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The *vimE* Gene Downstream of *vimA* Is Independently Expressed and Is Involved in Modulating Proteolytic Activity in *Porphyromonas gingivalis* W83

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Regulation/activation of the *Porphyromonas gingivalis* gingipains is poorly understood. A unique 1.3-kb open reading frame downstream of the *bcp-recA-vimA* transcriptional unit was cloned, insertionally inactivated with the *ermF-ermAM* antibiotic resistance cassette, and used to create a defective mutant by allelic exchange. In contrast to the wild-type W83 strain, the growth rate of the mutant strain (designated FLL93) was reduced, and when plated on *Brucella* blood agar it was nonpigmented and nonhemolytic. Arginine- and lysine-specific gingipain activities were reduced by approximately 90 and 85%, respectively, relative to activities of the parent strain. These activities were unaffected by the culture's growth phase, in contrast to the *vimA*-defective mutant *P. gingivalis* FLL92, which has increased proteolytic activity in stationary phase. Expression of the *rgpA*, *rgpB*, and *kgp* gingipain genes was unaltered in *P. gingivalis* FLL93 compared to that of the wild-type strain. Further, in extracellular protein fractions a 64-kDa band was identified that was immunoreactive with the RgpB-specific proenzyme antibodies. Active-site labeling with dansyl-glutamyl-glycyl-arginyl chloromethyl ketone or immunoblot analysis showed no detectable protein band representing the gingipain catalytic domain. In vitro protease activity could be slightly induced by a urea denaturation-renaturation cycle in an extracellular protein fraction, in contrast to the *vimA*-defective mutant *P. gingivalis* FLL92. Expression of flanking genes, including *recA*, *vimA*, and *Pg0792*, was unaltered by the mutation. Taken together, these results suggest that the *vimA* downstream gene, designated *vimE* (for virulence-modulating gene E), is involved in the regulation of protease activity in *P. gingivalis*.

The expression of extracellular proteolytic activities is highly regulated in both prokaryotic and eukaryotic systems (6, 40). This regulation usually occurs at the level of expression of the protease genes, secretion, and/or processing of an inactive secreted precursor to its active form. The multiple layers of regulation are vital to ensure that expression is tightly controlled in the appropriate temporal and spatial patterns. *Porphyromonas gingivalis*, a black-pigmented, gram-negative anaerobe, has been implicated as an important etiological agent in adult periodontitis (19, 23, 38). While several virulence factors, including hydrolytic enzymes, fimbriae, hemagglutinin, capsule, and lipopolysaccharide, have been implicated in the pathogenicity of *P. gingivalis*, the strong proteolytic abilities of this organism are the focus of much attention, as they are considered to play the most significant role in virulence (25). The major proteases, called gingipains, are both extracellular and cell associated. They consist of arginine-specific protease (Arg-gingipain [Rgp]) and lysine-specific protease (Lys-gingipain [Kgp]) (25). Although glycosylation appears to be important for their activation (10, 30), there remains a gap in our knowledge regarding the regulation/activation of the *P. gingivalis* gingipains.

It was previously reported that the *recA* locus can affect the phenotypic expression and distribution of the gingipains in *P. gingivalis* (1, 2, 27). Using the cloned *vimA* gene, which is downstream of the *recA* gene and is part of the *bcp-recA-vimA* transcriptional unit, a defective mutant was constructed by allelic exchange (1). The mutant strain, designated FLL92, did not have a black pigmentation and showed increased autoaggregration in addition to a significant reduction in proteolytic, hemolytic, and hemagglutinating activities (1). For in vivo experiments using a mouse model, *P. gingivalis* FLL92 had dramatically reduced virulence compared to that of the wild-type W83 strain (1). While a reduction in Arg-X- and Lys-X-specific proteolytic activities was observed in *P. gingivalis* FLL92, transcription of the gingipain genes was unaltered in this mutant compared to that of the wild-type strain (1). Furthermore, the partially processed RgpB proenzyme was secreted in *P. gingivalis* FLL92 (27). Collectively these observations suggest that the *vimA* gene in *P. gingivalis* may be involved in virulence modulation via an ability to affect protease activation/maturation. In addition, appearance of the gingipain proenzyme forms and the growth-phase-dependent activation of proteolytic (27) activity have raised the possibility of multiple mechanisms for gingipain activation/maturation involving multiple bacteria-specific factors.

We have further investigated a unique 1.3-kb gene downstream of the *vimA* gene to determine its relationship to the *bcp-recA-vimA* transcriptional unit and to evaluate its role, if any, in protease activation. In this report, we have created and characterized a *P. gingivalis* isogenic mutant (FLL93) defective in this gene, now designated *vimE*. While this gene was expressed independent of *vimA*, its inactivation resulted in reduced Arg-X- and Lys-X-specific proteolytic activities that were not affected by the phase of growth. In addition, in vitro

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TABLE 1. Plasmids and bacterial strains used in this study

Plasmid or strain	Phenotype or description	Source or reference
Plasmids		
pCR-2.1 TOPO	Ap^r, Km^r	Invitrogen
pFLL80	pCR 2.1-TOPO *Pg0791:Pg0792*	This study
pFLL81	pFLL80 with *Pg0791* interrupted with *ermF-ermAM*	This study
pVA2198	Sp^r, *ermF-ermAM*	1
P. gingivalis		
W83	Wild type	
FLL92	*vimA* defective	1
FLL93	*vimE* defective	This study
E. coli		
DH5α	F^- ϕ80d*lac*ZΔM15 Δ(*lacZYA-argF*) U169 *recA1 endA1 hsdR17* (r_K^- m_K^+) *phoA supE44* λ-*thi*-1 *gyrA96 relA1*	Invitrogen
Top10	F^- *mcrA*Δ(*mrr-hsdRMS-mcrBC*) ϕ80*lacZ*Δ M15 Δ*lacX74 recA1 ara139* Δ (*ara-leu*)7697 *galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen

protease activity was slightly activated by a urea denaturation-renaturation cycle. These results suggest an important role for the *vimE* gene in protease activation/maturation in *P. gingivalis* and further confirm the requirement of multiple specific host factors in this process.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains and plasmids used in this study are listed in Table 1. *P. gingivalis* strains were grown in brain heart infusion (BHI) broth supplemented with 0.5% yeast extract (both from Difco Laboratories, Detroit, Mich.), hemin (5 μg/ml), vitamin K (0.5 μg/ml), and cysteine (0.1%) (all from Sigma-Aldrich, St. Louis, Mo.). *Escherichia coli* strains were grown in Luria-Bertani broth. Unless otherwise stated, all cultures were incubated at 37°C. *P. gingivalis* strains were maintained in an anaerobic chamber (Coy Manufacturing, Ann Arbor, Mich.) in 10% H_2, 10% CO_2, 80% N_2. Growth rates for *P. gingivalis* and *E. coli* strains were determined spectrophotometrically (optical density at 600 nm [OD_{600}]). Antibiotics were used at the following concentrations: clindamycin, 0.5 μg/ml; erythromycin, 300 μg/ml; and carbenicillin, 100 μg/ml.

DNA isolation and analysis. *P. gingivalis* chromosomal DNA was prepared by the method of Marmur (22). For plasmid DNA analysis, DNA extraction was performed by the alkaline lysis procedure of Birnboim and Doly (4). For large-scale preparation, plasmids were purified by using the QIAGEN (Santa Clarita, Calif.) plasmid maxi kit. DNA was digested with restriction enzymes as specified by the manufacturer (Roche, Indianapolis, Ind.). For DNA subcloning, the desired fragments were isolated from 0.8% agarose gels run in Tris-acetate-EDTA (TAE) buffer and then were purified by using a Gene Clean kit as recommended by the manufacturer (QBiogene, Inc., La Jolla, Calif.).

Generation of *Pg0791* (*vimE*) mutant *P. gingivalis* strain. A 2.5-kb fragment carrying the intact *vimE* and *Pg0792* downstream genes was amplified by PCR using the P1 and P5 oligonucleotide primer (Table 2; Fig. 1). This fragment was cloned into the pCR 2.1-TOPO plasmid vector (Invitrogen, Carlsbad, Calif.) and was designated pFLL80. The *ermF-ermAM* cassette, which confers erythromycin/clindamycin resistance in *E. coli* and *P. gingivalis* (9), was PCR amplified from pVA2198 with Pfu turbo (Stratagene) and was ligated into the HincII restriction site of the *vimE* gene. The resultant recombinant plasmid, pFLL81, was used as a donor in electroporation of *P. gingivalis* W83.

Electroporation. Electroporation of cells was performed as previously reported (9). Briefly, 1 ml of an actively growing culture of *P. gingivalis* was used to inoculate 10 ml of BHI broth supplemented with yeast extract, hemin, and vitamin K, which was then incubated overnight at 37°C. Ninety milliliters of the same prewarmed (37°C) medium was then inoculated with 3 ml of the overnight culture and was incubated for an additional 4 h until an OD_{600} of 0.7 was obtained. The cells were then harvested by centrifugation at 3,000 × *g* for 15 min at 4°C and were washed twice in 50 ml of electroporation buffer (10% glycerol, 1 mM $MgCl_2$; filter sterilized and stored at 4°C). The final cell pellet was resuspended in 0.5 ml of electroporation buffer. A 100-μl sample of resuspended cells and 5 μg of DNA was placed in a sterile electrode cuvette (0.2-cm gap). The cells were then pulsed with a Bio-Rad (Hercules, Calif.) gene pulser at 2,500 V for 9.5 ms and then were incubated on ice for 5 min. The cell suspension was then added to 0.5 ml of BHI broth supplemented with yeast extract, hemin, and vitamin K and was incubated for approximately 16 h. A 100-μl sample was plated on solid medium containing erythromycin and was incubated anaerobically at 37°C for 5 to 10 days.

Growth curve analysis. Actively growing *P. gingivalis* W83, FLL92, and FLL93 cultures were inoculated at an initial OD_{600} of 0.2 in BHI broth supplemented with 0.5% yeast extract (both from Difco Laboratories), hemin (5 μg/ml), vitamin K (0.5 μg/ml), and cysteine (0.1%) (all from Sigma-Aldrich). Aliquots (500 μl) were taken every 4 h, and growth rate was determined by spectrometer.

Hemolytic activity assay. Hemolytic activity was determined as previously reported (11). Briefly, bacterial cells from overnight cultures were harvested by centrifugation (10,000 × *g* for 30 min) using a Sorvall RC5C centrifuge, washed three times with phosphate-buffered saline (PBS), and then resuspended to a final OD_{600} of 1.5. Sheep erythrocytes (Hemostat Laboratories, Dixon, Calif.) were harvested by centrifugation (4,400 × *g* for 25 min) and washed with 1× PBS

TABLE 2. Primers used in this study

Name	Description	Sequence
P1	*Pg0791* (*vimE*) forward	5′ATGAATACTGAAGTAATCCAC 3′
P2	*Pg0791* (*vimE*) reverse	5′ TTGGTCGATGGCCGTTTCGTA 3′
P3	*Pg0791* (*vimE*) reverse (intragenic)	5′ TCAGCACTTCCTTGCACAAC 3′
P4	*Pg0792* forward (intragenic)	5′ CAGTCGCACGGCGATGTGTTT 3′
P5	*Pg0792* reverse (intragenic)	5′ GCAGGATGGAGCGGGAGTATTC 3′
P6	*Pg0792* reverse	5′ TTCACGCGAAAAAGTGGAGAT 3′
P7	*vimA* forward	5′ ATGCCCATCCCTCTATACCTG 3′
P8	*vimA* reverse	5′ TACCTGTTTTTGCTGACCGG 3′
P9	*recA* forward	5′ ATGGCAGAAGAAAAGATACCC 3′
P10	*recA* reverse	5′ TGAATGTTTGTTGCGAATGG 3′
P11	*pro-kgp* forward	5′ ATGAGGAAATTATTATTGCTGATCG 3′
P12	*pro-kgp* reverse	5′ TTGAAGAGCTGTTTATAAGCTGTTT 3′
P13	*pro-rgpA* forward	5′ ATGAAAAACTTGAACAAGTTTGTTTC 3′
P14	*pro-rgpA* reverse	5′ CCGGTGTGTAACGCCCTG 3′
P15	*pro-rgpB* forward	5′ CGACCGATGAAGACTTCG 3′
P16	*pro-rgpB* reverse	5′ TGAAGACGCTCTTATAGATATCTT 3′
P17	*erythromycin* forward	5′ TATTAGGCCTATAGCTTCCGCTATT 3′
P18	*erythromycin* reverse	5′ AATAGGCCTTAGTAACGTGTAACTTT 3′

until the supernatant was visually free of hemoglobin pigment. The washed erythrocytes were suspended to a final concentration of 1% in 1× PBS. Hemolytic activity was determined by mixing an equal volume of bacterial cells with 1% erythrocytes in PBS. This mixture was then incubated at 37°C. Samples (500 μl) were withdrawn every 2 h and then were centrifuged (1,300 × *g* for 5 min) in an Eppendorf 5403 centrifuge. The OD_{405} was determined by spectrophotometry. As a negative control, erythrocytes were used alone.

Hemagglutination studies. Hemagglutinin activity was determined as previously reported (11). Twenty-four-hour cultures of *P. gingivalis* W83, FLL92, and FLL93 cells were harvested by centrifugation (10,000 × *g*, 15 min). Cells were washed twice in 1× PBS and were resuspended to a final OD_{600} of 1.5. Sheep erythrocytes were washed twice with 1× PBS and were resuspended to a final concentration of 1% in 1× PBS. An aliquot (100 μl) of the bacterial suspension was serially diluted twofold with 1× PBS in round-bottom 96-well microtiter plates. An equal volume (100 μl) of 1% sheep erythrocytes was mixed with each dilution and was incubated at 4°C for 3 h. Hemagglutination was visually assessed, and the hemagglutination titer was determined as the last dilution that showed complete hemagglutination.

Cell fraction preparation. One-liter cultures of *P. gingivalis* strains FLL92 and W83 were grown from actively growing cells. Preparations of whole-cell culture, cell-free medium, cell suspension, vesicles, and vesicle-free medium were made as previously reported (27). The whole-cell culture fraction is a sample of the culture after the bacterium has been grown to a specific growth phase. This sample has the bacterial cells suspended in the growth medium, and the enzyme activity includes the gingipains that are attached to the bacterial cell surface plus those that are secreted in the culture medium. After centrifugation the cell pellet was resuspended, and the enzyme activity in this sample (the cell suspension fraction) represents the gingipains that are attached to the bacterial cell surface, while the enzyme activity in the supernatant (the cell-free medium fraction) includes the gingipains that are secreted in the culture medium. Secreted gingipains can either be associated with vesicles or be soluble in the culture medium; thus, ultracentrifugation of the cell-free fractions will yield a vesicle pellet and a supernatant of soluble gingipains.

Preparation of *P. gingivalis* extracellular fractions. One-liter cultures of *P. gingivalis* strains FLL92, FLL93, and W83 were grown from actively growing cells. Cells were harvested by centrifugation at 10,000 × *g* for 30 min. The cell-free culture fluid was precipitated with 37.5 or 60% acetone (−20°C), and the protein pellet was resuspended in 7 ml of 100 mM Tris-HCl buffer (pH 7.4), dialyzed for 24 h against the same buffer, and then stored on ice or at 0°C. The presence of Arg-X- and Lys-X-specific cysteine protease activities was determined by microplate reader (Bio-Rad).

Production of rabbit polyclonal antibodies against the RgpB proenzyme. Extracellular proteins were prepared from the culture supernatant of *P. gingivalis* FLL92 (*vimA* defective) grown to exponential phase (OD_{600} of 0.8). The cells were harvested by centrifugation (10,000 × *g*, 30 min), and the supernatant was further clarified by filtration through a 0.45-μm-pore-size membrane (Millipore Corporation, Billerica, Mass.). The cell-free culture fluid was precipitated with 37.5% acetone (−20°C), and the protein pellet was resuspended in 7 ml of 100 mM Tris-HCl buffer (pH 7.4), dialyzed for 24 h against the same buffer, and then stored on ice or at 0°C. The protein concentration was determined as described below. The extracellular proteins (25 μg/lane) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using NuPAGE 4 to 12% Bis-Tris gels. A 64-kDa band representing the partially processed RgpB proenzyme (27) was excised with a gel cutter. A total of approximately 12 mg of the RgpB proenzyme protein was excised from gels, placed in 1× PBS, and stored at −80°C. The frozen gel slices were sent to Zymed Laboratories Inc. (South San Francisco, Calif.) for the production of polyclonal rabbit RgpB proenzyme antibodies by using the manufacturer's standard protocol. Dilutions and efficiency of the antibodies were tested in the laboratory with the purified RgpB proenzyme.

Protein concentration determination. Protein concentration was calculated spectrophotometrically by using the Warburg formula within the protein function of the Eppendorf Biophotometer (Brinkman, Westbury, N.Y.).

SDS-PAGE and immunoblot analysis. SDS-PAGE was performed with a 4 to 12% Bis-Tris separating gel in morpholinepropanesulfonic acid-SDS running buffer (NuPAGE Novex gels; Invitrogen) according to the manufacturer's instructions. Samples were prepared (65% sample, 25% 4× NuPAGE lithium dodecyl sulfate sample buffer, 10% NuPAGE reducing agent), heated at 72°C for 10 min, and then electrophoresed at 200 V for 65 min with a XCell SureLock Mini-Cell (Invitrogen). The separated proteins were then transferred to BioTrace nitrocellulose membranes (Pall Corporation, Ann Arbor, Mich.) and processed at 15 V for 25 min with a Semi-Dry Trans-blot apparatus (Bio-Rad). The blots were probed with antibodies against specific protease domains and species-specific secondary antibodies conjugated to horseradish peroxidase (Zymed Laboratories). Immunoreactive proteins were detected by the procedure described in the Western Lightning Chemiluminescence Reagent Plus kit (Perkin-Elmer Life Sciences, Boston, Mass.). The secondary antibody was immunoglobulin G (heavy plus light chains)-horseradish peroxidase conjugate (Zymed Laboratories, Inc.).

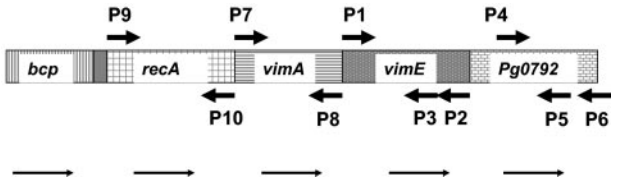

FIG. 1. Diagram of the *recA* locus and the two downstream genes. The diagram shows the open reading frame (from www.oralgen.lanl.gov) of the *recA* locus, and the two downstream genes are shown with the indicated primer locations.

Protease assays. The presence of Arg-X- and Lys-X-specific cysteine protease activities was determined with a microplate reader (Bio-Rad) by the method of Potempa et al. (30).

Analysis of *P. gingivalis Pg0791* mutant genes by RT-PCR. Total RNA was extracted from *P. gingivalis* strains grown to early stationary phase (OD_{600} of 1.2 to 1.3) using a RiboPure kit (Ambion, Austin, Tex.). The primers used for reverse transcription-PCR (RT-PCR) analysis (Table 2) were specific for the *recA*, *rgpA*, *rgpB*, *kgp*, *Pg0791*, or *Pg0792* genes. The RT-PCR (50 μl) contained 1 μg of template RNA in the Superscript One-Step RT-PCR mix (Invitrogen). Negative controls were reactions in the absence of reverse transcriptase.

Denaturation-renaturation procedure for in vitro protease activation. Extracellular protein fractions from *P. gingivalis* were mixed with 8 M urea and incubated at 4°C for 1 h as described previously (11). The urea was slowly removed from the mixture by centrifugation (10,000 × *g*) in a Millipore filtration unit (Biomax 10K NMWL membrane, 0.5-ml volume) with the addition of increasing volumes of 100 mM Tris-HCl buffer (pH 7.4).

Fluorescence labeling of proteases by using DNS-EGR-CK. Fluorescence labeling of proteases in the extracellular protein fraction from *P. gingivalis* was done as previously reported (3). Briefly, solutions of extracellular protein fractions from *P. gingivalis* were treated at 4°C for 10 min with equal volumes of 0.2 M Tris-HCl (pH 8.4) containing 20 mM $CaCl_2$ and 20 mM 2-mercaptoethanol. Dansyl-glutamyl-glycyl-arginyl chloromethyl ketone (DNS-EGR-CK) (0.25 mg; Calbiochem, San Diego, Calif.) was dissolved in 600 μl of 95% (vol/vol) aqueous ethanol just before use. Fifty microliters was added to the reduced protease solution, and the reaction was allowed to proceed at 4°C until the enzyme activity (monitored by *N*-α-benzoyl-DL-arginine-*p*-nitroanilide [BApNA] hydrolysis) was abolished. Samples of the protein solution were then dried in a SpeedVac concentrator (Thermo Savant, Holbrook, N.Y.). To identify the protease band unambiguously after SDS-PAGE, labeling was also performed in the presence of 50 μM leupeptin. The dried fluorescence-labeled proteases were then treated with NuPAGE SDS-PAGE sample buffer (Invitrogen) and were subjected to electrophoresis.

RESULTS

Inactivation of the *vimE* gene in *P. gingivalis* W83 by allelic exchange mutagenesis. Isogenic mutants of *P. gingivalis* W83 defective in the *Pg0791* (www.oralgen.lanl.gov) or open reading frame *Pg0883* (www.tigr.org) gene (designated *vimE*) were constructed by allelic exchange mutagenesis. The circular recombinant plasmid pFLL81, which carries the *ermF-ermAM* cassette in the unique HincII restriction site (base pair 909 of the open reading frame) of the *vimE* gene, was used as a donor in electroporation of *P. gingivalis* W83 (Fig. 2A). Because the plasmid was unable to replicate in *P. gingivalis*, we predicted that two double-crossover events between the regions flanking the *erythromycin* marker and the wild-type gene on the chromosome would result in replacement of a segment of the wild-type gene with a fragment conferring erythromycin resis-

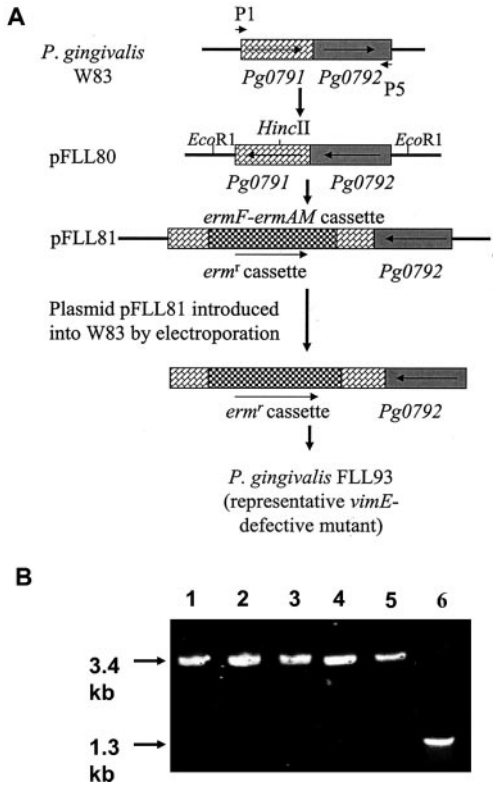

FIG. 2. Construction of a *vimE*-defective mutant by allelic exchange mutagenesis and confirmation by PCR analysis. pFLL81 contains the *vimE* gene interrupted by an *ermF-ermAM* cassette (the *vimE* gene with flanking sequences was amplified by PCR; the *ermF-ermAM* cassette was purified from pVA2198). The circular recombinant plasmid pFLL81 was integrally introduced into *P. gingivalis* W83 by electroporation. A reciprocal recombination event between areas of homology on the chromosome and regions flanking the *ermAM-ermF* cassette on pFLL81 replaced the intact *vimE* with an inactivated copy. (B) Oligonucleotide primers specific for the *vimE* gene (P1 and P2; Table 2) were then used to amplify that gene from total cellular DNA from *P. gingivalis*. Lane 1, *P. gingivalis* FLL93.1 (*vimE*::*ermF-ermAM*); lane 2, *P. gingivalis* FLL93.2 (*vimE*::*ermF-ermAM*); lane 3, *P. gingivalis* FLL93.3 (*vimE*::*ermF-ermAM*); lane 4, *P. gingivalis* FLL93.4 (*vimE*::*ermF-ermAM*); lane 5, *P. gingivalis* FLL93.5 (*vimE*::*ermF-ermAM*); and lane 6, *P. gingivalis* W83 (wild type).

tance. Following electroporation and plating on selective medium (BHI containing 10 μg of erythromycin/ml), we detected approximately 61 erythromycin-resistant colonies after a 5-day incubation period. To compare their phenotypic properties to those of wild-type strain W83, all mutants were plated on *Brucella* blood agar plates (Anaerobic Systems Inc., San Jose, Calif.). In contrast to the wild-type strain, all mutants displayed a non-black-pigmented, non-beta-hemolytic phenotype.

Confirmation of inactivation of *vimE* by PCR analysis. Chromosomal DNA from five randomly chosen clindamycin- and erythromycin-resistant colonies and the wild-type W83 strain were analyzed by PCR to confirm inactivation in the *vimE* gene. If the *vimE* gene was interrupted by the *ermF-ermAM* cassette, a 3.4-kb fragment was expected to be amplified by primers P1 and P2 (Table 2). The expected 3.4- and 1.3-kb fragments were observed in the five clindamycin-resistant strains and in wild-type W83, respectively (Fig. 2B). The orientation of the *ermF-ermAM* cassette was also confirmed by restriction digest (data not shown). Taken together these results indicated the insertional inactivation of the chromosomal *vimE* gene with the 2.1-kb *ermF-ermAM* antibiotic cassette was successful. One mutant, designated *P. gingivalis* FLL93, was randomly chosen for further study. A generation time of 3 h was determined for *P. gingivalis* W83 and *P. gingivalis* FLL92, in contrast to 4.75 h for *P. gingivalis* FLL93.

Hemolytic activity is reduced in *P. gingivalis* FLL93. Cell- and vesicle-associated hemolysins which will liberate hemoglobin from erythrocytes are produced in *P. gingivalis* apparently by two distinct genes (15). Further, other hemolysin-like genes have been identified from the *P. gingivalis* genome project (www.oralgen.lanl.gov) (26). *P. gingivalis* FLL93, FLL92, and W83 were evaluated for hemolysin activity by their ability to lyse erythrocytes. As shown in Fig. 3A, there was reduced hemolytic activity in *P. gingivalis* FLL93 compared to that in the wild-type *P. gingivalis* W83 strain. Further, this level of hemolytic activity was similar to that of the *vimA*-defective isogenic mutant *P. gingivalis* FLL92 or the negative control.

Hemagglutinin activity in *P. gingivalis* FLL93. We assessed the hemagglutination potential of *P. gingivalis* FLL93 in comparison to the potentials of *P. gingivalis* W83 and *P. gingivalis* FLL92, the nonproteolytic *vimA*-defective mutant. In contrast to the wild-type strain, there was a reduction in hemagglutinin activity in *P. gingivalis* FLL93 (Fig. 3B). This level of activity was similar to those of *P. gingivalis* FLL92 and the negative control.

Inactivation of the *vimE* gene affects proteolytic activity in *P. gingivalis*. It was previously reported that the proteolytic activity of the non-black-pigmented *recA*-defective mutant (*P. gingivalis* FLL32) or *vimA*-defective mutant (*P. gingivalis* FLL92) was reduced by more that 90% compared to levels of the wild-type strain (1, 2). In addition, a late onset of proteolytic activity was also reported for the *vimA* mutant compared to that of the wild-type strain (27). Because hemolysis and hemagglutinin activities can be associated with gingipain activity in this organism (21, 35), *P. gingivalis* FLL93 was assayed for proteolytic activity by using BApNA and acetyl-lysine-*p*-nitroanilide (ALNA). In late-exponential-growth-phase cultures, both Arg-X and Lys-X protease activities in *P. gingivalis* FLL93 were very similar to that of *P. gingivalis* FLL92, which was reduced by approximately 90% compared to the activity of the wild-type strain (Fig. 4). In stationary-phase cultures of *P. gingivalis* FLL93, there was just a slight increase in Lys-X activity; however, there was no change in Arg-X activity (Fig. 4). This is in contrast to the expected late onset of proteolytic activity that was observed in *P. gingivalis* FLL92 (Fig. 4). The proteolytic activity of the wild type remained constant throughout this growth phase. The proteolytic activity distribution pattern was the same for both *P. gingivalis* FLL93 and the wild type (Fig. 5). In exponential growth phase most of the activity was cell associated; this activity was reduced in stationary phase. Taken together, these data suggest that under the same physiological conditions, the proteolytic profile for *P. gingivalis* FLL93 can be severely altered by mutations affecting *vimE* gene expression in *P. gingivalis*.

Expression of the gingipain genes in the *vimE*-defective mutant. The reduced proteolytic activity in *P. gingivalis* strain FLL93 could have been the result of alteration in transcription

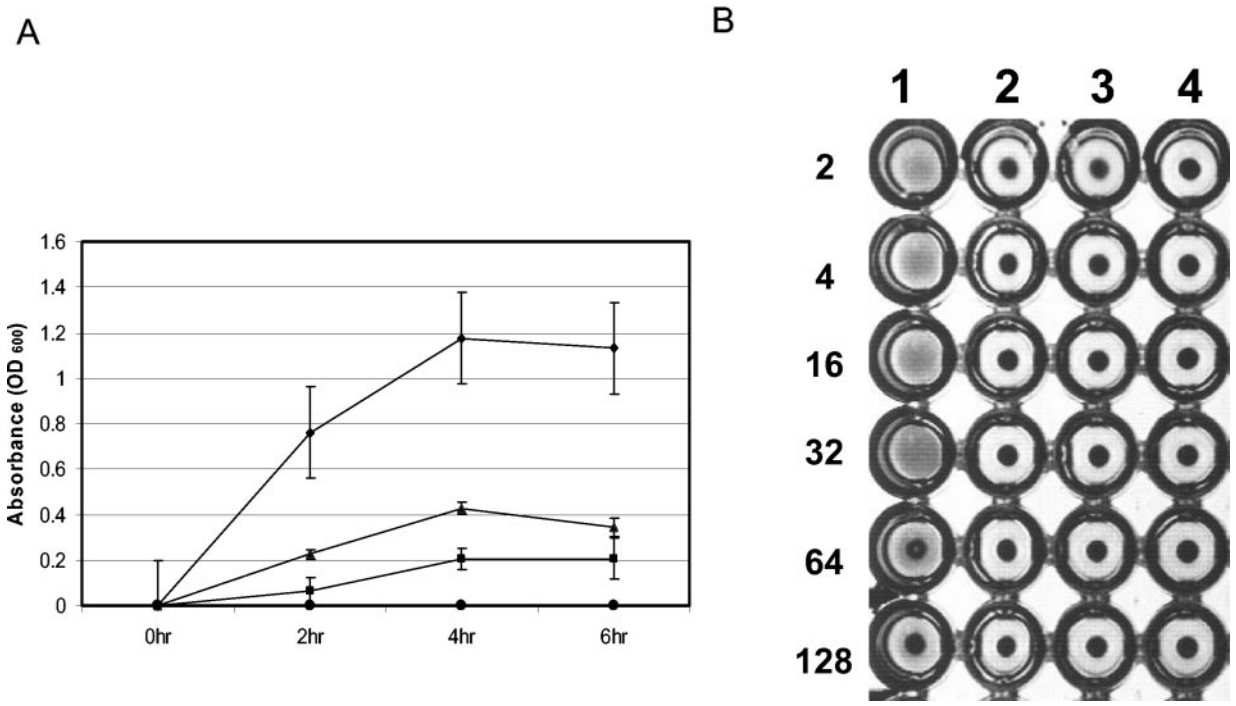

FIG. 3. Hemolytic and hemagglutinin activities of *P. gingivalis* FLL93 is reduced. Bacterial cells from overnight cultures were harvested by centrifugation, washed three times with 1× PBS, and then resuspended to a final OD_{600} of 1.5. Sheep erythrocytes were harvested by centrifugation and washed with 1× PBS until the supernatant was visually free of hemoglobin pigment. The washed erythrocytes were suspended to 1% in 1× PBS. (A) Hemolytic activity was determined by mixing equal volumes of bacterial cells with 1% erythrocytes in PBS. This mixture was then incubated at 37°C. Samples (500 μl) were withdrawn every 2 h and then were centrifuged. The OD_{405} was determined by spectrophotometry. As a negative control, erythrocytes were used alone. This experiment was done in triplicate, and average values are plotted (diamonds, W83; squares, FLL92; triangles, FLL93; circles, negative control). (B) Hemagglutination activity of *P. gingivalis* was performed on *P. gingivalis* W83, FLL92, and FLL93 cells that were serially diluted twofold in 1× PBS. Aliquots (100 μl) of the dilution were then mixed with an equal volume of 1% sheep erythrocytes and were incubated at 4°C for 3 h in a round-bottom microtiter plate. The hemagglutination titer was defined as the last dilution that showed full agglutination. Lane 1, W83; lane 2, FLL92; lane 3, FLL93; lane 4, negative control (sheep erythrocytes alone; Fig. 3B). Error bars indicate the standard error of the mean of three independent trials.

of the gingipain genes. There is also the possibility that the *vimE* gene may be involved in the posttranslational regulation of gingipain expression. In previous reports of mutations in two genes (*recA* and *vimA*) upstream of the *vimE* genes, a reduction in gingipain activity was also observed, although the expression of those genes was unaltered (1, 2). Furthermore, the partially processed RgpB gingipain proenzyme was identified in the *vimA*-defective mutant, suggesting a defect in gingipain biogenesis at the posttranslational level (27). To determine the presence of mRNA transcripts for the gingipain genes (*rgpB*,

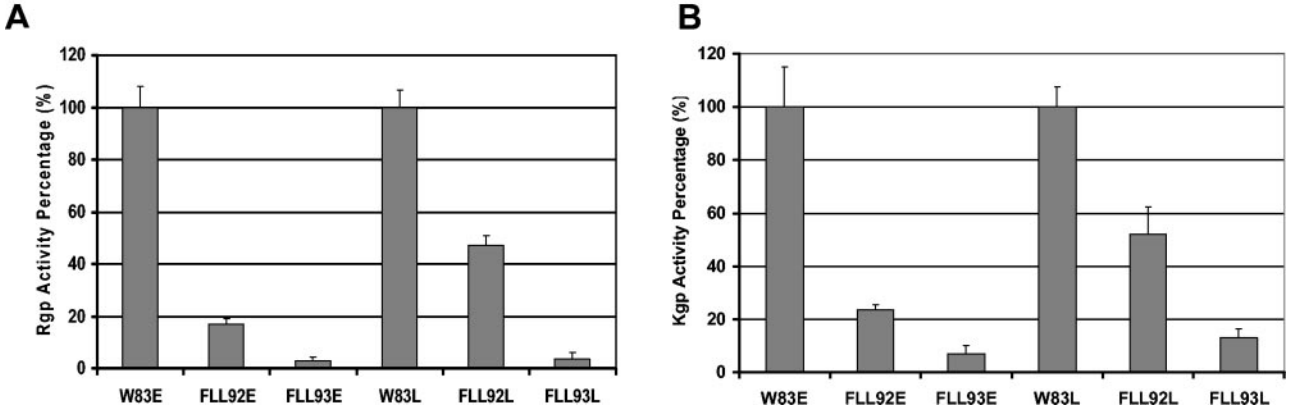

FIG. 4. Inactivation of *vimE* resulted in decreased proteolytic activity of *P. gingivalis*. *P. gingivalis* was grown to late log phase (OD_{600} of 0.7 to 0.8) or stationary phase (OD_{600} of 1.4 to 1.5) in BHI broth supplemented with yeast extract, hemin, and vitamin K. Activities against Rgp (A) and Kgp (B) were tested in whole-cell culture. The results shown are representative of three independent experiments in triplicate. E, exponential phase; L, stationary phase. Error bars indicate the standard errors of the means of three independent trials.

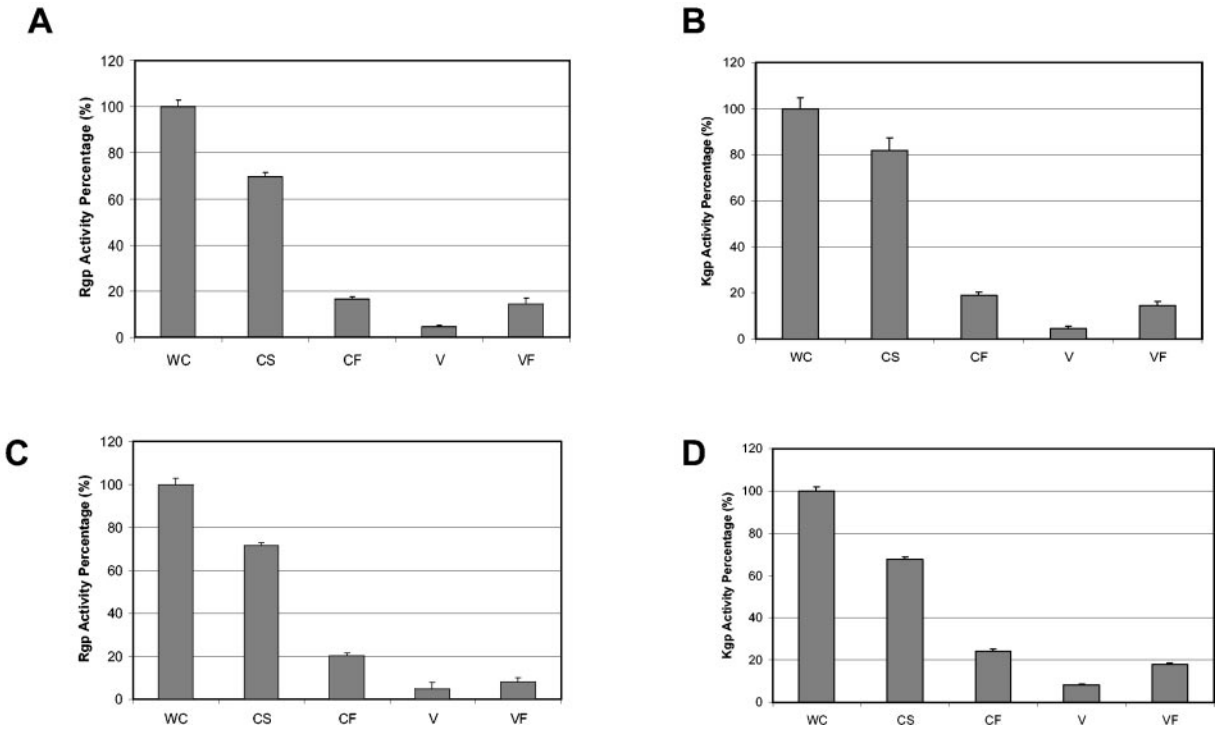

FIG. 5. Distribution of the proteases in *P. gingivalis* FLL93. Activities against BApNA (A and C) and ALNA (B and D) were tested in whole-cell culture (WC), cell-free medium (CF), cell suspension (CS), vesicle medium (V), and vesicle-free medium (VF) of *P. gingivalis* W83 (A and B) and *P. gingivalis* FLL93 (C and D) grown to stationary phase (OD_{600} of 1.4 to 1.5). The activity of WC for each strain was assumed to equal 100%. WC = CS + CF; CF = V + VF.

rgpA, and *kgp*), total RNA was isolated from the wild-type strain W83 and *P. gingivalis* FLL93 grown to stationary phase. Specific oligonucleotide primers as described in Table 2 for *rgpB*, *rgpA*, and *kgp* were used in RT-PCRs to amplify a predicted region of the gingipain gene transcripts. When the reverse transcriptase was present in the reaction, amplified products of the predicted size (0.8 kb for *rgpB* and 0.9 kb for *rgpA* and *kgp*) were observed for all three gingipain gene mRNA transcripts in both strains (Fig. 6).

Identification of gingipain proenzyme in *P. gingivalis* FLL93. The presence of mRNA transcript for the gingipain genes in *P. gingivalis* FLL93 may suggest a defect in gingipain biogenesis which could be similar to that of the *vimA*-defective (*P. gingivalis* FLL92) isogenic mutant previously reported (1). It was also shown previously that precipitation of extracellular proteins from *P. gingivalis* FLL92 with 37.5% acetone can enrich for the partially processed RgpB proenzyme (27). Extracellular fractions obtained from culture supernatants of *P. gingivalis* FLL93 grown to late exponential phase (OD_{600} of 0.8) were assessed by Western blot analysis using antibodies against the RgpB proenzyme. As shown in Fig. 7, the RgpB proenzyme-

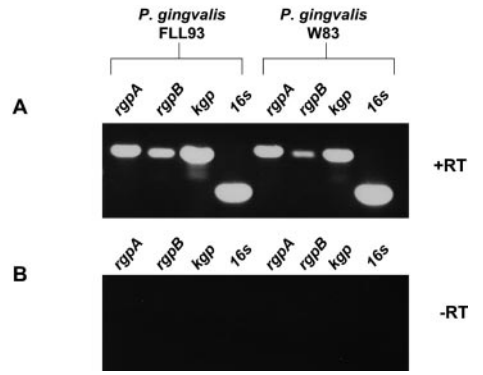

FIG. 6. Major gingipains are expressed in a *vimE*-defective mutant of *P. gingivalis*. DNase-treated total RNA extracted from *P. gingivalis* strains W83 and FLL93 grown to stationary phase was subjected to RT-PCR analysis.

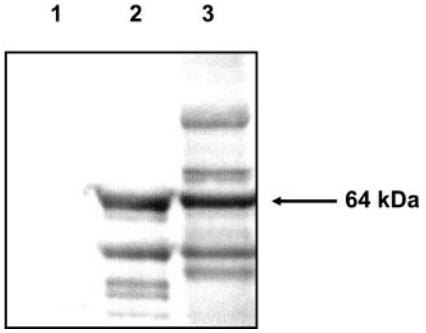

FIG. 7. Immunoreactivity of the RgpB proenzyme in *P. gingivalis* FLL93. Western blot analysis using specific RgpB proenzyme antibodies as probes was done on the extracellular fractions from *P. gingivalis* W83 (lane 1), FLL92 (lane 2), and FLL93 (lane 3). All lanes contained 20 μg of acetone (37.5%)-precipitated proteins from the supernatant fractions of cultures in BHI medium at exponential growth phase.

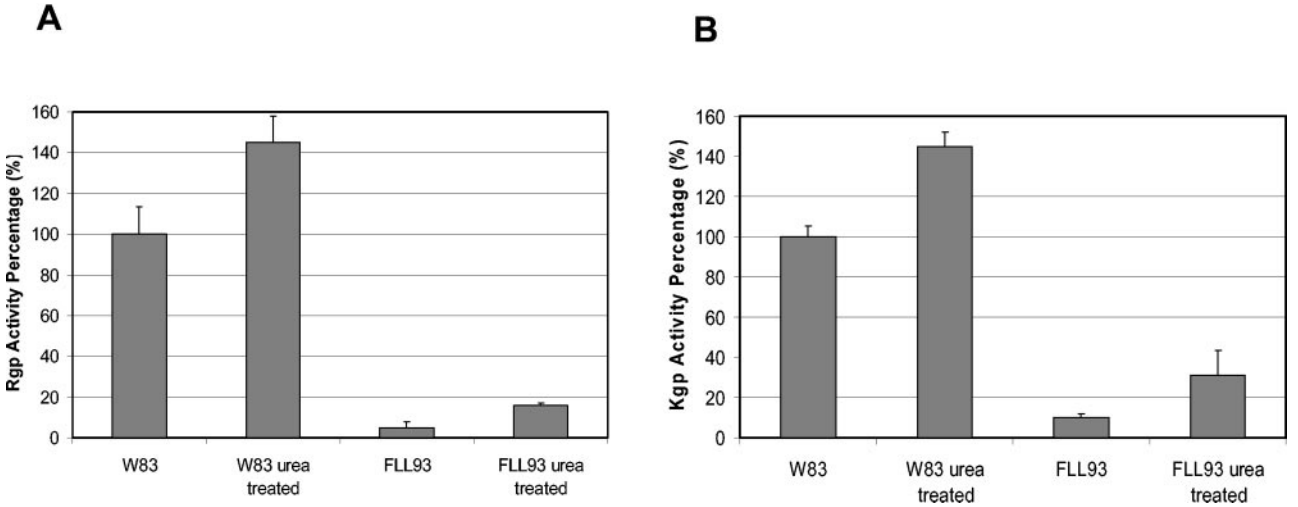

FIG. 8. Protease activation in the extracellular protein fraction from *P. gingivalis* FLL93. *P. gingivalis* was grown to stationary phase (OD_{600} of 1.3) in 1 liter of BHI broth supplemented with hemin and vitamin K. Acetone (37.5%)-precipitated proteins were mixed with 8 M urea and were incubated at 4°C for 1 h. The urea was slowly removed from the mixture by centrifugation (10,000 × *g*) in a Millipore filtration unit with the addition of increasing volumes of 100 mM Tris-HCl buffer (pH 7.4). Activities for Rgp and Kgp were tested. Error bars indicate the standard errors of the means of three independent trials.

specific antibodies revealed a 64-kDa immunoreactive band in *P. gingivalis* FLL93, which is similar in size to the RgpB proenzyme previously reported for the v*imA*-defective mutant FLL92 (27). It is also noteworthy that the antibodies do not immunoreact with any proteins present in the W83 extracellular fractions.

In vitro protease activation using urea. It was previously shown that in vitro protease activity could be induced by a urea denaturation-renaturation cycle in nonproteolytic extracellular protein fractions from *P. gingivalis* FLL92 grown to late exponential phase (27). This was consistent with the presence of a partially processed protease in those fractions. Proteolytic cleavage is a common mechanism that can generate an active enzyme from a larger proenzyme (17). While activation can be achieved by the action of other proteolytic enzymes, we cannot rule out the possibility that an inhibitor may be present at the catalytic site of the enzyme or the possibility that the tertiary structure of the proenzyme may be incompatible with activation. Because most proteins are denatured in the presence of high concentrations of urea and some will renature if the high concentration is slowly reduced, extracellular proteins from *P. gingivalis* FLL93 grown to late exponential phase or stationary phase were subjected to a urea denaturation-renaturation cycle. Figure 8 shows a slight activation of Rgp and Kgp in the extracellular protein fraction from *P. gingivalis* FLL93 grown to late exponential or stationary phase compared to activation of the wild type.

The Rgp active site is not labeled by DNS-EGR-CK in *P. gingivalis* FLL93. DNS-EGR-CK binds irreversibly to the active site of the arginine-specific proteases of *P. gingivalis* and can be detected by UV illumination following electrophoresis (3). To determine if the active site or catalytic domain is exposed or accessible in Arg-gingipain from *P. gingivalis* FLL93, extracellular fractions that were labeled with DNS-EGR-CK was subjected to SDS-PAGE and was analyzed with a UV transilluminator. As expected, DNS-EGR-CK-treated extracellular proteins from the wild-type strain generate fluorescent bands characteristic of the arginine-specific protease catalytic domain (Fig. 9). Similarly, DNS-EGR-CK treatment of extracellular proteins from *P. gingivalis* FLL92, grown to stationary phase, showed a 48-kDa fluorescent band (Fig. 9). However, there was no detectable specifically labeled protein that was similar in size to the 48-kDa band in the late-exponential- or stationary-phase extracellular protein fraction from *P. gingivalis* FLL93 (Fig. 9). There was also no detectable specifically labeled protein that was similar in size to the 64-kDa partially processed RgpB proenzyme in the late-exponential- or stationary-phase extracellular protein fraction.

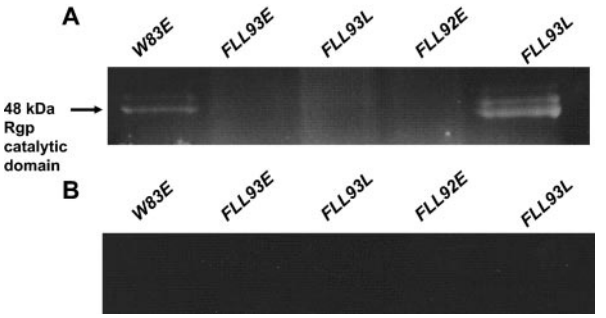

FIG. 9. The Rgp active site is not labeled by DNS-EGR-CK in *P. gingivalis* FLL93 extracellular fractions. Acetone-precipitated extracellular proteins from *P. gingivalis* W83, *P. gingivalis* FLL93, and *P. gingivalis* FLL92 were labeled with the fluorescent irreversible inhibitor DNS-EGR-CK and were subjected to SDS-PAGE on 4 to 12% NuPAGE gels (Invitrogen). The gels were viewed under UV light to visualize labeled proteins. (A) All the lanes were labeled with DNS-EGR-CK. (B) All the lanes were labeled with DNS-EGR-CK in the presence of 50 μM leupeptin. Each lane contains 20 μg of protein. The arrow indicates the DNS-EGR-CK-labeled protein band. E, exponential phase; L, stationary phase.

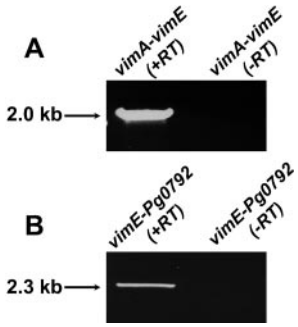

FIG. 10. *vimE* can be cotranscribed with *vimA* or *Pg0792.* One microgram of total DNase-treated RNA of *P. gingivalis* W83 from exponential phase (OD_{600} of 0.6) using a RiboPure kit (Ambion) was subjected to RT-PCR. (A) Cotranscription of *vimA* and *vimE.* Lane 1, P7 and P3 plus reverse transcriptase; lane 2, P7 and P3 minus reverse transcriptase. (B) Cotranscription of *vimE* and *Pg0792.* Lane 3, P1 to P5 plus reverse transcriptase; lane 4, P1 to P5 minus reverse transcriptase.

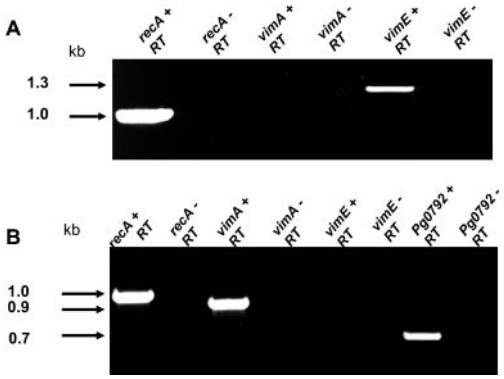

FIG. 11. *vimE* gene can be independently expressed. Total DNase-treated RNA was extracted from *P. gingivalis* FLL92 and *P. gingivalis* FLL93 grown to stationary phase (OD_{600} of 1.3) by using the RiboPure kit (Ambion). Samples (1 μg) were subjected to RT-PCR. (A) Expression of *vimA* flanking genes in *P. gingivalis* FLL92. (B) Expression of *vimE* flanking genes from *P. gingivalis* FLL93. +RT, with reverse transcriptase; −RT, without reverse transcriptase.

***vimE* can be independently expressed.** The similar phenotypic properties of the nonproteolytic *recA*-deficient mutant, *P. gingivalis* FLL32, and the *vimA*-defective mutant, *P. gingivalis* FLL92, may be explained by a polar effect (1). To determine if *vimE* is cotranscribed with *vimA*, RT-PCR using W83 RNA was performed with primers for *vimA* (P7) and *vimE* (P3). In the absence of reverse transcriptase, no amplified fragment was observed (Fig. 10). However, a 2.0-kb fragment was amplified, which is consistent with the expected size. To determine if *vimE* was cotranscribed with the downstream gene *Pg0792*, RT-PCR using W83 RNA and primers for *vimE* (P1) and *Pg0792* (P5) were used. As shown in Fig. 10B, a 2.3-kb fragment of the size expected was amplified. Taken together, these results indicate that *vimE* appears to be cotranscribed with *vimA* or *Pg0792* and may be a part of the *bcp-recA-vimA* transcriptional unit. In the absence of *vimE* there is little gingipain activation. Further, the late onset of gingipain activity in the *vimA*-defective mutant may suggest that the *vimE* gene could be independently expressed (1). RT-PCR of total RNA showed that the expected fragment for the *vimE* gene could be amplified from the *vimA*-defective mutant (Fig. 11A). In a similar experiment using RNA from the *vimE*-defective mutant, the downstream gene *Pg0792* was amplified (Fig. 11B).

DISCUSSION

We have used a genetic approach in this study to further assess the role of specific host factors in protease regulation/activation in *P. gingivalis*. Several recent studies (1, 12, 36; reviewed in reference 25) have identified nongingipain genes that are involved in the modulation of gingipain activity and other virulence factors in *P. gingivalis*. A comparison of several of the *P. gingivalis* mutants from these studies has raised the possibility of multiple mechanisms for gingipain activation. Mutation in the *vimA*, *porR*, or *gppX* gene has shown growth-phase-dependent activation of proteolytic activity that is mostly soluble (1, 12, 36). A mechanism of activation by these genes is presently not understood. Furthermore, it is unclear if they are part of a common pathway for gingipain activation. Although these mutants had a similar proteolytic profile, inactivation of each gene did not affect the expression of the others.

The *vimE* gene, which is downstream of *vimA*, does not appear to show any significant similarity to any known genes (www.oralgen.lanl.gov). *P. gingivalis* FLL93, the isogenic mutant defective in this gene, showed reduced proteolytic activity and reduced doubling time and was not black pigmented in this study. This phenotype of *P. gingivalis* FLL93 could be related to the reduced gingipain activity or, in particular, reduced membrane-associated activity. This would be similar to *P. gingivalis* FLL32, a *recA*-defective mutant (2), and *P. gingivalis* FLL92, a *vimA*-defective mutant (1). These results would also be consistent with other reports (8, 21, 28, 37) of the involvement of gingipains, especially Kgp, with hemoglobin binding, absorption, and heme accumulation.

Hemolysin activity appears to be modulated by the *vimE* gene in *P. gingivalis*. In this study, *P. gingivalis* FLL93, the *vimE*-defective mutant, showed no hemolytic activity when grown on blood plates or incubated with sheep erythrocytes. Because the protease Kgp has been shown to have hemoglobinase activity (21) and plays a role in erythrocyte degradation (34), our results are not surprising because of the reduced proteolytic activity in *P. gingivalis* FLL93. In addition to the effect of proteolytic activity on the hemolysin potential, several hemolysin-like genes have been identified by the *P. gingivalis* genome project (www.oralgen.lanl.gov) (26). Furthermore, there is genetic evidence for the involvement of two distinct hemolysins in the hemolysin activity of *P. gingivalis* (15). Taken together, these observations may suggest regulation of those genes or gene products by the *vimE* gene, although we cannot rule out the effect of the gingipains on their expression. There is evidence that the gingipains are involved in the processing of other proteins (14, 39).

The *vimE* gene in this study appears also to affect hemagglutination in *P. gingivalis*. Hemagglutination of sheep erythrocytes was reduced in *P. gingivalis* FLL93, the *vimE*-defective mutant. Again, these observations are not unexpected due to the reduced proteolytic activity of *P. gingivalis* FLL93. The proteases RgpA and Kgp have hemagglutinating activity (35).

Further, a monoclonal antibody (61BG1.3) that inhibited hemagglutination and selectively prevented the recolonization of *P. gingivalis* in periodontal patients was found to recognize a peptide within the adhesin domain encoded by *rgpA*, *kgp*, and *hagA* (5, 16). In addition to the association of the gingipains with hemagglutination in *P. gingivalis*, the presence of several genetically distinct genes, including hemagglutinin genes *hagB* and *hagC*, has been reported previously (20, 32). In this study, the effects of the *vimE* gene product on hemagglutination in *P. gingivalis* may also implicate the regulation of those genes or gene products by this gene.

Consistent with previous reports on two isogenic mutants (*P. gingivalis* FLL32, a *recA*-defective mutant, and *P. gingivalis* FLL92, a *vimA*-defective mutant) with reduced proteolytic activity, there was no detectable alteration of the gingipain genes in *P. gingivalis* FLL93. RgpB was secreted in an inactive form in the *vimA*-defective mutant, suggesting a role of the *vimA* gene in the posttranslational regulation of protease activity in *P. gingivalis* (27). A comparison of the extracellular proteins that used the RgpB proenzyme-specific antibodies revealed a 64-kDa immunoreactive band in *P. gingivalis* FLL93, which was similar in size to the partially processed RgpB proenzyme previously reported for the v*imA*-defective mutant *P. gingivalis* FLL92 (27). There was also an 80-kDa immunoreactive band which preliminary studies suggest is the full-length unprocessed RgpB that was absent in *P. gingivalis* W83 and *P. gingivalis* FLL92. Other bands may be intermediate products of the processing. In the process of maturation, the active site would become accessible. If there is a defect in this process in *P. gingivalis* FLL93, very little active-site labeling of the 48-kDa catalytic domain of RgpB by DNS-EGR-CK would be observed in cultures from exponential or stationary phase. As observed in this study, active-site labeling was undetected in *P. gingivalis* FLL93. This further confirms that there may be a defect in the activation/maturation of the gingipains. Collectively, these observations may suggest that the *vimE* gene product, similar to the case of VimA, is involved in the posttranslational regulation of protease activity in *P. gingivalis*.

An active enzyme can be generated from a larger polypeptide by autoprocessing or by the action of other proteolytic enzymes (17, 31). Several cysteine proteases are converted to their active forms by removal of the prosegment by an autocatalytic mechanism (17). There is accumulating evidence that a multicomponent maturation pathway(s), perhaps including an autolytic mechanism, may be involved in the production of Arg-X- and Lys-X-specific proteases in *P. gingivalis* (3, 24, 33). While it is clear that proteolytic processing of the gingipain precursors in *P. gingivalis* is required to produce the isoforms detected, it has been shown that denaturing and refolding of proteins can also induce the autoprocessing of RgpB (27). We have shown that there is very little activation after urea treatment in the *vimA*-defective mutant extracellular fraction. The presence of an 80-kDa proenzyme immunoreacting band, which was absent in the *vimA* mutant, may indicate that *vimE* is needed for the initial processing of RgpB. The processing of the 64-kDa RgpB proenzyme intermediate in *vimA* mutant could have been enhanced by the denaturing-renaturing experiments (27).

The distribution of the proteolytic activity in *P. gingivalis* FLL93 was similar to that of the wild-type strain and was in contrast to that of *P. gingivalis* FLL92, the *vimA*-defective mutant, that had mostly soluble proteolytic activity with little or no cell-associated activity (1, 27). While there was a unique late onset of Arg-X- and Lys-X-specific proteolytic activity in *P. gingivalis* FLL92 (27), there was little or no observed change of proteolytic activity in stationary phase in *P. gingivalis* FLL93, the *vimE*-defective mutant. Collectively, our observations could support a hypothesis that suggests that the regulation of proteolytic activity in *P. gingivalis* may occur by multiple mechanisms. During exponential growth phase, proteolytic activity in *P. gingivalis* may be regulated in a *vimA*-dependent manner. However, during stationary phase, an alternative pathway for protease activation may be upregulated to overcome or bypass the *vimA* mutation or may be activated in the absence of VimA. In addition, in the absence of the *vimE* gene product, as observed in this study, there is minimal activation of proteolytic activity during exponential or stationary growth phase. The specific functional role of VimE during protease activation is unclear and is being further investigated in our laboratory.

The *vimE* gene appears to be cotranscribed with either *vimA* or *Pg0792* and may be a part of the *bcp-recA-vimA* transcriptional unit. Further, its inactivation did not have any polar effect on the expression of the downstream gene. Because both the *vimE* and *vimA* genes have a similar effect on protease biogenesis in *P. gingivalis*, it is likely that they may be a part of the same or different pathway(s) that is involved in protein maturation, including protease activation/maturation. Presently, there is no information on the regulation of these genes. Because gingipain activation in the *vimE*-defective mutant is not growth phase related and because that gene product is required for activation/maturation, both *vimA* and *vimE* genes could be regulated differently. This question is under further evaluated in our laboratory.

The effect of proteolytic activity on virulence in *P. gingivalis* is well documented (7, 13, 18, 29). Inactivation of the gingipain genes has been demonstrated to reduce the virulence potential of *P. gingivalis*. Thus, modulation of virulence in *P. gingivalis* may be coordinated via an ability to modulate proteolytic activity. Although not directly tested in this study, the *vimE* gene may be an important virulence gene. Because inactivation of the *vimE* gene resulted in reduction in proteolytic activity and had a pleiotropic effect on other important virulence factors, *P. gingivalis* FLL93 would be expected to have a reduced pathogenic potential, in contrast to the wild-type strain. This would be consistent with the *vimA*-defective mutant that had a phenotype similar to that of *P. gingivalis* FLL93, and it was dramatically less virulent than the wild-type strain in the mouse model (1).

In summary, we have constructed an isogenic mutant of *P. gingivalis* that is defective in the *vimE* gene downstream of the *bcp-recA-vimA* transcriptional unit. While this mutant had reduced proteolytic activity, there was no detectable reduction of the gingipain gene transcription. Further, this mutant, in contrast to the wild-type strain, showed reduced hemolytic and hemagglutinating activities. The *vimE* gene appears to be independently expressed, and its identification represents a potentially new mechanism for regulating proteolytic activity and virulence in *P. gingivalis*. It is unclear, however, if there is any interaction between the *vimE* and *vimA* gene products; this is under further investigation in the laboratory.

ACKNOWLEDGMENTS

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