The role of PGN_0296 in *Porphyromonas gingivalis.*

Porphyromonas gingivalis における PGN_0296 遺伝子の役割の探求

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Introduction:

Periodontal disease is highly prevalent worldwide and therefore represents a major public health problem to countries[1)] and is one of the most frequently occurring infectious diseases in humans[2)]. It is a chronic inflammatory disease that causes the destruction of periodontal tissues and alveolar bone, is one of the two major infectious diseases together with dental caries in oral cavity[2,3)]. Moreover, the disease has been associated with some systemic diseases: for example, cardiovascular disease, vascular disease, aspiration pneumonia and diabetes[4-6)]. The anaerobic Gram-negative bacterium *Porphyromonas gingivalis* (*P. gingivalis*) is an etiologically important agent of periodontal disease and considered as to play an evidentiary role in the development of periodontitis[7)]. *P. gingivalis* possesses several virulence factors such as lipopolysaccharide, capsule, fimbriae, cysteine proteases (gingipains), and adhesion domains[8)]. Gingipains, the cysteine proteases from *P. gingivalis,* are extracellular and surface proteinases with high hydrolytic activities, are key players in subverting the various elements of periodontal tissues including extracellular matrix proteins, cytokines, complement proteins, antibodies, and proteinase inhibitors[9-12)]. Gingipains have two types, Arg-specific gingipain and Lys-specific gingipain, which are the products of 3 separate genes: *rgpA*, *rgpB*, and *kgp*[13,14)]. Some previous demonstrations had revealed the significant reduction of virulence in individual gingipain-deficient strain comparing to that of the parental strain in murine models[15-17)]. The *kgp* and *rgpA* genes encode polyproteins that comprise the signal peptide, propeptide, proteinase, and adhesion domains and the C-terminal domain (CTD). The *rgpB* gene encodes a protein consists of signal peptide, propeptide, proteinase domain and the CTD. *P. gingivalis* facilitates secretion of up to 35 cargos bearing the CTD, named as Type IX secretion system (T9SS) cargo proteins, many of which are implicated in bacterial pathogenicity including

the gingipains, RgpA, RgpB, and Kgp[18]. Other than the *P. gingivalis,* the CTD-containing proteins are also found in predicted proteins of other bacteria in the *Bacteroidetes* phylum, including *Prevotella intermedia* and *Tannerella forsythia*[19-21]. In cytoplasm, the Rgp and Kgp are equipped and synthesized with two sorting signals: N-terminal signal peptide (SP) directing the protein to the secretion system SecYEG and conserved CTD recognized by T9SS. After translocation through the inner membrane (IM), the Kgp and Rgp acquire their proper fold in the periplasm and then directed by the CTD for further translocation across the outer membrane (OM)[18,22]. After then the CTD region is removed by the C-terminal signal peptidase PG0026 (PorU) near the cell surface and subsequently, they are either secreted into the extracellular milieu as mature proteinases or are glycosylated with A-LPS and located on the cell surface[23].

T9SS is a unique secretion system for the *P. gingivalis* and facilitate disease through the delivery of virulence factors. T9SS translocates proteins, especially virulence factors, across the OM. Proteins destined for secretion bear a conserved CTD that directs the cargo to the OM translocon. At least 18 proteins are involved in this still enigmatic process including PorK, PorL, PorM, PorN, PorP, PorQ, PorT, PorU, PorV, PorW, PorZ, and Sov proteins with regulatory proteins PorX, PorY, and SigP[18,22,24-29].

We previously reported that OM protein 17 (Omp17), a 17-kDa immunoreactive protein that was detected mainly in the outer membrane fraction, is a Skp-like protein (also known as OmpH), encoded by PGN_0300 gene, is involved in the function of T9SS to transport CTD-proteins, and the deficiency of PGN_0300 gene shows loss of the protease activity of gingipains in this mutant[30]. Moreover, according to a tiling microarray analysis, it is likely that the genes from PGN_0296 gene to PGN_0301 gene form an operon on *P. gingivalis* genome[30]. The PGN_0296, PGN_0297, PGN_0298, and PGN_0299 gene products are annotated as a hypothetical protein, a membrane β-

barrel protein, an isoprenyl transferase, and an outer membrane protein assembly factor, respectively[18,31]. As the PGN_0296 gene is the first gene on the operon and its characteristics and functions have not been clarified. Therefore, we constructed the PGN_0296 gene deletion mutant to analyze its function in *P. gingivalis* cells in this study.

Materials and Methods

Bacterial strains and growth condition

Bacterial strains and plasmids used are listed in Tables 1 and 2 respectively. *E. coli* strains were grown aerobically in 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 FG250, Microbiology International, Frederick, MD, USA.) at 37°C, in enriched brain heart infusion (BHI) broth (Beckton, Dickinson and company, Sparks, MD, USA)[31], on enriched tryptic soy (TS) agar (Beckton, Dickinson and company)[31], and on blood agar prepared by adding hemolyzed defibrinated sheep blood (Nippon Bio-Test Laboratories Inc, Saitama, Japan) to enriched TS agar 5%. Antibiotics were used at the following concentrations: ampicillin (Ap; 100 μg/ml for *E. coli*), erythromycin (Em; 1.5μg/ml for *P. gingivalis*), and tetracycline (Tc: 1.0μg/ml for *P. gingivalis*).

Strain Construction

To disrupt PGN_0296, two fragments were PCR-amplified from the chromosomal DNA of *P. gingivalis* ATCC 33277, using PrimeSTAR® GXL DNA Polymerase (Takara Bio Inc. Shiga, Japan). The PGN_0296 fragment was amplified using the primers PGN_0296-F and PGN_0296-R, containing the sequences from the 5′ end of PGN_0296 and 3′ end of PGN_0296 including the ATG initiation codon along with its upstream and downstream region. Then the fragment was double digested with BamHI and EcoRI and ligated together into the BamHI/EcoRI site of pUC19. The open reading frame (ORF) of *ermF* was amplified from pKD355[32] using the primer set ermF-F plus ermF-R. Then the fragment was inserted in to the TOPO vector (Invitrogen™, Zero Blunt™ Topo™ PCR cloning kit by Thermo Fisher Scientific Inc. Waltham, MA, USA) according to the

manufacturer's instructions and then were double digested with BglII and ClaI and inserted into the BglII/ClaI site within PGN_0296 of this plasmid. The resulting plasmid, pUCPGN0296 was then linearized by BamHI and EcoRI digestion and introduced into *P. gingivalis* cells by electroporation[33], resulting in ΔPGN_0296. Correct gene replacement, which occurred through double crossover recombination events, were verified by PCR analysis.

To construct the complementation of PGN_0296, PGN_0296 was inserted into the PGN_1045 locus of the ΔPGN_0296. As the *P. gingivalis* does not exploit lactose and the PGN_1045 locus encodes ß-galactosidase, the effect on cell viability was not expected by the disruption of PGN_1045. To construct the complimentary strain, two DNA fragments were PCR-amplified from the chromosomal DNA of *P. gingivalis* ATCC 33277. The upstream DNA fragment amplified using the primers PGN_1045U-F and PGN_1045U-R containing the sequences from the 5′ end of PGN_1045, including the region upstream of the ATG initiation codon. The downstream DNA fragment was amplified using the primers PGN_1045D-F and PGN_1045D-R containing the sequences from the 5′ end of PGN_1045, including the region downstream of its stop codon. The upstream DNA fragment was double digested with XhoI and ClaI and inserted into the XhoI/ClaI site of pBluescript II SK(-) (pBSSK), resulting in p1045UA. Then the downstream DNA fragment was double digested with NotI and SacI and inserted into the NotI/SacI site of pBSSK, resulting into p1045DA. Next, a 2.7-kb BamHI-NotI *tetQ* DNA fragment from pKD375[34] using the primers tetQ-F and tetQ-R, was inserted into BamHI/NotI site of p1045DA to endure the plasmid pCPG. The promoter region *P. gingivalis fimA* was PCR-amplified from *P. gingivalis* ATCC 33277 chromosomal DNA using the primers fimA-F and fimA-R, digested with ClaI-EcoRI, and inserted into the ClaI/EcoRI region of p1045UA, resulting into p1045UAFIM. Next the coding of PGN_0296 was amplified from the *P. gingivalis* ATCC 33277 chromosomal DNA using the

primers PGN_296-F and PGN_296-R, digested with EcoRI-BamHI, and inserted into the EcoRI/BamHI site of p1045UAFIM which resulted into plasmid p1045UAFIM0296. Thereafter, the pCPG was digested with the BamHI and SacI and inserted into BamHI-SacI site of the p1045UAFIM0296 and resulted into the pCPG0296. Then the plasmid pCPG0296, which was containing the PGN_0296 ORF as the *fimA* promoter transcript, was linearized by XhoI and introduced into the ΔPGN_0296 cells by electroporation resulting into CPGN_0296 complimentary strain. Correct gene replacement, which occurred through double crossover recombination events, were verified by PCR analysis.

RT-PCR

Total RNA was isolated from *P. gingivalis* cells using the TRIzol®Plus RNA Purification Kit (Thermo Fisher Scientific Inc.) according to manufacturer's instructions. Then the RNA samples were incubated with DNase I (Takara Bio Inc.) at 37°C for 1 h. DNA contamination was checked by PCR. Two μg of total RNA was reverse transcribed into complimentary DNA (cDNA) with a random hexamer primer using the Superscript III First Strand Synthesis (Thermo Fisher Scientific Inc.), and the resulting cDNA were then used for PCR amplification.

Protease activity assay

For Kgp and Rgp activity assays, *P. gingivalis* cells were grown anaerobically on enriched BHI medium at 37°C for an 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 PBS. Kgp and Rgp activities were determined 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) in 20 mM sodium phosphate buffer (pH 7.5) containing 5 mM cysteine in a total volume of 1mL. After incubation at 40°C for 10 min, the reaction was terminated by adding 1mL of 10 mM iodoacetamide (pH 5.0), and the released 7-amino-4-methylcoumarin under these conditions. Kgp and Rgp activities are indicated as units per milliliter of culture supernatant. All cultures had similar cell densities at OD600 of approximately 1.0. Every experiment was done three times to check the viability, reliability, reproducibility and also to avoid the technical error.

Statistical analysis

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

Results

Construction of a PGN_0296-deleted mutant (ΔPGN_0296) and a complement strain CPGN_0296

To investigate the role of PGN_0296, we constructed a ΔPGN_0296 which PGN_0296 was replaced with the *ermF* cassette and a complimentary strain (CPGN_0296), in which the wild type PGN_0296 was introduced in to PGN_1045 locus of ΔPGN_0296. To confirm the presence of the flanking region and the absence of the PGN_0296 in the ΔPGN_0296, we performed PCR employed the genome DNA from ΔPGN_0296 as the template using two sets of primers PGN_0296 FW plus PGN_0296 RV and PGN_0297 FW plus PGN_0297 RV. As shown in Fig. 1A, the PGN_0296 gene fragments were also amplified in wild type ATCC 33277 and complimentary strain CPGN_0296 but not in gene disrupting mutant ΔPGN_0296. The PGN_0297 gene fragments were amplified in all strains (Fig. 1B). These results suggesting that PGN_0296 gene was successfully disrupted in Δ PGN_0296 and complemented in CPGN_0296.

The resulting colonies of all the strains, ATCC33277, ΔPGN_0296, and CPGN_0296, were showing the black pigmented colonies on the blood agar plates (Fig. 2), suggesting that the loss of PGN_0296 has no effect on the pigmentation of *P. gingivalis.*

Expression of PGN_0296 and PGN_0297 genes in ΔPGN_0296

In the previous study, a tiling microarray analysis showed that from PGN_0296 gene to PGN_0301 gene formed an operon in this order[30]. Therefore, there was a possibility that disruption of PGN_0296 gene effected the transcription of PGN_0297 gene to PGN_0301 gene. To rule out this possibility, we performed RT-PCR using the primers sets PGN_0296 FW plus

PGN_0296 RV and PGN_0297 FW plus PGN_0297. DNA fragments were amplified using the primers sets PGN_0296 FW plus PGN_0296 RV in ATCC33277 and CPGN_0296, but not in ΔPGN_0296 (Fig. 3A). On the other hands, DNA fragments were amplified in all three strains using the primers sets PGN_0297 FW plus PGN_0297 RV (Fig. 3B). These results suggested that PGN_0296 gene was correctly knocked out and the expression of PGN_0297 gene was unaffected in ΔPGN_0296.

Gingipain activities in ΔPGN_0296

Several studies have revealed that cell surface activities of proteinases Kgp and Rgp are associated with the colonial pigmentation on blood agar plates[30,34,35]. Then we determined the Kgp and Rgp activities in culture supernatants of ΔPGN_0296. The deletion of PGN_0296 gene (ΔPGN_0296) did not decrease the activity of Rgp in culture supernatant (Fig. 4A). The activity of Rgp in culture supernatant of ΔPGN_0296 higher than those in both ATCC 33277 and CPGN_0296 (Fig. 5B). These results showed that deletion of the PGN_0296 gene did not decrease the proteinase activities of both Kgp and Rgp.

Discussion

In this study, we established PGN_0296 gene deleted mutant, ΔPGN_0296, and a complementary strain of PGN_0296 (CPGN_0296). ΔPGN_0296 showed pigmentation on blood agar plates as well as ATCC3277 and CPGN_0296. We also found that deletion of PGN_0296 gene did not decrease the Kgp and Rgp activities. The black pigmentation on blood agar plates is caused by the accumulation of μ-oxo heme dimer on the cell surface and is linked with hemagglutination and activities of major proteinases. Kgp can degrade hemoglobin protein and a *kgp* mutant displayed a non-pigmented phenotype on blood agar plates[35,36,37,38].

It has been previously showed that 75 genes and intergenic regions involved in pigmentation[39] and PGN_0296 was not found among them. Our results were consistent with this result that the ΔPGN_0296 strain showed the pigmentation on blood agar plates.

In *P. gingivalis*, 18 genes have been identified for T9SS function[18]. Among them, 5 genes, *PorP*, *porK*, *porL*, *porM*, and *porN* consists a co-transcribed operon[40]. PGN_0297 (PG0189 in *P. gingivalis* strain W83) protein was found as a protein interacts with the PorKN outer membrane-associated complex of T9SS by a cross-linking study[41]. PorK, PorL, PorM, and PorN are components of a core membrane complex of T9SS from *P. gingivalis*[22]. PorK is a lipoprotein anchored to the outer membrane that interacts with the periplasmic protein PorN[41]. PorL and PorM are inner membrane proteins that interact via their trans-membrane segments. PorM interacts with both PorK and PorN complex, and therefore spans the entire periplasm by being anchored in the inner membrane and interacting with the outer membrane complex[40]. PorP is considered to interact with the PorKLMN complex labile or to associate with the PorKLMN complex under specific conditions[40]. In the previous study, we have demonstrated that the genes from PGN_0296 gene to PGN_0301 gene form an operon on *P. gingivalis* genome and PGN_0296 gene existed as the first

gene of this operon. Therefore, PGN_0296 gene product might functionally interact with gene products of other genes in this operon. The PGN_0296, PGN_0297, PGN_0298, and PGN_0299 gene products are annotated as a hypothetical protein, a membrane β-barrel protein, an isoprenyl transferase, and an outer membrane protein assembly factor, respectively[18,30,31,42]. 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[42], Omp17 protein coded by PGN_0300 gene is also localized to the outer membrane, and *omp17* mutant reduces proteolytic activity of the gingipains[30]. However, neither we found any reduced proteolytic activity of the gingipains of the ΔPGN_0296 nor we determine the localization of the hypothetical protein coded by PGN_0296. Further studies are needed to clarify this point.

Conclusion

PGN_0296 gene deletion mutant ΔPGN_0296 and its complement strain CPGN_0296 were constructed. ΔPGN_0296 and CPGN_0296 showed black pigmented colonies and the gingipain activities in these strains were comparative to those in the parent strain ATCC33277. PGN_0296 gene was not linked with activities of gingipain and has not any involvement in T9SS.

References

1. Petersen, PE., and Ogawa, H.: The global burden of periodontal disease: towards integration with chronic disease prevention and control: *Periodontol 2000*. **60**, 15-39, 2012.

2. Armitage, GC.: Periodontal diseases: diagnosis. *Ann. Periodontol*. **1**, 37-215, 1996.

3. Page, RC., Offenbacher, S., Schroeder, HE., Seymour, GJ., and Kornman, KS.: Advances in the pathogenesis of periodontitis: summary of developments, clinical implications and future directions. *Periodontol 2000*. **14**, 216-248, 1997.

4. Iacopino, AM., and Cutler, CW.: Pathophysiological relationship between periodontitis and systemic disease: recent concepts involving serum lipids. *J. Periodontol*. **71**, 1375-1384, 2000.

5. Lalla, E., and Papapanou, PN.: Diabetes mellitus and periodontitis: a tale of two common interrelated diseases. *Nat. Rev. Endocrinol*. **7**, 738-748, 2011.

6. Bansal, M., Khatri, M., and Taneja, V.: Potential role of periodontal infection in respiratory disease-a review. *J. Med. Life*. **6**, 244-248, 2013.

7. Holt, SC., and Ebersole, JL.: *Porphyromonas gingivalis*, *Treponema denticol*a, and *Tannerella forsythia*: the "red complex", a prototype polybacterial pathogenic consortium in periodontitis. *Periodontol 2000*. **38**, 72-122., 2005.

8. Nakayama, M., and Ohara, N.: Molecular mechanism of *Porphyromonas gingivalis*-host cell interaction on periodontal diseases. *Jpn. Dent. Sci. Rev*. **43**, 134-140, 2017.

9. Andrian, E., Mostefaoui, Y., Rouabhia, M., and Grenier, D.: Regulation of matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases by *Porphyromonas gingivalis* in an engineered human oral mucosa model. *J. Cell Physiol*. **211**, 56-62, 2007.

10. Curtis, MA., Kuramitsu, HK., Lantz, M., Macrina, FL., Nakayama, K., Potempa, J., Reynolds, EC., and Aduse-Opoku, J.: Molecular genetics and nomenclature of proteases of *Porphyromonas*

gingivalis. *J. Periodontal. Res*. **34**, 464-472, 1999.

11. Kadowaki, T., Nakayama, K., Okamoto, K., Abe, N., Baba, A., Shi, Y., Ratnayake, DB., and Yamamoto, K.: *Porphyromonas gingivalis* proteinases as virulence determinants in progression of periodontal diseases. *J. Biochem*. **128**, 153-159, 2000.

12. Potempa, J., Banbula, A., and Travis, J.: Role of bacterial proteinases in matrix destruction and modulation of host responses. *Periodontol 2000*. **24**, 153-192, 2000.

13. Nakayama, K., Kadowaki, T., Okamoto, K., and Yamamoto, K.: Construction and characterization of arginine-specific cysteine proteinase (Arg-gingipain)-deficient mutants of *Porphyromonas gingivalis*. Evidence for significant contribution of Arg-gingipain to virulence. *J. Biol. Chem*. **270**, 23619-23626, 1995.

14. Potempa, J., Pike, R., and Travis, J.: The multiple forms of trypsin-like activity present in various strains of *Porphyromonas gingivalis* are due to the presence of either Arg-gingipain or Lys-gingipain. *Infect. Immun*. **63**, 1176-1182, 1995.

15. O'Brien-Simpson, NM., Paolini, RA., Hoffmann, B., Slakeski, N., Dashper, SG., and Reynolds, EC.: Role of RgpA, RgpB, and Kgp proteinases in virulence of *Porphyromonas gingivalis* W50 in a murine lesion model. *Infect. Immun*. **69**, 7527–7534, 2001.

16. Pathirana, RD., O'Brien-Simpson, NM., Brammar, GC., Slakeski, N., and Reynolds, EC.: Kgp and RgpB, but not RgpA, are important for *Porphyromonas gingivalis* virulence in the murine periodontitis model. *Infect. Immun*. **75**, 1436–1442, 2007.

17. Curtis, MA., Aduse Opoku, J., Rangarajan, M., Gallagher, A., Sterne, JA., Reid, CR., Evans, HE., and Samuelsson, B.: Attenuation of the virulence of *Porphyromonas gingivalis* by using a specific synthetic Kgp protease inhibitor. *Infect. Immun*. **70**, 6968–6975, 2002.

18. Lasica, AM., Ksiazek, M., Madej, M., and Potempa, J.: The Type IX secretion system (T9SS):

highlights and recent insights into its structure and function. *Front. Cell Infect. Microbiol*. **7**, 215, 2017.

19. Nguyen, KA., Travis, J., and Potempa, J.: Does the importance of the C-terminal residues in the maturation of RgpB from *Porphyromonas gingivalis* reveal a novel mechanism for protein export in a subgroup of gram-negative bacteria? *J. Bacteriol*. **189**, 833-843, 2007.

20. Tomek, MB., Neumann, L., Nimeth, I., Koerdt, A., Andesner, P., Messner, P., Mach, L., Potempa, JS., and Schaffer, C.: The S-layer proteins of *Tannerella forsythia* are secreted via a type IX secretion system that is decoupled from protein O-glycosylation. *Mol. Oral. Microbiol*. **29**, 307-320, 2014.

21. Narita, Y., Sato, K., Yukitake, H., Shoji, M., Nakane, D., Nagano, K., Yoshimura, F., Naito, M., and Nakayama, K.: Lack of a surface layer in *Tannerella forsythia* mutants deficient in the type IX secretion system. *Microbiology*. **160**, 2295-2303, 2014.

22. Sato, K., Naito, M., Yukitake, H., Hirakawa, H., Shoji, M., McBride, MJ., Rhodes, RG., and Nakayama, K.: A protein secretion system linked to bacteroidete gliding motility and pathogenesis. *Proc. Natl. Acad. Sci. USA*. **107**, 276-281, 2010.

23. Kadowaki, T., Yukitake, H., Naito, M., Sato, K., Kikuchi, Y., Kondo, Y., Shoji, M., and Nakayama, K.: A two-component system regulates gene expression of the type IX secretion component proteins via an ECF sigma factor. *Sci. Rep*. **6**, 23288, 2016.

24. Glew, MD., Veith, PD., Peng, B., Chen, YY., Gorasia, DG., Yang, Q., Slakeski, N., Chen, D., Moore, C., Crawford, S., and Reynolds, EC.: PG0026 is the C-terminal signal peptidase of a novel secretion system of *Porphyromonas gingivalis*. *J. Biol. Chem*. **287**, 24605-24617, 2012.

25. Nakayama K: *Porphyromonas gingivalis* and related bacteria.: from colonial pigmentation to the type IX secretion system and gliding motility. *J. Periodontal. Res*. **50**, 1-8, 2015.

26. Saiki, K., and Konishi, K.: Identification of a *Porphyromonas gingivalis* novel protein *sov* required for the secretion of gingipains. *Microbiol. Immunol*. **51**, 483-449, 2007.

27. Sato, K., Sakai, E., Veith, PD., Shoji, M., Kikuchi, Y., Yukitake, H., Ohara, N., Naito, M., Okamoto, K., Reynolds, EC., and Nakayama, K.: Identification of a new membrane-associated protein that influences transport/maturation of gingipains and adhesins of *Porphyromonas gingivalis*. *J. Biol. Chem*. **280**, 8668-8677, 2005.

28. Veith, PD., Nor Muhammad, NA., Dashper, SG., Likic, VA., Gorasia, DG., Chen, D., Byrne, SJ., Catmull, DV., and Reynolds, EC.: Protein substrates of a novel secretion system are numerous in the *Bacteroidetes* phylum and have in common a cleavable C-terminal secretion signal, extensive post-translational modification, and cell-surface attachment. *J. Proteome. Res*. **12**, 4449-4461, 2013.

29. Heath, JE., Seers, CA., Veith, PD., Butler, CA., Nor Muhammad, NA., Chen, YY., Slakeski, N., Peng, B., Zhang, L., Dashper, SG., Cross, KJ., Cleal, SM., Moore, C., Reynolds, EC.: PG1058 is a novel multidomain protein component of the bacterial type IX secretion system. *PLoS One*. **11**, e0164313, 2016.

30. Taguchi, Y., Sato, K., Yukitake, H., Inoue, T., Nakayama, M., Naito, M., Kondo, Y., Kano, K., Hoshino, T., Nakayama, K., Takashiba, S., and Ohara, N.: Involvement of an Skp-like protein, PGN_0300, in the type IX secretion system of *Porphyromonas gingivalis*. *Infect. Immun*. **84**, 230-240, 2015.

31. https://www.ncbi.nlm.nih.gov/nuccore/NC_010729.1.

32. Ueshima, J., Shoji, M., Ratnayake, DB., Abe, K., Yoshida, S., Yamamoto, K., and Nakayama, K.: Purification, gene cloning, gene expression, and mutants of Dps from the obligate anaerobe *Porphyromonas gingivalis*. *Infect. Immun*. **71**, 1170-1178, 2003.

33. Tagawa, J., Inoue, T., Naito, M., Sato, K., Kuwahara, T., Nakayama, M., Nakayama, K., Yamashiro, T., Ohara, N.: Development of a novel plasmid vector pTIO-1 adapted for electrotransformation of *Porphyromonas gingivalis*. *J. Microbiol. Methods*. **105**, 174-179, 2014.

34. Shi, Y., Ratnayake, DB., Okamoto, K., Abe, N., Yamamoto, K., and Nakayama, K.: Genetic analyses of proteolysis, hemoglobin binding, and hemagglutination of *Porphyromonas gingivalis*. Construction of mutants with a combination of *rgpA*, *rgpB*, *kgp*, and *hagA*. *J. Biol. Chem*. **274**, 17955-17960, 1999.

35. Yamaguchi, M., Sato, K., Yukitake, H., Noiri, Y., Ebisu, S., and Nakayama, K.: A *Porphyromonas gingivalis* mutant defective in a putative glycosyltransferase exhibits defective biosynthesis of the polysaccharide portions of lipopolysaccharide, decreased gingipain activities, strong autoaggregation, and increased biofilm formation. *Infect. Immun*. **78**, 3801–3812, 2010.

36. Okamoto, K., Nakayama, K., Kadowaki, T., Abe, N., Ratnayake, DB., and Yamamoto, K.: Involvement of a lysine-specific cysteine proteinase in hemoglobin adsorption and heme accumulation by *Porphyromonas gingivalis*. *J. Biol. Chem*. **273**, 21225–21231, 1998.

37. Nakayama, M., Inoue, T., Naito, M., Nakayama, K., and Ohara, N.: Attenuation of the phosphatidylinositol 3-kinase/Akt signaling pathway by *Porphyromonas gingivalis* gingipains RgpA, RgpB, and Kgp., *J. Biol. Chem.*, **290**, 5190-5202, 2015.

38. Vanterpool, E., Roy, F., and Fletcher, HM.: Inactivation of *vimF*, a putative glycosyltransferase gene downstream of *vimE*, alters glycosylation and activation of the gingipains in *Porphyromonas gingivalis.,* W83. *Infect. Immun*. **73**, 3971-3982, 2005.

39. Klein, BA., Cornacchione, LP., Collins, M., Malamy, MH., Duncan, MJ., and Hu, LT.: Using Tn-seq to identify pigmentation-related genes of *Porphyromonas gingivalis*: Characterization of the role of a putative glycosyltransferase. *J. Bacteriol*. **199**, e00832-16, 2017.

40. Vincent, MS., Canestrari, MJ., Leone, P., Stathopulos, J., Ize, B., Zoued, A., Cambillau, C., Kellenberger, C., Roussel, A., and Cascales, E.: Characterization of the *Porphyromonas gingivalis* type IX secretion trans-envelope PorKLMNP core complex. *J. Biol. Chem*. **292**, 3252-3261, 2017.

41. Gorasia, DG., Veith, PD., Hanssen, EG., Glew, MD., Sato, K., Yukitake, H., Nakayama, K., and Reynolds, EC.: Structural insights into the PorK and PorN components of the *Porphyromonas gingivalis* type IX secretion system. *PLoS Pathog*. **12**, e1005820, 2016.

42. Veith, PD., Chen, YY., Gorasia, DG., Chen, D., Glew, MD., O'Brien-Simpson, NM., Cecil, JD., Holden, JA., and Reynolds, EC.: *Porphyromonas gingivalis* outer membrane vesicles exclusively contain outer membrane and periplasmic proteins and carry a cargo enriched with virulence factors. *J. Proteome. Res*. **13**, 2420-2432, 2014.

Figure legends

Figure 1. PCR analysis of ΔPGN_0296. Two sets of gene specific primers, for PGN_0296 and the PGN_0297 respectively were used for PCR. Showing the absence of PGN_0296 gene in ΔPGN_0296 (panel A) and the presence of PGN_0297 gene in ΔPGN_0296 (panel B).

Figure 2. PCR analysis of CPGN_0296. Two sets of gene specific primers, for PGN_0296 and the PGN_0297 respectively were used for PCR. Showing the presence of both the PGN_0296 (panel A) and the PGN_0297 gene (panel B) CPGN_0296.

Figure 3. Colonies of the mutants. Colonial pigmentation of *P. gingivalis* WT (33277), ΔPGN_0296 and CPGN_0296 cells. The cells were grown anaerobically (10%CO_2,10%H_2, 80%N_2) at 37ºC on blood agar plates for 10 days.

Figure 4. Expression of PGN_0296 gene in the *P. gingivalis* WT (33277), ΔPGN_0296 and CPGN_0296 by reverse transcription polymerase chain reaction (RT-PCR). RT-PCR were done by using the gene specific primers for the PGN_0296 gene (panel A) and PGN_0297 gene (panel B).

Figure 5. Measurement of the Rgp and Kgp activities in ΔPGN_0296. *P. gingivalis* cells were grown anaerobically in enriched BHI medium at 37ºC for 36 h. Rgp (panel A) and Kgp (panel B) activities of the culture supernatants of ΔPGN_0296 were measured. Groups with a letter (a, b, c) in common are not statistically different, with no common letter are statistically different (P<0.05, Tukey test).

KYT-36: inhibitor for Lys-gingipain.

KYT-1: inhibitor for Arg-gingipain.

Table 1. Bacterial strains and plasmids used in this study

Name	Description	Source or reference
E. coli strain		
DH5a	General purpose host strain for cloning	Nippongene, Toyama, Japan
P.gingivalis strain		
ATCC 33277	Wild type	American Type Culture Collection
ΔPGN_0296	ATCC 33277 ΔPGN_0296; Em^r	This study
CPGN_0296	ΔPGN_0296 harboring PGN_0296, Em^r Tc^r	This study
Plasmid		
pBluescript II SK(+)	Ap^r, cloning vector	Stratagene
pUC19	Ap^r, cloning vector	Takara Bio
pUCPGN0296	Ap^r Em^r, pUC19-PGN_0296::*ermF*	This study
pCPG	Ap^r Em^r, pBSSK containing PGN_1045::*tetQ*	This study
pCPG0296	Ap^r Tc^r pBSSK containing PGN_1045::p-*fimA*-PGN_0296-*tetQ*	This study
pKD355	Ap^r Em^r, contains the *ermF ermAM* DNA cassette in pUC19	Ueshima *et al*. (32)
pKD375	Ap^r Tc^r, contains the *tetQ* DNA cassette in pUC19	Shi *et al*. (34)

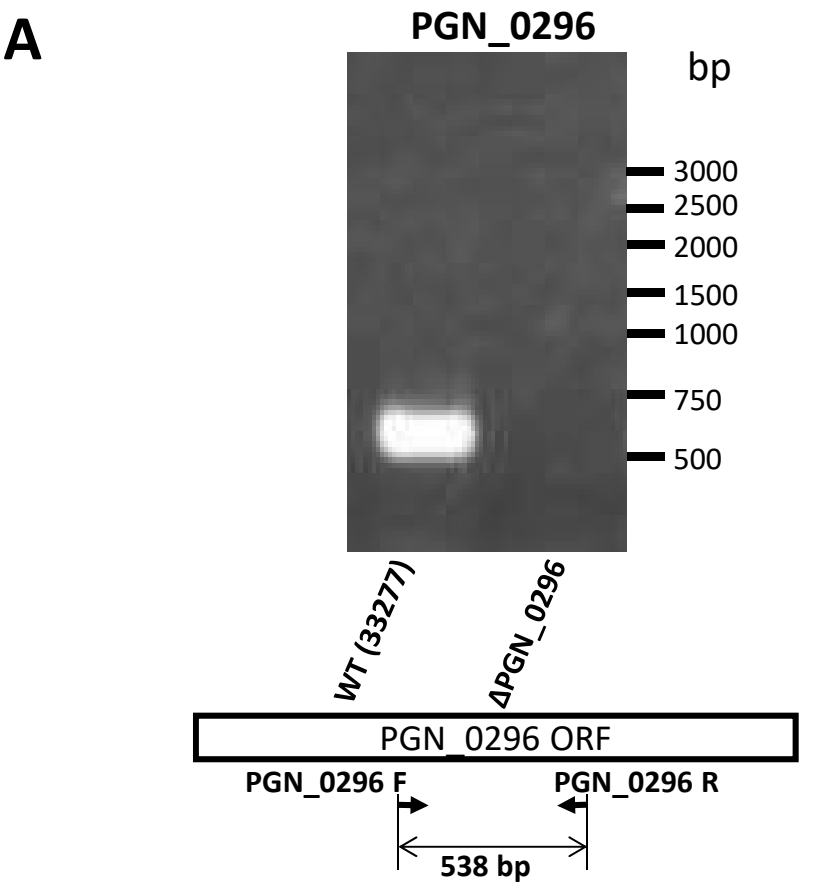
Table 2. Oligonucleotide primers used in this study

Primers	Oligonucleotide sequences
*PGN_0296-F	GTGAAGTAAGCGGATCAGCACGTGC
*PGN_0296-R	GGCCATTACCATCCATGACCAAAGCG
**PGN_0296 F	GGGAAGCTTATGTGTAAGAAACATTTCATCC
**PGN_0296 R	CCTTGTCAGCATGCGTTACAACATCG
**PGN_0297 F	GGTTCATCCGGAGTTGGTTTGGAG
**PGN_0297 R	GAGGCGGCCGCATTGTTTATTACAAAAAGTCTTACG
ermF-F	GCGAGATCTCATGACAAAAAAGAAATTGCCC
ermF-R	CCATCGATTACGAAGGATGAAATTTTTCAG
PGN_1045U-F	GGG CTCGAG TAGCGCATTGATGGGAGCAG
PGN_1045U-R	GGC ATCGAT ACGGGCATCGGGAGAATAAC
PGN_1045D-F	GAA GCGGCCGC GTCTTGCGCTTCGACTATAT
PGN_1045D-R	GGG GAGCTC CATCAGTCATTCAAGGAAG
tetQ-F	CCCGGGGATCCTCTAGAGTCTC
tetQ-R	GGAAGCGGCCGCCAGTGAATTCGAGCTCGTC
fimA-F	GGA ATCGAT ATATGCCTACAGCGAAAAATGG
fimA-R	GCG GAATTC ATGCTGATGGTGGCATTACCTT

Underlined nucleotides indicate recognition sites of restriction enzymes.

*The primers used for the amplification of PGN_0296 from ATCC 33277.

**The primers used to detect the PGN_0296 and PGN_0297 genes respectively.



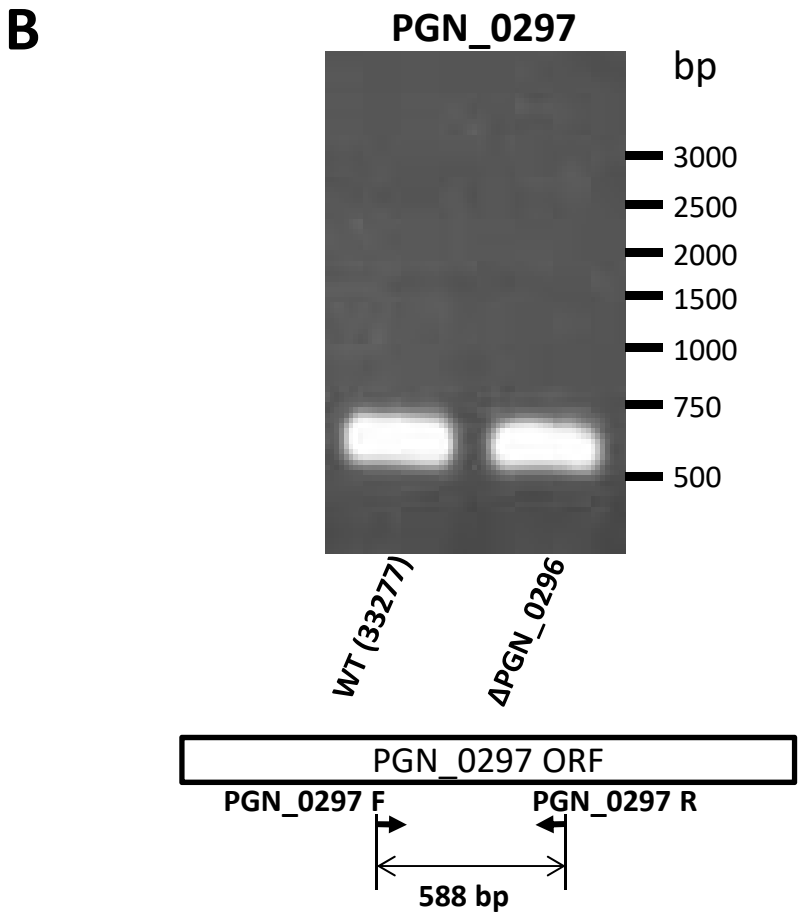
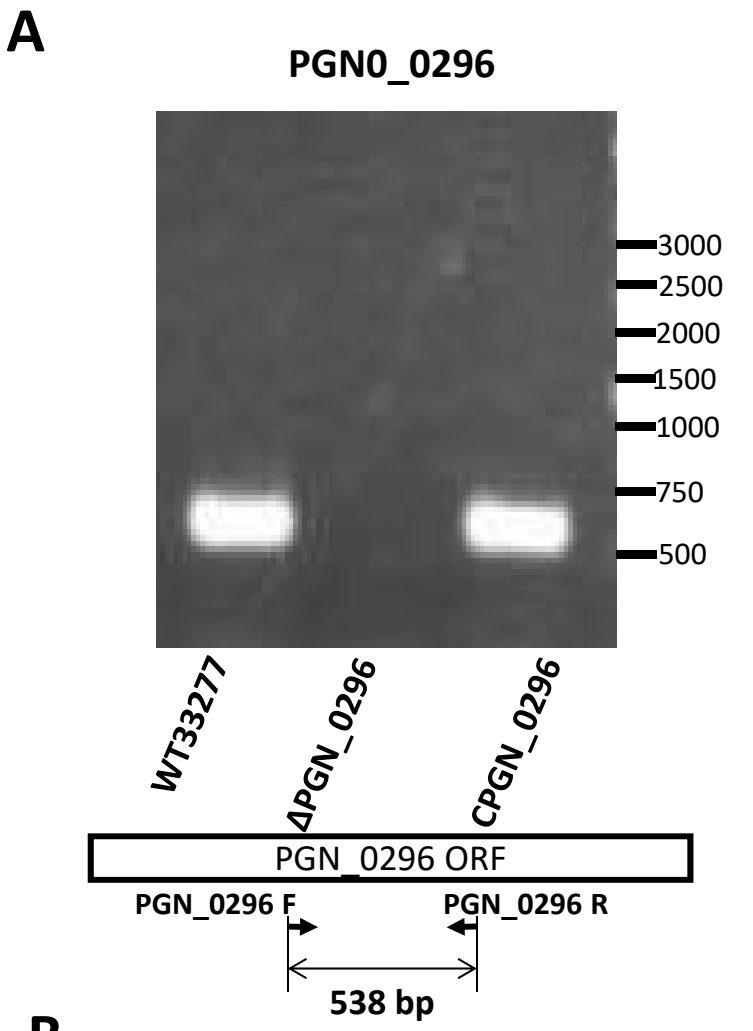


Figure 1 Shahriar



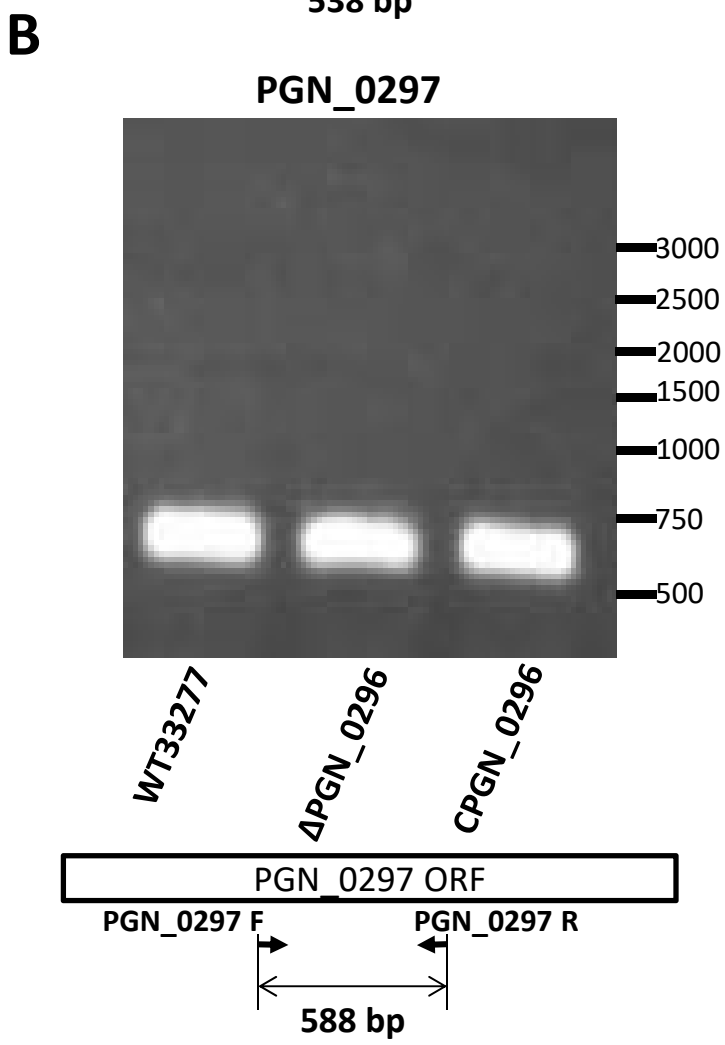


Figure 2 Shahriar

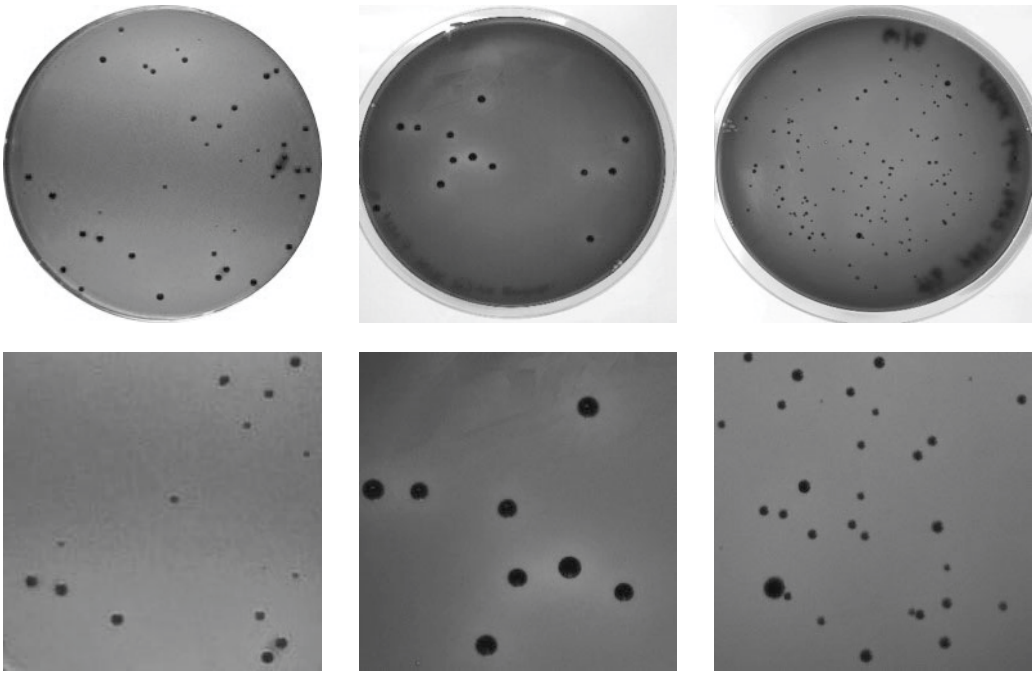
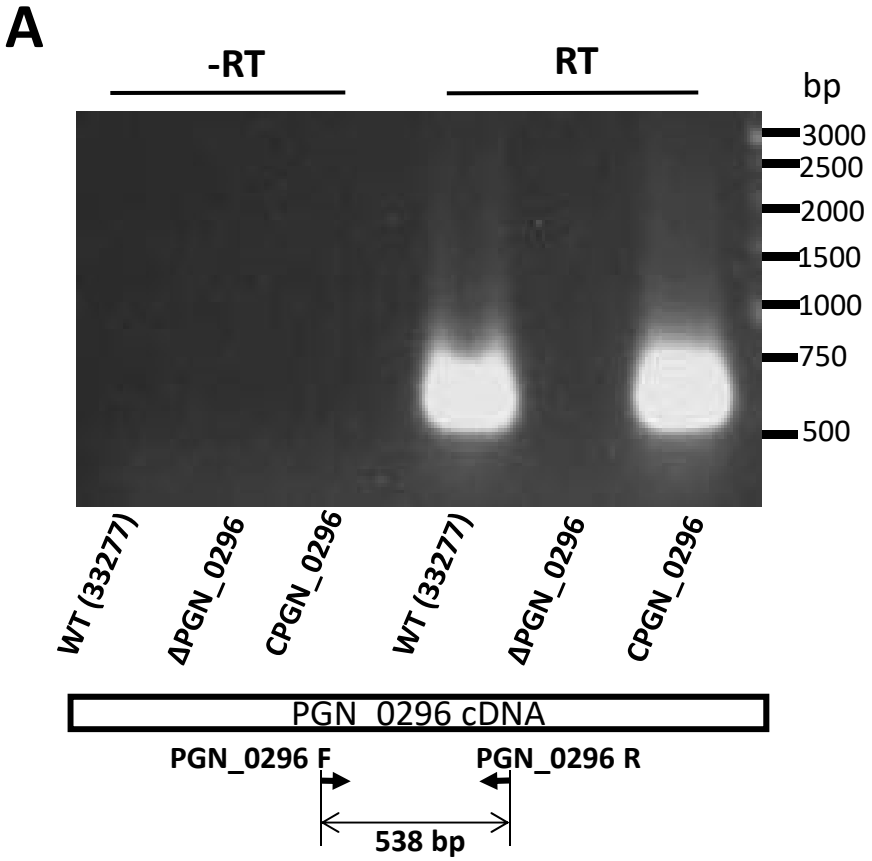


Figure 3 Shahriar



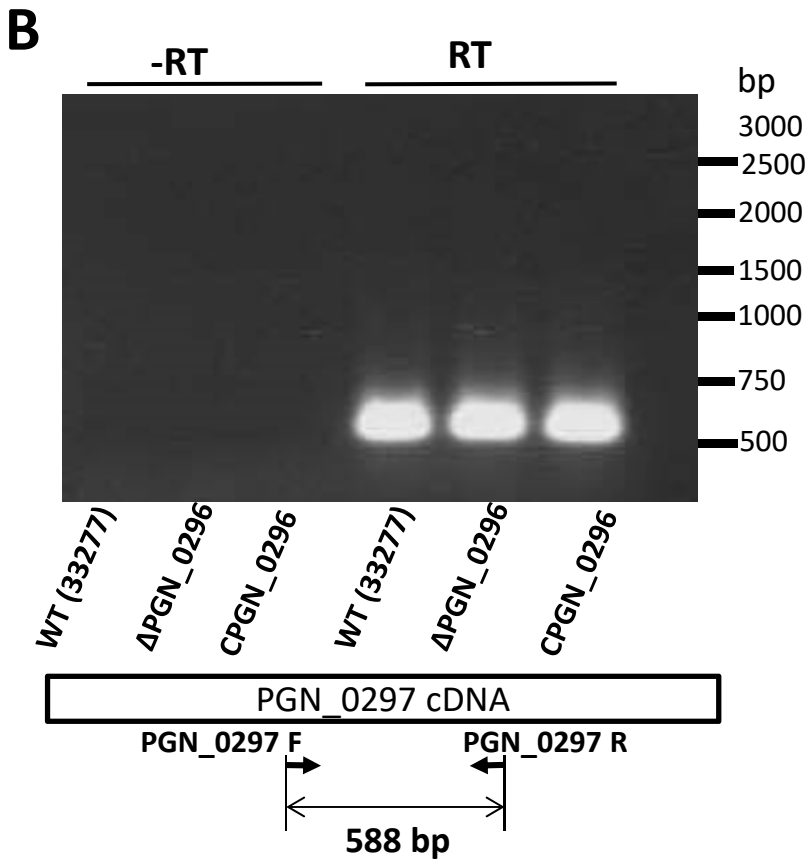
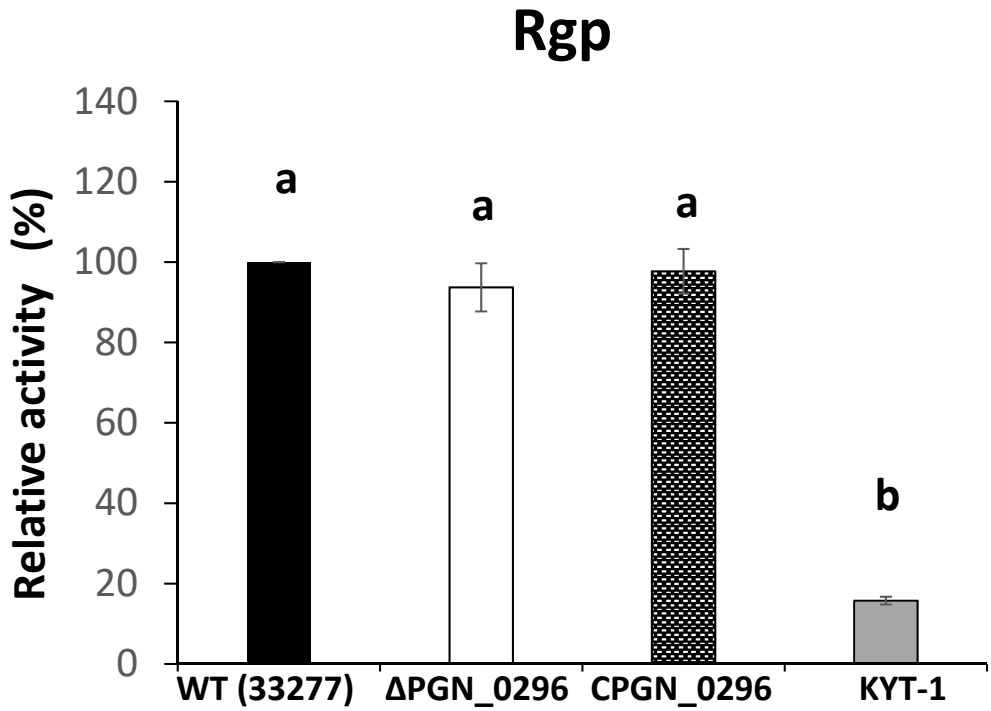


Figure 4 Shahriar

A



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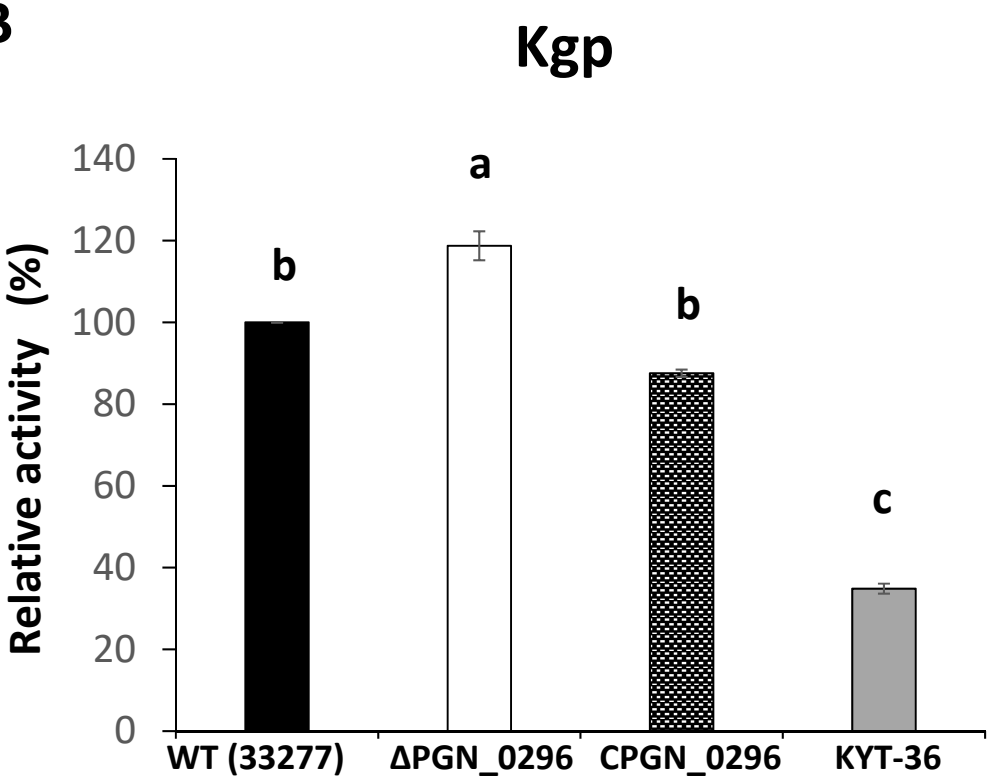

Figure 5 Shahriar