Construction and Characterization of the PGN_0296 Mutant of *Porphyromonas gingivalis*

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Abstract

The periodontal pathogen *Porphyromonas gingivalis* produces gingipains (Kgp, RgpA, and RgpB), cysteine proteases involved in the organism's virulence, and pigmentation. We previously showed that deletion of the PGN_0297 and PGN_0300 genes reduced the proteolytic activity of gingipains. The role of the PGN_0296 gene, consisting of an operon with the PGN_0297 and PGN_0300 genes, is unclear. Herein, we examined the effect of PGN_0296 gene deletion on the proteolytic activity. Although the proteolytic activity of the gingipains did not decrease in the culture supernatant of a PGN_0296 gene deletion mutant (ΔPGN_0296), the growth was delayed.

Keywords

Porphyromonas gingivalis, Type XI secretion system, gingipain, C-terminal domain

1. Introduction:

The anaerobic gram-negative bacterium *Porphyromonas gingivalis* is an etiologically important agent for periodontal disease [1]. *P. gingivalis* possesses several virulence factors such as the fimbriae, hemagglutinins, lipopolysaccharides, capsules, and proteinases [2]. Gingipains, the extracellular and surface proteinases with high hydrolytic activities, are particularly significant virulence factors as they can destroy the periodontal tissues directly and/or indirectly [3-5]. *P. gingivalis* possesses two Arg-specific gingipains, RgpA and RgpB, and a Lys-specific gingipain, Kgp, encoded by *rgpA*, *rgpB*, and *kgp*, respectively [6-8].

The gingipains contain the C-terminal domain (CTD), which interacts with components of the Type IX secretion system (T9SS) to transport across the outer membrane. The *P. gingivalis* genome encodes approximately 34 CTD-containing proteins [9,10] and the T9SS is present in the *Fibrobacteres-Chlorobi-Bacteroidetes* superphylum (CFB group) [9].

The T9SS is a complex system involving many proteins [11,12]. Many studies have focused on analyzing the details of the T9SS and the role of each component such as PorE, PorK, PorL, PorM, PorN, PorQ, PorU, PorV, PorZ, and SprA (Sov). A two-component system, PorX-PorY, is known to regulate the operon of *por* genes (*porP*, *porK*, *porL*, *porM*, and *porN*) [13,14]. In addition, PorP, PorT, PorW, Omp17, PorF, and PorG are known to be involved in the T9SS, but their functions have not been revealed [15]. Among them, PorG (PGN_0297) and Omp17 (PGN_0300) consist of an operon with PGN_0296, PGN_0298, PGN_0299, and PGN_0301 (PGN_0297) [16]. PGN_0299 encodes Omp85 proteins, involved in biofilm formation [17], and PGN_0301 is predicted to encode OmpH-like domain proteins [16]. The PGN_0296 and PGN_0298 gene products are annotated as outer membrane protein (Omp) and undecaprenyl pyrophosphate synthase, respectively; however, their functions are unknown. As the PGN_0296 gene is the first gene of this operon, there is a possibility of its involvement in T9SS function.

To determine whether PGN_0296 contributes to the secretion of CTD-containing proteins via the T9SS, we constructed a PGN_0296 gene deletion mutant (ΔPGN_0296), determined the effects of the protease activities of gingipains on ΔPGN_0296, and observed its growth.

2. Materials and methods

2.1. Bacterial strains and growth condition

Escherichia coli were grown in Luria-Bertani (LB) medium at 37°C. *P. gingivalis* cells were grown anaerobically (10% CO_2, 10% H_2, 80% N_2) at 37°C in enriched brain heart infusion (BHI) broth [18] and enriched tryptic soy (TS) agar [18]. Antibiotics were used at the following concentrations: ampicillin (Ap; 100 μg/mL for *E. coli*), erythromycin (Em; 10 μg/mL for *P. gingivalis*), and tetracycline (Tc: 0.7 μg/mL for *P. gingivalis*).

2.2. Strain construction

The PGN_0296 fragment was PCR-amplified from the chromosomal DNA of *P. gingivalis* ATCC33277 to disrupt PGN_0296, using the primers 0295F (5'-GAGGGATCCGTGAAGTAAGCGGATCAGCACGTGC-3') and 0298R (5'-GAGGAATTCGGCCATTACCATCCATGACCAAAGCG-3') (Figure 1A). Then, the fragment was double-digested with BamHI and EcoRI and ligated into the BamHI/EcoRI site of pUC19, resulting in p0296A. The Em resistance gene, *ermF*, was amplified from pKD355 [19] using the primer set ermF-F (5'-GCGAGATCTCATGACAAAAAAGAAATTGCCC-3') plus ermF-R (5'- CCATCGATTACGAAGGATGAAATTTTTCAG-3'). Following this, the fragment was double-digested with BglII and ClaI and inserted into the BglII/ClaI site within PGN_0296 of p0296A. The resulting plasmid was then linearized by BamHI and EcoRI digestion and introduced into *P. gingivalis* cells by electroporation [16]. A recombinant where the double crossover recombination event occurred correctly, was designated as ΔPGN_0296.

To construct the complementation of PGN_0296, PGN_0296 was inserted into the PGN_1045 locus of ΔPGN_0296 using the techniques described by Taguchi et al. [16]. Briefly, the coding region of PGN_0296 was amplified using the primers 0296OF (5'-GAGGAATTCGGGAAGCTTATGTGTAAGAAACATTTCATCC-3') and 0296OR (5'-GAGGGATCCTTGTCAGCATGCGTTACAACATCG-3'), digested with EcoRI-BamHI, and ligated just downstream of *the promoter region of fimA* of pCPG [16]. The resulting plasmid was linearized by XhoI digestion and introduced into ΔPGN_0296 cells by electroporation, resulting in ΔPGN_0296/ΔPGN_0296^{+}.

2.3. Reverse transcription PCR (RT-PCR)

Total RNA was prepared and cDNA synthesis was performed as previously described [16]. The primers used for PCR amplification were 0296OF plus 0296PR (5'-CCTTGTCAGCATGCGTTACAACATCG-3') and 0297PF (5'-GGTTCATCCGGAGTTGGTTTGGAG-3') plus 0297PR (5'-ATTGTTTATTACAAAAAGTCTTACG -3') for the PGN_0296 and PGN _0297 genes, respectively.

2.4. Protease activity assay

Determination of Kgp and Rgp activities in the culture supernatants of *P. gingivalis* cells was performed by the methods described by Ono et al. [20] using the synthetic substrates benzyloxycarbonyl-L-histidyl-Lglutamyl-L-lysine-4-methyl-coumaryl-7-amide (Z-His-Glu-Lys-MCA) and benzyloxycarbonyl-L-phenylalanyl-L-arginine-4-methylcoumary-7-amide (Z-Phe-Arg-MCA). All the cultures used had similar cell densities at OD600 nm of approximately

1.0. Every experiment was performed thrice to check the viability, reliability, and reproducibility, and to avoid technical errors.

2.5. Statistical analysis

The results of Kgp and Rgp activities were statistically analyzed using one-way ANOVA followed by the Tukey's test. Statistical significance was set at $P<0.05$.

3. Results

3.1. Construction of a PGN_0296-deficient mutant (ΔPGN_0296)

To confirm the absence of PGN_0296 and the expression of the flanking gene PGN_0297 in ΔPGN_0296, we performed RT-PCR using the cDNA from ΔPGN_0296. As shown in Fig. 1B, the PGN_0296 gene fragments were amplified in wild-type ATCC33277 and the complimentary strain ΔPGN_0296/PGN_0296$^+$, but not in ΔPGN_0296. On the other hand, the PGN_0297 gene fragments were amplified in all strains. These results suggested that the PGN_0296 gene was successfully disrupted in ΔPGN_0296 and that the expression of the PGN_0297 gene was unaffected in ΔPGN_0296. The black-pigmented colonies on the blood agar plates were produced by ΔPGN_0296, ATCC33277, and ΔPGN_0296/PGN_0296$^+$ (data not shown).

3.2. Gingipain activities in ΔPGN_0296

As shown in Figure 2, the Kgp and Rgp activities in culture supernatants of ΔPGN_0296 were comparable to those in culture supernatants of ATCC33277. These results suggested that the deletion of the PGN_0296 gene (ΔPGN_0296) did not affect the activity of both Kgp and Rgp.

3.3. Effect of deletion of PGN_0296 on the growth of *P. gingivalis* cells

The growth of ΔPGN_0296 was slower than that of ATCC33277 in enriched BHI broth (Figure 3). Introducing the PGN_0296 gene into ΔPGN_0296 (ΔPGN_0296/PGN_0296$^+$) partially restored the growth speed. However, the cell density of ΔPGN_0296 was comparable to those of ATCC33277 and ΔPGN_0296/PGN_0296$^+$ after 40 h.

4. Discussion

The PGN_0296 operon consists of six genes from PGN_0296 to PGN_0301. Among them, the PorG (PGN_0297) and Omp17 (PGN_0300) genes contribute to T9SS function [16,20,21].

Disruption of these genes results in no pigmentation along with the reduction of gingipain activities. The lack of black pigmentation in *P. gingivalis* was due to reduction in gingipain activity and loss of function of the T9SS [11,22]. The other four genes in this operon also probably contribute to T9SS function and characterization of these genes is required to fully understand the role of T9SS. Therefore, we constructed a PGN_0296 gene deletion mutant (ΔPGN_0296). Our results clearly demonstrated that deletion of the PGN_0296 gene from *P. gingivalis* did not change gingipain activity. Thus, the PGN_0296 gene may not contribute to T9SS function, although we cannot completely exclude the relationship between PGN_0296 and T9SS function.

The growth of ΔPGN_0296 was slower than that of ATCC33277 and the reason for this was not revealed. However, PGN_0296 is annotated as an outer membrane protein and the surface structure of the cells was probably affected. This should be analyzed with the localization of PGN_0296 in further studies.

The information obtained in this study may contribute to enhancing the understanding of the role of the PGN_0296 operon in T9SS function.

5. Conclusions

A PGN_0296 gene deletion mutant, ΔPGN_0296, was constructed, possessing gingipain activity comparable to that of the parent strain ATCC33277, and PGN_0296 genes were not directly involved in the T9SS. ΔPGN_0296 showed delayed growth.

Ethics approval

Ethical approval was not required for this manuscript.

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Conflicts of interest

All the authors have declared no conflict of interest.

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Figure legends

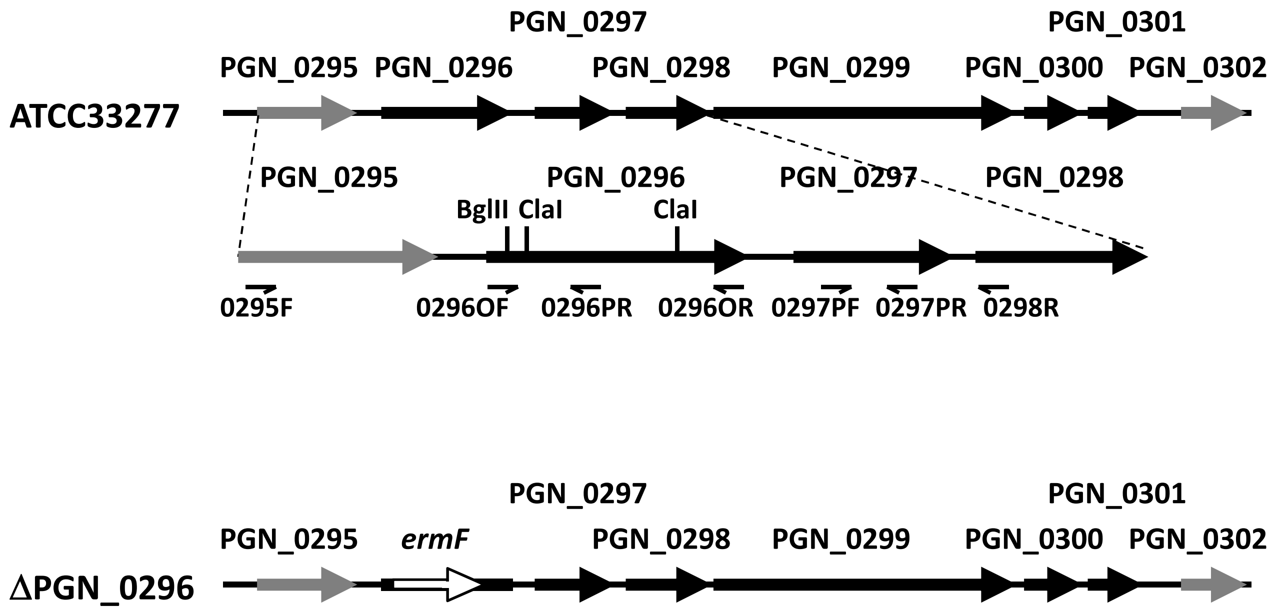
Figure 1. Expression of PGN_0296 and PGN_0297 genes. (A) Gene organization of the PGN_0296 operon locus in ATCC33277 and ΔPGN_0296 and the position of primers used in this study are shown. (B) RT-PCR analysis of *P. gingivalis* strains. –RT, without reverse transcription; +RT, with reverse transcription.

Figure 2. Rgp and Kgp activities of the culture supernatants. Differences between the groups were analyzed using one-way ANOVA followed by Tukey test (*$P<0.05$). KYT-36 and KYT-1 are inhibitors for Lys- and Arg-gingipain, respectively.

Figure 3. Growth of *P. gingivalis* strains. All the strains were grown anaerobically in enriched BHI broth. All the cells were adjusted at OD600 nm of 0.2 and growth of *P. gingivalis* strains was monitored at 600 nm every 4 h for 60 h.

Figure 1

A



B

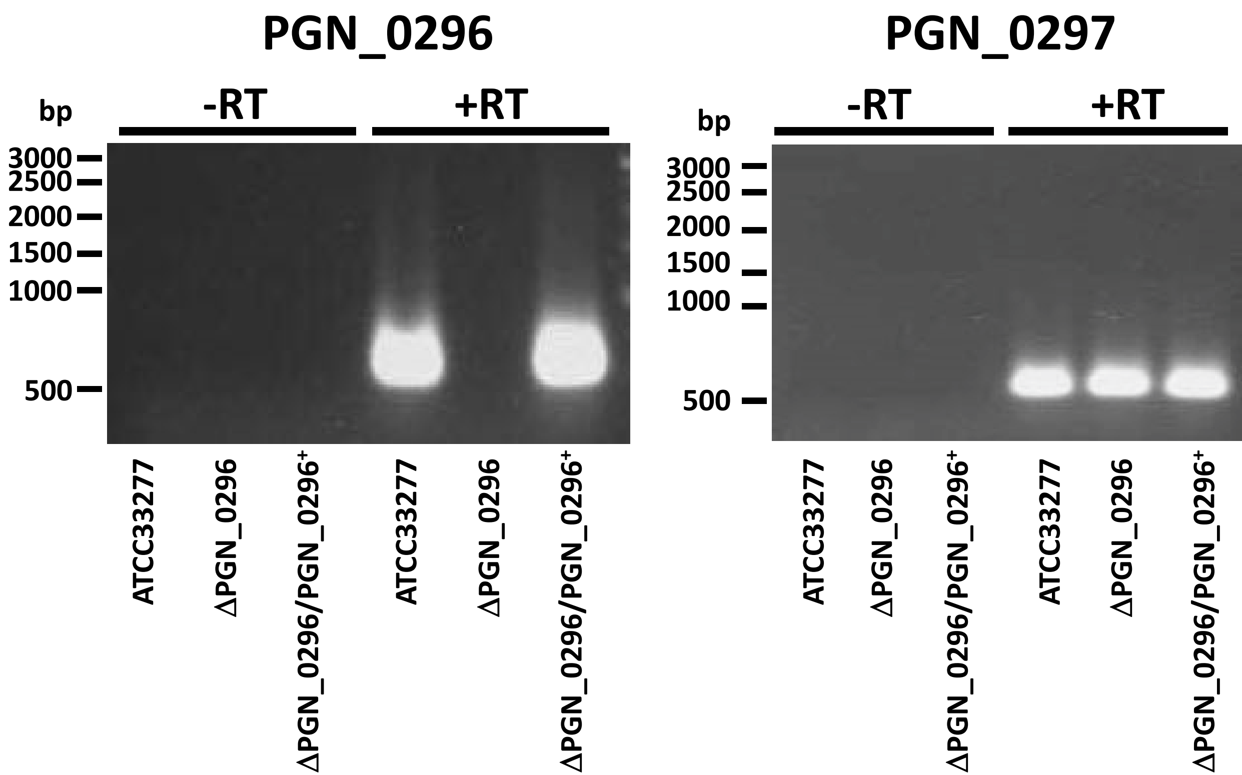


Figure 2

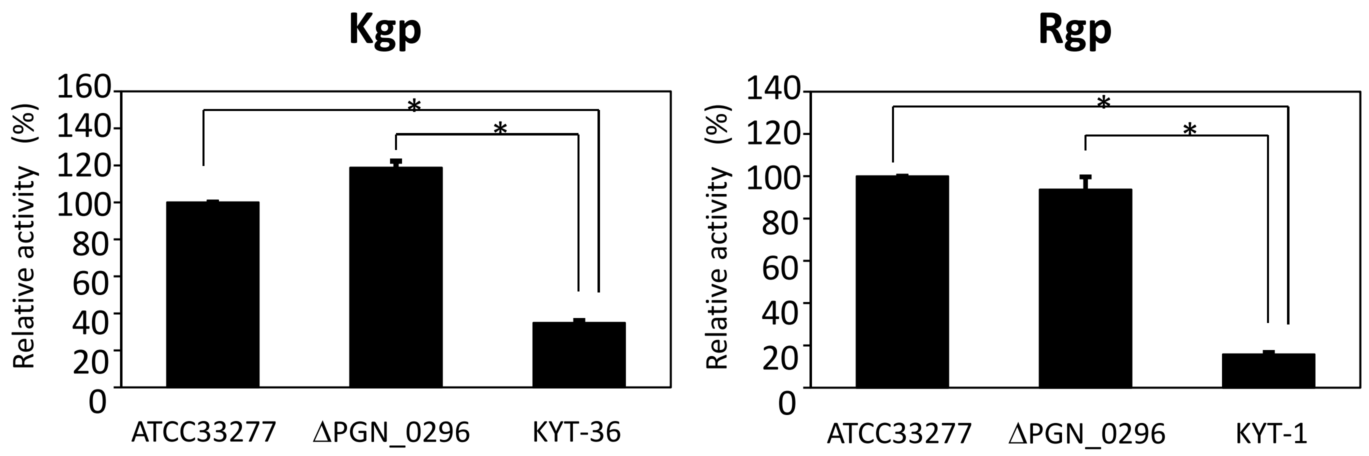


Figure 3