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

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Received 9 February 2000/Returned for modification 15 March 2000/Accepted 18 October 2000

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 activities 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). Activation 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.
**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 Quagen (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) and 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 DNA 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 dye-deoxy-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.** After PCR amplification, the vimA gene including its putative ribosome binding (Shine-Dalgarno) site was inserted into pT7-5 digested with SacI and EcoRI (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 μg of amino acid mixture without methionine, 20 μl of S30 premix, 1 μl of [35S]methionine (1,200 Ci/mmol at 15 μCi/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 recE gene (48). The reaction mixture (50 μl), containing 1 μl of template DNA (0.5 μg), 1 μM each primer, and 0.2 mM deoxyribonucleoside triphosphates in 1× Expand High Fidelity system buffer (Boehringer Mannheim), was denatured for 2 min at 94°C, and then 1.75 U of Expand High Fidelity system enzyme was added. The PCR program consisted of 40 cycles with a

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

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype and phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em>, NM522</td>
<td>*supE thi Δ(lac-proAB) Δ(hsdS-McrB5) (F′ proAB’ lacZΔM15)</td>
<td>17</td>
</tr>
<tr>
<td><em>P. gingivalis</em></td>
<td>Wild type (black pigmented, proteolytic)</td>
<td>1</td>
</tr>
<tr>
<td>W83</td>
<td><em>recA::ermAM</em> (non-black pigmented, nonproteolytic)</td>
<td>14</td>
</tr>
<tr>
<td>FLL92</td>
<td><em>vima::ermAM</em> (non-black pigmented, nonproteolytic)</td>
<td>This work</td>
</tr>
<tr>
<td>FLL92.1</td>
<td><em>vima::ermAM</em> (non-black pigmented, nonproteolytic)</td>
<td>This work</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pT7-5</td>
<td>Ap’ T7 Δ10</td>
<td>61</td>
</tr>
<tr>
<td>pFLL97</td>
<td>pT7-5 carrying the vimA gene</td>
<td>This work</td>
</tr>
<tr>
<td>pUC19</td>
<td>Ap’lacPOZ’</td>
<td>66</td>
</tr>
<tr>
<td>pVA2198</td>
<td>Sp’ Em’</td>
<td>15</td>
</tr>
<tr>
<td>pFLL90</td>
<td>Ap’, pUC19 carrying 1.6-kb DNA fragment including the intact vimA gene</td>
<td>This work</td>
</tr>
<tr>
<td>pFLL91</td>
<td>Ap’, pFLL90 with vimA gene interrupted with <em>ermA::ermAM</em> cassette</td>
<td>This work</td>
</tr>
</tbody>
</table>
temperature profile of 30 °C 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 purified 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 (OD<sub>600</sub> 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 ppRII (rggA) (3), ppRII (rggB) (56), and pTP (kpg) (5). The reaction mixture (50 μl) contained 1 μl of template RNA, 1 μl each primer, 0.2 mM deoxynucleoside triphosphates, 1 mM MgSO<sub>4</sub>, 0.1 U of avian myeloblastosis virus reverse transcriptase for first-strand DNA synthesis, and 0.1 U of thermostable Thermus flavus polymerase (from Clontech) as recommended by the manufacturer. Autoradiography was performed (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 MgCl<sub>2</sub>; filter sterilized; stored at 4°C), and the pellet was resuspended in 0.5 ml of electroporation buffer A 1000 bacteria per ml. Sheep erythrocytes (Hemostat Laboratories, Dixon, Calif.) were harvested by centrifugation (10 min × g for 30 min) using a Sorvall RC5C centrifuge, washed three times with NBC buffer (5 mM sodium citrate, 0.9% NaCl [pH 6.9]), and then resuspended to a final concentration of 2 × 10<sup>8</sup> bacteria per ml. Sheep erythrocytes (Hemostat Laboratories, Dixon, Calif.) were harvested by centrifugation (4,400 × g for 25 min) and washed with NBC buffer until the supernatant was visually free of hemoglobin pigments. 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 gene with an inactivated copy.

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 × g for 30 min) in a Sorvall RCSC centrifuge, washed three times with NCN buffer, and then resuspended to a final concentration of 2 × 10^9 bacteria per ml. For each test, 100 μl of each sample were mixed in a 96-well microtiter plate with 900 μl of a 1% suspension of sheep erythrocytes. 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 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 (P2 and 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 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
the Kyte-Doolittle scale (PROTScale) predicted the putative vimA gene product to be hydrophilic. NNPREDICT and DAS queries indicated 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 amp gene. Molecular masses are shown on the left. Lanes: A, pT7-5; B, pFLL97 carrying the vimA gene. Proteins were labeled with [35S]methionine and visualized by autoradiography.

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 BamHI 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 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 rgaA gene flanking region, was used as a donor in electroporation of P. gingivalis W83. Inactivation in the rgaA 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 32P-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 1.9, 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 *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* FLL92, 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. 5](http://iai.asm.org/)  
**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 32P-labeled intragenic region of the *recA* gene. Sizes of the transcripts are given at the left.

![FIG. 6](http://iai.asm.org/)  
**FIG. 6.** Kinetic analysis of the hemolytic activity of whole cells of *P. gingivalis* W83 (○) and derivative mutants strains FLL32 (▼) and FLL92 (■). 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 (○), erythrocytes were used alone. Each value indicates average ± 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.
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. gingivalis* 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
that gene was further examined in \textit{P. gingivalis} FLL92. In Northern blot analysis as shown in Fig. 9, there was no observed difference between the wild type and \textit{P. gingivalis} FLL92 when probed with the \textit{kpg} gene. These data are consistent with previous results for \textit{P. gingivalis} FLL32, a \textit{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 \textit{P. gingivalis} W83, the \textit{recA}-defective mutants FLL32 and FLL33, and the \textit{vimA}-defective mutant FLL92. While the activity of \textit{P. gingivalis} FLL33 (a proteolytic \textit{recA} mutant) was similar to the wild-type level, the hemagglutination capability of \textit{P. gingivalis} FLL32 (a nonproteolytic \textit{recA} mutant) and \textit{P. gingivalis} FLL92 (\textit{vimA}:\textit{ermF}-\textit{ermAM}) was reduced (Fig. 10).

**Analysis of isogenic mutants for the presence all known \textit{P. gingivalis} IS elements.** Some of the phenotypic properties of the \textit{vimA}-defective mutant are similar to the IS element-induced properties of \textit{P. gingivalis} spontaneous mutants previously described (9, 32, 55, 65). To determine the movement of any IS element in \textit{P. gingivalis} FLL92, chromosomal DNA isolated from two \textit{vimA} mutants (\textit{P. gingivalis} FLL92 and \textit{P. gingivalis} FLL92.1 [from two independent experiments]), \textit{P. gingivalis} FLL32 (\textit{recA}:\textit{ermF}-\textit{ermAM}), \textit{P. gingivalis} FLL33 (\textit{recA}:\textit{ermF}-\textit{ermAM}), and \textit{P. gingivalis} W83 was digested with \textit{BamH}I and probed with \textit{32P}-labeled intragenic DNA from PG182 (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, \textit{P. gingivalis} FLL92 and \textit{P. gingivalis} FLL92.1 exhibited similar profiles, for all IS elements compared to the wild-type strain W83 or the \textit{recA}-defective mutants \textit{P. gingivalis} FLL32 and \textit{P. gingivalis} FLL33. Taken together, these data suggest that no detectable intragenomic changes due to the movement of the known \textit{P. gingivalis} IS elements have occurred in the isogenic mutants.

**Virulence testing of \textit{P. gingivalis} FLL92.** The virulence of \textit{P. gingivalis} FLL92 was assessed, as reduced proteolytic activity in \textit{P. gingivalis} can give rise to attenuation in virulence in a mouse model (15). At 36 h, two of five mice challenged with \textit{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 \textit{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 \textit{recA} locus in \textit{P. gingivalis}. Several studies have reported that the \textit{recA} gene in some bacteria can be associated with other genes in an operon structure (40, 42, 62). Northern blot analysis of the \textit{P. gingivalis} \textit{recA} region using the \textit{recA} gene as a probe revealed a 2.8-kb transcript for the wild-type strain W83. Since \textit{recA} and the putative downstream gene are 1 and 0.9 kb, respectively, this result suggested that \textit{recA} is part of a multigene operon. Furthermore, the \textit{recA}-defective mutants \textit{P. gingivalis} FLL32 and \textit{P. gingivalis} FLL33 and the \textit{vimA}-defective mutant \textit{P. gingivalis} FLL92 exhibited transcripts of 1 and 1.9 kb, respectively, suggesting that \textit{recA} and \textit{vimA} genes are associated with a third gene that should be localized upstream of \textit{recA}. A 0.5-kb open reading frame was detected upstream of the \textit{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 \textit{recA} operon and examination of its functional role in \textit{P. gingivalis} are in progress.

The \textit{recA} gene in bacteria can be associated with genes of different functions, such as resistance to low pH in \textit{Helicobacter pylori} (62), competence induction in \textit{Streptococcus pneumoniae} (42), or regulation of \textit{RecA} function in mycobacteria (40). In \textit{P. gingivalis}, however, the \textit{recA} gene may be associated with a \textit{bcp}-like gene (upstream) and the \textit{vimA} gene (downstream), which shows no significant similarity with any known genes. The \textit{vimA} gene is not involved in DNA repair, instead \textit{P. gingivalis} FLL92, the isogenic mutant defective in that gene, showed reduced proteolytic activity and was non-black pigmented. This phenotype of \textit{P. gingivalis} FLL92, which is similar
could suggest a role for the pains but lacking proteolytic activity (data not shown). This proteins immunoreacting with antibodies against the gingipain protoporphyrin IX on bacterial cell surface (31). This plays a role in iron uptake by effecting the accumulation of iron in (21, 37, 39). More recently, KGP, the Lys-X-specific protease 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 (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. Posttranslation 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 potential, 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 hagD (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 iso- genic 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 FLL32, 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 coordi- nated 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 peri- odontal pocket. It is interesting that recA, which plays a role in DNA repair, is upstream of a gene that is involved in modu- lating proteolytic activity. Such an association may be signifi- cant, 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 detect- able modification of the gingipain genes. Further, this mutant, in contrast to the wild-type strain, showed increased autoag- gregation, 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.

ACKNOWLEDGMENTS

We thank Zubaida Jhuma for technical assistance.

This work was supported by the Loma Linda University School of Medicine and National Institute of Dental Research grant DE11864-01A2 (to H.M.F.).

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