved in physiologic housekeeping activities. No other obvious phenotypic changes for V2296 apart from growth on antibiotics were detected.

In in vivo experiments using the mouse model, V2296 appeared to be dramatically reduced in virulence compared with wild-type W83 (Table 2). The virulence profile of W83 was identical to that described by Neiders et al. (27) in that no primary lesions or ulcerations were observed when the mice were challenged with 10^{10} W83 cells. Furthermore, secondary lesions on the abdomen appeared after 24 h and none of the animals survived (Table 2). In contrast to that of W83, the behavior of V2296 was similar to that of noninvasive strains (10, 27). All animals challenged with 10^{10} bacteria survived. Further, there was no apparent effect at a challenge dose of 5 × 10^9 bacteria, which was sufficient to cause spreading lesions and death in the case of mice challenged with W83. We propose that the decrease in virulence is related to the decreased proteolytic activity of the mutant. Although it is tempting to conclude that reduced virulence of V2296 can be attributed

solely to the inactivation of the *prtH* gene, we cannot rule out the possibility that the virulence phenotype may be the result of secondary effects caused by loss of the *prtH*-encoded protease.

Virulence of *P. gingivalis* is believed to be associated with failure to accumulate opsonins, which contributes to invasiveness (35). Further, van Steenbergen et al. (46) found that the most virulent strains of *P. gingivalis* are not readily phagocytized and are the least sensitive to the killing effects of complement and antibody. Cutler et al. (5) have demonstrated that inhibition of C3 and immunoglobulin G proteolysis enhances phagocytosis of *P. gingivalis*. Sundqvist et al. (44) have shown that there is no correlation between phagocytosis of strains of *P. gingivalis* and virulence in a mouse model of bacterial invasiveness. In preliminary experiments W83 failed to accumulate C3-derived opsonins, while V2296 showed accumulation levels similar to those observed for a noninvasive strain (data not shown). These observations suggest a role for the *prtH* protease in contributing to virulence.

A survey of 10 clinical isolates of *P. gingivalis* and *P. gingivalis* ATCC 33277 performed by using the *prtH* gene as a probe revealed that *prtH* sequences were conserved among several of the isolates (Fig. 6). The recognition of multiple fragments of differing sizes among the strains suggests that the gene may exist in several copies or that the probe is recognizing a related gene(s). Alternatively, the probe might be recognizing conserved regions of other genes. In preliminary studies, when the 0.6-kb hybridizing fragment (Fig. 6, lane A) was cloned and its nucleotide sequence was determined, repeated sequences of 66 and 32 bp that were identical to repeated sequences present at the amino-terminal end of the *prtH* gene were found (data not shown). When the 0.6-kb fragment was used as a probe, a hybridization profile similar to that seen with the *prtH* gene probe was observed (data not shown). Further, the repeated sequences appear to be associated with the protease coding capability in the genome of *P. gingivalis*, and this relationship is the focus of current investigations.

There was no hybridization of the *prtH* probe with two of the clinical isolates. Furthermore, in preliminary experiments, these two isolates showed reduced proteolytic activity and had a virulence profile comparable to that of V2296, the *prtH*-deficient mutant (data not shown). These observations suggest the existence of naturally occurring protease-deficient mutants which are likely the result of gene deletion. Our proposed repeated sequences could be the source of protease gene deletions that may occur in nature. This notion is under further investigation in our laboratory.

In the present study we constructed an allelic exchange mutant that is defective in a protease gene that degrades the C3 complement protein. We have demonstrated that this mutant is significantly less virulent than the wild-type strain. To our knowledge these experiments represent the first study elucidating the role of a complement-degrading protease in the pathogenesis of *P. gingivalis*. Further characterization of the protease should give us insight into its possible role in the tissue destruction typical of periodontal disease.

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