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Inactivation of *vimF*, a Putative Glycosyltransferase Gene Downstream of *vimE*, Alters Glycosylation and Activation of the Gingipains in *Porphyromonas gingivalis* W83

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Regulation/activation of the *Porphyromonas gingivalis* gingipains is poorly understood. A 1.2-kb open reading frame, a putative glycosyltransferase, downstream of *vimE*, was cloned, insertionally inactivated using the *ermF-ermAM* antibiotic resistance cassette, and used to create a defective mutant by allelic exchange. In contrast to the wild-type W83 strain, this mutant, designated *P. gingivalis* FLL95, was nonpigmented and nonhemolytic when plated on Brucella blood agar. Arginine- and lysine-specific gingipain activities were reduced by approximately 97% and 96%, respectively, relative to that of the parent strain. These activities were unaffected by the growth phase, in contrast to the *vimA*-defective mutant *P. gingivalis* FLL92. Expression of the *rgpA*, *rgpB*, and *kgp* gingipain genes was unaffected in *P. gingivalis* FLL95 in comparison to the wild-type strain. In nonactive gingipain extracellular protein fractions, multiple high-molecular-weight proteins immunoreacted with gingipain-specific antibodies. The specific gingipain-associated sugar moiety recognized by monoclonal antibody 1B5 was absent in FLL95. Taken together, these results suggest that the *vimE* downstream gene, designated *vimF* (*vi*rulence *m*odulating gene *F*), which is a putative glycosyltransferase group 1, is involved in the regulation of the major virulence factors of *P. gingivalis*.

As a common theme, the expression of extracellular proteolytic activities is highly regulated in both prokaryotic and eukaryotic systems (reviewed in references 11 and 53). This regulation can occur at multiple levels including expression of the protease genes, secretion, processing of an inactive secreted precursor to its active form, and/or the posttranslational glycosylation of the proteins (16, 49). 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 (reviewed in references 26, 32, and 48). While several virulence factors have been implicated in the pathogenicity of *P. gingivalis*, the high proteolytic abilities of this organism are the focus of much attention as they are considered to play the most significant role in virulence (34). The major proteases, called gingipains, are both extracellular and cell associated. They consist of arginine-specific proteases (Arg-gingipain [Rgp]) and lysine-specific protease (Lys-gingipain [Kgp]) (34). Although glycosylation appears to be important for their activation (reviewed in references 16 and 41), there remains a gap in our knowledge about the regulation/activation of the *P. gingivalis* gingipains.

Previously, we have reported that the *recA* locus can affect the phenotypic expression and distribution of the gingipains in *P. gingivalis* (1, 2, 37). Using the cloned *vimA* gene, which is downstream of the *recA* gene and part of the *bcp-recA-vimA* transcriptional unit, a defective mutant was constructed by allelic exchange (1). The mutant strain, designated FLL92, was non-black-pigmented and showed increased autoaggregration in addition to a significant reduction in proteolytic, hemolytic, and hemagglutinating activities (1). Expression of the *rgpA*, *rgpB*, and *kgp* gingipain genes in *P. gingivalis* FLL92 was similar to expression in the wild-type strain (1). Furthermore, the partially processed RgpB proenzyme was secreted in *P. gingivalis* FLL92 (37). The appearance of the gingipain proenzyme forms and the growth phase-dependent activation of proteolytic (37) activity have raised the possibility of multiple mechanisms for gingipain activation. In other studies, we have also demonstrated that another gene, designated *vimE*, which is downstream of the *vimA* gene, is important in gingipain activation in *P. gingivalis*. Inactivation of this gene resulted in a similar phenotype as the *vimA*-defective mutant; however, gingipain activities were unaffected by the growth phase. Furthermore, in contrast to the *vimA*-defective mutant *P. gingivalis* FLL92, the gingipains, although inactive, were membrane associated in strain FLL93, the *vimE*-defective mutant (51). It has been suggested that *vimE* is needed for proper carbohydrate biogenesis (51). Collectively, these observations suggest that multiple bacteria-specific factors in *P. gingivalis* are involved in gingipain biogenesis.

Glycosylation, which is one of the important means in which protein maturation and other cellular processes are regulated, may be important in gingipain biogenesis in *P. gingivalis*. In this process, different glycosyltransferases catalyze the transfer of different carbohydrate moieties from active donors to specific acceptors (including lipids, proteins, and nucleic acids) (9). Glycosyltransferases have been classified on the basis of the reaction catalyzed, substrate specificity, and homology to other glycosyltransferases (9). In eukaryotes, it has been established that glycosylation occurs in the endoplasmic reticulum or Golgi apparatus (16, 20). However, the localization or the mechanism of glycosylation in prokaryotes has yet to be established.

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TABLE 1. Plasmids and bacterial strains used in this study

Plasmid or strain	Phenotype and description	Source
Plasmids		
pCR-2.1 TOPO	Ap^r, Km^r	Invitrogen
pFLL80	pCR 2.1-TOPO PG0791:PG0792	50
pFLL86	pUC 19: PG0791:PG0792	This study
pFLL87	pUC19: PG0791:PG0792:: *ermF-ermAM*	This study
pVA2198	Sp^r, *ermF-ermAM*	15
Bacterial strains		
P. gingivalis		
W83	Wild type	1
FLL93	*vimE*-defective	50
FLL95	*vimF*-defective	This study
E. coli		
DH5α	F^-ϕ80d*lacZ*Δ M15 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* (StrR) *endA1 nupG*	Invitrogen

It has been shown in eukaryotes that the glycosylation of proteins plays a role in protein folding, protein stabilization, and protein conformation by sugar-sugar interaction or sugar-protein interactions (8, 16, 20). In addition, it plays a regulatory role in the activation of protein precursors or zymogens, resulting in active proteins. Only recently, the roles of glycosylation and glycosyltransferases in prokaryotes have been shown to be involved in the attachment of membrane proteins to bacterial membrane, for fimbriae maturation, host-cell adhesion, and also maturation of bacterial proteins (5, 43, 49).

In this report we have investigated a 1.2-kb gene downstream of the *vimE* gene to determine its role in protease activation in *P. gingivalis*. This gene, which shares homology with glycosyltransferase 1 genes from several bacteria, was inactivated in *P. gingivalis* by allelic exchange mutagenesis. The *P. gingivalis* isogenic mutant designated FLL95 exhibited reduced Arg-X- and Lys-X-specific proteolytic activities that were not affected by the phase of growth. The glycosylation of the gingipains was altered in this mutant. These results suggest an important role for this putative glycosyltransferase gene, now designated *vimF*, in protease maturation/activation in *P. gingivalis* and further confirm the requirement of multiple specific host factors in this process.

MATERIALS AND METHODS

Bacteria 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 (Difco Laboratories, Detroit, MI) supplemented with hemin (5 μg/ml), vitamin K (0.5 μg/ml), and cysteine (0.1%). *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, MI) 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, 50 to 100 μg/ml.

Bioinformatic analysis of *vimF*. The protein sequence of *vimF*, obtained from the *P. gingivalis* genome (www.oralgen.lanl.gov), was analyzed for any homology to other proteins or any predicted class or functions using the analysis tool functions at the website. Clusters of orthologous groups (COG) searches, Pfam protein family database searches (with a cutoff strategy using an E value of 1.0), and BLAST searches were performed at the website.

DNA isolation and analysis. *P. gingivalis* chromosomal DNA was prepared by the method of Marmur (31). For plasmid DNA analysis, DNA extraction was performed by the alkaline lysis procedure of Birnboim and Doly (6). For large-scale preparation, plasmids were purified using the QIAGEN (Santa Clarita, CA) plasmid maxi kit.

Generation of *vimF* mutant *P. gingivalis* strain. A 2.5-kb fragment carrying the intact *vimE* and *vimF* downstream genes was amplified by PCR using the P1 and P6 oligonucleotide primers (Table 2). This fragment was cloned into the pCR 2.1-Topo plasmid vector (Invitrogen, Carlsbad, CA) and designated pFLL80 (51). The 2.5-kb fragment was then isolated from the EcoRI-digested pFLL80 and ligated to pUC19 linearized with EcoR1. The new recombinant plasmid was designated pFLL86. Orientation was confirmed by restriction analysis. The *ermF-ermAM* cassette which confers erythromycin/clindamycin resistance in *E. coli* and *P. gingivalis* (15) was PCR amplified from pVA2198 using Pfu Turbo (Stratagene, La Jolla, CA) and inserted into the EcoRV restriction site of the *vimF* gene. The resultant recombinant plasmid, pFLL87, was used as a donor in electroporation of *P. gingivalis* W83 as previously reported (15).

Hemagglutination studies. Hemagglutinin activity was determined as previously reported (17). Twenty-four-hour cultures of *P. gingivalis* W83, FLL93, and FLL95 cells were harvested by centrifugation (10,000 × *g* for 15 min). Cells were washed twice in 1× phosphate buffered saline (PBS; 0.147 m NaCl, 0.01 M sodium phosphate) and resuspended to a final OD_{600} of 1.5. Sheep erythrocytes were washed twice with PBS and resuspended in 1% in PBS. An aliquot (100-μl volume) of the bacterial suspension was serially diluted twofold with PBS in round-bottom 96-well microtiter plates. An equal volume (100 μl) of 1% sheep erythrocytes was mixed with each dilution and 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.

Preparation of *P. gingivalis* extracellular fractions and protease assays. One-liter cultures of *P. gingivalis* strains FLL93, FLL95, and W83 were grown for 24 h

TABLE 2. Primers used in this study

Primer	Description	Sequence
P1	*vimE* forward	5′ ATGAATACTGAAGTAA TCCAC 3′
P2	*vimE* reverse	5′ TTGGTCGATGGCCGTT TCGTA 3′
P3	*vimE* reverse (intragenic)	5′ TCAGCACTTCCTTGCA CAAC 3′
P4	*vimF* (forward)	5′ ATTCTACAAAGAAGCG GGGA 3′
P5	*vimF* reverse (intragenic)	5′GCAGGATGGAGCGGG AGTATTC 3′
P6	*vimF* reverse	5′ TTCACGCGAAAAAGT GGAGAT 3′
P7	*vimA* forward	5′ ATGCCCATCCCTCTAT ACCTG 3′
P8	*vimA* reverse	5′ TACCTGTTTTTGCTGA CCGG 3′
P10	*pro-kgp* forward	5′ ATGAGGAAATTATTAT TGCTGATCG 3′
P11	*pro-kgp* reverse	5′ TTGAAGAGCTGTTTAT AAGCTGTTT 3′
P12	*pro-rgpA* forward	5′ ATGAAAAACTTGAAC AAGTTTGTTTC 3′
P13	*pro-rgpA* reverse	5′ CCGGTGTGTAACGC CCTG 3′
P14	*pro-rgpB* forward	5′ CGACCGATGAAGAC TTCG 3′
P15	*pro-rgpB* reverse	5′ TGAAGACGCTCTTATA GATATCTT 3′
P16	*erythromycin* forward	5′ TATTAGGCCTATAGCT TCCGCTATT 3′
P17	*erythromycin* reverse	5′ AATAGGCCTTAGTAAC GTGTAACTTT 3′

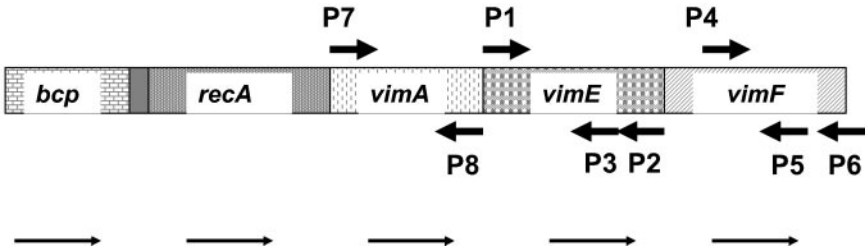

FIG. 1. Diagram of the *recA* locus and the two downstream genes. Diagram shows open reading frames (taken from www.oralgen.lanl.gov.) of the *recA* locus and the two downstream genes, *vimE* and *vimF*. Open reading frames are shown with given primer locations. Arrows indicate gene orientation.

in BHI supplemented with hemin, cysteine, and yeast extract. Cells were harvested by centrifugation at 10,000 × *g* for 30 min. The cell-free culture fluid was precipitated with cold (−20°C) 37.5% acetone, 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 with a microplate reader (Bio-Rad Laboratories, Hercules, CA) as previously reported (44).

Preparation of membrane fraction. One-liter cultures of *P. gingivalis* strains FLL93, FLL95, and W83 were grown to OD_{600} of 1.3 to 1.4. Cells were harvested by centrifugation at 10,000 × *g* for 30 min. Membrane fractions were prepared by lysing the cells using a French pressure cell press (American Instrument Company, Silver Spring, MD) for three cycles at 109 MPa. The lysed cells were then centrifuged at 27,000 × *g* for 1 h. The supernatant was subjected to ultracentrifugation at 100,000 × *g* for 1 h. The pellet, designated as the membrane fraction, was resuspended in 100 mM Tris HCl, pH 7.4, containing 1 mM *N*α-*p*-tosyl-L-lysine chloromethyl ketone. The remaining supernatant was considered the cytosolic fraction.

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

SDS-PAGE and immunoblot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a 4 to 12% bis-Tris separating gel in MOPS (morpholinepropanesulfonic acid)-SDS running buffer (NuPAGE Novex gels; Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Samples were prepared (65% sample, 25% 4× NuPAGE LDS sample buffer, and 10% NuPAGE reducing agent), heated at 72°C for 10 min, and then electrophoresed at 200 V for 65 min with the XCell SureLock Mini-Cell (Invitrogen, Carlsbad, CA). The separated proteins were then transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) 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 (40) or HagA-specific antibodies (generously donated by the Progulske-Fox group). Immunoreactive proteins were detected by the procedure described in the Western Lightning Chemiluminescence Reagent Plus kit (Perkin-Elmer Life Sciences, Boston, MA). The secondary antibody was immunoglobulin G (heavy plus light chains)-horseradish peroxidase conjugate (Zymed Laboratories, Inc., South San Francisco, CA).

Fibronectin cleavage using *P. gingivalis*. *P. gingivalis* W83 and isogenic mutants FLL92, FLL93, and FLL95 cells grown to early stationary phase (OD_{600} of 1.1) were harvested, washed, and resuspended in PBS to a final OD_{600} of 1.5. Fibronectin (75 μg) resuspended in PBS was incubated for 30 min at 37°C with 50 μl of *P. gingivalis* strains. The reaction mixture volume was 500 μl. After a 30-min incubation, reaction samples were centrifuged at 10,000 × *g* for 5 min, and cell-free supernatant from samples was analyzed by SDS-PAGE and stained with Simply Blue Safe Stain (Invitrogen, Carlsbad, CA).

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

LPS and polysaccharide isolation and silver staining. Overnight cultures of *P. gingivalis* W83 and FLL95 were grown to stationary phase. Isolation of lipopolysaccharides (LPS) was done according to the manufacturer's protocol (Intron Biotechnology, Republic of Korea). Polysaccharides (PS) were separated from lipid A by treating the LPS preparations with 2% acetic acid for 2 h at 100°C. The soluble fraction containing the PS was purified using a Superdex 75 column (Amersham Biosciences, Piscataway, NJ). LPS were subjected to SDS-PAGE on 4 to 12% bis-Tris gel using the MOPS running buffer. PS were subjected to SDS-PAGE on 4 to 12% bis-Tris gel using the morpholineethanesulfonic acid (MES) running buffer. LPS and PS were visualized using a SilverQuest Silver Staining Kit according to manufacturer's instructions (Invitrogen, Carlsbad, CA).

RESULTS

Bioinformatic analysis of VimF. It has been demonstrated that the *vimA* and *vimE* genes play important roles in gingipain maturation/activation (50, 51). It has also been reported that *vimE* can be cotranscribed with *vimF* and *vimA* (50). In addition, it has been shown that the *vimA* and *vimE* mutants display carbohydrate biogenesis defects, which may be correlated with the altered gingipain maturation/activation (51). Thus, it is our hypothesis that inactivation of a glycosyltransferase needed for modification of the gingipains would result in a phenotype similar to what is seen in the *vimA*- and/or *vimE*-defective mutants. The *vimE* downstream gene, *vimF*, may play a role in gingipain maturation/activation. A COG search on the gene sequence of *vimF* (Fig. 1) from www.ncbi.nih.gov revealed a COG of glycosyltransferase type 1 (COG0438) of class M, which is involved in cell envelope and outer membrane biogenesis. In addition, PSI (position-specific iterating) or local BLAST searches from tool analysis function on the www.oralgen.lanl.gov website showed that *vimF* has homology to other glycosyltransferases including those from the organisms *Methanococcus* sp. (26% identity; 42% positives), *Geobacter metallireducence* (26% identity; 42% positives), *Haloarcula marismortui* (24% identity; 44% positives), *Bacillus cereus* (24% identity; 42% positives), and *Bacillus anthracis* (24% identity; 41% positives) (PSI-BLAST). A Pfam search of *vimF* showed a model and description of glycosyltransferase group 1 having a significant E value of less than 0.5.

Inactivation of the *vimF* gene in *P. gingivalis* W83 by allelic exchange mutagenesis. Isogenic mutants of *P. gingivalis* W83 defective in the PG0792 gene (designated *vimF*) were constructed by allelic exchange mutagenesis. The circular recombinant plasmid pFLL87, which carries the *ermF-ermAM* cassette in the unique EcoRV restriction site (bp 909 of the open reading frame) of the *vimF* gene, was used as a donor in electroporation of *P. gingivalis* W83 (Fig. 2A). Following electroporation and plating on selective medium (BHI containing 10 μg/ml erythromycin), we detected approximately 89 erythromycin-resistant colonies after a 5-day incubation period. To compare their phenotypic properties with those of wild-type strain W83, 20 mutants were plated on *Brucella* blood agar

plates. In contrast to the wild-type strain, all selected mutants displayed a non-black-pigmented, non-beta-hemolytic phenotype.

Confirmation of inactivation of *vimF* by PCR analysis. Chromosomal DNA from two randomly chosen clindamycin/erythromycin-resistant colonies and the wild-type W83 strain were analyzed by PCR to confirm the inactivation in the *vimF* gene. If the *vimF* gene was interrupted by the *ermF-ermAM* cassette, a 3.1-kb fragment was expected to be amplified using *vimF* primers P4 and P5 (Table 2). In addition, a 2.1-kb fragment should be amplified from the mutant using the *ermF-ermAM* primers (Table 2). The expected 3.1-kb (using *vimF* primers) and 2.1-kb (using erythromycin primers) fragments were observed only in the erythromycin-resistant strains (Fig. 2B) in contrast to the wild-type (data not shown). The orientation of the *ermF-ermAM* cassette was also confirmed by restriction digest (data not shown). To further confirm the absence of the *vimF* transcript in the erythromycin-resistant mutant, DNase treated RNA for *P. gingivalis* FLL95 was subjected to RT-PCR. As shown in Fig. 2C, no *vimF* transcript was detected from the mutant in comparison to the wild-type W83. Using *kgp*-specific primers (Table 2) as a control, the expected 0.8-kb fragment was amplified from all the *P. gingivalis* strains. There was no amplified fragment observed from reactions in the absence of reverse transcriptase. Taken together, these results indicated that the insertional inactivation of the chromosomal *vimF* gene with the 2.1-kb *ermF-ermAM* antibiotic cassette was successful. One mutant designated *P. gingivalis* FLL95 was randomly chosen for further study. In both the wild-type and the *vimF*-defective mutant, a generation time of 3 h was determined.

The *vimE* and *vimA* genes are transcribed in *P. gingivalis* FLL95. It has been shown that *vimE* and its downstream gene, *vimF*, can be cotranscribed (50). To ensure that the inactivation of the *vimF* gene did not prevent the transcription of *vimE* or *vimA*, DNase-treated RNA from the *P. gingivalis* isogenic mutant and wild type was subjected to RT-PCR. As shown in Fig. 3, the *vimA* and *vimE* fragments were amplified from the *vimF*-defective mutant in comparison to the wild-type W83. No amplified fragments were observed for either the wild-type or the mutant when reverse transcriptase was absent from the reaction mixture. Using 16S RNA-specific primers as a positive control, fragments of expected size were amplified from both the wild-type and the isogenic mutant.

Hemagglutinin activity in *P. gingivalis* FLL95. We assessed the hemagglutination potential of *P. gingivalis* FLL95 in comparison to *P. gingivalis* W83 and *P. gingivalis* FLL93, the nonproteolytic *vimE*-defective mutant. In contrast to the wild-type strain, there was a reduction in hemagglutinin activity in *P. gingivalis* FLL95 (Fig. 4A). This level of activity was similar to *P. gingivalis* FLL93 and the negative control as previously reported (51). Immunoblot analysis of membrane and extracellular fractions using monoclonal antibodies raised against HagA showed the presence of multiple high-molecular-weight immunoreactive bands ranging between 45 kDa to >191 kDa in the *vimE*- and *vimF*-defective mutant membrane fractions (Fig. 4B) and immunoreactive bands ranging between 25 to 185 kDa in the extracellular fraction (Fig. 4B). However, lower immunoreactive bands ranging between 19 to 64 kDa were observed in the wild-type membrane and extracellular fractions (Fig. 4B).

Inactivation of the *vimE* gene affects proteolytic activity in *P. gingivalis*. We previously reported that the proteolytic activity of the non-black-pigmented *recA*-defective mutant (*P. gingivalis* FLL32), the *vimA*-defective mutant (*P. gingivalis* FLL92), or the *vimE*-defective mutant (FLL93) was reduced by more that 90% in comparison to the wild-type strain (1, 2, 50). In addition, a late onset of proteolytic activity was also reported in the *vimA* mutant relative to the wild-type strain (37). Because hemagglutinin activities can be associated with gingipain activity in this organism (28, 45), *P. gingivalis* FLL95 was assayed for proteolytic activity using *N*-α-benzoyl-DL-arginine-*p*-nitroanilide for Rgp activity and acetyl-lysine-*p*-nitroanilide for Kgp activity. In late-exponential growth-phase cultures, both Arg-X and Lys-X protease activity levels in *P. gingivalis* FLL95 were very similar to activity in *P. gingivalis* FLL93 (51), which was reduced by approximately 94 to 96% compared to the wild-type strain (data not shown). In stationary phase cultures of *P. gingivalis* FLL95, there was a slight increase in Lys-X activity relative to Rgp activity (Fig. 5) and similar to that of FLL93 (51). This is in contrast to the expected late onset of proteolytic activity that was observed in *P. gingivalis* FLL92 (37). Taken together, these data suggest that under the same physiological conditions, the proteolytic profile for *P. gingivalis* FLL95 is dramatically altered by inactivation of the *vimF* gene in *P. gingivalis*.

Expression of the gingipain genes in the *vimF*-defective mutant. The reduced proteolytic activity in the *P. gingivalis* FLL95 strain could have been the result of an alteration in the transcription of the gingipain genes. There is also the possibility that the *vimF* gene may be involved in the posttranslational regulation of gingipain expression. In previous reports of mutations in two genes (*vimA* and *vimE*) upstream of the *vimF* gene, a reduction in gingipain activity was observed although the gingipains were normally expressed (1, 2, 50). Furthermore, the partially processed RgpB gingipain proenzyme was identified in the *vimA*-defective mutant, suggesting a defect in gingipain biogenesis at the posttranslational level (37). To determine the presence of mRNA transcripts for the gingipain genes (*rgpB*, *rgpA*, and *kgp*), total RNA was isolated from the wild-type W83 strain 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-PCR to amplify a predicted region of the 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 transcripts in both strains (Fig. 6). This suggests that the gingipains were being expressed in *P. gingivalis* FLL95 in comparison to the wild type.

Extracellular proteins from *P. gingivalis* FLL95. Even though there was a dramatic reduction in proteolytic activity in *P. gingivalis* FLL95, the gingipains appeared to be expressed. This may suggest a posttranscriptional regulation of gingipain maturation/activation. To examine any changes in extracellular proteins from *P. gingivalis* FLL95 in comparison to the wild-type W83 and *vimE*-defective mutant FLL93, extracellular fractions from stationary growth phase of all three strains were analyzed by SDS-PAGE. As shown in Fig. 7A, there were multiple high-molecular-weight protein bands in *P. gingivalis*

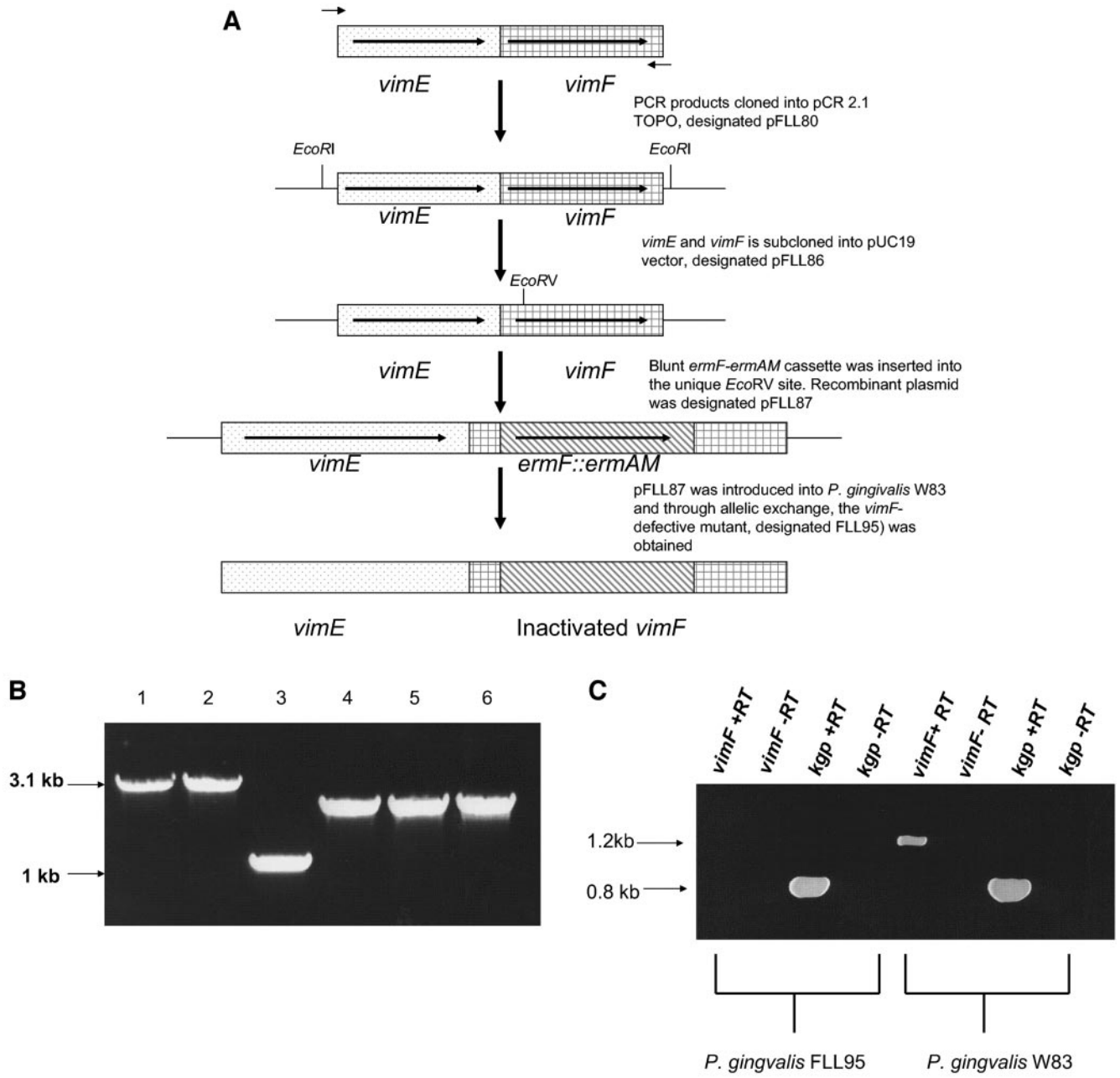

FIG. 2. Construction and confirmation of the *vimF*-defective mutant. (A) Construction of mutant. Plasmid pFLL87 contains the *vimF* gene interrupted by an *ermF-ermAM* cassette (the *vimF* gene with flanking sequences was amplified by PCR; the *ermF-ermAM* cassette was purified from pVA2198). The circular recombinant plasmid pFLL87 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 pFLL87 replaced the intact *vimF* with an inactivated copy. (B) PCR analysis of allelic exchange mutants of *P. gingivalis* carrying the *vimF* gene inactivated with the *ermF-ermAM* cassette. Oligonucleotide primers specific for the *vimF* gene or erythromycin cassette were used to amplify that gene or erythromycin from total chromosomal DNA from *P. gingivalis*. Lane 1, *P. gingivalis* FLL95.1 (*vimF*::*ermF-ermAM*; *vimF* primers); lane 2, *P. gingivalis* FLL95.2 (*vimF*::*ermF-ermAM*; *vimF* primers); lane 3, *P. gingivalis* W83 (wild type); lane 4, *P. gingivalis* FLL95.1 (*vimF*::*ermF-ermAM*; erythromycin primers); lane 5, *P. gingivalis* FLL95.2 (*vimF*::*ermF-ermAM*; erythromycin primers); lane 6, pVA2198 plasmid (plasmid carrying the erythromycin cassette; erythromycin primers) as control. (C) Transcriptional profile of *vimF*-defective mutant. Total RNA isolated from *P. gingivalis* W83 and the *vimF*-defective mutant isolated from stationary phase (OD_{600} of 1.4 to 1.5) was subjected to RT-PCR using primers for *vimF* and *pro-kgp*. No transcription or *vimF* was detected in *P. gingivalis* FLL95.

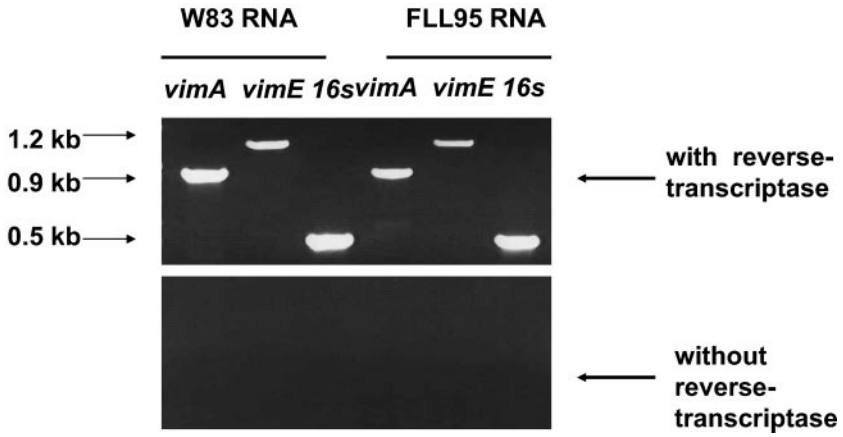

FIG. 3. Expression of *vimE* and *vimA* in *vimF*-defective mutant. Total RNA isolated from *P. gingivalis* W83 and the *vimF*-defective mutant was subjected to RT-PCR using primers for *vimE*, *vimA*, and 16S RNA as a control.

FLL95 that were absent in the wild type. Specific antibodies against RgpB, RgpA, and Kgp were used to determine the presence of the gingipain immunoreactive proteins in *P. gingivalis* FLL95. Similar to the *vimE*-defective mutant, the extracellular proteins from *P. gingivalis* FLL95 showed multiple unique immunoreactive bands to RgpB (Fig. 7B), RgpA (Fig. 7C), and Kgp (Fig. 7D) in contrast to the wild-type strain. A 48-kDa band representing the catalytic domain of RgpB was detected in the wild-type strain but was absent in *P. gingivalis* FLL95 (Fig. 7B). It is noteworthy that other immunoreactive bands representing the processed mature gingipains were absent in *P. gingivalis* FLL93 unlike the *vimA*-defective mutant. Similar to the *vimE*-defective mutant, when the *vimF*-defective mutant was probed with antibodies against RgpA, high-molecular-weight protein bands were seen. A 185-kDa band that was absent in the *vimF* mutant was present in the *vimE* mutant (Fig. 7C). Also, immunoblot analysis using anti-RgpB antibodies showed an 80-kDa immunoreacting band consistent with the size of the RgpB proenzyme (Fig. 7B).

Membrane protein profile of *P. gingivalis* FLL95. Gingipain-dependent protein processing is documented in *P. gingivalis* (22). Thus, a reduction in functional or active gingipains may result in unprocessed proteins on the membrane of *P. gingivalis*. As shown in Fig. 8A, SDS-PAGE analysis of membrane preparations from *P. gingivalis* strains W83, FLL93, and FLL95 shows that there are multiple high-molecular-weight protein bands that are present in the membrane preparation of *P. gingivalis* FLL93 and FLL95 that are missing in the wild type. To determine the presence of the inactive cell-associated gingipain species in *P. gingivalis* FLL95, membrane preparations of *P. gingivalis* W83, FLL93, and FLL95 were immunoreacted with antibodies against RgpA, RgpB, and Kgp. As shown in

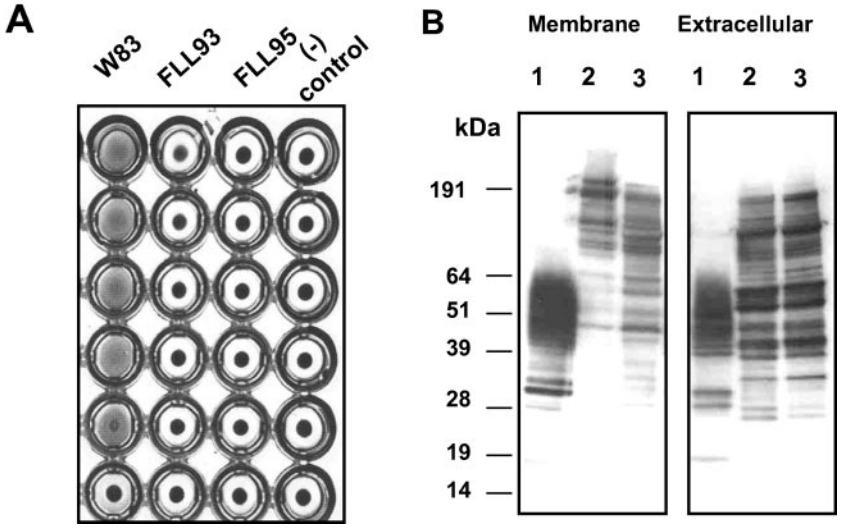

FIG. 4. Hemagglutinin analysis of the *vimF*-defective mutant. (A) Hemagglutinin activities of *P. gingivalis* were assessed in *P. gingivalis* W83, FLL93, and FLL95 cells serially diluted and incubated with sheep erythrocytes at 4°C for 3 h in a round-bottom microtiter plate. The titer was defined as the last dilution that showed full agglutination. (B) Membrane and extracellular fraction preparations were assessed as described for *P. gingivalis* W83, FLL93, and FLL95. Proteins were analyzed by immunoblot analysis using monoclonal antibodies raised against hemagglutinin protein HagA. Lanes 1, *P. gingivalis* W83; lanes 2, *P. gingivalis* FLL93; lanes 3, *P. gingivalis* FLL95. Molecular mass markers are at left.

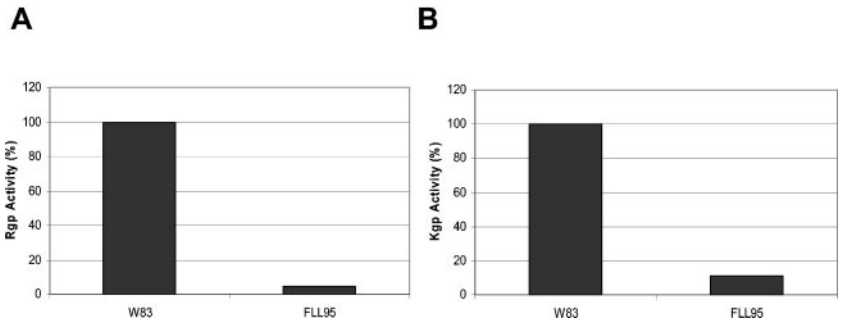

FIG. 5. Proteolytic activity of *P. gingivalis vimF*-defective mutant. *P. gingivalis* was grown to stationary phase in BHI broth supplemented with hemin and vitamin K. Activities against Rgp (A) and Kgp (B) were tested in whole-cell culture.

Fig. 8, there were high-molecular-weight immunoreactive bands in the membrane preparations from *P. gingivalis* FLL93 and FLL95 when antibodies against RgpB (Fig. 8B), RgpA (Fig. 8C), or Kgp (Fig. 8D) were used. It is noteworthy that, in contrast to the wild-type strain, there were unique high-molecular-weight bands in *P. gingivalis* FLL95 that immunoreacted with antibodies against RgpA, RgpB, and Kgp. Taken together, these data may suggest altered processing of the gingipains on the membrane of the *vimE*-defective mutant *P. gingivalis* FLL93 or the *vimF*-defective mutant FLL95. In contrast to the wild type, all the gingipains, although inactive, were present in the membrane fraction of the *vimE*-defective mutant *P. gingivalis* FLL93 and *vimF*-defective mutant FLL95.

***P. gingivalis* FLL95 has reduced ability to cleave fibronectin.** The gingipains of *P. gingivalis* have been shown to cleave extracellular matrix components, such as fibronectin, in vitro (39). A defect in gingipain maturation should abolish or reduce the potential of the organism to cleave fibronectin. To test this hypothesis, 75 μg of fibronectin was incubated for 30 min at 37°C with *P. gingivalis* W83 or isogenic mutant FLL92, FLL93, or FLL95 cells washed with and resuspended in PBS. Incubation of fibronectin with the *vimA*-defective mutant of *P. gingivalis* demonstrated no visible cleavage bands (Fig. 9); however, incubation with the *vimE*-defective mutant resulted in a slight cleavage band at 50 kDa, and incubation with the *vimF*-defective mutant demonstrated slight cleavage bands ranging between 39 to 52 kDa (Fig. 9). After incubation with *P. gingivalis* W83, the fibronectin was completely degraded in contrast to results in the isogenic mutants of *P. gingivalis* (Fig. 9).

Immunoblot analysis using MAb 1B5. Monoclonal antibody (Mab) 1B5 has been demonstrated to immunoreact with membrane-associated Rgp (mt-Rgp) and certain LPS modifications in *P. gingivalis* (13, 16). The polysaccharide profiles of the wild-type and the *vimF*-defective mutant are similar (data not shown). However, as shown in Fig. 10, there was no detectable immunoreactivity in membrane preparations from *P. gingivalis* FLL93 or FLL95 compared to the wild type.

DISCUSSION

We have used a genetic approach in this study to further assess the role of specific host factors in protease regulation/activation. Several recent studies (1, 19, 46; reviewed in reference 34) 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 delayed activation of proteolytic activity that is mostly soluble (1, 19, 46). Currently, a mechanism of activation by these genes is not understood; furthermore, it is unclear if they are part of a common pathway for gingipain activation. Although these mutants had similar proteolytic profiles, inactivation of each gene did not affect the

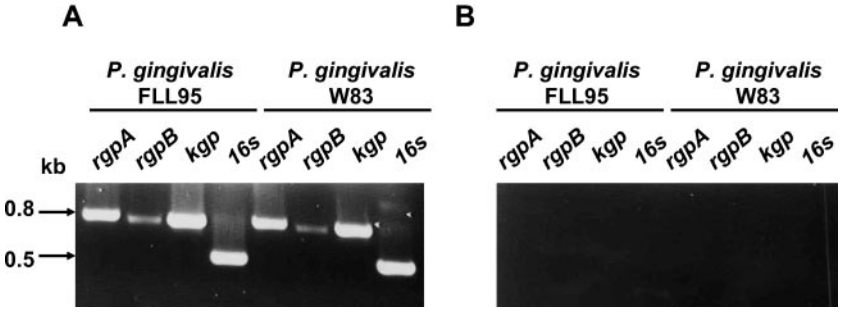

FIG. 6. Expression of the gingipains in the *vimF*-defective mutant. DNase-treated total RNA for *P. gingivalis* W83, FLL93, and FLL95 was grown to stationary phase and subjected to reverse transcriptase PCR. (A) Performed in the presence of reverse transcriptase. (B) Performed in the absence of reverse transcriptase.

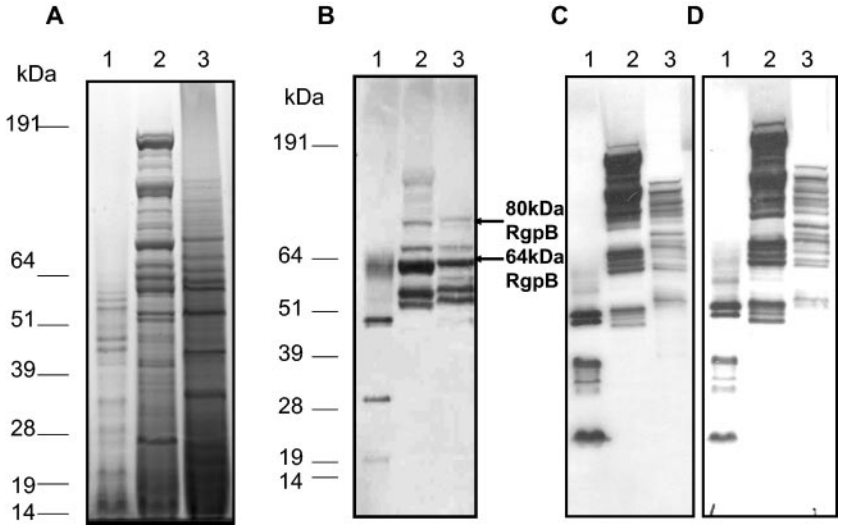

FIG. 7. Extracellular protease profile of *P. gingivalis* FLL93 and FLL95 from stationary phase. Acetone-precipitated proteins isolated from culture supernatants grown to an OD_{600} of 1.4 to 1.5 were analyzed by SDS-PAGE and stained with Simply Blue safe stain (A) or analyzed by immunoblotting using gingipain-specific antibodies (B to D). Approximately 20 to 25 μg of protein was loaded in each lane. Lanes 1, *P. gingivalis* W83; lanes 2, *P. gingivalis* FLL93; lanes 3, *P. gingivalis* FLL95. Antibodies: panel B, rabbit anti-RgpB; panel C, chicken anti-RgpA; panel D, chicken anti-Kgp. Arrows indicate the unprocessed and partially processed RgpB; molecular mass markers are shown at left.

expression of the others. This raises the possibility that regulation of virulence factors of *P. gingivalis* W83 may occur through the function of gene products that may affect carbohydrate biogenesis.

The *vimF* gene, which is downstream of *vimE*, does appear to show similarity with glycosyltransferase 1 genes from several organisms (www.oralgen.lanl.gov). *P. gingivalis* FLL95, the isogenic mutant defective in this gene, showed reduced proteolytic activity and was non-black-pigmented. This is also similar to a mutant of *P. gingivalis* that was defective in the glycosyl (rhamnosyl) transferase gene (10). Other glycosyltransferases in *P. gingivalis* including NahA and GtfA have been identified (29, 30, 35). They were demonstrated to be involved in the maturation of fimbriae and the attachment of the bacteria to epithelial cells. While the effect of NahA on protease activation was not evaluated (30), the *gtfA*-defective mutant was pigmented, which may suggest that the mutation had no effect on gingipain activity (35). Collectively, these observations would support an important role for specific glycosyltransferases in the expression of gingipain activity in *P. gingivalis*.

Because the posttranslational addition of carbohydrates to the gingipains is highly variable, it may implicate a role for

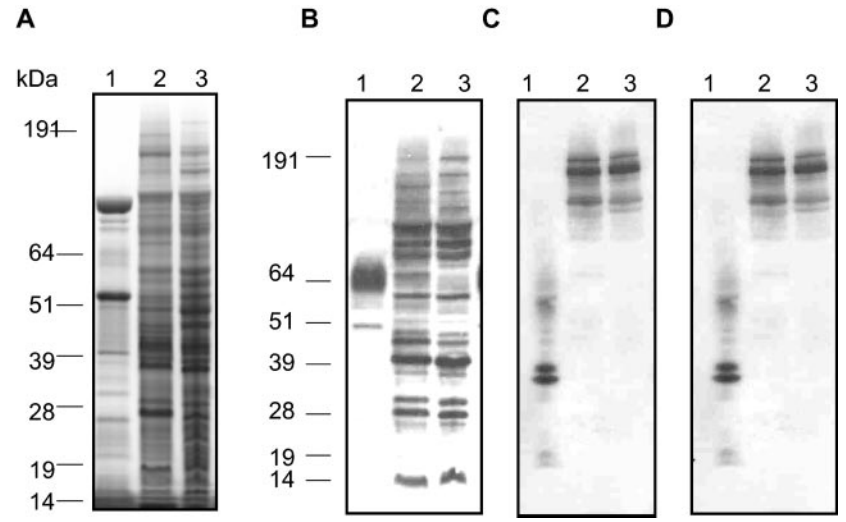

FIG. 8. Membrane protease profile of *P. gingivalis* FLL93 and FLL95. Membrane preparations taken from *P. gingivalis* strains W83, FLL93, and FLL95 taken from stationary phase were analyzed by SDS-PAGE and stained with Simply Blue Safe stain (A) or analyzed by immunoblotting using gingipain-specific antibodies (B to D). Approximately 20 to 25 μg of protein was loaded in each lane. Lanes 1, *P. gingivalis* W83; lanes 2, *P. gingivalis* FLL93; lanes 3, *P. gingivalis* FLL95. Antibodies: panel B, rabbit anti-RgpB; panel C, chicken anti-RgpA; panel D, chicken anti-Kgp. Molecular mass markers are shown at left of panels A and B.

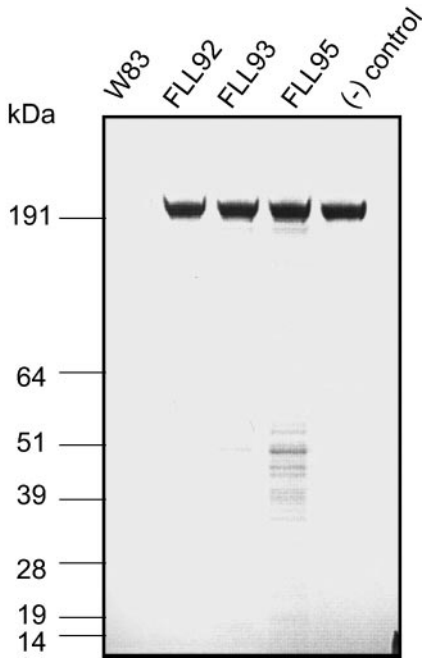

FIG. 9. Reduced ability to cleave fibronectin in isogenic mutants of *P. gingivalis*. Fibronectin was incubated for 30 min with *P. gingivalis* W83 and isogenic mutants FLL92, FLL93, and FLL95 grown of to early stationary phase (OD_{600} of 1.1), harvested, washed, and resuspended in 1× PBS to a final OD_{600} of 1.5. Cell-free supernatant from samples was analyzed by SDS-PAGE and stained with Simply Blue Safe Stain. Molecular mass markers are shown at left.

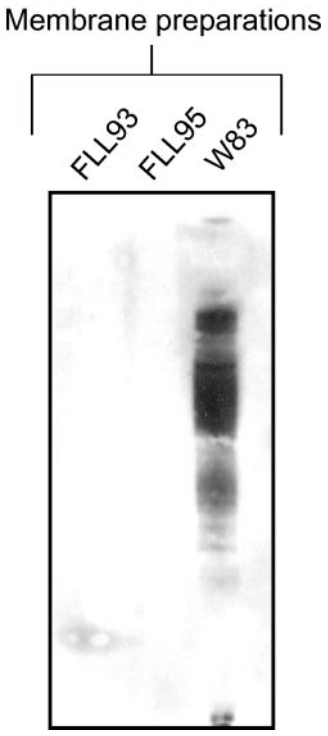

FIG. 10. Immunoblot analysis of membrane using MAb 1B5. Membrane preparations from *P. gingivalis* W83 and isogenic mutants FLL93 and FLL95 were separated by SDS-PAGE and analyzed for certain carbohydrate modifications of mt-Rgps and/or LPS modifications of membrane proteins using MAb 1B5. All lanes contained 20 μg of protein.

multiple genes in this process. In *P. gingivalis* FLL95, the *vimF*-defective mutant, membrane-associated RgpA, RgpB, and Kgp were detected. Based on the high molecular weight of immunoreacting bands to antibodies against these gingipains, the membrane-associated forms could represent the unprocessed or partially processed gingipains. Further, no immunoreactivity was detected using MAb 1B5, which is specific for carbohydrate modifications of membrane-associated Rgp and LPS. While we cannot rule out an alteration in the carbohydrate composition of the LPS in *P. gingivalis* FLL95, its mobility profile on SDS-PAGE appeared to be similar to the wild-type strain. This may correlate with the anchorage of the gingipains in the membrane of FLL95 and may suggest the ability of VimF to affect the gingipains more directly. This is in contrast to a mutation in the *porR* locus, which is associated with the synthesis of the O-antigen side chains of the LPS (16, 46). In the *porR*-defective mutant, there was reduced membrane-associated gingipain activity and a late onset of protease activity. The cell surface polysaccharide profile of this mutant was different from the wild-type strain. This defective polysaccharide may have prevented the correct distribution and activation of the gingipain. If the inactive gingipains are cell-associated as observed in *P. gingivalis* FLL95, it may be possible that VimF could function independently of PorR and that it is essential for gingipain activation. A specific mechanism for the role of the *vimF* gene in protease activation is currently being investigated in the laboratory.

The phenotype of *P. gingivalis* FLL95 could be related to the reduced gingipain activity and, in particular, reduced membrane-associated activity. This would be similar to *P. gingivalis* FLL32, a *recA*-defective mutant (2), *P. gingivalis* FLL92, a *vimA*-defective mutant (1), and *vimE*-defective mutant FLL93 (51). These results would also be consistent with other reports (14, 28, 38, 47) on the involvement of gingipains, especially Kgp, with hemoglobin binding, absorption, and heme accumulation. Hemolysin activity could also be modulated by the *vimF* gene in *P. gingivalis*. In this study, *P. gingivalis* FLL95, the *vimF*-defective mutant, showed no hemolytic activity when grown on blood plates. Because the gingipain Kgp has been shown to have hemoglobinase activity (28) and plays a role in erythrocyte degradation (44), our results are not surprising due to the reduced proteolytic activity in *P. gingivalis* FLL95. In addition to the effect of proteolytic activity on the hemolysin potential, several hemolysin-like genes have been identified from the *P. gingivalis* genome project (36; www.oralgen.lanl.gov). Furthermore, there is genetic evidence for the involvement of two distinct hemolysins in the hemolysin activity of *P. gingivalis* (23). Taken together, these observations may suggest regulation of these genes or gene products by the *vimF* 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 (22, 52).

The *vimF* gene in this study appears to also affect hemagglutination in *P. gingivalis*. Hemagglutination of sheep erythrocytes was reduced in *P. gingivalis* FLL93, the *vimE*-defective mutant and the *vimF*-deficient mutant FLL95. Again, these

observations are not unexpected due to the reduced proteolytic activity in *P. gingivalis* FLL95. The gingipains RgpA and Kgp have hemagglutinating activity (45). Further, a monoclonal antibody (MAb 61BG1.3) that inhibited the 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* (7, 24). In addition to the association of the gingipains with hemagglutination in *P. gingivalis*, the presence of several genetically distinct genes including the hemagglutinin genes *hagB* and *hagC* has been reported (27, 42). In this study, the effects of the *vimF* gene product on hemagglutination in *P. gingivalis* may also implicate the regulation of these genes or gene products by this gene. The HagA protein appears to range in size from 210 to 283 kDa (210 kDa [www.tigr.org], 233 kDa [www.oralgen.lanl.gov], or 283 kDa [18]). Analysis of the hemagglutinin protein HagA from the *vimE*- and *vimF*-defective mutants demonstrated multiple high-molecular-weight bands in both the membrane and extracellular fractions that were absent in the wild type. The high-molecular-weight band greater than 191 kDa in the membrane of the *vimE*- and the *vimF*-defective mutants, which are absent from the wild type, may represent the unprocessed or partially processed HagA protein. The other, multiple immunoreactive bands that were present in the isogenic mutants may represent the multiple intermediate HagA species. Collectively, these data suggest that *vimF* is needed for the proper processing of the hemagglutinin protein HagA.

The formation of zymogens is an important strategy that organisms use to regulate the activation of several enzymes. Thus, the gingipain zymogen may be crucial in the timely activation of the gingipains. Consistent with previous reports on two isogenic mutants (*P. gingivalis* FLL92, a *vimA*-defective mutant, and *P. gingivalis* FLL93, a *vimE*-defective mutant) with reduced proteolytic activity, there was expression of the gingipain genes in *P. gingivalis* FLL95 (1, 50). The gingipain RgpB was secreted in an inactive form in the *vimA*- and *vimE*-defective mutants, suggesting a role of the *vimA* and *vimE* genes in the posttranslational regulation of protease activity in *P. gingivalis* (37). A comparison of the extracellular proteins using the RgpB proenzyme-specific antibodies revealed a 64-kDa and an 80-kDa immunoreactive band in *P. gingivalis* FLL95, which is similar in size to the RgpB proenzyme previously reported (37). Consistent with the lack of protease activation in this isogenic mutant, this protein was present in stationary phase culture medium, unlike the *vimA*-defective mutant that demonstrated a late onset of proteolytic activity (1). In contrast to the wild type, high-molecular-weight immunoreactive protein bands that could represent the unprocessed gingipains were also secreted in *P. gingivalis* FLL95. In addition, there appeared to be RgpB-immunoreactive bands between 80 and 54 kDa, which may represent an intermediate, processed species. Furthermore, the multiple immunoreactive bands to RgpA and Kgp may represent the gingipain precursors and other inactive gingipain intermediates. Collectively, these data have confirmed a defect in the maturation of the gingipains in *P. gingivalis* FLL95.

Fibronectin plays important roles in cellular signal transduction by its interaction with the integrins (3). It has been documented that the incubation of culture supernatants of *P. gingivalis* with fibroblasts resulted in the disappearance of fibronectin and the integrin subunits (4); however, incubation with culture supernatants of the Rgp-deficient mutant showed very little change in the adhesion molecules (4). In this study, incubation of *P. gingivalis* W83 cells with fibronectin resulted in complete degradation of the fibronectin. As expected, due to reduced gingipain activity in the *vimA*-, *vimE*-, or *vimF*-defective mutants, there was a significant reduction in cleavage of the fibronectin when these isogenic strains were incubated with fibronectin. The absence of fibronectin cleavage when the *vimA*-defective mutant was incubated with fibronectin could be explained by the absence of Kgp and RgpA on the surface of the cell (51). The *vimE*- and *vimF*-defective mutants have low cell-associated gingipain activity. We cannot rule out the possibility that other cell-associated protease(s) involved in fibronectin degradation might be present in the *vimE*- and *vimF*-defective mutants. This is under further investigation in the laboratory.

It is possible that a defect in the proper glycosylation of proteins, including the gingipains, may contribute to the abnormal maturation of the gingipains. In the *vimA*-defective mutant FLL92, the 64-kDa RgpB (37, 51) may be partially processed, resulting in an inactive intermediate of the full-length 80-kDa RgpB proenzyme. The initial cleavage of the 80-kDa RgpB and 185-kDa RgpA proenzyme probably occurs at a normal rate. However, in the *vimE* and *vimF* mutants, the presence of the 80-kDa RgpB and/or the 185-kDa RgpA (51) and the 64-kDa partially processed RgpB proenzyme intermediate (51) suggests that there may be a slow processing of the full-length RgpB and RgpA, possibly due to aberrant modification of the proteins. VimA, VimE, and/or VimF are possible factors needed for carbohydrate biogenesis involved in the anchorage and/or activation of the gingipains. In contrast, the absence of the 80-kDa RgpB in *P. gingivalis* FLL92 suggests that *vimA* may not be needed for the initial step of activation (51). Further, in the *vimA* mutant, the normal activation mechanism may be disrupted, resulting in a alternate pathway of activation, similar to that described by Mikolajczyk et al. (33). Since purified RgpB has been shown to activate the Rgps but not Kgp in vitro (51), we can envision a scenario where, in the *vimA* mutant, the concentration of the secreted 64-kDa RgpB reaches a high, critical concentration in stationary phase, such that RgpB is activated by proximity-induced activation and subsequently activates RgpA. The activated Rgps may then be able to process other proteins or factors needed for Kgp activation. In contrast to the wild type, the drastically reduced proteolytic activities of the *vimE* and *vimF* mutants may be the result of a defect in the initial activation step of the RgpB proenzyme. Taken together, these data suggest that the VimE and VimF proteins in *P. gingivalis* may be involved in protease activation upstream of VimA.

The modulation of virulence in *P. gingivalis* may be coordinated via an ability to modulate proteolytic activity (reviewed in references 12, 21, 25, and 39). Although not directly tested in this study, the *vimF* gene may be an important virulence gene. Because inactivation of the *vimF* gene resulted in a reduction in proteolytic activity and had a pleiotropic effect on other important virulence factors, *P. gingivalis* FLL95 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 similar phenotype as *P. gingivalis* FLL93 and FLL95 and was dramatically less virulent that the wild-type strain in the mouse model (1).

In summary, we have constructed an isogenic mutant of *P. gingivalis* that is defective in a putative glycosyltransferase gene. While this mutant had reduced proteolytic activity, there was expression of the gingipain genes. Further, this mutant in contrast to the wild-type strain showed reduced hemolytic and hemagglutinating activities. Because glycosyltransferases are important in carbohydrate biosynthesis and posttranslational glycosylation, further study of this process in bacteria, in addition to its ability to modulate virulence factors in pathogenic bacteria, is of significance. Identification of the *vimF* gene represents a potentially new mechanism for regulating proteolytic activity and virulence in *P. gingivalis* and could possibly be an important therapeutic target.

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