Construction and Characterization of Arginine-specific Cysteine Proteinase (Arg-gingipain)-deficient Mutants of Porphyromonas gingivalis

EVIDENCE FOR SIGNIFICANT CONTRIBUTION OF Arg-GINGIPAIN TO VIRULENCE*

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Arginine-specific cysteine proteinase (Arg-gingipain; formerly, argingipain) is one of the major extracellular proteinases produced by the oral anaerobic bacterium Porphyromonas gingivalis. To determine whether Arg-gingipain is important for periodontopathogenicity of the organism, Arg-gingipain-deficient mutants were constructed via gene disruption by use of suicide plasmid systems. First, Southern hybridization analyses suggested that two separate Arg-gingipain-encoding genes designated rgpA and rgpB existed on 12.5- and 7.8-kilobase pair HindIII chromosomal fragments of P. gingivalis ATCC33277, respectively. rgpA and rgpB single mutants were constructed by mobilization of a suicide plasmid. Then, an rgpA rgpB double mutant was isolated by electroporation with a second suicide plasmid. No proteolytic activity for Arg-gingipain was observed in either the cell extract or the culture supernatant of the rgpA rgpB mutant. The chemiluminescence response of polymorphonuclear leukocytes, which is closely related to their bactericidal function, was not inhibited by the culture supernatant of the rgpA rgpB mutant, while the wild type parent showed a significant inhibition of the response. The result suggests that Arg-gingipain is responsible for disruption of the function of polymorphonuclear leukocytes. In addition, the rgpA rgpB double mutations caused a marked decrease in the hemagglutination of P. gingivalis, indicating that a major part of the hemagglutinin activity of the organism is associated with the two genes. These findings demonstrate that Arg-gingipain makes a significant contribution to the virulence of P. gingivalis.

The oral anaerobic bacterium Porphyromonas (Bacteroides) gingivalis, which belongs to the bacteroides-flavobacterium phyllum of the bacterial phylogenetic tree (1), has been implicated as one of the major causative agents for advanced adult periodontitis (2-4). The microorganism possesses several potential virulence factors for periodontopathogenicity (5). Among these factors the proteolytic enzymes are of special importance, since some of them have the abilities to destroy periodontal tissue directly or indirectly (6, 7), to activate or degrade host inflammatory proteins (8-11), and to disturb host defense mechanisms (12-14). There have been many attempts to isolate a variety of proteinases produced by P. gingivalis in both cell-free and cell-associated forms (see Ref. 15 for a review). In addition, several genes encoding proteinases have been cloned from P. gingivalis (16-21). Although it has been found that the multiple forms of trypsin-like activity of P. gingivalis are due to the presence of either Arg-gingipain or Lys-gingipain (22), the number and properties of proteinases that are actually associated with virulence of the organism remain to be clarified.

Protoheme is an absolute requirement for growth of P. gingivalis (23-25), and it is probably derived from erythrocytes in the natural niche for the organism. Therefore, it is particularly important for the organism to agglutinate and lyse erythrocytes in order to survive in vivo (26, 27). The dose relationship between hemagglutinin and cysteine proteinase has been pointed out by several researchers (19, 28-31). However, there has been little agreement as yet on the identity of the two molecules. Nishikata and Yoshimura (29) found that one molecule possesses both the proteinase and hemagglutinin activities. On the other hand, Pike et al. (30) and Shah et al. (31) reported that the proteinase and hemagglutinin are separate molecules, although the two molecules are noncovalently bound to each other. Recently, based on the cloning and sequence analysis of the gene encoding arginine-specific cysteine proteinase (arginipain) (19), we have suggested that the enzyme results from processing of a 109-kDa preproenzyme comprising four domains, i.e. the signal peptide, the amino-terminal domain, the proteinase domain, and the carboxyl-terminal hemagglutinin domain. This finding is consistent with the results of Ciborowski et al. (28), who have suggested that the proteinase and hemagglutinin molecules are formed by processing of the primary product from the same gene.

Pavloff et al. (21) also have sequenced the gene encoding arginine-specific cysteine proteinase (Arg-gingipain-1) from a different P. gingivalis strain. Comparison of the amino acid sequences deduced from the nucleotide sequences of argingipain and Arg-gingipain-1 genes have revealed that they are essentially identical, except that the argingipain gene lacks a sequence intervening between direct repeats in the carboxy-terminal domain. Especially, the proteinase domains of argingipain and Arg-gingipain-1 genes were completely identical. Therefore, the arginine-specific cysteine proteinase “arginipain” was renamed “Arg-gingipain” in the present study to avoid redundancies in nomenclature.

We have shown previously that Arg-gingipain is a major cysteine proteinase of P. gingivalis, a part of which is secreted...
extracellularly, and have strongly suggested that the enzyme is directly involved in the destruction of periodontal tissue (32). Furthermore, based on its strong inhibition of the chemiluminescence (CL) response of polymorphonuclear leukocytes and its ability to degrade human immunoglobulins G and A, we have proposed that the enzyme can impair host defense mechanisms (32). However, we do not understand to what extent Arg-gingipain contributes to the virulence of P. gingivalis. Also, there is little information available on the physiological significance of the enzyme in the organism. To gain some insights into these questions, it is necessary to undertake the molecular genetic approach. For this, we constructed Arg-gingipain-deficient mutants via disruption of the Arg-gingipain gene by use of suicide plasmid systems (33). In the course of construction, we found that two different Arg-gingipain-deficient mutants exist on the chromosome of P. gingivalis ATCC33277. The results obtained with the Arg-gingipain-deficient mutants provide the evidence that Arg-gingipain is a major virulence factor of P. gingivalis.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Plasmids—**P. gingivalis ATCC33277, P. gingivalis 381, and Escherichia coli DH5α were used. Plasmids pUC18 (34), pKDCMZ (33), pJF-3 (35), and R751 (36) were used for construction of suicide/integration plasmids for P. gingivalis. Plasmid P.g./pUC118 (19) was used as a source of the Arg-gingipain gene. Plasmids pKD274, pKD279, pKD280, and pKD290 and P. gingivalis mutants KDP110, KDP111, and KDP112 were obtained in this study.

**Chemicals and Proteins—**Proteinase inhibitors, Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK) and leupeptin were purchased from Sigma and Peptide Institute Inc. (Osaka, Japan). Synthetic substrates, carbobenzoxy-L-phenylalaninyl-L-arginine 4-methyl-7-coumarylamide (Z-Phe-Arg-MCA) and seryl-L-arginine 4-methyl-7-coumarylamide (Boc-Phe-Ser-Arg-MCA) were obtained from Peptide Institute Inc. (Osaka, Japan), respectively. Synthetic substrates, carbobenzoxy-L-phenylalaninyl-L-arginine 4-methyl-7-coumarylamide (Z-Phe-Arg-MCA) and t-butyloxy carbonyl-L-phenylalaninyl-L-arginine 4-methyl-7-coumarylamide (Boc-Phe-Phe-Arg-MCA) were obtained from Peptide Institute Inc. (Osaka, Japan). Casein, bovine hemoglobin, oyster glycogen, and zymosan A were obtained from Sigma. DNA restriction enzymes and T4 ligase were added to the media at the following concentrations (ampicillin, 50 μg/ml; chloramphenicol, 25 μg/ml; erythromycin, 10 μg/ml; gentamicin, 100 μg/ml; and tetracycline, 1 μg/ml).

**Media and Conditions for Cell Growth—**P. gingivalis cells were grown anaerobically (10% CO2, 10% H2, 80% N2) in enriched BHI broth (containing, per liter, 37 g of brain heart infusion (Difco), 5 g of yeast extract (Difco), 1 g of cysteine 5 g of hemin, and 1 mg of vitamin K1) and on enriched tryptic soy agar (containing, per liter, 40 g of tryptic soy agar (Nissui, Tokyo, Japan), 5 g of brain heart infusion, 1 g of cysteine, 5 mg of hemin, and 1 mg of vitamin K1) L broth (containing, per liter, 10 g of tryptone (Difco), 5 g of yeast extract, and 5 g of sodium chloride) was used for growing E. coli cells. For selection or maintenance of the antibiotic-resistant strains, antibiotics were added to the media at the following concentrations (ampicillin, 50 μg/ml; chloramphenicol, 25 μg/ml; erythromycin, 10 μg/ml; gentamicin, 100 μg/ml; and tetracycline, 1 μg/ml).

**DNA Manipulations—**Plasmid DNA was purified from E. coli cells by using the Wizard DNA purification system (Promega, Madison, WI). Chromosomal DNA was isolated from P. gingivalis cells by the guanidine isothiocyanate method (37) with the IsoQuick DNA extraction kit (MicroProbe, Garden Grove, CA).

Plasmid Construction—Suicide plasmids constructed in this study are depicted in Fig. 1. A 1.0-kbp Smal-BamHI fragment of P.g./pUC118 plasmid was ligated with a HindIII-BamHI digest of pUC18. The resulting plasmid was then digested with EcoRV and ligated with a Black BglII linker (digonucleotide to convert the EcoRV site to BglII, giving rise to plasmid pKD274). Plasmid pKD274 was digested with BglII and BamHI and self-ligated to yield pKD279. A 0.7-kbp BglII-PstI fragment of pKD274, which corresponded to the EcoRV-PstI fragment within the Arg-gingipain gene, was ligated with a BamHI-PstI digest of pKDCMZ.
plasmid to yield pkD280. A 0.7-kbp HindIII-Bgl II fragment of pkD274 was ligated with a HindIII-BamHI digest of plasmid F-3 into plasmid pkD290.

DNA Probes and Southern Blot Hybridization—A 2.8-kbp Smal fragment of P.g./pUC118 (probe I) and a 0.7-kbp PstI-EcoRI fragment of pkD279 (probe II) were labeled with digoxigenin-dUTP (Boehringer GmbH, Mannheim, Germany). Southern blotting was performed on a nylon membrane (Hybond™-N: Amersham) essentially according to Southern (38). Hybridization with probes I and II was done by using the Boehringer nonradioactive DNA labeling and detection kit, and the ECL 3-oligolabeling and detection systems (Amersham) were used for Southern analyses with the oligonucleotide probes.

Mobilization of a Suicide Plasmid (pkD280) from E. coli to P. gingivalis—The procedure for the mobilization was described previously (33). Briefly, the culture of E. coli DH5 harboring pkD280 and R751 plasmids was mixed with an equal volume of the culture of P. gingivalis ATCC33277 and the cultures were harvested by centrifugation. The cell pellet was resuspended in prewarmed enriched BHI broth and spotted on enriched tryptic soy agar containing erythromycin and gentamicin, and incubated anaerobically at 37°C for 4 days. The resulting colonies were picked and used for further experiments.

Electrotransformation of P. gingivalis with pkD290 Plasmid DNA—P. gingivalis cells were anaerobically grown to 6 × 10^8 cells/ml at 37°C in enriched BHI broth. The cells were then harvested by centrifugation, washed with the electroporation solution (300 µl), and resuspended in 0.1 vol of the same solution. Fifteen microliters of pkD290 plasmid DNA solution (270 µg of DNA/ml in TE buffer) were added to 100-µl aliquots of cell suspensions, which were then mixed with 100-µl aliquots of a nylong membrane (Hybond™-N: Amersham) essentially according to Southern (38). Hybridization with probes I and II was done by using the Boehringer nonradioactive DNA labeling and detection kit, and the ECL 3-oligolabeling and detection systems (Amersham) were used for Southern analyses with the oligonucleotide probes.

RESULTS

Southern Hybridization Analyses with DNA Probes for the P. gingivalis Arg-gingipain Gene

The Arg-gingipain-encoding gene has been cloned from P. gingivalis 381, and its nucleotide sequence has been determined (19). Since it was difficult to use this strain for construction of Arg-gingipain-deficient mutants because of its low efficiency in mobilization and electroporation, we chose P. gingivalis ATCC33277 from which we had isolated a superoxide dismutase-deficient mutant by mobilization of a suicide plasmid (33). To examine whether the Arg-gingipain gene is located on the chromosome of P. gingivalis ATCC33277, Southern hybridization of its chromosomal DNA was performed with a 2.8-kbp Smal fragment of P.g./pUC118 corresponding to the Smal-PvuI region of Arg-gingipain gene (probe I) as a DNA probe (Fig. 2). Interestingly, probe I DNA hybridized to four separate HindIII fragments of the chromosomes of P. gingivalis ATCC33277 and 381, respectively. Since probe I DNA has no HindIII site, the result indicates that P. gingivalis chromosome may possess several regions which share homology with the probe DNA. Then, we analyzed the chromosome of ATCC33277 with other Arg-gingipain gene-associated DNA probes. A 0.7-kbp PstI-EcoRI fragment of pkD279, which corresponded to the Smal-EcoRV region encoding the amino-terminal region of the proteinase domain (probe II) and an oligonucleotide probe encoding a putative catalytic site for the proteinase (probe III) (19), hybridized to two separate HindIII fragments, suggesting that P. gingivalis ATCC33277 may possess
Fig. 2. Southern blot analyses of P. gingivalis ATCC33277 chromosomal DNA with the Arg-gingipain and Arg-gingipain-1 gene probes. Panel A, restriction maps of Arg-gingipain gene of P. gingivalis 381 and Arg-gingipain-1 gene of P. gingivalis H66 and location of the DNA regions complementary to their DNA probes. The restriction maps of Arg-gingipain and Arg-gingipain-1 genes are according to Okamoto et al. (19) and Pavloff et al. (21), respectively. Open boxes above restriction maps show Arg-gingipain DNA probes I and II. Triangles indicate the DNA regions complementary to the oligonucleotide probes III, IV, V, and VI. The dark, dotted, open, and hatched regions of Arg-gingipain gene represent the signal sequence, the amino-terminal domain, the proteinase domain, and the carboxyl-terminal domain, respectively. Black regions of Arg-gingipain and Arg-gingipain-1 genes represent a direct repeat encoding 17 amino acids starting from YYTYVYRDF. Restriction sites: B, BamHI; E, EcoRV; Ps, PsI; Pv, PvuII; S, Smal. Panel B, Southern blots. The chromosomal DNA of P. gingivalis was digested with several restriction enzymes. The digested DNA was subjected to agarose gel electrophoresis and Southern blot hybridization with the indicated DNA probes. a, chromosomal DNA of ATCC33277 (lanes 1 and 3) and 381 (lanes 2 and 4) was digested with HindIII (lanes 1 and 2) and PvuII (lanes 3 and 4). b, chromosomal DNA of ATCC33277 was digested with HindIII (lane 1), PvuII plus PsI (lane 2), PvuII plus EcoRV (lane 3), PvuII (lane 4), and Stul (lane 5). c-f, chromosomal DNA of ATCC33277 was digested with HindIII (lane 1), Stul (lane 2), PvuII (lane 3), PvuII plus PsI (lane 4), and PvuII plus EcoRV (lane 5). The blots (c and d) were subjected to rehybridization with probe VI (e) and probe V (f), respectively, after removal of probes III and IV. Two different chromosomal loci encoding Arg-gingipain (Fig. 2). One of the gene loci (12.5-kbp HindIII fragment) and the other (7.8-kbp HindIII fragment) were tentatively designated rgpA and rgpB, respectively. The rgpA locus was also hybridized with two oligonucleotide probes for the carboxyl-terminal domain of Arg-gingipain (probe IV) and for the region downstream from the Arg-gingipain gene (probe V), whereas the rgpB locus was hybridized with neither of them (Fig. 2). The result suggests that the rgpA locus may encode the carboxyl-terminal domain of Arg-gingipain in addition to the proteinase domain, while the rgpB locus may not. Recently, Pavloff et al. (21) have sequenced the Arg-gingipain-1 gene (rgp1) of P. gingivalis H66. Comparison between the amino acid sequences of Arg-gingipain gene of 381 and Arg-gingipain-1 gene of H66 revealed that their proteinase domains were identical but their carboxyl-terminal domains were different (Fig. 2). The Arg-gingipain-1 gene contains three direct repeats of the nucleotide sequence that encodes 17 amino acids starting from YYTYVYRDF in its carboxyl-terminal region. A DNA region intervening between the first repeating sequence and the third one is completely deleted from the carboxyl-terminal region of the Arg-gingipain gene. To determine whether the rgpA locus of ATCC33277 contains this intervening sequence, the oligonucleotide probe for the intervening sequence (probe VI) was used. The rgpA locus was hybridized with this probe, and the restriction maps of the rgpA locus of ATCC33277 and the rgp1 locus of H66 were so far identical, suggesting that the rgpA of ATCC33277 is probably equivalent to the rgp1 of H66. From these Southern analyses, we also found that at least two separate chromosomal regions other than the rgpA locus might encode the carboxyl-terminal domain of Arg-gingipain-1.

Construction of Arg-gingipain-deficient Mutants

To determine the importance of Arg-gingipain for pathogenicity of P. gingivalis, we constructed Arg-gingipain-deficient mutants via gene disruption by use of a suicide plasmid containing an internal DNA fragment of the Arg-gingipain gene. Thus, Em" transconjugants were obtained after mobilization of pKD280 containing the 0.7-kbp EcoRV-PstI fragment of the Arg-gingipain gene into P. gingivalis ATCC33277. Southern hybridization analysis of the chromosomes of the Em" transconjugants showed that two classes of transconjugants were obtained with respect to the location of the integrated plasmid. In one class (a representative strain, KDP110) the rgpA locus is disrupted, while in the other class (a representative strain, KDP111) disruption occurred at the rgpB locus (Figs. 3 and 4). Thus, integration of pKD280 plasmid DNA into the chromosome at the rgpA locus resulted in the disappearance of the 12.5-kbp hybridizing HindIII fragment and the appearance of
Southern blot hybridization with probe II. These digested DNA were subjected to agarose gel electrophoresis and revealed bands of molecular masses of 76 and 70 kDa, but not 44 kDa. Moreover, KDP121 (rgpB) showed complete loss of the hydrolytic activity in both its culture supernatant and cell extract (Table I). Residual activities observed in KDP110 and KDP111 were inhibited by leupeptin, TLCK, and EDTA (data not shown). The residual activities of the single mutants apparently did not balance. The marked and disproportional decrease in the proteolytic activities of Arg-gingipain in the single mutants suggests the possibility that the disruption of one rgp gene has an inhibitory effect on expression from the other gene. When the protein substrates casein and hemoglobin were used in assay, only 5–10% of the hydrolytic activity of the culture supernatant of ATCC33277 was observed in that of KDP112 (rgpA rgpB) (Table I). These results indicate that Arg-gingipain is a major extracellular proteinase of P. gingivalis.

Effect on CL Response of PMNs—The culture supernatant of P. gingivalis possesses a potent virulence factor, which disrupts the bactericidal function of PMNs (14). The virulence factor was successfully purified and turned out to be arginine-specific cysteine proteinase (Arg-gingipain) (32). To determine whether the virulence factor in the culture supernatant is attributable to Arg-gingipain, we examined the rgp mutants for the effect of their supernatants on CL response of PMNs (Table I). Residual activities observed in KDP110 (rgpA) and KDP111 (rgpB) partially lost the effect (Fig. 6). The result suggests the possibility that the effect of the culture supernatant of P. gingivalis on CL response of PMNs is mainly due to Arg-gingipain.

Hemagglutination—We found in the previous study (19) that the carboxyl-terminal domain of Arg-gingipain contained the sequence identical to the amino-terminal sequence of a hemagglutinin fraction suggested by Pike et al. (30). To determine whether the rgp genes are related in hemagglutination of P. gingivalis, we examined the rgp mutants for hemagglutination. KDP110 (rgpA) and KDP111 (rgpB) showed only a little decrease of hemagglutination, whereas the rgpA rgpB double mutant (KDP112) showed a greater decrease of hemagglutination (Fig. 7). These results indicate that the rgp genes are directly or indirectly involved in the hemagglutination activities of P. gingivalis.

**DISCUSSION**

We constructed rgpA and rgpB single mutants of Arg-gingipain by integration of the Em' suicide plasmid pDCMZ containing an internal DNA fragment of the gene. To construct the rgpA rgpB double mutant, we needed to use a second suicide plasmid carrying a different antibiotic resistance gene and having little or no homology with pKDCMZ. The shuttle vector pMJ F-3 contains a Tcr gene, which functions in plasmid pKDCMZ DNA. Therefore, this vector was used to construct the second suicide plasmid pKD290 for construction of the double knockout mutant. We used electroporation to introduce pKD290 into P. gingivalis.
gingivalis cells, although P. gingivalis strains including ATCC33277 were thought to possess DNA restriction systems (43). All the Tc' transformants analyzed contained the suicide plasmid DNA in the homologous chromosomal region. This result indicates that electroporation can be applied to construct gene-disrupted mutants in P. gingivalis.

Several lines of evidence show that P. gingivalis ATCC33277 possesses two separate genes responsible for arginine-specific cysteine proteinase activity in its chromosome. First, the DNA probes for the proteinase domain of Arg-gingipain hybridized to two different restriction fragments of the chromosomal DNA. Second, homologous recombination took place between each of the two genetic loci (rgpA and rgpB) on the chromosome and the Arg-gingipain DNA on the suicide plasmids. Third, the rgpA and the rgpB single mutants showed the marked reduction of the hydrolytic activity on the two synthetic substrates for arginine-specific proteinase but still retain some of the activity; however, the rgpA rgpB double mutant showed no hydrolytic activity on the substrates. Fourth, residual proteinase activity seen in the culture supernatants and the cell extracts of the rgpA and rgpB single mutants was sensitive to leupeptin, TLCK, and EDTA, all of which are potent inhibitors for Arg-gingipain. Fifth, the culture supernatants and cell extracts of the rgpA and rgpB single mutants still contained proteins that immunoreacted with anti-Arg-gingipain antibody. However, the amounts of immunoreacting protein made by the mutants were much less than that of the wild type strain, and the profiles of the immunoblots were also different from that of the wild type strain. These immunoreacting substances almost disappeared in the rgpA rgpB mutant.

Gene duplication has been observed in other prokaryotic systems (44–46). Among them, the cholera toxin operon (ctxAB) in Vibrio cholerae is a good example with respect to the direct correlation between copy number and virulence (46). The proteinase domain region of Arg-gingipain gene was probably duplicated at some time in the evolution of P. gingivalis, and a strain possessing two or more Arg-gingipain-related genes may selectively survive in conflict with its host and other microorganisms.

Although the rgpA and rgpB genes are very similar to each

![Diagram](https://via.placeholder.com/150)
A role for Arg-gingipain in P. gingivalis hemagglutination is indicated by the marked decrease in this activity in the rgpA rgpB double mutant, although we should reserve the possibility that hemagglutinin genes could be located downstream of both of the rgpA and rgpB genes and simultaneously inactivated in the double mutant by integration of the suicide plasmids. Pike et al. (30) found that the high molecular mass Arg-gingipain has hemagglutinin activity that is inhibited by arginine, whereas the low molecular mass Arg-gingipain is devoid of the activity. We also found that the extracellular Arg-gingipain (44 kDa) purified from the culture supernatant has no hemagglutinin activity that is inhibited by arginine, because no stable plasmid has been found in this organism.

Hydrolytic activities on casein and hemoglobin were significantly reduced in the culture supernatant of the rgpA rgpB mutant, indicating that Arg-gingipain is particularly important among extracellular proteinases secreted from P. gingivalis for general protein degradation. In addition, Arg-gingipain has the specific ability to disturb the function of PMNs. The present study indicates that the inhibitory effect of the culture supernatant of P. gingivalis on CL response of PMNs is mainly attributable to the presence of Arg-gingipain in the supernatant. Thus, it is feasible to consider that Arg-gingipain plays an important role in periodontopathogenicity of P. gingivalis.

Several proteinases with specificities similar to Arg-gingipain have been purified from various P. gingivalis strains (20, 28, 30, 47–52). In this study, we found that all of the enzymatic activity for arginine-specific cysteine proteinase in P. gingivalis ATCC33277 might be derived from the rgpA and rgpB, as suggested by the total loss of the activity in the rgpA rgpB mutant. Therefore, some of the proteinases previously described may be homologs of the rgpA or rgpB gene product and others may not be virtually expressed in the P. gingivalis cells, although we cannot rule out the possibility that a protease gene(s) could be located downstream of rgpA or rgpB and inactivated by polar effect of plasmid integration. Usually, complementation of a mutation by a plasmid carrying a wild type gene can be used in many microorganisms to prove the cause and effect relationship. However, it is difficult to apply complementation to P. gingivalis because no stable plasmid has been found in this organism.

The hemagglutinating titers of P. gingivalis ATCC33277, KDP110, KDP111, and KDP112 are 32, 16, 16, and 1, respectively.
tinin activity. These results suggest that the carboxyl-terminal domains of Arg-gingipain and Arg-gingipain-1 (starting from SGQAEIVL) could be responsible for the hemagglutinin activity; however, the possibility that the proteinase domain is also necessary for the activity cannot be excluded since the rgpB gene seems not to possess the same carboxyl-terminal domain. In this connection, we found in this study that there were at least two other chromosomal regions which may share homology with the carboxyl-terminal domain, suggesting that these regions might encode other hemagglutinins.

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