

HGP44 Induces Protection against *Porphyromonas gingivalis*-Induced Alveolar Bone Loss in Mice[∇]

Kyotaro Muramatsu,^{1,2} Eitoyo Kokubu,^{1,3} Takahiko Shibahara,^{1,2}
Katsuji Okuda,³ and Kazuyuki Ishihara^{1,3*}

Oral Health Science Center,¹ Department of Oral Surgery,² and Department of Microbiology,³
Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba 261-8502, Japan

Received 24 December 2010/Returned for modification 16 February 2011/Accepted 11 March 2011

The protective effect of DNA vaccines expressing the Arg-gingipain A domain against bone loss induced by *Porphyromonas gingivalis* infection was investigated in a murine model. phgp44, which expresses the 44-kDa adhesion/hemagglutinin domain of Arg-gingipain A, prevented *P. gingivalis*-induced alveolar bone loss. The results indicate that phgp44 could be a candidate antigen for a vaccine against *P. gingivalis* infection.

Periodontitis, a highly prevalent chronic inflammatory disease which causes irreversible destruction of the supporting tissue of the teeth, affects more than 30% of the adult population (19). Periodontitis has also been reported to be involved in the development of systemic diseases such as bacterial endocarditis, atherosclerosis, and diabetes (13).

Porphyromonas gingivalis is a major pathogen of chronic periodontitis (5, 21). *P. gingivalis* expresses several virulence factors, including proteases, fimbriae, and endotoxin (1, 9, 18). Arg-gingipain A (RgpA) is a major virulence factor of *P. gingivalis* (16). RgpA is involved in activation of complement and bradykinin and degradation of C3b, interleukin 8 (IL-8), IgG, and monocyte chemoattractant protein 1 (MCP-1) (9). These activities may play an important role in the virulence of *P. gingivalis*. RgpA consists of a preproprotein, a catalytic domain, and an adhesin/hemagglutinin (HA) domain, which consists of HGP44, HGP15, HGP17, and HGP27 (Fig. 1). This HA domain has similarity to hemagglutinin A (HagA) genes and the HA domains of the Lys-gingipain (Kgp) (16). Antibody against gingipain was reported to have a protective effect against infection by *P. gingivalis* (7, 15, 23). In the present study, we investigated the protective effect of *rgpA* domain DNA vaccines against *P. gingivalis*-induced alveolar bone loss, with the aim of clarifying their potential as candidate antigens for a novel vaccine.

We investigated the protective effect of immunization with *rgpA* domain vaccines (Fig. 1) containing the *rgpA* catalytic domain (pcat), the HGP44 domain-coding region (phgp44), the HGP15-27 domain-coding region (phgp15-27), or the N-terminal (phgp44H) or C-terminal (phgp44T) half of the HGP44 domain-coding region against infection by *P. gingivalis*. All vaccines were constructed by self-ligation of amplified fragments from the *rgpA* DNA vaccine (23) with the primers described in Table 1, using LA *Taq* DNA polymerase (Takara Bio, Inc., Shiga, Japan). The plasmid used for construction of

these vaccines, pVAX1 (Invitrogen, Carlsbad, CA), was used as a control.

Immunization with *rgpA* domain DNA vaccines was carried out as described previously (23). Briefly, 6-week-old female BALB/c mice were separated into 8 groups of 4 mice each: a nonimmunized group and groups immunized with 2.5 μ g *rgpA* DNA vaccine, pcat, phgp44, phgp15-27, phgp44H, phgp44T, or pVAX1 alone via the skin of the abdomen by a Gene Gun (Bio-Rad Laboratories, Hercules, CA) weekly for 5 weeks. Additional immunization with phgp44H and phgp44T was performed at 6 weeks, as the antibody titers for the mice immunized with the DNA vaccines had not reached a plateau at 5 weeks. The levels and reactivity of antibodies against RgpA at days 0, 28, 35, and 42 after immunization were determined by an enzyme-linked immunosorbent assay (ELISA) and immunoblotting. Approval to conduct these studies was obtained from the Animal Use Committee of Tokyo Dental College (Chiba, Japan).

The protective effect of the vaccinations against *P. gingivalis* infection was investigated according to the method of Baker et al. (2). Briefly, the mice were challenged with *P. gingivalis* at 6 weeks after the first immunization. Initially, the mice were given 5 mg each of kanamycin and ampicillin by gavage once a day for 4 days. After a 3-day antibiotic-free period, all mice except the nonimmunized control mice were orally infected with 1×10^9 CFU *P. gingivalis* ATCC 33277 in 2% carboxymethylcellulose. Challenge was carried out 3 times at 2-day intervals. Forty-two days after the last challenge, the mice were sacrificed and alveolar bone loss at the defined landmark sites on the maxillary molars was assessed as described previously (8). We performed measurements (14 sites) on each skull from the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC) with a stereomicroscope. Measurements were made under a dissecting microscope fitted with a video image maker measurement system, MS-803 (Moritex Co., Tokyo, Japan), standardized to yield measurements in millimeters. The 4 non-infected and nonimmunized mice were used to determine the baseline value for the region from the CEJ to the ABC in normal mice. The experiments were repeated to confirm reproducibility, and a one-way analysis of variance (ANOVA) and the Turkey *post hoc* test were used to make multiple

* Corresponding author. Mailing address: Department of Microbiology, Tokyo Dental College, 1-2-2 Masago, Mihama-ku, Chiba 261-8502, Japan. Phone: 81-43-270-3741. Fax: 81-43-270-3744. E-mail: ishihara@tdc.ac.jp.

[∇] Published ahead of print on 23 March 2011.

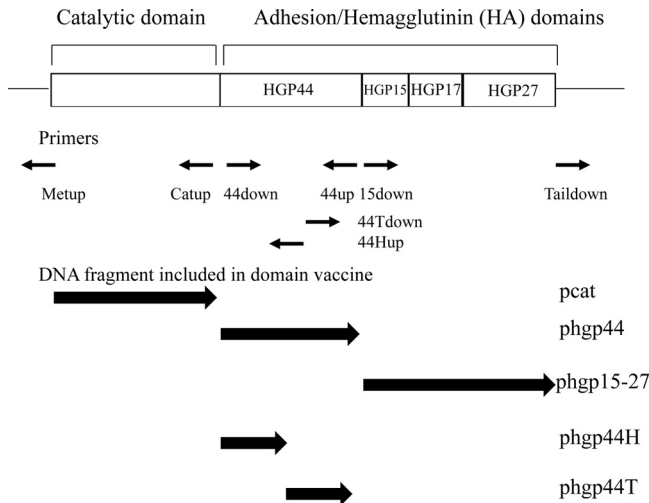


FIG. 1. Map of cloned *rgpA* in the *rgpA* DNA vaccine and primers used to construct the *rgpA* domain DNA vaccines. Small arrows below the map indicate the locations of primers used in this study. Large arrows indicate fragments expressed by the DNA vaccines.

comparisons between groups in terms of antibody titers and protective effects against bone loss using the pooled data from the experiments.

Antibody titers elicited by the pcat, phgp44, or phgp15-27 DNA vaccine plasmid are shown in Fig. 2A. Significant elevation of specific IgGs against *P. gingivalis* was observed, reaching similar levels in mice immunized with the *rgpA* DNA vaccine and in those immunized with phgp44. Only a small increase was seen in antibody levels obtained with phgp15-27, and the level obtained with pcat was almost the same as that in the controls. As shown in Fig. 2B, the specificities of IgG in mice immunized with phgp44 or the *rgpA* DNA vaccine were evaluated by immunoblot analyses. In sera from mice immunized with the *rgpA* DNA vaccine, 52.6-, 43.8-, 40.8-, 33.5-, and 14.5-kDa bands were observed. In sera from mice immunized with phgp44, 43.8-, 33.5-, and 14.5-kDa bands were observed. These multiple protein bands may have been degraded fragments of RgpA, Kgp, and HagA, which share antigenicity with HGP44. In both groups, the predominant band was the 43.8-kDa band, suggesting high immunogenicity for HGP44. This agrees with the results for earlier reports (10, 12). The epitope for the protective antibody was reported to be located within HGP44 (12). Twenty-one of 25 amino acid residues of the epitope were contained in phgp44H. The antibody titer for mice immunized with phgp44H was significantly high at week 5 ($2.6 \pm 1.43 \log_2$)

TABLE 1. Primers used in this study

Primer name	Sequence ^a
Metup.....	5'-CATGGTCGCTAGCTAGCCAGCTTGGGT-3'
Taildown.....	5'-TAATTCTGTCTTGGACTCGGAGCTCGAGTCTAG-3'
44down.....	5'-AGCGGTCAGGCCGAGATTGTTCTTGAA-3'
Catup.....	5'-GCGAAGAAGTTCGGGGGCATCGCTGACTGACA-3'
15down.....	5'-CGCAGACTTCACGGAAACGTTTCGAGTCTTCTAC-3'
44up.....	5'-CGCTTGCCGTTGGCCTTGATCTCAACCTCATCA-3'
44Tdown.....	5'-CAGAACCTGACCGGTAGTGCAGTCGCCAGA-3'
44Hup.....	5'-TACAGGAGCAAATTCATTGGATCCTTCTACC-3'

^a Underlining indicates the start codon and the termination codon in Metup and Taildown, respectively.

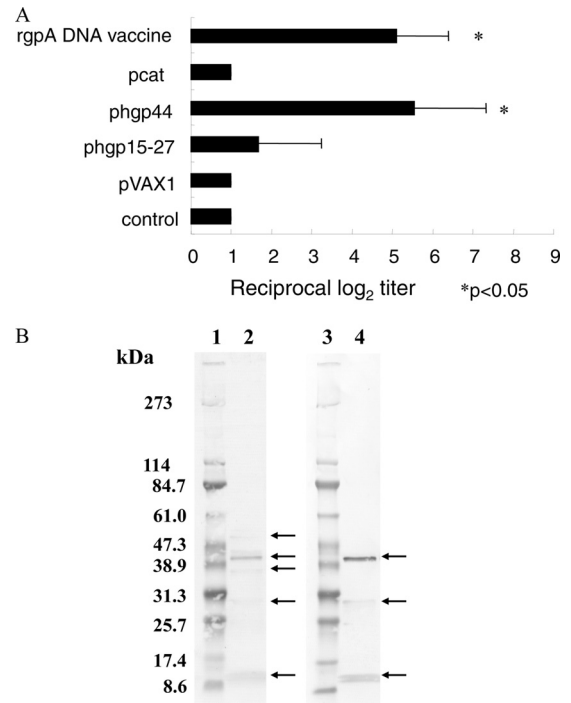


FIG. 2. (A) Induction of *P. gingivalis*-specific IgGs in mice immunized with *rgpA* domain DNA vaccines. Titers of IgG against sonicates of *P. gingivalis* in sera from mice were determined on day 42 after primary immunization, and endpoint titers were evaluated by measuring serially diluted sera. Results represent means \pm standard deviations for log₂ ELISA antibody titers. *, $P < 0.05$ by one-way ANOVA for comparison with mice immunized with pVAX1. (B) Immunoblot analysis with sera from mice immunized with the *rgpA* DNA vaccine or phgp44. Sonicates of *P. gingivalis* were separated by SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. Blotted membranes were immunostained using sera from mice immunized with the *rgpA* DNA vaccine or phgp44. Lanes 1 and 3, molecular size markers; lane 2, sera from mice immunized with the *rgpA* DNA vaccine; and lane 4, sera from mice immunized with phgp44. Molecular mass markers are shown in kilodaltons.

(see Fig. 4A), and the reciprocal titer reached $3.9 \pm 1.64 \log_2$ at week 6. These results suggest that the N-terminal half of HGP44 is potentially a potent epitope in the induction of protective antibody by RgpA, although further study is required to confirm this.

The effects of immunization with *rgpA* domain vaccines on *P. gingivalis*-induced alveolar bone loss are shown Fig. 3. The infected mice showed significantly greater alveolar bone loss in the maxillary molar area than did the uninfected control mice. Alveolar bone loss was reduced significantly in both the *rgpA* DNA vaccine-immunized group and the phgp44-immunized group, whereas the pcat- and phgp15-27-immunized groups showed no protection against alveolar bone loss. These results suggest that the HGP44 domain-coding region plays a predominant role in the *rgpA* DNA vaccine. The pattern of protection observed in the present study is in accordance with earlier results showing that passive immunization with monoclonal antibody against *P. gingivalis* protected against recolonization by *P. gingivalis* (3). Specific IgGs against *P. gingivalis* RgpA protected against colonization and alveolar bone loss in a murine model (3, 6, 7, 15, 23). The HGP44 domain was involved

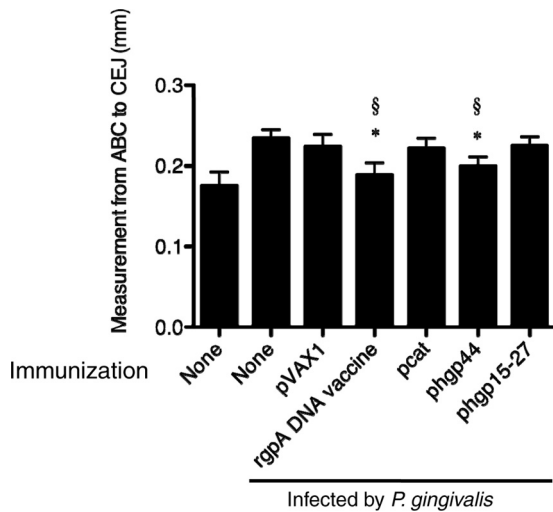


FIG. 3. Levels of alveolar bone loss elicited following *P. gingivalis* oral challenge after immunization with the *rgpA* domain DNA vaccine. BALB/c mice were immunized with *rgpA* DNA or *rgpA* domain DNA vaccines. The control groups consisted of age-matched, nonvaccinated mice and pVAX1-immunized mice. After immunization, mice were orally challenged with *P. gingivalis* ATCC 33277. At 42 days after oral challenge, all mice were sacrificed. *, $P < 0.05$ by one-way ANOVA for comparison with mice infected by *P. gingivalis* without immunization. §, $P < 0.05$ by one-way ANOVA for comparison with mice infected by *P. gingivalis* and immunized with pVAX1.

in adherence to epithelial cells (4) and *Treponema denticola* (11). Moreover, antibody elicited by an *rgpA* DNA vaccine inhibited binding of *P. gingivalis* to collagen sponges and hemagglutination of *P. gingivalis* (23). Antibody induced by a repeated sequence in the HGP44 domain inhibited binding of the RgpA-Kgp complex to fibrinogen, fibronectin, and collagen type IV (17). Antibody against anti-HGP44 also enhanced opsonization and killing of both invasive and noninvasive strains of *P. gingivalis* (22). These results agree with the protective effects we demonstrated with phgp44.

In this study, phgp44H was observed to exert a protective effect in comparison with the level for the nonimmunized mice but not in comparison with the level for pVAX-immunized mice (Fig. 4B). The antibody titer for phgp44H-immunized mice was lower than that for phgp44-immunized mice (Fig. 4A). Further study is required to determine the dose and rate of immunization required to induce protection against infection by *P. gingivalis*. Taken together with the results of an earlier study, the present results suggest that antibody against the N-terminal half of the HGP44 domain has a major protective effect.

The protective effect of phgp44 in the present experiments was somewhat lower than that of the *rgpA* DNA vaccine, although the difference was not significant. Kuboniwa et al. (14) reported that a DNA vaccine encoding the catalytic subunits of Rgp and Kgp elicited antibody production. The antibody attenuated protease activity and showed a protective effect against lethal challenge by *P. gingivalis*. Genco et al. (6) reported that an N-terminal peptide of the RgpA catalytic domain showed a protective effect against colonization by *P. gingivalis*. One study reported that a T cell epitope in the catalytic domain of Kgp induced Th2 responses (20). It is

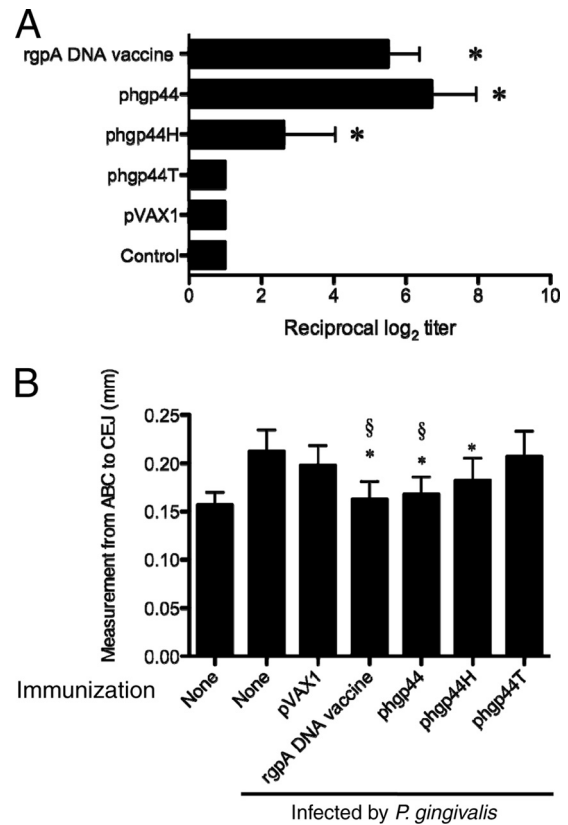


FIG. 4. (A) Induction of *P. gingivalis*-specific IgGs in mice immunized with *rgpA* domain DNA vaccines. Titers of IgG against sonicates of *P. gingivalis* in sera from mice were determined on day 43 after primary immunization, and endpoint titers were evaluated by measuring serially diluted sera. Results represent means \pm standard deviations for log₂ ELISA antibody titers. *, $P < 0.05$ by one-way ANOVA for comparison with mice immunized with pVAX1. (B) Levels of alveolar bone loss elicited following *P. gingivalis* oral challenge after immunization with the phgp44 derivative. BALB/c mice were immunized with *rgpA* DNA or *rgpA* domain DNA vaccines. The control groups consisted of age-matched, nonvaccinated mice and pVAX1-immunized mice. After immunization, mice were orally challenged with *P. gingivalis* ATCC 33277. At 42 days after oral challenge, all mice were sacrificed. *, $P < 0.05$ by one-way ANOVA for comparison with mice infected by *P. gingivalis* without immunization. §, $P < 0.05$ by one-way ANOVA for comparison with mice infected by *P. gingivalis* and immunized with pVAX1.

possible that an additional effect in response to the catalytic subunit is necessary to elicit the same protective effect as that obtained with the *rgpA* DNA vaccine. HGP44 was reported to be involved in the adherence by this microorganism (4, 11). Colonization by *P. gingivalis* was also reported to occur in the mouse model (2). It is possible that the protection against colonization by *P. gingivalis* plays a major role in the inhibition of bone loss in the present study. Based upon our results, we cannot exclude the possibility that the proteolytic activity of RgpA plays an important role in bone loss since protection mediated by the adhesive and proteolytic domains may not be additive. Therefore, further analysis is required to investigate the effects of antibody against the catalytic subunit on bone loss.

In the present study, the HGP44 domain-coding DNA vac-

cine could account for the protective effect of the *rgpA* DNA vaccine. The results of the present study indicate that phgp44 has the ability to induce protective immunity against *P. gingivalis*-induced alveolar bone loss and that the HGP44-coding region is a candidate antigen for a DNA vaccine.

We thank Howard K. Kuramitsu for critical review of the manuscript and Jeremy Williams for his assistance with the development of the manuscript.

This study was supported by grant HRC7 from the Oral Health Science Center of Tokyo Dental College and a High-Tech Research Center Project for Private Universities Matching Fund Subsidy from MEXT (2006 to 2010).

REFERENCES

- Amano, A., I. Nakagawa, N. Okahashi, and N. Hamada. 2004. Variations of *Porphyromonas gingivalis* fimbriae in relation to microbial pathogenesis. *J. Periodontol. Res.* **39**:136–142.
- Baker, P. J., R. T. Evans, and D. C. Roopenian. 1994. Oral infection with *Porphyromonas gingivalis* and induced alveolar bone loss in immunocompetent and severe combined immunodeficient mice. *Arch. Oral Biol.* **39**:1035–1040.
- Booth, V., F. P. Ashley, and T. Lehner. 1996. Passive immunization with monoclonal antibodies against *Porphyromonas gingivalis* in patients with periodontitis. *Infect. Immun.* **64**:422–427.
- Chen, T., K. Nakayama, L. Belliveau, and M. J. Duncan. 2001. *Porphyromonas gingivalis* gingipains and adhesion to epithelial cells. *Infect. Immun.* **69**:3048–3056.
- Darveau, R. P. 2010. Periodontitis: a polymicrobial disruption of host homeostasis. *Nat. Rev. Microbiol.* **8**:481–490.
- Genco, C. A., B. M. Odusanya, J. Potempa, J. Mikolajczyk-Pawlinska, and J. Travis. 1998. A peptide domain on gingipain R which confers immunity against *Porphyromonas gingivalis* infection in mice. *Infect. Immun.* **66**:4108–4114.
- Gibson, F. C., III, and C. A. Genco. 2001. Prevention of *Porphyromonas gingivalis*-induced oral bone loss following immunization with gingipain R1. *Infect. Immun.* **69**:7959–7963.
- Gonzalez, D., A. O. Tzianabos, C. A. Genco, and F. C. Gibson III. 2003. Immunization with *Porphyromonas gingivalis* capsular polysaccharide prevents *P. gingivalis*-elicited oral bone loss in a murine model. *Infect. Immun.* **71**:2283–2287.
- Guo, Y., K. A. Nguyen, and J. Potempa. 2010. Dichotomy of gingipains action as virulence factors: from cleaving substrates with the precision of a surgeon's knife to a meat chopper-like brutal degradation of proteins. *Periodontol.* **2000** **54**:15–44.
- Inagaki, S., K. Ishihara, Y. Yasaki, S. Yamada, and K. Okuda. 2003. Antibody responses of periodontitis patients to gingipains of *Porphyromonas gingivalis*. *J. Periodontol.* **74**:1432–1439.
- Ito, R., K. Ishihara, M. Shoji, K. Nakayama, and K. Okuda. 2010. Hemagglutinin/adhesin domains of *Porphyromonas gingivalis* play key roles in coaggregation with *Treponema denticola*. *FEMS Immunol. Med. Microbiol.* **60**:251–260.
- Kelly, C. G., et al. 1997. The relationship between colonization and haemagglutination inhibiting and B cell epitopes of *Porphyromonas gingivalis*. *Clin. Exp. Immunol.* **110**:285–291.
- Kinane, D., and P. Bouchard. 2008. Periodontal diseases and health: consensus report of the Sixth European Workshop on Periodontology. *J. Clin. Periodontol.* **35**:333–337.
- Kuboniwa, M., A. Amano, S. Shizukuishi, I. Nakagawa, and S. Hamada. 2001. Specific antibodies to *Porphyromonas gingivalis* Lys-gingipain by DNA vaccination inhibit bacterial binding to hemoglobin and protect mice from infection. *Infect. Immun.* **69**:2972–2979.
- Miyachi, K., K. Ishihara, R. Kimizuka, and K. Okuda. 2007. Arg-gingipain A DNA vaccine prevents alveolar bone loss in mice. *J. Dent. Res.* **86**:446–450.
- Nakayama, K. 2003. Molecular genetics of *Porphyromonas gingivalis*: gingipains and other virulence factors. *Curr. Protein Pept. Sci.* **4**:389–395.
- O'Brien-Simpson, N. M., et al. 2005. An immune response directed to proteinase and adhesin functional epitopes protects against *Porphyromonas gingivalis*-induced periodontal bone loss. *J. Immunol.* **175**:3980–3989.
- Ogawa, T., Y. Asai, Y. Makimura, and R. Tamai. 2007. Chemical structure and immunobiological activity of *Porphyromonas gingivalis* lipid A. *Front. Biosci.* **12**:3795–3812.
- Pihlstrom, B. L., B. S. Michalowicz, and N. W. Johnson. 2005. Periodontal diseases. *Lancet* **366**:1809–1820.
- Tam, V., N. M. O'Brien-Simpson, R. D. Pathirana, L. T. Frazer, and E. C. Reynolds. 2008. Characterization of T cell responses to the RgpA-Kgp proteinase-adhesin complexes of *Porphyromonas gingivalis* in BALB/c mice. *J. Immunol.* **181**:4150–4158.
- Teles, R. P., A. D. Haffajee, and S. S. Socransky. 2006. Microbiological goals of periodontal therapy. *Periodontol.* **2000** **42**:180–218.
- Yasaki-Inagaki, Y., S. Inagaki, S. Yamada, K. Okuda, and K. Ishihara. 2006. Production of protective antibodies against *Porphyromonas gingivalis* strains by immunization with recombinant gingipain domains. *FEMS Immunol. Med. Microbiol.* **47**:287–295.
- Yonezawa, H., K. Ishihara, and K. Okuda. 2001. Arg-gingipain a DNA vaccine induces protective immunity against infection by *Porphyromonas gingivalis* in a murine model. *Infect. Immun.* **69**:2858–2864.