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Original Article

Construction and Characterization of a PGN_0297 Mutant of *Porphyromonas gingivalis*: Evidence of the Contribution of PGN_0297 to Gingipain Activity

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The periodontal pathogen *Porphyromonas gingivalis* shows colonial pigmentation on blood agar and produces gingipains (Kgp, RgpA, and RgpB), cysteine proteases involved in an organism's virulence and pigmentation. We showed previously that deletion of the PGN_0300 gene abolished the pigmentation activity and reduced the proteolytic activity of gingipains. The role of the PGN_0297 gene, which consists of an operon with the PGN_0300 gene, is unclear. Herein we examined the effect of PGN_0297 gene deletion on the pigmentation and proteolytic activities and transcriptional levels of gingipains. A PGN_0297 gene deletion mutant (ΔPGN_0297) did not exhibit the pigmentation. The proteolytic activity of the gingipains was decreased in the culture supernatant and on the cell surface of ΔPGN_0297. The mutant ΔPGN_0297 failed to attenuate Akt phosphorylation at Thr308 and Ser473, but both phosphorylations were attenuated in the wild-type and its complementation strain. The deletion of PGN_0297 gene did not substantially affect the transcriptional levels of the gingipain genes *kgp*, *rgpA*, and *rgpB*. Taken together, these results indicate that PGN_0297 is closely involved in the secretion and maturation of gingipains.

Key words: periodontitis, *Porphyromonas gingivalis*, gingipain, C-terminal domain, secretion system

The anaerobic Gram-negative bacterium *Porphyromonas gingivalis* is a major oral pathogen that is frequently isolated from infectious lesions of periodontal disease, which is a chronic inflammatory disease involving multiple infectious agents [1,2]. *P. gingivalis* is thus considered to play a crucial role in the development of periodontitis [3]. *P. gingivalis* possesses two Arg-specific gingipains, RgpA and RgpB, are encoded by *rgpA* and *rgpB*, respectively, and the Lys-specific gingipain, Kgp, is encoded by a single gene, *kgp* [4-6]. Gingipains are a particularly significant virulence factor that may provoke the destruction of periodontal tissue directly or indirectly [7-10], and individual gingipain-deficient strains of *P. gingivalis* exhibited significantly reduced virulence compared to the parental strain in murine models [11-13].

Several studies have described the activities of gingipains. Kgp and Rgp have been associated with colonial pigmentation on blood agar plates [14-18]. The contribution of gingipains to the virulence of *P. gingivalis* has been demonstrated in both a murine model [11,12] and in host cellular models such as macrophages [19,20] and fibroblasts [21-23]. In our study using human gin-

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gival epithelial cells, we observed the effect of protease activity of gingipains on the disturbance of host cell signal transduction (*i.e.*, the PI3K/Akt signaling pathway) in *P. gingivalis* infection [24].

P. gingivalis produces approx. 34 C-terminal domain (CTD)-containing proteins [25] that are also found among the predicted proteins of other bacteria in the *Bacteroidetes* phylum, including *Prevotella intermedia* and *Tannerella forsythia* [26-28]. Gingipains have a CTD that is translocated from the cytoplasm to the outer membrane via the type IX secretion system (T9SS), resulting in the secretion and maturation of gingipains on the cell surface and into the extracellular milieu [25,29,30]. The T9SS consists of the proteins PorK, PorL, PorM, PorN, PorP, PorQ, PorT, PorU, PorV, PorW, PorZ, and Sov along with the regulatory proteins PorX, PorY, and SigP [29,31-36]. We reported that the Omp17 protein encoded by the PGN_0300 gene is an outer membrane protein that contributes to the processing and modification of CTD-containing proteins [37]. We observed that a mutant with the PGN_0300 gene deleted from the genome did not show pigmentation on blood agar and demonstrated reduced proteolytic activity of gingipains. CTD-containing proteins were released from bacterial cells without cleavage of the CTDs in the PGN_0300 gene deletion mutant, and in our mouse subcutaneous infection experiment the PGN_0300 gene mutant was less virulent than the wild type.

The PGN_0300 gene forms an operon with the PGN_0296, PGN_0297, PGN_0298, PGN_0299, and PGN_0301 genes [37,38]. It was predicted that the PGN_0297 protein is a β-barrel protein [39] localized to the outer membrane of *P. gingivalis* cells and that this protein interacts with the T9SS components PorK and PorN [40]. Klein *et al.* newly found 75 genes and intergenic regions involved in the pigmentation of *P. gingivalis* using Tn-seq, but the PGN_0297 gene was not included in their screening [41].

Thus, the function of PGN_0297 has not been elucidated. In the present study we focused on the colonial pigmentation and proteolytic activity of gingipains, which are generally thought to be involved in the characterization and virulence of *P. gingivalis*. To determine whether the PGN_0297 protein contributes to the secretion of CTD-containing proteins such as gingipains via the T9SS, we investigated several points as follows. (i) We constructed a PGN_0297 gene deletion mutant (ΔPGN_0297); (ii) we determined the effects of the protease activities of gingipains on ΔPGN_0297; and (iii) we evaluated the phosphorylation level of Akt in ΔPGN_0297-infected host cells to reveal the effect of PGN_0297-gene deletion from *P. gingivalis* on the host cell signal transduction.

Materials and Methods

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used are listed in Table 1. *Escherichia coli* DH5α was grown aerobically in

Table 1 Bacterial strains and plasmids used in this study

Name	Description	Source or reference
E. coli strain		
DH5α	General purpose host strain for cloning	Nippongene, Toyama, Japan
P. gingivalis strain		
ATCC 33277	Wild type	American Type Culture Collection
ΔPGN_0297	ATCC 33277 ΔPGN_0297; Em^r	This study
ΔPGN_0297::pPGN_0297	ΔPGN_0297 harboring pPGN_0297, Em^r Tc^r	This study
plasmid		
pBluescript II SK(+)	Ap^r, cloning vector	Stratagene
pBSPGN_0297	Ap^r Em^r, pBlueScriptII SK(+) containing PGN_0297::*ermF*	This study
pTIO-1	Ap^r Em^r, *E. coli-P. gingivalis* shuttle vector	Tagawa *et al.* (43)
pTIO-1T	Ap^r Tc^r, *E. coli-P. gingivalis* shuttle vector	This study
pPGN_0297	Ap^r Tc^r, pTIO-1T containing PGN_0297 with the promoter region of *rgp*B	This study
pKD355	Ap^r Em^r, contains the *ermF ermAM* DNA cassette in pUC19	Ueshima *et al.* (42)
pKD375	Ap^r Tc^r, contains the *tetQ* DNA cassette in pUC19	Shi *et al.* (14)

Luria-Bertani (LB) medium (Nacalai Tesque, Kyoto, Japan) at 37°C. *P. gingivalis* cells were grown anaerobically (10% CO_2, 10% H_2, 80% N_2) using an anaerobic cabinet (Whitley Workstation DG250; Microbiology International, Frederick, MD, USA) at 37°C in enriched brain heart infusion (BHI) broth [4], on enriched tryptic soy (TS) agar [4], and on blood agar prepared by adding hemolyzed defibrinated sheep blood (Nippon Bio-Test Laboratories, Saitama, Japan) to enriched TS agar at 5%. 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*).

General genetic procedures. Unless otherwise stated, standard procedures were used for the preparation and handling of DNA and RNA. Plasmids and DNA fragments were introduced into *P. gingivalis* cells by electroporation using an electroporator (#ECM399; BTX, Holliston, MA).

Construction of a PGN_0297 gene-deletion mutant. The construction of *P. gingivalis* deletion mutants was performed as described previously [37] with several modifications. The upstream and downstream fragments of the PGN_0297 gene were amplified using the primer set PGN_0297UF (5'-CCAATATCGATGGAATCCATTCTTGGCAGG-3'; the underlining indicates a *Cla*I site) plus PGN_0297UR (5'-CCGGAATTCAGTTTCAGGATACCCTTCC-3'; the underlining indicates an *Eco*RI site) and the primer set PGN_0297DF (5'-GCCGAATTCATGAAAACAATTAGTAAG-3'; the underlining indicates an *Eco*RI site) plus PGN_0297DR (5'-CGGACTAGTGGAATGCCGGCGGTGGAAAGATG-3'; the underlining indicates a *Spe*I site), respectively. The upstream and downstream fragments were double digested with *Cla*I-*Eco*RI and *Eco*RI-*Spe*I, respectively, and ligated together into the *Cla*I/*Spe*I site of pBlueScriptII SK(+) (pBSSK) (Stratagene, La Jolla, CA, USA).

The *ermF* cassette was PCR-amplified from pKD355 [42] using the primer set ErmF-F (5'-GCCGAATTCATGACAAAAAAGAAATTGCCCG-3'; the underlining indicates an *Eco*RI site) plus ErmF-R (5'-GCCAAGAATTCTGAACTACGAAGGATG-3'; the underlining indicates an *Eco*RI site) and inserted into the *Eco*RI site within the PGN_0297 gene of this plasmid. The resulting plasmid pBSPGN0297 (Fig. 1A), in which the direction of *ermF* was the same as that of the PGN_0297 gene, was linearized by *Cla*I-*Spe*I digestion and introduced into *P. gingivalis* ATCC 33277 cells by electroporation as described previously [43], resulting in ΔPGN_0297. Correct gene replacement, which occurred through double crossover recombination events, was verified by a polymerase chain reaction (PCR) analysis (data not shown).

Construction of a plasmid for gene expression. To obtain pTIO-1T, *ermF* was removed from pTIO-1 [43] by *Sac*I-*Xba*I digestion. The *tetQ* cassette, which was PCR-amplified from pKD375 [14] using primers (5'-GGGTCTAGACTAAATTTAAATATAAACAACG-3'; the underlining indicates an *Xba*I site) and (5'-GGGGAGCTCGTCTATTTTTTTATTGCC-3'; the underlining indicates a *Sac*I site), was inserted into the same site. Since *rgpB* is highly and constitutively expressed in *P. gingivalis* cells [44], we used the *rgpB* promoter to express the PGN_0297 gene in this study. The promoter region of *rgpB* and the open reading frame (ORF) of the PGN_0297 gene were PCR-amplified using the primer sets rgpBPF (5'-GAAGAAGTCGACTGCTCCGCTCGTTCGCAG-3'; the underlining indicates a *Sal*I site) plus rgpBPR (5'-CGGCCTGCAGCTTGAATTAGTTT-3'; the underlining indicates a *Pst*I site) and the primer sets PGN_0297ORFF (5'-GCCGCTGCAGATGAAAACAATTAG -3'; the underlining indicates a *Pst*I site) plus PGN_0297ORFR (5'-CCCATGGCGGCCGCACTATTGTTTATTACAA-3'; the underlining indicates a *Not*I site), respectively. The promoter region of *rgpB* and the PGN_0297 ORF fragments were double digested with *Sal*I-*Pst*I and *Pst*I-*Not*I, respectively, and ligated together into the *Xho*I/*Not*I site of pTIO-1T, resulting in pPGN_0297 (Fig. 1B).

RNA purification and reverse transcription. Log-phase cultures (OD_{600} of 0.6-0.8) of *P. gingivalis* strains were used to extract total RNA using a TRIzol® Plus RNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. The RNA samples were then incubated with DNase I (Takara Bio, Shiga, Japan) at 37°C for 1 h. DNA contamination was checked by PCR. One microgram of total RNA was reverse transcribed into cDNA with a random hexamer primer using the PrimeScript®II 1st strand cDNA Synthesis Kit (Takara Bio).

Quantitative real-time-PCR (qRT-PCR) analysis. qRT-PCR was carried out on a CFX Connect™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA,

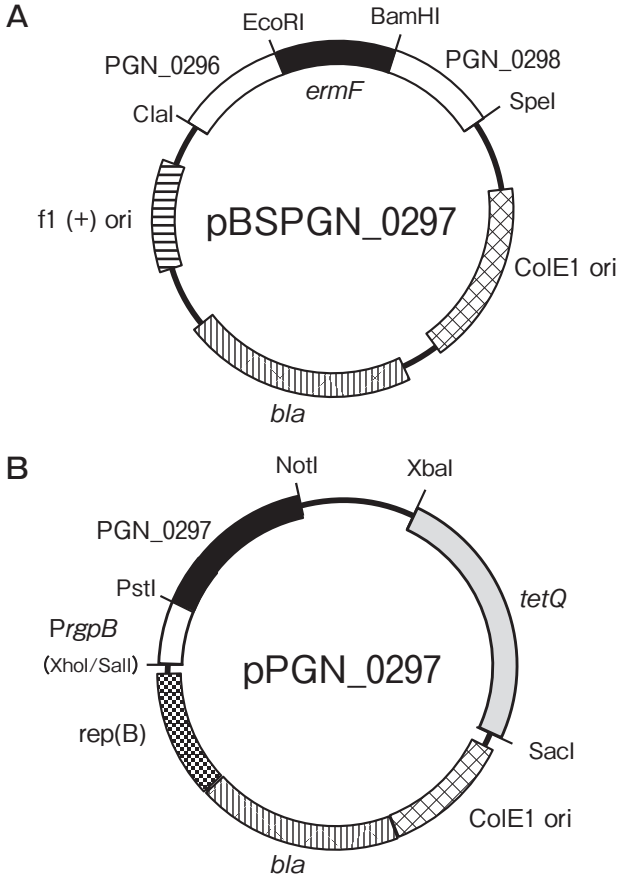

Fig. 1 Functional map of the suicide vector pBSPGN_0297 (**A**) and the expression plasmid pPGN_0297 (**B**). *bla*: The beta-lactamase gene. ColE1 ori: The ColE1 origin of replication. *ermF*: The EM-resistant gene derived from transposon Tn4351. f1 (+) ori: The origin of bacteriophage f1 DNA replication. PGN_0296: The partial sequence of the PGN_0296 gene. PGN_0297: The open reading frame of the PGN_0297 gene. PGN_0298: The partial sequence of the PGN_0298 gene. P*rgpB*: The promoter region of the *rgpB* from *P. gingivalis* ATCC 33277. rep (B): The replication origin from *Bacteroides*. *tetQ*: The Tc-resistant gene derived from *Bacteroides*.

USA) using a KAPA SYBR® FAST Universal kit (Nippon Genetics, Tokyo). The primer sets used were as follows: Kgp-RTF (5'-GAAGTGCCAGCAGTTAGG AAGT-3') and Kgp-RTR (5'-ACTTCATCAGCTCCTT GAAAGC-3') for *kgp*, RgpA-RTF (5'-CCTACGCTT CCCATTCTATCAC-3') and RgpA-RTR (5'-CAACG TCTTTGTCACAGGGTTA-3') for *rgpA*, RgpB-RTF (5'-CGATCGTAGCATTCTCCTCTCT-3') and RgpB-RTR (5'-GGATTTGATCAGGATTTTCAGC-3') for *rgpB*, and 16SrRNA-V3F (5'-CCTACGGGAGGC AGCAG-3') and 16SrRNA-V4R (5'-GACTACCAG GGTATCTAATCC-3') for the 16S rRNA gene.

The melting curve profiles were reviewed to verify single peaks for individual samples. Each experiment was done in triplicate, and the experiments were independently performed three times with comparable results. The 16S rRNA gene was used as a reference to normalize gene expression. We used the $2^{-\Delta\Delta CT}$ method [45] to evaluate the relative gene expressions of *kgp*, *rgpA*, and *rgpB* in different strains.

Protease activity assay. Kgp and Rgp activity assays were performed as described previously [35]. *P. gingivalis* cells were grown anaerobically in enriched BHI medium at 37°C overnight. Bacterial cells and culture supernatants were separated by centrifugation at 10,000 *g* for 10 min at 4°C. Cells were suspended in the original volume of phosphate-buffered saline (PBS). A portion of each cell suspension was lysed by sonication and then centrifuged to separate it into a soluble fraction and an insoluble fraction. The soluble cell extracts were used as cell lysates.

Kgp and Rgp activities were determined using the synthetic substrates benzyloxycarbonyl-L-histidyl-L-glutamyl-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) in 20 mM sodium phosphate buffer (pH 7.5) containing 5 mM cysteine in a total volume of 1 mL. After incubation at 40°C for 10 min, the reaction was terminated by adding 1 mL of 10 mM iodoacetamide (pH 5.0), and the released 7-amino-4-methylcoumarin was measured at 460 nm (excitation at 380 nm). One unit of enzyme activity was defined as the amount of enzyme required to release 1 nmol of 7-amino-4-methylcoumarin under these conditions. Kgp and Rgp activities are indicated as units per mL of cell suspension or culture supernatant. All cultures had similar cell densities at the OD_{600} of approx. 1.0.

Effect of* P. gingivalis *infection on the phosphorylation level of Akt in Ca9-22 cells. The human gingival epithelial cell line Ca9-22 was obtained from the Culture Collection of the Health Science Research Resources Bank, Japan Health Sciences Foundation, and the cells were grown in MEMα medium (Wako, Osaka, Japan) containing 10% fetal calf serum (FCS). Ca9-22 cells were incubated with *P. gingivalis* ATCC 33277, ΔPGN_0297, and ΔPGN_0297:: pPGN_0297, which had been grown anaerobically at 37°C on TS agar for 48 h, at the multiplicity of infection

(MOI) of 100 for 2 h. The infected cells were lysed, and the cell lysates were subjected to SDS-PAGE and Western blotting.

The total Akt and phosphorylation levels of Akt in these cells were probed with primary antibodies: total Akt (#4691), phopho-Thr308 Akt (#13038), and phopho-Ser473 Akt (#4060) produced by Cell Signaling Technologies (Danvers, MA, USA). Secondary antibodies were used for detection with goat anti-rabbit HRP-conjugated antibodies (Agilent, Santa Clara, CA, USA).

Statistical analysis. The statistical analysis of the data was performed using Student's *t*-test.

Results

A PGN_0297-deficient mutant showed no pigmentation on blood agar plates. To investigate the role of PGN_0297, we cultured ΔPGN_0297 on blood agar plates. ΔPGN_0297 exhibited no pigmentation (Fig. 2). We then cultured a complemented strain (ΔPGN_0297::pPGN_0297) to clarify the relationship between PGN_0297 and colonial pigmentation. The complemented strain showed pigmentation on blood agar plates similar to the wild type. Our results thus demonstrated that PGN_0297 is associated with black pigmentation in *P. gingivalis* cells.

The reduction of gingipain activities in the PGN_0297 mutant. Based on the results illustrated in Fig. 2, we then determined the activities of Kgp and Rgp in cell lysates, intact cells, and culture supernatants of ΔPGN_0297. The Kgp activities in ΔPGN_0297 were significantly decreased in the cell lysates, intact cells, and culture supernatants. The Rgp activity in the intact cells and in the culture supernatants in ΔPGN_0297 was also significantly reduced. The Rgp activity in cell lysates in ΔPGN_0297 showed a tendency to be decreased, but not significantly.

The activities of Kgp and Rgp were moderately restored in ΔPGN_0297::pPGN_0297 (Fig. 3). The mRNA levels of *kgp*, *rgpA*, and *rgpB* in ΔPGN_0297 were comparable to those in the wild-type strain, suggesting that a decrease of Rgp and Kgp activities of ΔPGN_0297 cannot be explained by the altered expressions of *rgpA*, *rgpB*, and *kgp* at the transcriptional level (Fig. 4).

The PI3K/Akt signaling pathway in the PGN_0297 mutant-infected host cells. Based on the results of our previous study, we next examined whether ΔPGN_0297 would impact the phosphorylation levels of Akt [24]. Ca9-22 cells were incubated with *P. gingivalis* ATCC 33277, ΔPGN_0297, and ΔPGN_0297::pPGN_0297 for 2 h, and the phosphorylation levels of Akt at Thr308 and Ser473 were measured in these cells. The phosphorylation level of Akt at both sites by the infection with ΔPGN_0297 was almost the same as the levels of the controls and that observed with the use of inhibitors specific for Rgp (KYT-1) and Kgp (KYT-36) in ATCC 33277 infection. In contrast, infection with ATCC 33277 and ΔPGN_0297::pPGN_0297 remarkably decreased the Akt phosphorylation (Fig. 5). These results support the possibility that the deletion of PGN_0297 decreases in the protease activity of gingipains.

Discussion

Our present experiments demonstrated that a

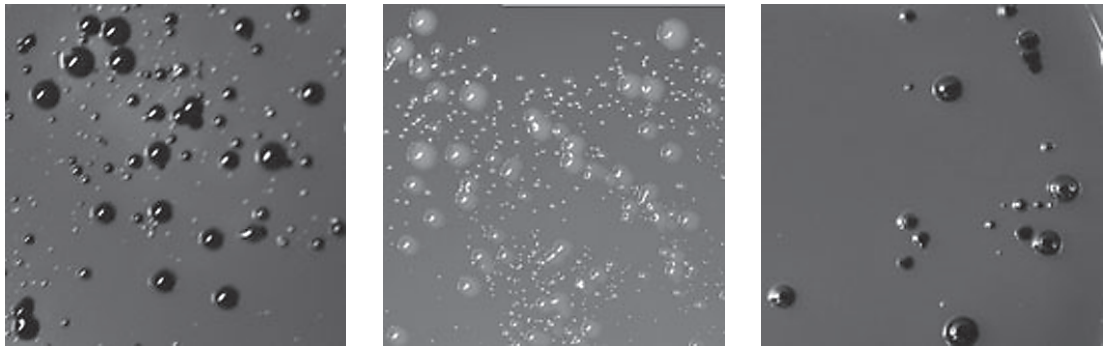

Fig. 2 Colonial pigmentation. *P. gingivalis* cells were grown anaerobically on blood agar plates at 37°C.

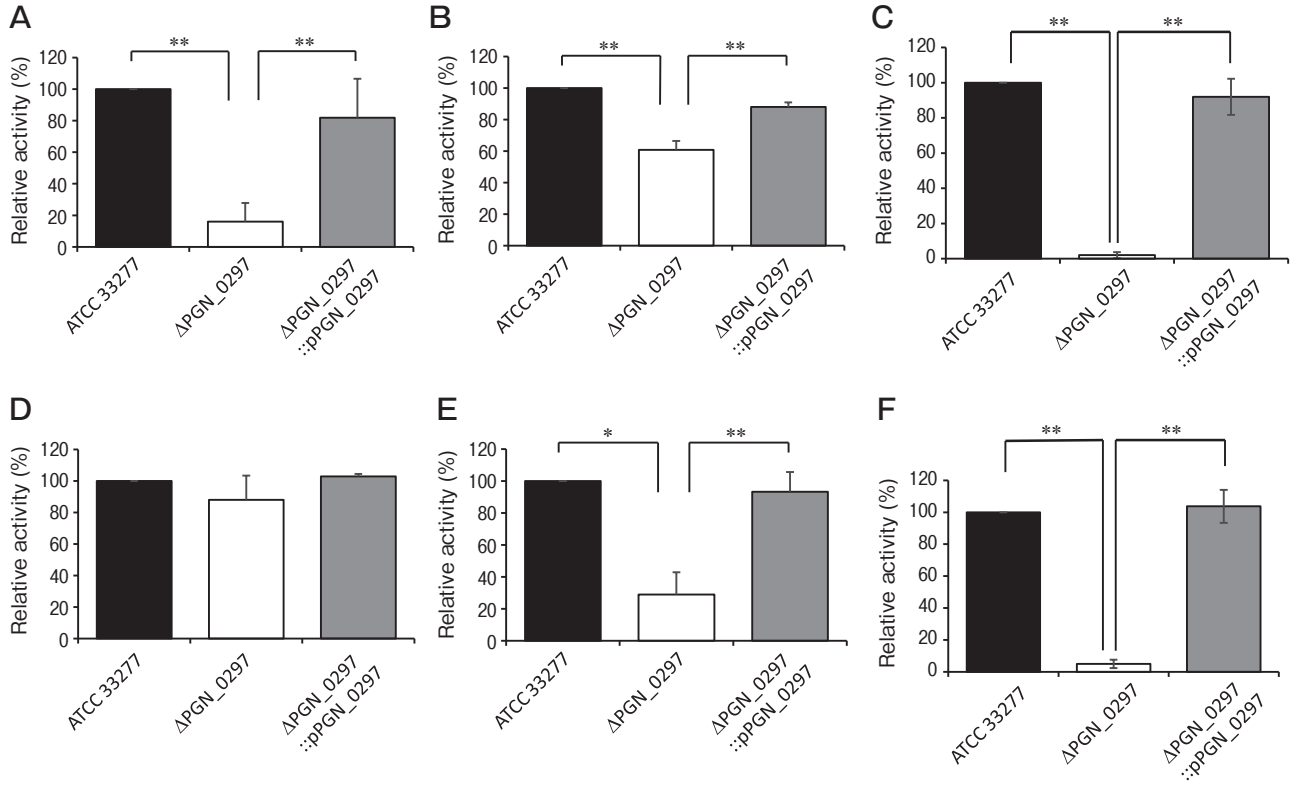

Fig. 3 Kgp and Rgp activities in the PGN_0297 mutant. *P. gingivalis* cells were grown anaerobically in enriched BHI medium at 37°C for 36 h. We measured the Kgp (panels **A**, **B**, **C**) and Rgp (panels **D**, **E**, **F**) activities of the cell lysates (A, D), of the culture supernatants (B, E), and on the intact cells (C, F). $^{*}p < 0.05$, $^{**}p < 0.01$.

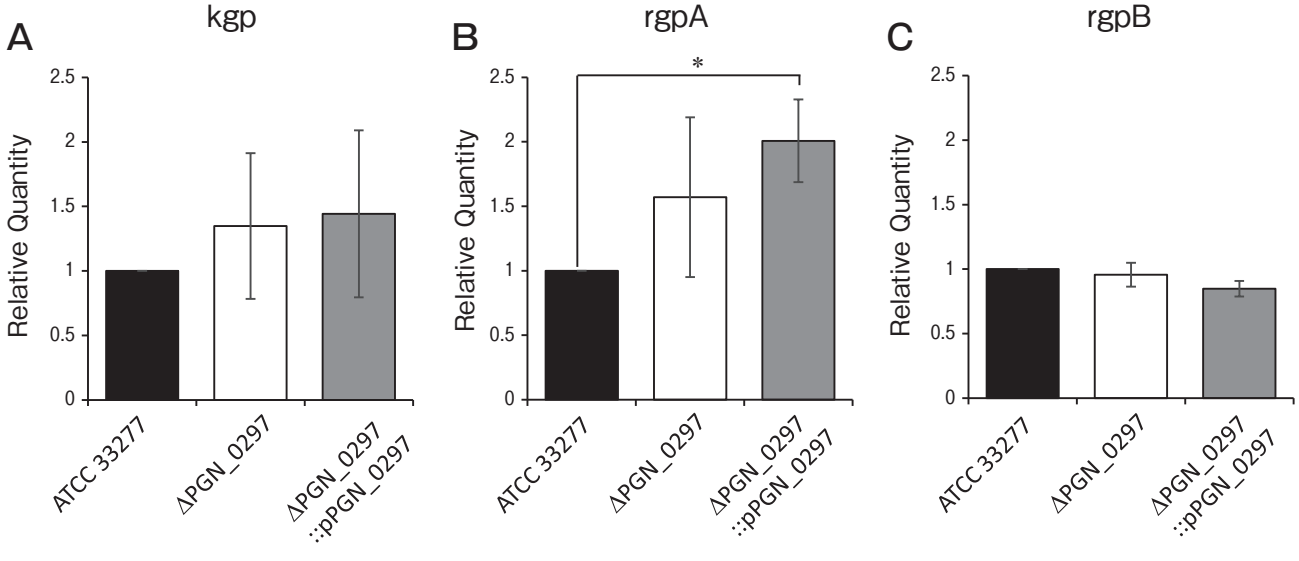

Fig. 4 The transcriptional levels of *kgp*, *rgpA*, and *rgpB* in the PGN_0297 mutant. Total RNA was prepared from log-phase cultures of *P. gingivalis* strains and was reverse transcribed into cDNA with a random hexamer primer. Then qRT-PCR was carried out using the gene-specific primers. The 16S rRNA gene was used as a reference to normalize gene expression. The $2^{-\Delta\Delta CT}$ method was used to evaluate the relative gene expressions of *kgp* (**A**), *rgpA* (**B**), and *rgpB* (**C**) in different strains.

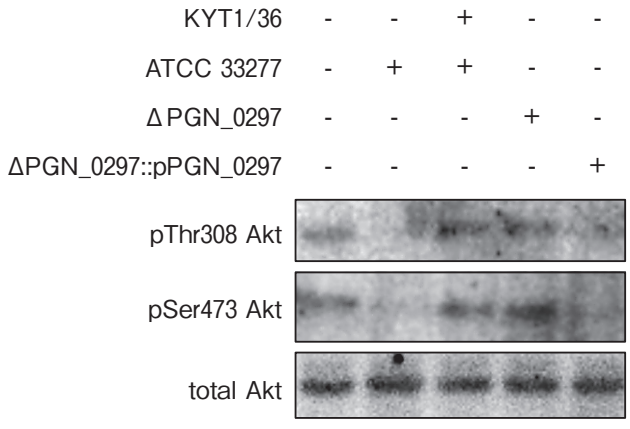

Fig. 5 Effect of *P. gingivalis* infection on Akt phosphorylation in Ca9-22 cells. Ca9-22 cells were infected with *P. gingivalis* ATCC 33277, ΔPGN_0297, and ΔPGN_0297::pPGN_0297 at an MOI of 100 and ATCC 33277 treated with 5 μM of the Rgp-specific inhibitor KYT-1 and 5 μM of the Kgp-specific inhibitor KYT-36 (KYT1/36) for 2 h. After infection, the cells were lysed and analyzed by SDS-PAGE and probed by Western blotting with antibodies against phospho-Akt at Thr308 and Ser473, and total Akt. These results were independently demonstrated on three separate experiments.

PGN_0297 gene-deleted mutant, ΔPGN_0297, showed no pigmentation on blood agar plates and reduced the proteinase activity of Kgp and Rgps. We also observed that disturbance of the PI3K/Akt signaling pathway by gingipains did not occur in ΔPGN_0297-infected Ca9-22 cells. These results suggest that the PGN_0297 gene is probably involved in the secretion and maturation of gingipains.

P. gingivalis exhibits a black pigmentation on blood agar plates that is caused by the accumulation of μ-oxo heme dimer on the cell surface and that is linked with hemagglutination and proteolytic activities of gingipains [14,15,17,18]. Our previous findings suggested that PGN_0300 is closely associated with the colonial pigmentation and proteolytic activity of gingipains and the function of T9SS, presumably contributing to the virulence of *P. gingivalis* [37]. In the present investigation we focused on the operons formed in the range from PGN_0296 to PGN_0301, and we observed that deletion of PGN_0297 from the genome did not result in pigmentation on blood agar plates and that the deletion reduced the proteolytic activity of the gingipains. According to our results, it is likely that PGN_0297 is a novel gene involved in the pigmentation.

Although Klein *et al.* performed a screening of the genes and intergenic regions that are involved in pigmentation by Tn-seq, the PGN_0297 gene was not identified in their screening [41]. This discrepancy indicates the possibility that their experiments did not fully cover all of the genes involved in the pigmentation of *P. gingivalis*.

Our present findings revealed that PGN_0297 is involved in the pigmentation of gingipains and decreased the proteolytic activity of gingipains. The transcriptional levels of *rgpA*, *rgpB*, and *kgp* in ΔPGN_0297 were comparable to those in the parent strain ATCC 33277, and Kgp activity was significantly reduced in the cell lysates, culture supernatants, and intact cells. The reduced levels were higher in the cell lysates than in the culture supernatants. These results suggested that Kgp was released outside of *P. gingivalis* cells after being synthesized. Rgp activity was also significantly reduced in the culture supernatants but not in the cell lysates; this indicates that RgpA and RgpB were synthesized but accumulated in the cytoplasm and/or in the periplasmic space in ΔPGN_0297.

It remains unclear why RgpA and RgpB, but not Kgp, were accumulated in the cytoplasm and/or in the periplasmic space; further studies are needed to clarify this point. The activities of both Kgp and Rgp in the intact cells of ΔPGN_0297 were at basal levels, suggesting that Kgp, RgpA, and RgpB did not attach to the cell surface in ΔPGN_0297. For their anchoring to the bacterial cell surface, gingipains are thought to be modified by anionic polysaccharide (A-LPS) after removal of the CTD region [30,46]. It is thus possible that CTD regions were not removed from Kgp, RgpA, and RgpB; another possibility is that A-LPS modification did not occur in Kgp, RgpA, and RgpB. Further studies are needed to clarify this point.

Next, based on our above-described findings, we infected Ca9-22 gingival epithelial cells with ΔPGN_0297 to confirm that gingipains lack proteolytic activity when functioning as virulence factors in the host cell model. We found that ΔPGN_0297 did not cause a decrease in the phosphorylation levels of Akt at Thr308 and Ser473, suggesting that PGN_0297 deletion had an effect on the proteolytic activity of gingipains both on the cell surface and in the extracellular milieu.

In *P. gingivalis*, 18 genes have been identified for T9SS function to secrete CTD-containing proteins [32]. Among them, the five genes *PorP*, *porK*, *porL*, *porM*, and *porN* comprise a co-transcribed operon [47]. PorK,

PorL, PorM, and PorN are components of a core membrane complex of T9SS from *P. gingivalis* [29]. PorK is a lipoprotein anchored to the outer membrane that interacts with the periplasmic protein PorN [40]. PorL and PorM are inner membrane proteins that interact via their trans-membrane segments. PorM interacts with both the PorK and PorN complex, and it therefore spans the entire periplasm by being anchored in the inner membrane and interacting with the outer membrane complex [47]. PorP is speculated to interact with the labile PorKLMN complex or to associate with the PorKLMN complex under specific conditions [47].

PGN_0297 protein (PG0189 in strain W83) was detected by a cross-linking study as a protein that interacts with the PorKN outer membrane-associated complex of T9SS [40]. In consideration of all the above-described findings, along with our present results that the PGN_0297 deletion mutant had no pigmentation on blood agar plates and decreased the enzyme activity of gingipains in the extracellular supernatant and on the intact cells, we conclude that PGN_0297 may have an essential role in the secretion of the CTD-containing proteins via T9SS. It should be pointed out that the PGN_0297 protein is predicted to be a β-barrel protein localized to the outer membrane of *P. gingivalis* cells [39]; in addition, the Omp17 protein coded by PGN_0300 gene is also localized to the outer membrane [37], and the *omp17* mutant reduces the proteolytic activity of the gingipains [37]. These findings suggest the possibility that the PGN_0297 protein might interact with the Omp17 protein functionally. In conclusion, all of our data indicate a novel function of PGN_0297; that is, PGN_0297 plays a significant role in the secretion and maturation of gingipains via T9SS, and it may contribute to the virulence of *P. gingivalis*.

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