

vimA Gene Downstream of *recA* Is Involved in Virulence Modulation in *Porphyromonas gingivalis* W83

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A 0.9-kb open reading frame encoding a unique 32-kDa protein was identified downstream of the *recA* gene of *Porphyromonas gingivalis*. Reverse transcription-PCR and Northern blot analysis showed that both the *recA* gene and this open reading frame are part of the same transcriptional unit. This cloned fragment was insertionally inactivated using the *ermF-ermAM* antibiotic resistance cassette to create a defective mutant by allelic exchange. When plated on *Brucella* blood agar, the mutant strain, designated *P. gingivalis* FLL92, was non-black pigmented and showed significant reduction in beta-hemolysis compared with the parent strain, *P. gingivalis* W83. Arginine- and lysine-specific cysteine protease activities, which were mostly soluble, were approximately 90% lower than that of the parent strain. Expression of the *rgpA*, *rgpB*, and *kgp* protease genes was the same in *P. gingivalis* FLL92 as in the wild-type strain. In contrast to the parent strain, *P. gingivalis* FLL92 showed increased autoaggregation in addition to a significant reduction in hemagglutinating and hemolysin activities. In *in vivo* experiments using a mouse model, *P. gingivalis* FLL92 was dramatically less virulent than the parent strain. A molecular survey of this mutant and the parent strain using all known *P. gingivalis* insertion sequence elements as probes suggested that no intragenomic changes due to the movement of these elements have occurred in *P. gingivalis* FLL92. Taken together, these results suggest that the *recA* downstream gene, designated *vimA* (virulence-modulating gene), plays an important role in virulence modulation in *P. gingivalis* W83, possibly representing a novel posttranscriptional or translational regulation of virulence factors in *P. gingivalis*.

Porphyromonas gingivalis, a black-pigmented, gram-negative anaerobic bacterium, is an important etiological agent of chronic adult periodontitis, a chronic inflammatory condition affecting tooth-supporting tissues (reviewed in references 29, 36, and 60). This organism possesses several putative virulence factors, including proteases, adhesins, endotoxins, and cytotoxins, that can directly affect the periodontium or elicit host functions that result in destruction typical of advanced periodontitis (16, 51). The high proteolytic abilities of this organism have been the focus of much attention and appear to play an important role in virulence. 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) (45). RGP is encoded by *rgpA* and *rgpB*, and KGP is encoded by *kgp* (38, 41). Several reports (reviewed in reference 26) have documented the multiple effects of proteases, which, in addition to being essential for growth, play a role in complement and immunoglobulin degradation, inactivation of cytokines and their receptors, platelet aggregation, attenuation of neutrophil antibacterial activities, increase in vascular permeability, and prevention of blood clotting. Recently, the proteases which are not coordinately regulated (63) have been shown to act as major processing enzymes for various cell surface proteins, including individual proteases (2, 4, 21).

An ability to overcome oxidative stress is vital for colonization and survival of *P. gingivalis* in an inflammatory environment such as the periodontal pocket. Protease activity in *P. gingivalis* may play a role in oxidative stress. Some reports (25, 39) have suggested a role for *P. gingivalis* proteases in hemoglobin binding, adsorption, and heme accumulation. Iron accumulated on the cell surface can bind oxygen and its toxic derivatives (58). This could limit or reduce cell damage by facilitating maintenance of a local anaerobic environment and forming a protective barrier against oxygen and oxygen radicals generated by neutrophils and occasional exposure to air. *P. gingivalis* cells carrying a surface layer of bound heme are less sensitive to killing by toxic oxygen derivatives (57).

Previously, we reported the nucleotide sequence of the *P. gingivalis* W83 *recA* homolog and construction of a *recA*-defective mutant by allelic exchange mutagenesis (14). While the *recA* mutation resulted in increased sensitivity to UV irradiation, we found that it did not affect the virulence of *P. gingivalis* FLL33, a *recA*-defective mutant, in a murine model (14). Inactivation of the *recA* gene in *P. gingivalis* also produced a mutant (FLL32) that was nonpigmented and lacked beta-hemolytic activity on blood agar, in contrast to the wild-type strain and *P. gingivalis* FLL33 (1). Analysis of the nucleotide sequence of the *recA* gene together with its downstream flanking region (14) did not indicate the presence of a transcriptional terminator sequence. Thus, we could not rule out that a downstream gene is somehow responsible for the phenotype observed in FLL32. While *P. gingivalis* FLL32 had reduced Arg-X- and Lys-X-specific proteolytic activities, there was no change in the transcription of those genes compared to *P.*

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TABLE 1. *E. coli* and *P. gingivalis* strains and plasmids used in this study

Strain or plasmid	Genotype and phenotype	Reference
Strains		
<i>E. coli</i> , NM522	<i>supE thi Δ(lac-proAB) Δ(hsdMS-mcrB)5 (F' proAB⁺ lacI^q lacZΔM15)</i>	17
<i>P. gingivalis</i> W83	Wild type (black pigmented, proteolytic)	
FLL32	<i>recA::ermF-ermAM</i> (non-black pigmented, nonproteolytic)	1
FLL33	<i>recA::ermF-ermAM</i> (black pigmented, proteolytic)	14
FLL92	<i>vimA::ermF-ermAM</i> (non-black pigmented, nonproteolytic)	This work
FLL92.1	<i>vimA::ermF-ermAM</i> (non-black pigmented, nonproteolytic)	This work
Plasmids		
pT7-5	Ap ^r T7 φ10	61
pFLL97	pT7-5 carrying the <i>vimA</i> gene	This work
pUC19	Ap ^r lacI ^{POZ'}	66
pVA2198	Sp ^r Em ^r	15
pFLL90	Ap ^r , pUC19 carrying 1.6-kb DNA fragment including the intact <i>vimA</i> gene	This work
pFLL91	Ap ^r Em ^r , pFLL90 with <i>vimA</i> gene interrupted with <i>ermF-ermAM</i> cassette	This work

gingivalis W83 and FLL33 (1). There was also little or no membrane-bound Arg-X- and Lys-X-specific cysteine protease activity in *P. gingivalis* FLL32. This mutant was significantly less virulent than the wild-type strain in a murine model and partially protected the animals against a subsequent lethal challenge by the wild-type strain (1). *P. gingivalis* FLL32 is similar in proteolytic and virulence properties to spontaneous mutants of *P. gingivalis* described by others (9, 11, 32). In contrast to *P. gingivalis* FLL32, however, molecular characterizations of some of those mutants show alterations in the *kgp* protease gene which in those reports are explained by either a deletion (9) or a transposition event (32). To further examine the possibility that the phenotype in *P. gingivalis* FLL32 may be the result of polar mutation and not a spontaneous mutation, we undertook this investigation to determine the role of the *recA* locus in the proteolytic and virulence properties of this organism. Here we report the genetic analysis of a gene downstream of *recA* that encodes a unique 32-kDa protein. Using this cloned downstream gene, a defective mutant was constructed by allelic exchange. This strain demonstrated reduced Arg-X- and Lys-X-specific proteolytic activities, was nonpigmented, and lacked beta-hemolytic and hemagglutinating activities. Further, we found that this mutant showed increased autoaggregation and was nonvirulent when tested in the mouse model of virulence. There was no intragenomic movement of any of the known insertion sequence (IS) elements in this mutant compare to the wild-type strain. Our results suggest that the gene downstream of *recA* plays an important role in modulating the virulence potential of *P. gingivalis*; thus, we propose that this gene be designated *vimA* (virulence modulating).

MATERIALS AND METHODS

Bacterial strains and culture 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, Mich.) supplemented with hemin (5 μg/ml), vitamin K (0.5 μg/ml), and cysteine (1%). *Escherichia coli* strains were grown in Luria-Bertani broth (49). 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% H₂-10% CO₂-80% N₂. Growth rates for *P. gingivalis* and *E. coli* strains were determined spectrophotometrically (optical density at 600 nm [OD₆₀₀]). 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 (35). 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 a Qiagen (Santa Clarita, Calif.) plasmid maxi kit. DNA was digested with restriction enzymes as specified by the manufacturer (Boehringer Mannheim Corp., Indianapolis, Ind.). For DNA subcloning, the desired fragments were isolated from 0.7% agarose gels run in Tris-acetate-EDTA buffer (49) then purified using a Gene Clean kit as recommended by the manufacturer (Bio 101, Inc., La Jolla, Calif.). Southern blot transfer was done by the method of Smith and Summers (59). DNA hybridization, DNA labeling, and autoradiography were done as previously described (15). For the detection of IS elements, the IS DNA probes were amplified by PCR using specific primers (Table 2). These probes were ³²P labeled using the Prime-a-Gene labeling system (Promega Corporation, Madison, Wis.). Hybridization was carried out in ExpressHyb hybridization solution (Clontech, Palo Alto, Calif.) according to the manufacturer's recommendations.

DNA sequencing. Nucleotide sequences were determined by the dideoxy-chain termination method (50) with a Perkin-Elmer (Foster City, Calif.) DNA sequencing kit and analyzed on an Applied Biosystems (Foster City, Calif.) model 373A DNA sequencing system at the DNA core facility of Loma Linda University (Loma Linda, Calif.); overlapping oligonucleotide primers were synthesized by the same facility. Nucleotide sequences were analyzed using the Genetics Computer Group sequence analysis software package (13).

In vitro transcription and translation of the cloned *vimA* gene. Following PCR amplification, the *vimA* gene including its putative ribosome binding (Shine-Dalgarno) site was inserted into pT7-5 digested with *SacI-SmaI* (61). To inactivate the *amp* gene, whose product (β-lactamase; 31.5 kDa) could mask the *vimA* product after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the recombinant plasmid designated pFLL97 was digested with *ScaI*. The linear pFLL97 plasmid was purified as described above. For in vitro transcription and translation we used the Promega *E. coli* S30 extract system for linear templates according to the manufacturer's instructions. The reaction mixture (50 μl) consisted of the purified linear DNA fragment, 5 μl of amino acid mixture without methionine, 20 μl of S30 premix, 1 μl of [³⁵S]methionine (1,200 Ci/mmol at 15 mCi/ml), and 15 μl of the *E. coli* S30 extract. Nuclease-free water was added to a final volume of 50 μl. The reaction mixture was incubated at 37°C for at least 2 h and then placed in an ice bath for 5 min. The samples were then suspended in 2% SDS buffer and separated by SDS-PAGE on a 15% polyacrylamide gel by the method of Laemmli (27). To ensure good band resolution on SDS-PAGE, polyethylene glycol contained in the *E. coli* S30 extract was removed by acetone precipitation.

PCR analysis of RNase-treated chromosomal DNA from *P. gingivalis*. PCR amplification was performed with a Perkin-Elmer Cetus (Norwalk, Conn.) DNA thermal cycler. The primers used (Table 2) were specific for a 0.8-kb intragenic region of *recA* (14), a 2.1-kb *recA* and downstream region (this study), and the 5' region of the *ermF* gene (48). The reaction mixture (50 μl), containing 1 μl of template DNA (0.5 μg), 1 μM each primer, and 0.2 mM deoxynucleoside triphosphates in 1× Expand High Fidelity system buffer (Boehringer Mannheim), was denatured for 2 min at 94°C, and then 1.73 U of Expand High Fidelity system enzyme was added. The PCR program consisted of 40 cycles with a

TABLE 2. Oligonucleotide primers used in this study

Primer	Oligonucleotide sequence	Characteristic	Reference
P1	5' TTCCTCAGGATAAGGAGGGC 3'	Upstream <i>recA</i> right	14
P2	5' GAGAAAGAACTGCAAGCCCTG 3'	Intragenic <i>recA</i> right	14
P3	5' AGCTGAACCACGATCCGCT 3'	Intragenic <i>recA</i> left	14
P4	5' TGAATGATGATGAAGCAACGC 3'	Downstream 1 <i>recA</i> right	This work
P5	5' CCATCCGTACGACATCGA 3'	Downstream 1 <i>recA</i> left	This work
P6	5' CGGTATCATGAAAGGCTCG 3'	Downstream 2 <i>recA</i> left	This work
P7	5' GCGAATGATGTAGTGGATTA 3'	Downstream 3 <i>recA</i> left	This work
P8	5' ATGACAAAAAGAAATTGCC 3'	<i>ermF</i> right	48
P9	5' ATGAAAACTTGAACAAGTT 3'	<i>rgpA</i> right	3
P10	5' CGTTGTAGTGGTCATTACCT 3'	<i>rgpA</i> left	3
P11	5' ATGAAAAAGAATTTTAGCAG 3'	<i>rgpB</i> right	54
P12	5' CGACCGATGAAGACTTCG 3'	<i>rgpB</i> left	56
P13	5' ATGAGGAAATTTATTGCT 3'	<i>kgp</i> right	5
P14	5' CTGTAATACAAGTCGGTA 3'	<i>kgp</i> left	5
P15	5' ATGTATCATTCTCTATTGAA 3'	PGIS2 right	65
P16	5' ATTAATAATTGTATTGTGATTA 3'	PGIS2 left	65
P17	5' ATGAGTACAAATATAAGCCTA 3'	ISPg4 right	F. F. Dewhirst
P18	5' CTATGTGCCCTAAAAGAGCTGA 3'	ISPg left	F. F. Dewhirst ^a
P19	5' ATGCAACCAGTATCCATTATGGCA 3'	ISPg5 right	8
P20	5' TCTAATTTCCGTTGACACGAGCAA 3'	ISPg5 left	8
P21	5' ATGGATGTACAAGTTTATTTTCT 3'	ISPg6 right	F. F. Dewhirst
P22	5' TTCAGAGCAGCTTTGAACCAA 3'	ISPg6 left	F. F. Dewhirst
P23	5' AGCTTTCATGGCTCTTTTGAA 3'	ISPg7 right	F. F. Dewhirst
P24	5' TATTGAGCAAATTAGTTGCGAAA 3'	ISPg7 left	F. F. Dewhirst
P25	5' ATGAACACAAATATAGTTGA 3'	IS195 right	32
P26	5' ATCTAAGCGGAAATAGGAAA 3'	IS195 left	32
P27	5' ATGACGTGATTCTCTTATTCA 3'	IS1126 right	34
P28	5' TCAAGAATGTTTGGAGTA 3'	IS1126 left	34

^a Unpublished data.

temperature profile of 30 s at 94°C, 1 min at 55°C, and 2 min at 72°C, with a final extension at 72°C for 7 min. The PCR-amplified DNA was then identified by 0.7% agarose gel electrophoresis.

RT-PCR and Northern blot analysis of DNase-treated RNA extracted from *P. gingivalis* strains. Total RNA was extracted from *P. gingivalis* strains grown to mid-log phase (OD₆₀₀ of 0.3 to 0.4) using a Qiagen RNeasy midi kit. The primers used for reverse transcription-PCR (RT-PCR) analysis (Table 2) were specific for a 0.8-kb intragenic region of *recA* (14), a 1-kb intragenic region of *pppRI* (*rgpA*) (3), *pppRII* (*rgpB*) (56), and *prtP* (*kgp*) (5). The reaction mixture (50 µl) contained 1 µg of template RNA, 1 µM each primer, 0.2 mM deoxynucleoside triphosphates, 1 mM MgSO₄, 0.1 U of avian myeloblastosis virus reverse transcriptase for first-strand DNA synthesis, and 0.1 U of thermostable *Tfl* DNA polymerase (from *Thermus flavus*) for second-strand cDNA synthesis and DNA amplification (Access RT-PCR system; Promega). The reverse transcription reaction was performed at 48°C for 45 min. and then stopped by raising the temperature 94°C for 2 min. PCR amplification was performed with a Perkin-Elmer Cetus DNA thermal cycler, using 40 cycles of 30 s at 94°C, 1 min at 60°C, and 2 min at 68°C. The final products were analyzed by 0.7% agarose gel electrophoresis.

For Northern blot analysis, RNA samples (5 µg) were separated by 1% agarose gel electrophoresis and then transferred to a nylon membrane (Hybond-N; Amersham Pharmacia Biotech) according to the method of Sambrook et al. (49). The probes were ³²P labeled using the Promega Prime-a-Gene labeling system. Hybridization was carried out in ExpressHyb hybridization solution (Clontech) as recommended by the manufacturer. Autoradiography was done as previously described (15).

Mutagenesis of the cloned *vimA* gene in *P. gingivalis*. A 2.1-kb fragment carrying the intact *recA* and downstream gene was amplified by PCR using the P1 and P7 oligonucleotide primers (Table 2; Fig. 1). This fragment was treated with Klenow enzyme and then digested with *SaI*. The resulting 1.6-kb fragment was subcloned into pUC19 which was digested with *SmaI* and *SaI* in order to remove the *Bam*HI site contained in the multiple cloning site. The *ermF-ermAM* cassette (which confers erythromycin/clindamycin resistance in *E. coli* and *P. gingivalis*) was isolated from pVA2198 digested with *PstI* and *SacI* (14) and treated with Klenow enzyme to fill in the single-stranded ends. The resulting plasmid, pFLL90, which carried a part of the *recA* gene and the intact *vimA* gene, was linearized at a unique *Bam*HI site located within codon 55 of the *vimA* open

reading frame and then ligated with the *ermF-ermAM* cassette. The resultant recombinant plasmid, pFLL91, was used as a donor in electroporation of *P. gingivalis* W83.

Electroporation. Electroporation of cells was performed as previously reported (14). Briefly, 1 ml of an actively growing culture of *P. gingivalis* was used to inoculate 10 ml of BHI broth supplemented with hemin and vitamin K, which was then incubated overnight at 37°C. Seventy milliliters of the same prewarmed medium (37°C) was then inoculated with 3 ml of the overnight culture and incubated for an additional 4 h. The cells were harvested by centrifugation at 2,600 × g for 7 min at 4°C and washed in 70 ml of electroporation buffer (10% glycerol, 1 mM MgCl₂; filter sterilized; stored at 4°C), and the pellet was resuspended in 0.5 ml of electroporation buffer. A 100-µl sample of cells to which 1 µg of DNA was added was placed in a sterile electrode cuvette (0.2-cm gap). The cells were pulsed with a Bio-Rad gene pulser at 2,500 V for 9.5 ms and then incubated on ice for 3 min. The cell suspension was then added to 0.5 ml of BHI broth supplemented with hemin and vitamin K and incubated for approximately 16 h. A 100-µl sample was plated on solid medium containing clindamycin and incubated anaerobically at 37°C for 7 to 10 days.

UV sensitivity measurements. Aliquots (0.1 ml) of exponentially growing cells at a 10⁻⁵ dilution were plated on BHI agar, placed in the dark, and irradiated with increasing doses (from 500 to 2,000 µJ) of UV using a Stratallinker-2400 (Stratagene, La Jolla, Calif.). To avoid photoactivation, the plates were immediately wrapped in foil and incubated for 7 to 10 days at 37°C in 10% H₂-10% CO₂-80% N₂.

Fraction preparation and protease assay. Whole-cell culture, cell-free medium, cell suspension, vesicles, and particle-free medium were prepared as previously reported (46). The presence of Arg-X- and Lys-X-specific cysteine protease activities was determined using a microplate reader (Bio-Rad) by the method of Potempa et al. (44).

Hemolytic activity assay. Hemolytic activity was determined as previously reported (10). 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 NCN buffer (3 mM sodium citrate, 0.9% NaCl [pH 6.9]), and then resuspended to a final concentration of 2 × 10¹⁰ bacteria per ml. Sheep erythrocytes (Hemostat Laboratories, Dixon, Calif.) were harvested by centrifugation (4,400 × g for 25 min) and washed with NCN buffer until the supernatant was visually free of hemoglobin pigment. The washed erythrocytes were sus-

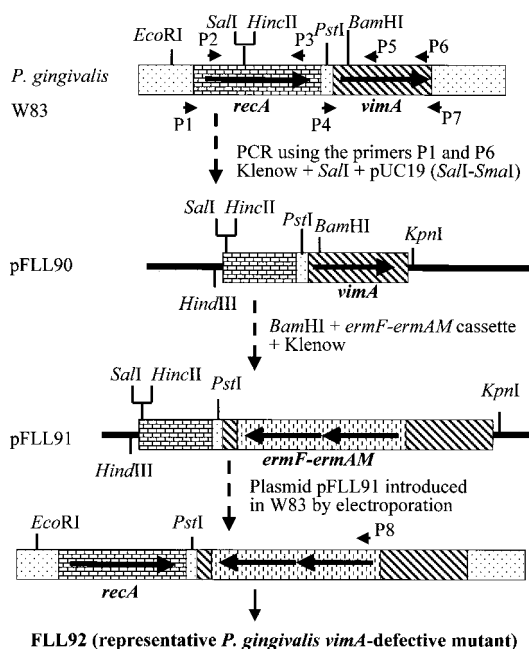


FIG. 1. Construction of site-specific mutations by allelic exchange. pFLL91 contains the *vimA* gene interrupted by an *ermF-ermAM* cassette. The *vimA* gene and flanking sequences were amplified by PCR; the *ermF-ermAM* cassette was purified from pVA2298 (15). The circular recombinant plasmid pFLL91 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 pFLL91 replaced the intact *vimA* with an inactivated copy.

pended in NCN buffer to a concentration of 2×10^9 /ml. Hemolytic activity was determined by mixing 2×10^{10} bacterial cells with 2×10^9 erythrocytes in NCN buffer. This mixture was then slowly mixed in a water bath at 37°C. Samples (100 μ l) were withdrawn every 2 h, immediately diluted with 900 μ l of NCN buffer, and then centrifuged ($1,300 \times g$ for 5 min) in an Eppendorf 5403 centrifuge. The resulting supernatant was diluted another 10-fold with NCN buffer, and the OD was determined by spectrophotometry at 405 nm. As a negative control, erythrocytes were used alone.

Hemagglutination assays. Hemagglutination activity was assayed by the method of Chu et al. (10). Bacterial cells from overnight cultures were harvested by centrifugation ($10,000 \times g$ for 30 min) in a Sorvall RC5C centrifuge, washed three times with NCN buffer, and then resuspended to a final concentration of 2×10^{10} bacteria per ml. The cells (100- μ l volumes) were twofold serially diluted in 96-well microtiter plates. To each dilution, 100 μ l of 1% sheep erythrocyte suspension was added. Sheep erythrocytes (Hemostat Laboratories) were harvested by centrifugation ($3,800 \times g$ for 25 min) in an Eppendorf 5403 centrifuge and then washed with NCN buffer until the supernatant was visually free of hemoglobin pigment. The washed erythrocytes were suspended in NCN buffer to a 1% suspension. The plates were incubated at 5°C for 3 h. Hemagglutination was assessed visually, and a positive reaction was taken as the reciprocal of the last dilution showing complete hemagglutination.

Virulence testing. *P. gingivalis* wild-type strain W83 and mutant strain FLL92 were tested for invasiveness in a murine model as previously described (15). These experiments were performed under authorization of an institutionally approved animal use protocol (33).

Nucleotide sequence accession number. The nucleotide sequence reported here has been assigned GenBank accession number AF064682.

RESULTS

Nucleotide sequencing of the gene downstream of the *recA* homolog gene in *P. gingivalis*. Inactivation of the *recA* gene in

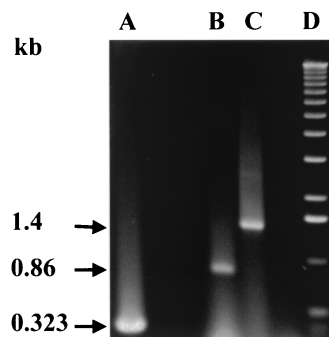


FIG. 2. RT-PCR analysis of DNase-treated RNA extracted from *P. gingivalis*. Total RNA was extracted from W83 grown to mid-log phase (OD_{600} of 0.3 to 0.4) using a Qiagen RNeasy midi kit. Samples (1 μ g) were subjected to RT-PCR (Access RT PCR system; Promega). Lanes: A, Promega kanamycin positive control RNA; B, intragenic primers for *recA* (P2 and P3 Table 2; Fig. 1); C, forward primer from *recA* and reverse primer from the downstream gene (P2 and P5 (Table 2; Fig. 1); D, 1-kb ladder. Expected sizes of the transcripts are 0.323, 0.86, and 1.4 kb for the positive control, *recA*, and *recA*/downstream genes, respectively. Sizes of the PCR products are given at the left.

P. gingivalis FLL32 not only affected the capacity for DNA repair but also resulted in increased autoaggregation, decreased protease activity, and reduced virulence in a mouse model (1). Analysis of the *recA* and flanking downstream sequences did not show any putative transcription terminator (14), which suggests that the *recA* gene could be part of an operon resulting in a polar mutation affecting a downstream gene(s). Using a forward oligonucleotide primer from the *recA* gene (P1 [Table 2; Fig. 1]) in combination with a reverse primer from the downstream flanking region (P5 [Table 2; Fig. 1]), a 1.4-kb fragment was amplified by RT-PCR using total DNase-treated RNA from the wild-type strain W83 (Fig. 2, lane C). As expected, the intragenic primers of the *recA* gene (P2 and P3 [Table 2; Fig. 1]) led to the amplification of a 0.86-kb fragment (Fig. 2, lane B). No amplified fragments were observed when reverse transcriptase was omitted from the reaction (data not shown). The putative *recA* gene is 1 kb and thus may be associated with some other gene(s) on the same transcriptional unit.

Phage L10, which is part of a *P. gingivalis* W83 genomic lambda library, carries the *recA* gene together with its downstream region (14). Using overlapping oligonucleotide primers, both strands of a 1.2-kb region downstream of the *recA* gene were sequenced. A putative 0.9-kb open reading frame (called *vimA*) corresponding to a 32-kDa protein was detected 113 bases downstream of the stop codon of the *recA* gene. This sequence was confirmed by comparison with the *P. gingivalis* genome sequence in the databank (Forsyth Dental Center/The Institute of Genome Research). A purine-rich sequence found in *E. coli* ribosome binding sites was also seen one base upstream from the translation initiation site. Further, partial direct repeat sequences (bp 2820 to 2832 and 2848 to 2860) starting 6 bp downstream of the translation stop were observed. The calculated G+C content for this gene was 52%, which is close to the value of 46 to 48% previously reported for *P. gingivalis* genomic DNA (53). Hydrophobicity analysis using

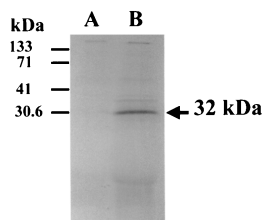


FIG. 3. In vitro transcription-translation of the linear pFLL97 plasmid carrying the *vimA* gene. Five microliters of each 50- μ l reaction mix was subjected to acetone precipitation and analyzed by SDS-PAGE. The plasmid vector (pT7-5 [61]) and the recombinant plasmid pFLL97 were linearized by *ScaI* to prevent expression of the *amp* gene. Molecular masses are shown on the left. Lanes: A, pT7-5; B, pFLL97 carrying the *vimA* gene. Proteins were labeled with [35 S]methionine and visualized by autoradiography.

the Kyte-Doolittle scale (PROTSKALE) predicted the putative *vimA* gene product to be hydrophilic. NNPREDPIC and DAS queries indicted no transmembrane and DNA binding domains, respectively. Comparison of the nucleotide and amino acid sequences of this gene with entries in the National Center for Biotechnology Information data bank did not reveal any significant similarity with any known genes.

In vitro expression of the *vimA* gene. To confirm the predicted size of the VimA protein, chromosomal DNA from the wild type was subjected to PCR analysis using two primers (P4 and P7; Table 2; Fig. 1) that would amplify the open reading frame plus the putative ribosome binding site. This purified DNA fragment was inserted into pT7-5 under control of the ϕ 10 promoter. The recombinant plasmid pFLL97 was linearized at the *ScaI* site contained in the *amp* gene and then expressed in an in vitro transcription-translation system. As shown in Fig. 3 (lane B), the predicted 32-kDa protein was observed. In contrast, no protein was seen with the nonrecombinant plasmid pT7-5 (lane A). Taken together, these results suggest that the *vimA* gene that is present downstream of the *recA* gene encodes a 32-kDa protein.

Inactivation of the *vimA* gene in *P. gingivalis* W83 by allelic exchange mutagenesis. Isogenic *vimA*-defective derivatives of *P. gingivalis* W83 were constructed by allelic exchange mutagenesis. The circular recombinant plasmid pFLL91, which carries the *ermF-ermAM* cassette in the unique *Bam*HI restriction site (bp 165 of the open reading frame) of the *vimA* gene, was used as a donor in electroporation of *P. gingivalis* W83 (Fig. 1). Because the plasmid was unable to replicate in *P. gingivalis*, we predicted that two double-crossover events between the regions flanking the *erm* 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 clindamycin resistance.

Following electroporation and plating on selective medium, we detected 200 clindamycin-resistant colonies after a 7-day incubation period. To compare their phenotypic properties with 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. These

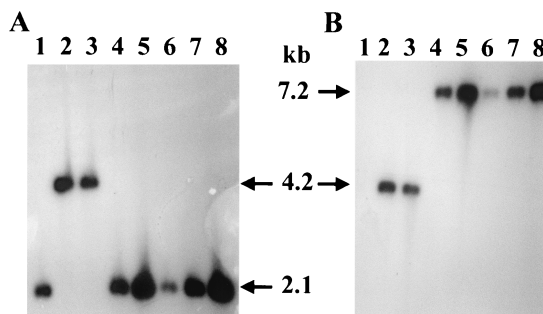


FIG. 4. Southern blot analysis of allelic exchange mutants of *P. gingivalis*. Total chromosomal DNA from *P. gingivalis* was cleaved with *EcoRI* and *PstI*, electrophoresed through 0.7% agarose, and bidirectionally transferred to a nitrocellulose membrane. Lanes: 1, W83; 2, FLL32; 3, FLL33; 4 to 8, *vimA*-defective mutants. The probes used were 32 P-labeled fragments representing an intragenic region of the *recA* gene (A) and erythromycin cassette purified from plasmid pVA2198 (12) (B). A 2.1-kb fragment was detected in strains W83 and FLL92, which have the intact *recA* gene. A 4.2-kb fragment was detected in FLL32 and FLL33, which contain the *recA* gene inactivated with the *ermF-ermAM* cassette, in contrast to a 7.2-kb fragment in the *vimA* mutants.

mutant strains grown in BHI broth showed increased autoaggregation, in contrast to the wild-type strain.

The non-black-pigmented, non-beta-hemolytic phenotype of the *vimA* mutants shows some similarity to the phenotype of spontaneous mutants of *P. gingivalis* (9, 11, 32) and thus could have arisen by a similar mechanism. To determine the likelihood for selection of this phenotype independent of inactivation of the *vimA* gene or to rule out the possibility of the effect of electroporation on selection of this phenotype, the recombinant plasmid pVA2295 (15), which carries the *ermF-ermAM* cassette with the *rgpA* gene flanking region, was used as a donor in electroporation of *P. gingivalis* W83. Inactivation in the *rgpA* gene does not give rise to mutants with a non-black-pigmented and non-beta-hemolytic phenotype (15), in contrast to *kgp*-defective mutants, which are non-black pigmented and non-beta-hemolytic (9, 32). We detected 80 clindamycin-resistant colonies following a 7-day incubation. When plated on *Brucella* blood agar plates, all of these mutants displayed a black-pigmented, beta-hemolytic phenotype similar to that of the wild type. When the recombinant plasmid pFLL91, which carries the *ermF-ermAM* cassette in the *vimA* gene, was used as a donor in electroporation of *P. gingivalis* W83 in an independent experiment, all of the clindamycin-resistant mutants again displayed a non-black-pigmented and non-beta-hemolytic phenotype. Growth of these mutant strains in BHI broth also resulted in increased autoaggregation compared to the wild-type strain. Five mutant strains were randomly chosen from the two independent experiments and used for further studies.

Characterization of *vimA*-defective *P. gingivalis*. To confirm the changes in the *vimA* gene and to verify the sequence of the intact *recA* gene, chromosomal DNA from five strains (from two independent experiments), *P. gingivalis* FLL32 (*recA::ermF-ermAM*), *P. gingivalis* FLL33 (*recA::ermF-ermAM*), and the wild type were probed with 32 P-labeled *recA* or *ermF-ermAM* fragments. A predicted 2.1-kb fragment should be seen in the wild-type and *vimA*-defective mutants when digested with *EcoRI* and *PstI* and probed with the *recA*-specific frag-

ment. Since the *ermF-ermAM* cassette is missing *EcoRI* and *PstI* sites, a 4.2-kb fragment should be seen in the *recA*-defective mutants as previously reported (1). As shown in Fig. 4A, the wild type (lane 1) and the *vimA*-defective mutants (lanes 4 to 8) showed a 2.1-kb DNA fragment. In contrast, a 4.2-kb fragment was present only in the *recA* mutants that contained the 2.1-kb *ermF-ermAM* cassette in the *recA* gene (*P. gingivalis* FLL32 [lane 2] and *P. gingivalis* FLL33 [lane 3]) as reported previously (1). A similar blot probed with the *ermF-ermAM* cassette (Fig. 4B) revealed an identical 4.2-kb hybridizing band in the *recA* mutants (*P. gingivalis* FLL32) [lane 2] and *P. gingivalis* FLL33 [lane 3]); however, a 7.2-kb hybridizing fragment was present in the *vimA*-defective mutants (lanes 4 to 8). No hybridizing sequences were seen in the wild-type strain (lane 1). The *ermF-ermAM* cassette and its orientation in the *vimA* gene were further verified by PCR analysis (data not shown). These data indicated that the predicted recombination had occurred and *vimA* was interrupted by the *ermF-ermAM* cassette. One strain from the five *vimA*-defective mutants, designated *P. gingivalis* FLL92, was randomly chosen for further studies.

The *recA* gene in *P. gingivalis* plays a role in DNA repair (14). To confirm the integrity of the RecA protein function in *P. gingivalis* FLL92, cells from *P. gingivalis* FLL92 and wild-type strain W83 were exposed to UV irradiation. Both *P. gingivalis* W83 and *P. gingivalis* FLL92 showed 77% survival after 1,000 μJ and 43% survival after 2,000 μJ of UV irradiation (data not shown). However, in the previous study, less than 1% of the *recA*-defective mutants survived after a 2,000- μJ dose of UV irradiation (1).

To confirm the presence of the *recA* transcript, oligonucleotide primers (P2 and P3) specific for an intragenic region of the *recA* gene (Table 2; Fig. 1) were used in RT-PCR to amplify that gene. In contrast to the *recA*-defective mutants (*P. gingivalis* FLL32 and *P. gingivalis* FLL33) that did not show any amplified fragments, the *recA* intragenic primers amplified a 0.86-kb region in both *P. gingivalis* FLL92 and the wild-type strains (data not shown). These data confirm that the function of RecA protein is intact in *P. gingivalis* FLL92 compared to the *recA*-defective mutants (*P. gingivalis* FLL32 and *P. gingivalis* FLL33).

Northern blot analysis of the *P. gingivalis recA* locus. To confirm that the *recA* and *vimA* genes are part of the same transcriptional unit, total RNA was isolated from the wild-type strain W83, the isogenic *recA*-defective mutants, and the *vimA*-defective mutant grown to mid-log phase. Intragenic probes from the *recA* and *vimA* genes were amplified by PCR from the wild-type W83 chromosomal DNA, using the primers listed in Table 2. Since the sizes of the *recA* and *vimA* genes are 1.0 and 0.9 kb, respectively, a 1.9-kb transcript is expected. As shown in Fig. 5 (lane A), hybridizing bands of approximately of 1, 1.9, and 2.8 kb were observed in the wild-type strain when the *recA* gene was used as a probe. For the *recA*-defective mutants (lanes B and C), a hybridizing band of approximately 1 kb was observed. In contrast, a 1.9-kb band was observed for a mutant that carried a defect in the *vimA* gene. A similar hybridization profile was observed when the *vimA* gene was used as a probe (data not shown). Taken together, these results suggest that the *recA* and *vimA* genes are a part of the same transcriptional unit. Sequence analysis of the DNA region upstream of the

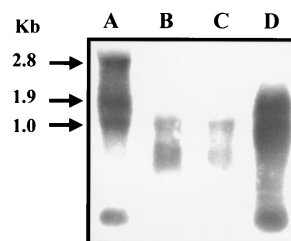


FIG. 5. Autoradiograph of Northern hybridization filter of total RNA from mid-log-phase *P. gingivalis* isogenic strains. Lanes: A, *P. gingivalis* W83; B, *recA*-defective mutant *P. gingivalis* FLL32; C, *recA*-defective mutant *P. gingivalis* FLL33; D, *vimA*-defective mutant *P. gingivalis* FLL92. The probe used was the ^{32}P -labeled intragenic region of the *recA* gene. Sizes of the transcripts are given at the left.

recA gene identified a putative 0.5-kb open reading frame with the stop codon located 20 bp from the *recA* start codon. No typical transcriptional stop was observed. Preliminary in silico analysis of this open reading frame, using Blast and Alignment (<http://www.ncbi.nlm.nih.gov>), suggests that this gene may encode a 18-kDa protein with homology to the bacterioferritin comigratory protein (BCP) (20).

Hemolytic activity of *P. gingivalis* FLL92. Cell- and vesicle-associated hemolysins which will liberate hemoglobin from erythrocytes are produced in *P. gingivalis* apparently by two distinct genes (10, 21). The effects of *P. gingivalis* W83, *P. gingivalis* FLL32, and *P. gingivalis* FLL92 on erythrocyte lysis are shown in Fig. 6. *P. gingivalis* W83 showed a significant increase in hemolytic activity. However, similar to results for the negative control, hemolytic activity was missing in *P. gingivalis* FLL32 and *P. gingivalis* FLL92.

Proteolytic activity of *P. gingivalis* FLL92. The proteolytic activity of *P. gingivalis* FLL32, a *recA*-defective mutant that was non-black pigmented, was more than 90% lower than that of the wild-type strain (1). Thus, it is possible that the *vimA* locus affects proteolytic activity in *P. gingivalis*. *P. gingivalis* strains

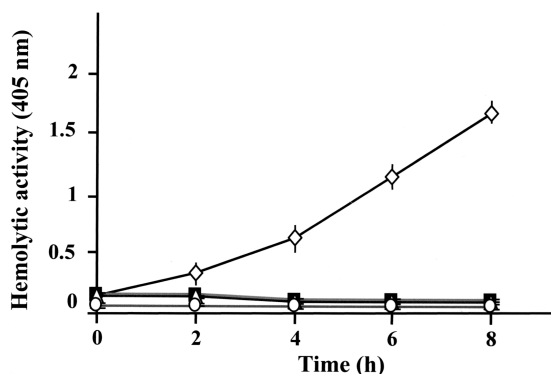


FIG. 6. Kinetic analysis of the hemolytic activity of whole cells of *P. gingivalis* W83 (\diamond) and derivative mutants strains FLL32 (\triangle) and FLL92 (\blacksquare). Erythrocytes were prepared by the method of Chu et al. (9). Bacterial cells were grown, pelleted, and assayed for hemolytic activity as described in the text. As a negative control (\circ), erythrocytes were used alone. Each value indicates average \pm standard deviation (assays were performed in triplicate). Using Student's *t* test (Statistical Package for the Social Sciences, version 9), there is a significance difference ($P > 0.05$) between the wild-type strain and isogenic mutants.

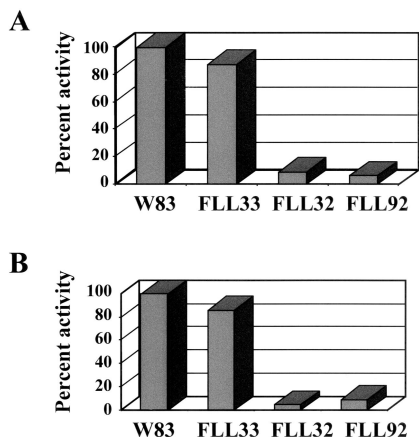


FIG. 7. Proteolytic activity of *P. gingivalis* mutants. *P. gingivalis* was grown to late log phase (OD₆₀₀ of 1.1) in 500 ml of BHI broth supplemented with hemin and vitamin K. Activity against BAPNA (A) and Z-lysine-DL-p-nitroanilide (B) was tested in whole-cell culture by the method of Potempa et al. (38). The results shown are representative of four independent experiments.

W83, FLL32, and FLL92 were assayed for proteolytic activity using *N*- α -benzoyl-DL-arginine *p*-nitroanilide (BAPNA) and Z-lysine-DL-*p*-nitroanilide. In late-exponential-growth-phase cultures, proteolytic activity in *P. gingivalis* FLL92, as in *P. gingi-*

valis FLL32, was almost 90% lower than the wild-type level (Fig.7). In addition, most of the Arg-X (Fig. 8A) and Lys-X (Fig. 8B) specific activity in *P. gingivalis* FLL92 as in *P. gingivalis* FLL32 (Fig. 8C and D) was found to be soluble. In contrast, most of the Arg-X (Fig. 8E) and lysine-X (Fig. 8F)-specific activity in *P. gingivalis* W83 was membrane bound. Taken together, these data suggest that under the same physiological conditions, the proteolytic profiles for *P. gingivalis* FLL32 and *P. gingivalis* FLL92 were similar, and this proteolytic activity could be severely altered by mutations affecting the *vimA* gene expression in *P. gingivalis*.

Transcription of the gingipain genes in strain FLL92 is similar to that in the wild-type strain. The reduced proteolytic activity in *P. gingivalis* FLL92 could have been the result of alteration in transcription of the gingipain genes. To determine the presence of mRNA transcripts for the major protease genes, total RNA was isolated using a Qiagen RNeasy kit from the wild-type strain W83 and *P. gingivalis* FLL92 grown to mid-log phase. Unique oligonucleotide primers for *prtP* (*kgp*) (5) *prpRI* (*rgpA*) (3), and *prRII* (*rgpB*) (56) (Table 2) were used in RT-PCR to amplify a 1-kb region of the transcripts. When reverse transcriptase was present in the reaction, amplified products of the predicted size (1.0 kb) were observed for all three protease gene transcripts in both strains (data not shown). Since non-black pigmentation in *P. gingivalis* can arise from a defect in the *kgp* gene (9, 32), the level of expression of

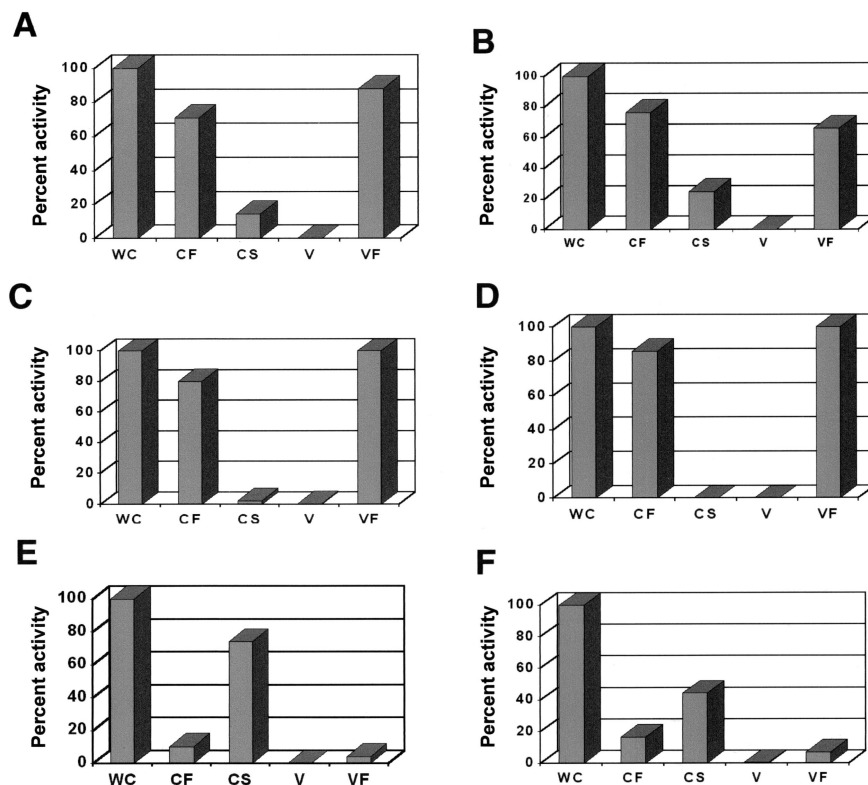


FIG. 8. Distribution of cysteine protease activity in *P. gingivalis*. Activities against BAPNA (A, FLL92; C, FLL32; E, W83) and Z-lysine-DL-*p*-nitroanilide (B, FLL92; D, FLL32; F, W83) were tested in whole-cell culture (WC), cell-free medium (CF), cell suspension (CS), vesicles (V), and particle-free medium (VF). The fractions were prepared by the method of Potempa et al. (38). The activity of each strain in the whole-cell fraction was assumed to be 100%; this activity measured in *P. gingivalis* FLL32 and FLL92 represents 10% of the activity measured in the wild-type strain W83. The results are representative of three independent experiments performed on late-log-phase (OD₆₀₀ = 1.1) growth cultures.

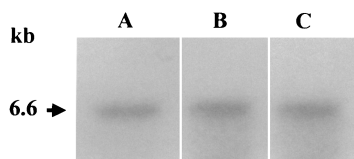


FIG. 9. Northern blot analysis of the *kgp* protease gene from isogenic mutants of *P. gingivalis*. Lanes: A, *P. gingivalis* W83; B, *P. gingivalis* FLL32; C, *P. gingivalis* FLL92. The probe used was the 32 P-labeled intragenic region of the *kgp* gene. The size of the corresponding *kgp* transcript is given at the left.

that gene was further examined in *P. gingivalis* FLL92. In Northern blot analysis as shown in Fig. 9, there was no observed difference between the wild type and *P. gingivalis* FLL92 when probed with the *kgp* gene. These data are consistent with previous results for *P. gingivalis* FLL32, a *recA*-defective mutant which was non-black pigmented and had reduced proteolytic activity compared to the wild-type strain (1).

Hemagglutination studies. We assessed the hemagglutination potential of *P. gingivalis* W83, the *recA*-defective mutants FLL32 and FLL33, and the *vimA*-defective mutant FLL92. While the activity of *P. gingivalis* FLL33 (a proteolytic *recA* mutant) was similar to the wild-type level, the hemagglutination capability of *P. gingivalis* FLL32 (a nonproteolytic *recA* mutant) and *P. gingivalis* FLL92 (*vimA::ermF-ermAM*) was reduced (Fig. 10).

Analysis of isogenic mutants for the presence all known *P. gingivalis* IS elements. Some of the phenotypic properties of the *vimA*-defective mutant are similar to the IS element-induced properties of *P. gingivalis* spontaneous mutants previously described (9, 32, 55, 65). To determine the movement of any IS element in *P. gingivalis* FLL92, chromosomal DNA isolated from two *vimA* mutants (*P. gingivalis* FLL92 and *P.*

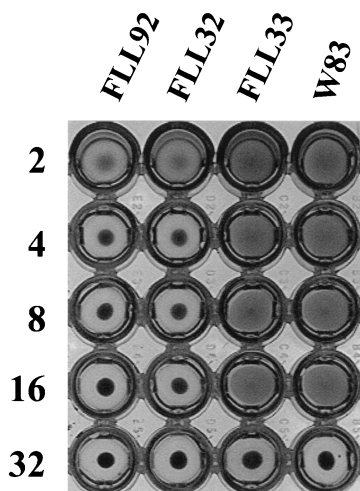


FIG. 10. Hemagglutinating activity of *P. gingivalis*. Titers were determined by twofold serial dilution of 100- μ l volumes of suspension in microtiter plates as previously described (9). To each dilution, 100 μ l of 1% sheep erythrocyte suspension was added. The plates were incubated at 5°C for 3 h. Reciprocals of the dilutions are shown at the left; the strains tested are indicated above the columns of wells. The experiments were carried out at least three times; the results reported are representative of four independent experiments.

gingivalis FLL92.1 [from two independent experiments]), *P. gingivalis* FLL32 (*recA::ermF-ermAM*), *P. gingivalis* FLL33 (*recA::ermF-ermAM*), and *P. gingivalis* W83 was digested with *Bam*HI and probed with 32 P-labeled intragenic DNA from PGIS2 (65), ISPg4 (F. F. Dewhirst, unpublished data), ISPg5 (8), ISPg6 (Dewhirst, unpublished), ISPg7 (Dewhirst, unpublished), IS195 (32) and IS1126 (34). As shown in Fig. 11, *P. gingivalis* FLL92 and *P. gingivalis* FLL92.1 exhibited similar profiles, for all IS elements compared to the wild-type strain W83 or the *recA*-defective mutants *P. gingivalis* FLL32 and *P. gingivalis* FLL33. Taken together, these data suggest that no detectable intragenomic changes due to the movement of the known *P. gingivalis* IS elements have occurred in the isogenic mutants.

Virulence testing of *P. gingivalis* FLL92. The virulence of *P. gingivalis* FLL92 was assessed, as reduced proteolytic activity in *P. gingivalis* can give rise to attenuation in virulence in a mouse model (15). At 36 h, two of five mice challenged with *P. gingivalis* W83 (wild type) at a dose of 10^{10} bacteria/animal died. Surviving animals appeared cachectic and hunched, with ruffled hair. Although the mice did not display lesions at the dorsal surface site of injection, all had developed spreading, ulcerative abdominal lesions. By 48 h all animals had died. In contrast, all of the five mice challenged with *P. gingivalis* FLL92 at a dose of 10^{10} bacteria/animal survived the 14-day observation period. No lesions of any sort appeared, and none of the animals in this group appeared cachectic.

DISCUSSION

In this study we used a genetic approach to further examine the *recA* locus in *P. gingivalis*. Several studies have reported that the *recA* gene in some bacteria can be associated with other genes in an operon structure (40, 42, 62). Northern blot analysis of the *P. gingivalis* *recA* region using the *recA* gene as a probe revealed a 2.8-kb transcript for the wild-type strain W83. Since *recA* and the putative downstream gene are 1 and 0.9 kb, respectively, this result suggested that *recA* is part of a multigene operon. Furthermore, the *recA*-defective mutants *P. gingivalis* FLL32 and *P. gingivalis* FLL33 and the *vimA*-defective mutant *P. gingivalis* FLL92 exhibited transcripts of 1 and 1.9 kb, respectively, suggesting that *recA* and *vimA* genes are associated with a third gene that should be localized upstream of *recA*. A 0.5-kb open reading frame was detected upstream of the *recA* gene. In silico analysis of this open reading frame has shown that the putative gene may encode a 18-kDa protein with homology to BCP, which is known to have hydroperoxide peroxidase activity (20). Further confirmation of this gene as part of the *recA* operon and examination of its functional role in *P. gingivalis* are in progress.

The *recA* gene in bacteria can be associated with genes of different functions, such as resistance to low pH in *Helicobacter pylori* (62), competence induction in *Streptococcus pneumoniae* (42), or regulation of RecA function in mycobacteria (40). In *P. gingivalis*, however, the *recA* gene may be associated with a *bcp*-like gene (upstream) and the *vimA* gene (downstream), which shows no significant similarity with any known genes. The *vimA* gene is not involved in DNA repair, instead *P. gingivalis* FLL92, the isogenic mutant defective in that gene, showed reduced proteolytic activity and was non-black pigmented. This phenotype of *P. gingivalis* FLL92, which is similar

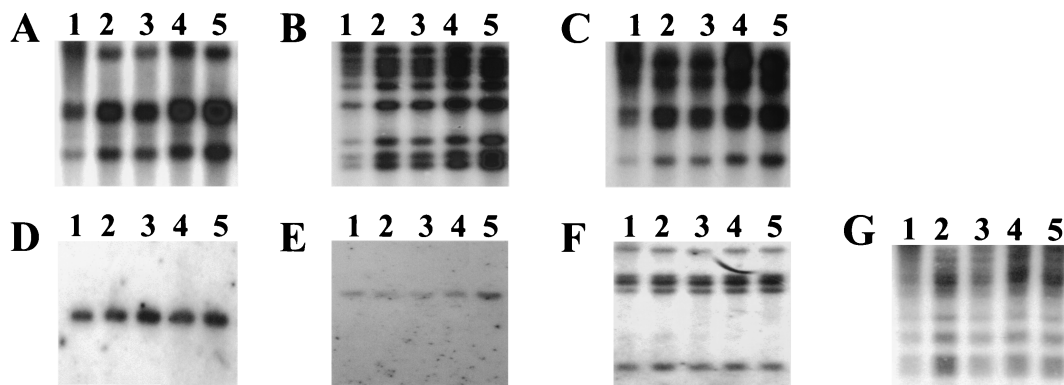


FIG. 11. Southern blot analysis of isogenic mutants of *P. gingivalis* to determine intragenomic movement of IS elements. Total chromosomal DNA from *P. gingivalis* was cleaved with *Bam*HI, electrophoresed through 0.7% agarose, and bidirectionally transferred to nitrocellulose. Lanes: 1, *P. gingivalis* W83; 2, *P. gingivalis* FLL32 (*ermF-ermAM::recA*); 3, *P. gingivalis* FLL33 (*ermF-ermAM::recA*); 4, *P. gingivalis* FLL92 (*ermF-ermAM::vimA*); 5, *P. gingivalis* FLL92.1 (*ermF-ermAM::vimA*). The probes used were the ³²P-labeled intragenic region of the IS elements. (A) PGIS2; (B) ISPg4; (C) ISPg5; (D) ISPg6; (E) ISPg7; (F) IS195; (G) IS1126.

to that of *P. gingivalis* FLL32, a *recA*-defective mutant (1), could be related to its reduced proteolytic activity and, in particular, reduced membrane-bound activity. This would be consistent with other reports of the involvement of gingipains with hemoglobin binding, absorption, and heme accumulation (21, 37, 39). More recently, KGP, the Lys-X-specific protease in *P. gingivalis* has been shown to be a hemoglobinase which plays a role in iron uptake by effecting the accumulation of iron protoporphyrin IX on bacterial cell surface (31).

Consistent with a previous report on *P. gingivalis* FLL32, a *recA*-defective mutant with reduced proteolytic activity (1), there was no detectable alteration of the gingipain genes in *P. gingivalis* FLL92. It is unclear whether the low activity is a result of an inefficient processing of the gingipains or other proteases (12) that could lead to inefficient substrate cleavage. However, as has been suggested for *P. gingivalis* FLL32 (1), the decreased proteolytic activity observed in *P. gingivalis* FLL92 may result from posttranscriptional regulation of those genes. Furthermore, in contrast to extracellular protein extracts from the wild-type strain, preliminary experiments in the laboratory have demonstrated *P. gingivalis* FLL92 high-molecular-weight proteins immunoreacting with antibodies against the gingipains but lacking proteolytic activity (data not shown). This could suggest a role for the *vimA* gene in posttranslational regulation of the gingipains. Posttranslational modification of proteases has been documented in *P. gingivalis* W50/BE1, an avirulent spontaneous mutant that is non-black pigmented with reduced Arg-X-specific protease activity (11). The source of the mutation in *P. gingivalis* W50/BE1 is unknown.

The *vimA* gene appears to also modulate hemolysin activity in *P. gingivalis*. *P. gingivalis* FLL92, the *vimA*-defective mutant used in this study, showed no hemolytic activity when grown on *Brucella* blood plates or incubated with sheep erythrocytes. These results are not surprising due to the reduced proteolytic activity in *P. gingivalis* FLL92. Gingipains, especially the Lys-X-specific gingipain, play a role in erythrocyte degradation (52). Mutations abolishing Lys-X-specific activity have resulted in loss of black pigmentation and a reduction in the hemolytic potential of the mutant strains (31). In addition to the effects of the proteolytic activity in *P. gingivalis* on its hemolysin po-

tential, the presence of two genetically distinct hemolysins have been documented (22). Thus, the reduced proteolytic and hemolysin activities in *P. gingivalis* FLL92 may implicate the regulation of those genes or gene products by the *vimA* gene.

The ability for hemagglutination is a significant characteristic of *P. gingivalis* (reviewed in reference 28). The *vimA* gene analyzed in this study appears to also affect hemagglutination in *P. gingivalis*. Hemagglutination of sheep erythrocytes was reduced in *P. gingivalis* FLL92, the *vimA*-defective mutant used in this study. Again, these results are not surprising due to the reduced proteolytic activity in *P. gingivalis* FLL92. The RGP-adhesin and KGP-adhesin complexes have hemagglutinating activity (43). Han et al. (19) reported that the adhesin domain of the gingipains was also encoded by *hagA*, a hemagglutinin gene in *P. gingivalis*. 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* (7, 23). In addition to the association of the gingipains with hemagglutination in *P. gingivalis*, the presence of several genetically distinct genes including hemagglutinin genes *hagB*, *hagC*, and *hagD* has been reported (18, 30, 47). Shi et al. (54) reported construction of an *rgpA kgp hagA* triple mutant and confirmed that the *rgpA*-, *kgp*-, and *hagA*-encoded adhesin domains play a role in the hemagglutination of *P. gingivalis*. The effects of the *vimA* gene product on hemagglutination in *P. gingivalis* may implicate the regulation of those genes or gene products by this gene.

In contrast to *P. gingivalis* W50/BE1, a spontaneous mutant which did not show autoaggregation (M. A. Curtis, personal communications), *P. gingivalis* FLL92 grown in BHI broth showed increase autoaggregation compare to the parent strain W83. This phenotype is similar to that of isogenic *recA*-defective mutants previously reported (1). Inactivation of the *vimA* gene suggests that *vimA* plays a role in autoaggregation. It is noteworthy that inactivation of the *vimA* gene also resulted in reduced membrane-bound proteolytic activity. While the reduced activity could give rise to incomplete processing of surface membrane proteins resulting in aggregation, our results are in contrast to a previous report of an *rgpA*-defective mutant

(64). This mutant displayed approximately 40% of the Arg-X-specific cysteine protease activity of the wild-type strain and was severely retarded in its ability to hemagglutinate and autoaggregate. Our results, however, do not rule out the possibility that the *vimA* gene is a regulatory gene whose product could negatively affect the expression of other genes that facilitate autoaggregation.

The correlation of intragenomic changes due to the movement of IS elements and their effect on the phenotypic properties of *P. gingivalis* is well documented (9, 32, 55, 65). While most of the *P. gingivalis* spontaneous mutants that were non-black pigmented and had reduced proteolytic activities and virulence were IS element induced (9, 32), no movement of the these elements was observed in the *vimA*-defective mutant compared to the wild-type strain. Although we cannot rule out any other genetic rearrangement in the *vimA*-defective isogenic mutants, these results suggest that the phenotypic changes observed in *P. gingivalis* FLL92 could be due to the lack of expression of the *vimA* gene. While in vitro translation of the *vimA* gene in *E. coli* may verify the predicted size in that system, it is still unclear whether a similar product is made in *P. gingivalis*. Also, we cannot rule out the possibility that the *vimA*-induced effects observed in *P. gingivalis* FLL92 could be due to a regulatory RNA molecule transcribed from that gene. Complementation is needed to confirm the role of *vimA* in the observed phenotype of *P. gingivalis* FLL92.

Reduced proteolytic activity is correlated with reduced virulence in *P. gingivalis* (15). Consistent with this report, *P. gingivalis* FLL92 has shown lower virulence in a murine model than the wild-type W83 strain. These results have also confirmed that the *vimA* gene is involved in virulence modulation in *P. gingivalis*. Based on the properties of *P. gingivalis* FLL92 in this study, the phenotype of *P. gingivalis* FLL32, a non-black-pigmented non-virulent *recA*-defective mutant, might be the result of a polar mutation (1). This possibility raises questions as to the source of the mutation of *P. gingivalis* FLL33, another isogenic *recA*-defective mutant that was black pigmented and similar in virulence to the wild-type strain (15). While both isogenic *recA*-defective mutants appear to have the *ermF-ermAM* cassette in similar locations (1), it is possible that differences during the recombination event could give rise to a gene product of the *recA* locus that has partial activity in *P. gingivalis* FLL33. Further, we cannot rule out the occurrence in *P. gingivalis* FLL33 of a suppressor mutation that has restored a wild-type phenotype. Complete molecular characterization of the *recA* locus is under way in our laboratory.

The modulation of virulence in *P. gingivalis* may be coordinated via an ability to modulate proteolytic activity, although we cannot rule out any other direct or indirect effect of *vimA* on the expression of other virulence factors. It is possible, therefore, that the *recA* locus is important for the survival of *P. gingivalis* in the inflammatory microenvironment of the periodontal pocket. It is interesting that *recA*, which plays a role in DNA repair, is upstream of a gene that is involved in modulating proteolytic activity. Such an association may be significant, since a response to oxidative stress involves binding of oxygen and its toxic derivatives to iron accumulated on the cell surface (58). The bound heme can be involved in the catalytic destruction of the toxic oxygen derivative species (57). Since proteolytic activity in *P. gingivalis* is associated with heme ac-

cumulation, it may be an important strategy for the organism to coordinate its oxidative stress and proteolytic activities.

We have constructed an isogenic mutant of *P. gingivalis* that is defective in *vimA*, a gene, downstream of *recA*. While this mutant had reduced proteolytic activity, there was no detectable modification of the gingipain genes. Further, this mutant, in contrast to the wild-type strain, showed increased autoaggregation, reduced hemolytic and hemagglutinating activities, and no virulence when tested in a mouse model. Identification of the *vimA* gene represents a potentially new avenue for regulating virulence in *P. gingivalis*.

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