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PGN_0297 is an essential component of the type IX secretion system (T9SS) in *Porphyromonas gingivalis*: Tn-seq analysis for exhaustive identification of T9SS-related genes

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Abstract

The type IX secretion system (T9SS) was originally discovered in *Porphyromonas gingivalis*, one of the pathogenic bacteria associated with periodontal disease and is now known to be present in many members of the phylum *Bacteroidetes*. The T9SS secretes a number of potent virulence factors, including the highly hydrolytic proteases called gingipains, across the outer membrane in *P. gingivalis*. To understand the entire machinery of T9SS, an exhaustive search for T9SS-related genes in *P. gingivalis* using the mariner family transposon (Tn) and Tn-seq analysis was performed. Seven hundred and two Tn insertion sites in Tn mutants with no colony pigmentation that is associated with Lys-gingipain (Kgp) defectiveness were determined, and it was found that the Tn was inserted in the *kgp* gene and 54 T9SS-related candidate genes. Thirty-three out of the 54 genes were already known as T9SS-related genes. Furthermore, deletion mutant analysis of the remaining 21 genes revealed that they were not related to the T9SS. The 33 T9SS-related genes include a gene for PGN_0297, which was found to be associated with the T9SS components PorK and PorN. A PGN_0297 gene deletion mutant was constructed, and it was found that the mutant showed no colony pigmentation, hemagglutination or gingipain activities, indicating that PGN_0297 was an essential component of the T9SS. The 33 genes did not include the six genes (*gppX*, *omp17*, *porY*, *rfa*, *sigP* and *wzx*) that were also reported as T9SS-related genes. *gppX* deletion and insertion mutants were constructed, and it was found that they did not show deficiency in the T9SS.

KEYWORDS
colony pigmentation, *Porphyromonas gingivalis*, Tn-seq analysis, type IX secretion system

Abbreviations: Ap, ampicillin; CDS, coding sequence; Em, erythromycin; ITR, inverted terminal repeat sequence; Kgp, Lys-gingipain; PBS, phosphate-buffered saline; Rgp, Arg-gingipain; Tc, tetracycline; Tn, transposon; T9SS, type IX secretion system.

1 | INTRODUCTION

Bacterial infections have been serious health problems to date. Many pathogenic bacteria have systems to secrete various virulence factors across their cell membrane(s). Especially, the Gram-negative bacteria have a number of secretion systems across their bilayer membranes [1]. Among such bacterial secretion systems, we found the type IX secretion system (T9SS), by comparative genome analysis, in *Porphyromonas gingivalis*, one of the pathogenic bacteria associated with periodontal disease [2]. This system secretes many potent virulence factors (T9SS cargo proteins) across the outer membrane depending on the presence of a conserved C-terminal domain in the T9SS cargo proteins [3,4]. These virulence factors include the highly catalytic proteases known as gingipains, the Arg-gingipain (Rgp) and Lys-gingipain (Kgp) [5], hemin-binding protein HBP35 [6], CPG70 [7] and peptidylarginine deiminase [8]. Moreover, the T9SS is indirectly involved in the pathogenesis of *P. gingivalis* because gingipains play a role in the polymerization of pilus subunit proteins to form the type V pili [9]. *P. gingivalis* type V pili (Fim and Mfa pili) are also key virulence factors that are essential for colonization on the host cells, evasion of innate defenses and co-aggregation with other pathogens [10,11]. T9SS is found in bacteria belonging to the Bacteroidetes phylum including the other periodontal pathogens, *Tannerella forsythia* and *Prevotella intermedia* [12,13], suggesting the significance of T9SS in periodontal disease.

Translocation of Kgp to the cell surface via the T9SS is a prerequisite for colony pigmentation of *P. gingivalis* on the blood agar plate [14,15]. We had identified *porR* and *porT* mutants as non-pigmented mutants [16,17]. Thereafter, using the whole-genome comparison, we identified nine T9SS-related genes (*porK*, *porL*, *porM*, *porP*, *porQ*, *porU*, *porW*, *porX* and *porY*) [2]. A number of other genes have been reported as T9SS-related genes [18–24]; however, it has not been determined how many genes are actually involved in the T9SS.

Transposon (Tn) mutagenesis is an attractive tool for identifying the genes responsible for a given phenotype. The mariner family Tn has the advantage that it shows very little insertion site specificity, requiring only the dinucleotide TA in the target sequence. Very recently, Klein *et al*. [25] identified pigmentation-related genes of *P. gingivalis* using Tn-seq analysis with the mariner family Tn. However, they isolated mutants of only five genes (*gtfC*, *kgp*, *porL*, *porT* and *waaL*) among the 40 genes that have been reported to be related to colony pigmentation, suggesting that their Tn-seq analysis was incomplete. To understand the entire machinery of the T9SS, we performed an exhaustive search for T9SS-related genes in *P. gingivalis* using the mariner family Tn.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table S1. *P. gingivalis* strains were maintained in tryptic soy broth medium (30 g/L tryptic soy broth supplemented with 2.5 g/L yeast extract, 1 g/L cysteine, 5 μg/mL hemin and 5 μg/mL menadione) and TS agar plates (40 g/L tryptic soy agar base supplemented with 10 g/L of yeast extract, 1 g/L cysteine, 5 μg/mL hemin and 5 μg/mL menadione). To prepare blood agar plates, 5% defibrinated sheep blood was added to the TS agar. *Escherichia coli* was maintained on LB agar plates. 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 | Mariner-based Tn mutagenesis

The plasmids and oligonucleotides used in this study are listed in Tables S2 and S3, respectively. Mariner-based Tn mutagenesis in *P. gingivalis* was carried out as previously described for *Bacteroides* [26]. The plasmid pMI07 harbors the mariner family Tn, *Himar1* transposase and the delivery gene cassette. This gene cassette consists of the Em-resistant gene (*ermF*) and the inverted terminal repeat sequence (ITR) recognized by *Himar1* transposase. Briefly, this plasmid was introduced into *P. gingivalis* ATCC 33277 by electro-transformation [27]. Electro-competent cells were prepared from cultures at stationary phase (48 hr culture). The *ermF*-ITR insertion mutants were selected on blood agar plates supplemented with 10 μg/mL Em. T9SS-related mutants were detected as non-pigmented mutants on the blood agar plate after 10 days of anaerobic culturing. Individual non-pigmented mutants were seeded on the blood agar plates for 10 days and re-examined for colony pigmentation. Genomic DNA was isolated from the selected colonies and then the sites of *ermF*-ITR insertion were amplified by nested arbitrarily-primed PCR as previously described [26]. The oligonucleotides used in this study are listed in Table S3. Briefly, the first round PCR was performed with a random primer (AR8) and *ermF*-ITR PCR-specific primer (mariner-A); then, the second round PCR was performed with AR2 primer and mariner-B primer (*ermF*-ITR PCR-specific primer designed within the mariner-A). The resulting product was purified and sequenced from the left arm of the *ermF*-ITR cassette into the *P. gingivalis* genomic region using the primer mariner-S. Sequences obtained were compared with the *ermF*-ITR and genome sequence of *P. gingivalis* ATCC 33277 (GenBank accession no. AP009380) [28] by BLASTN search, resulting in the determination of the insertion sites in the genome.

2.3 | Construction of *P. gingivalis* strains

P. gingivalis mutants were generated by double recombination of the target genes and the introduction of *ermF* (Figure S1), as previously described [2]. The targeting DNA was constructed as follows. The upstream and downstream regions of the target genes were amplified with two pairs of primers (geneX-Up-F/geneX-Up-R; geneX-Dw-F/geneX-Dw-R, where "geneX" indicates the name of the target gene and "Up", "Dw", "F" and "R" indicate upstream, downstream, forward and reverse, respectively). The *ermF* region in the *ermF* DNA cassette was amplified with ErmF-F/ErmF-R from pTIO-1. Using the three purified products, further PCR was performed with geneX-Up-F/geneX-Dw-R. Finally, the desired PCR product was purified and introduced into *P. gingivalis* ATCC 33277 by electroporation. Transformants were selected on blood agar plates containing Em. This procedure yielded deletion mutant strains, KDP1030 (*ΔgppX::ermF*) and KDP1031 (*ΔporG::ermF*). Correct deletion of the target genes was verified by PCR (Figure S2).

To create a shuttle plasmid for gene complementation, the *tetQ* gene fragment was amplified with tetQ-F/tetQ-R from pTCB. Using infusion enzyme (Takara Bio, Kusatsu, Japan), the amplified fragment was ligated with pTIO-1, which had been digested with SacI and SacII to yield pTIO-*tetQ*. The promoter region of *Porphyromonas gulae* catalase was amplified from pKD954 with the primer pair Pcat-F/Pcat-R. The PGN_0297-encoding gene (*porG*) was amplified from *P. gingivalis* genomic DNA with the primer pair, N0297-CDS-F/N0297-CDS-R. Using these two purified products, further PCR was performed with Pcat-F/N0297-CDS-R. Finally, the desired PCR product and pTIO-*tetQ* were digested with SalI and PstI, and then ligated together to yield pTIO-*tetQ-porG*$^{+}$. The plasmids pTIO-*tetQ* and pTIO-*tetQ-porG*$^{+}$ were introduced into the *porG* deletion mutant KDP1031 by electroporation, resulting in KDP1032 and KDP1033, respectively. Transformants were selected on blood agar plates containing Tc. Correct gene complementation of *porG* was verified by PCR (Figure S2).

2.4 | Enzymatic assay

Kgp and Rgp activities were determined using the synthetic substrates benzyloxycarbonyl-L-histidyl-L-glutamyl-L-lysine-4-methyl-coumaryl-7-amide (Peptide Institute, Ibaraki, Japan) and benzyloxycarbonyl-L-phenyl-L-arginine-4-methyl-coumaryl-7-amide (Peptide Institute), respectively, as previously described [29]. Cultures of *P. gingivalis* strains at OD_{600} of 1.0 were centrifuged to separate the culture supernatant and cells. Precipitated cells were resuspended with an original volume of PBS. Each 10 μL of bacterial cell suspension and culture supernatant were added to the reaction mixture (0.25 mL) containing 5 mM cysteine, 20 mM sodium phosphate buffer, pH 7.5, and 10 mM each of the fluorogenic substrate. After 10 min incubation at 40°C, the reaction was terminated by adding 100 mM sodium acetate buffer, pH 5.0, containing 10 mM iodoacetic acid (0.25 mL). The released 7-amino-4-methyl-coumarine was measured at 460 nm (excitation at 380 nm) by fluorescence spectrophotometer (DTX 800; Beckman Coulter, Brea, CA, USA). The significance of all described comparisons was established using two-tailed unpaired Student's *t*-test on triplicate samples.

2.5 | Hemagglutinating activity

Hemagglutinating activity was determined as previously described [29]. The erythrocyte cells were precipitated from defibrinated sheep blood by centrifugation and washed with PBS. Overnight culture of *P. gingivalis* strains in TS medium was centrifuged, washed and resuspended in PBS to yield a bacterial cell suspension with an optical density value of 3.0 at OD_{600}. The bacterial cell suspension was then diluted in twofold series with PBS. Each diluted suspension (0.1 mL) was mixed with an equal volume of the washed erythrocyte suspension (1% in PBS) in round-bottomed microtiter plates, and then incubated at room temperature for 1 hr.

3 | RESULTS

3.1 | Mariner-based Tn mutagenesis for the exhaustive isolation of T9SS-related genes

The *ermF*-ITR insertion mutants were successfully obtained from *P. gingivalis* ATCC 33277. Approximately 2000–6000 insertion mutants were obtained when 10 μg of pMI07 DNA was used. We generated 55 000 insertion mutants that theoretically covered all coding sequences (CDS) of *P. gingivalis* (2090 CDS). Approximately 1.6% of the insertion mutants showed non-pigmented colonies on blood agar plates. Among the non-pigmented colonies, 724 non-pigmented clones were randomly selected and subjected to Tn-seq analysis to determine their *ermF*-ITR insertion sites. Insertion sites were successfully identified in 702 mutants among them. Tn insertion had occurred at different positions in these mutants at a high frequency (85.5%). The *ermF*-ITR insertion sites were found mostly in the coding regions, and some in the 5′-intergenic regions, of the 55 genes including *kgp* (Tables 1, 2) (2, 6, 14, 16–24, 29–45).

Among them, 21 were newly identified genes (Table 2). Five out of the 21 genes were located in regions upstream of known T9SS-related genes. An average of 5.8 Tn mutants were isolated from the five genes upstream of the T9SS-related genes, whereas an average of 1.1 Tn mutants were isolated from the remaining 16 genes. To determine whether non-pigmentation of the 21 mutants is derived from the insertion of *ermF*-ITR, mutants of the 21 genes were reconstructed. We designed deletion mutants to avoid a polar

TABLE 1 Number of Tn mutant clones showing Tn insertion in the T9SS-related genes previously reported

	Locus	No. of Tn mutant clones	Gene name	Product	Reference
1	PGN_0002	5	*wbpD*	Wbp pathway protein WbpD	[29]
2	PGN_0022	38	*porU*	T9SS component protein PorU	[2]
3	5′-intergenic region of PGN_0023	1	*porV/pg27/lptO*	T9SS component protein PorV/Pg27/LptO	[18,19]
4	5′-intergenic region of PGN_0168 PGN_0168	3 20	*wbpB*	Wbp pathway protein WbpB	[29,30]
5	PGN_0242	14	*gtfG*	Glycosyltransferase group 1	[6,31]
6	PGN_0297	2	*porG*	T9SS component protein PorG	[32]
7	PGN_0361	12	*gtfC*	Glycosyltransferase family 2	[33]
8	5′-intergenic region of PGN_0509 PGN_0509	1 36	*porZ*	T9SS component protein PorZ/immunoreactive 84 kDa antigen PG93	[22]
9	PGN_0613	22	*ugdA/wbpA*	UDP-glucose 6-dehydrogenase/Wbp pathway protein WbpA	[34]
10	PGN_0645	10	*porQ*	T9SS component protein PorQ	[2]
11	PGN_0778	9	*porT*	T9SS component protein PorT	[17]
12	PGN_0832	84	*sov*	T9SS component protein Sov	[35]
13	PGN_1019	2	*porX*	Two-component system response regulator PorX for expression of T9SS component genes	[2]
14	PGN_1054	14	*vimF*	Virulence modulating gene F/glycosyltransferase family 4	[36,37]
15	PGN_1055	17	*vimE*	Virulence modulating gene E	[38]
16	5′-intergenic region of PGN_1056 PGN_1056	1 24	*vimA*	Virulence modulating gene A	[39]
17	PGN_1236	30	*porR/wbpE*	Wbp pathway protein PorR/WbpE	[16]
18	PGN_1239	5	*gtfD*	Glycosyltransferase family 2	[33]
19	PGN_1240	20	*gtfE*	Glycosyltransferase group 1	[33]
20	PGN_1242	16	*wzy*	O-antigen polymerase Wzy	[6,40]
21	PGN_1251	15	*gtfB*	Glycosyltransferase group 1	[34,41]
22	PGN_1296	22	*porE*	T9SS component protein PorE/putative OmpA family protein	[23]
23	PGN_1302	41	*waaL*	O-antigen ligase waaL	[29,42]
24	PGN_1437	7	*porF*	T9SS component protein PorF/putative lipoprotein	[20]
25	PGN_1668	13	*gtfF*	Glycosyltransferase family 2	[33]
26	PGN_1673	7	*porN*	T9SS component protein PorN	[2]
27	PGN_1674	12	*porM*	T9SS component protein PorM	[2]
28	PGN_1675	5	*porL*	T9SS component protein PorL	[2]
29	5′-intergenic region of PGN_1676 PGN_1676	1 10	*porK*	T9SS component protein PorK	[2]
30	PGN_1677	5	*porP*	T9SS component protein PorP	[2]
31	5′-intergenic region of PGN_1728 PGN_1728	3 79	*kgp* †	Kgp gingipain/lysine-specific cysteine protease	[14]

(Continues)

TABLE 1 (Continued)

	Locus	No. of Tn mutant clones	Gene name	Product	Reference
32	PGN_1877	22	*porW*	T9SS component protein PorW	[2]
33	PGN_1896	26	*wbaP*	Phosphoglycosyltransferase	[43,44]
34	PGN_2005	2	*wzzP*	O-antigen chain length regulator WzzP	[43]
35	PGN_0274	0	*sigP*	ECF sigma factor SigP for expression of T9SS component genes	[24]
36	PGN_0300	0	*omp17*	Skp-like protein Omp17	[21]
37	PGN_1033	0	*wzx*	O-antigen flippase Wzx	[43]
38	PGN_1255	0	*rfa*	Glycosyltransferase family 9	[34]
39	PGN_1768	0	*gppX* ‡	Two-component system for regulation of gingipains and black pigmentation in Porphyromonas gingivalis	[45]
40	PGN_2001	0	*porY*	Two-component system sensor kinase PorY for expression of T9SS component genes	[2]

†Encoding the Kgp gingipain which is one of the T9SS cargo proteins and its activity is a prerequisite for colony pigmentation on blood agar plates.
‡Showing no relationship to colony pigmentation, as revealed by gene deletion mutant analysis in this study.

TABLE 2 Number of Tn mutant clones showing no relationship to the T9SS by deletion mutant analysis

	Locus	No. of Tn mutant clones	Product	Downstream T9SS-related gene
1	PGN_0056	1	Conserved hypothetical protein found in conjugate transposon	
2	PGN_0128	1	Immunoreactive 53 kDa antigen	
3	PGN_0148	1	Conserved hypothetical protein	
4	PGN_0211	1	Conserved hypothetical protein	
5	PGN_0289	1	Conserved hypothetical protein	
6	5′-intergenic region of PGN_0538	1	Conserved hypothetical protein	
7	PGN_0589	1	Conserved hypothetical protein	
8	5′-intergenic region of PGN_0612	2	Phosphoserine aminotransferase	
9	PGN_0617	1	Conserved hypothetical protein	
10	PGN_0700	1	Putative oxidoreductase Gfo/Idh/MocA family	
11	PGN_0786	1	Conserved hypothetical protein	
12	PGN_0973	1	Conserved hypothetical protein	
13	PGN_1195	1	Conserved hypothetical protein	
14	PGN_1238	2	Conserved hypothetical protein	PGN_1236/*porR*
15	PGN_1303	7	Hypothetical protein	PGN_1302/*waaL*
16	PGN_1304	5	Conserved hypothetical protein	PGN_1302/*waaL*
17	PGN_1364	1	Probable peptidyl-prolyl cis-trans isomerase cyclophilin-type	
18	PGN_1666	13	Phosphoribosylformylglycinamidine synthase	PGN_1668/*gtfF*
19	PGN_1667	2	Putative DNA processing Smf-like protein	PGN_1668/*gtfF*
20	PGN_1808	1	Conserved hypothetical protein	
21	PGN_1821	1	Conserved hypothetical protein	

effect on the downstream genes. All the deletion mutants of the 21 genes formed black-pigmented colonies on the blood agar plates, indicating that they were not related to the T9SS (data not shown).

The remaining 34 genes identified in this study were T9SS-related genes that were previously reported (33 genes) and the *kgp* (Table 1). An average of 17.4 Tn mutants were isolated from the 33 T9SS-related genes. In 82 Tn mutants, the *ermF*-ITR was inserted in the *kgp* gene. Interestingly, the insertion sites in *kgp* were found in the 5′ half of *kgp* and not in the 3′ half (Figure 1). The 5′ half of *kgp* gene encodes the protease domain. The result correlates well with the indispensable role of Kgp enzymatic activity in colony pigmentation. Involvement of PGN_1296 and PGN_1437 in the T9SS was found by previous mutant studies [20,23]. The present Tn-seq study confirmed their findings: the genes for PGN_1296 and PGN_1437 were then tentatively designated *porE* and *porF*, respectively.

Among the 33 T9SS-related genes that were determined in this study, all the genes except the PGN_0297-encoding gene were confirmed to be required for colony pigmentation and expression of gingipains by mutant analysis. The PGN_0297 protein was reported to be associated with PorK and PorN proteins [32]; however, involvement of PGN_0297 in the T9SS has not been previously determined in any mutant study. In addition, pigmentation-related genes found by the Tn-seq analysis in this study do not include the six T9SS-related genes previously reported by our group and others (*gppX*, *omp17*, *porY*, *rfa*, *sigP* and *wzx*). Among those genes, we found that *omp17*, *porY*, *rfa*, *sigP* and *wzx* contribute to the T9SS by mutant analysis [2,21,24,34]. Hasegawa *et al.* [45] reported that *gppX* mutants showed deficiency in cell-associated gingipain activities and no colony pigmentation, suggesting that the phenotype of the *gppX* mutant is similar to that of the *porR* and *vimA* mutants. Previous studies [16,46] revealed that the *porR* and *vimA* gene products are related to A-LPS formation; however, the relationship between *gppX* and A-LPS has not been elucidated. Then, we attempted to elucidate the roles of PGN_0297 and *gppX* in the T9SS.

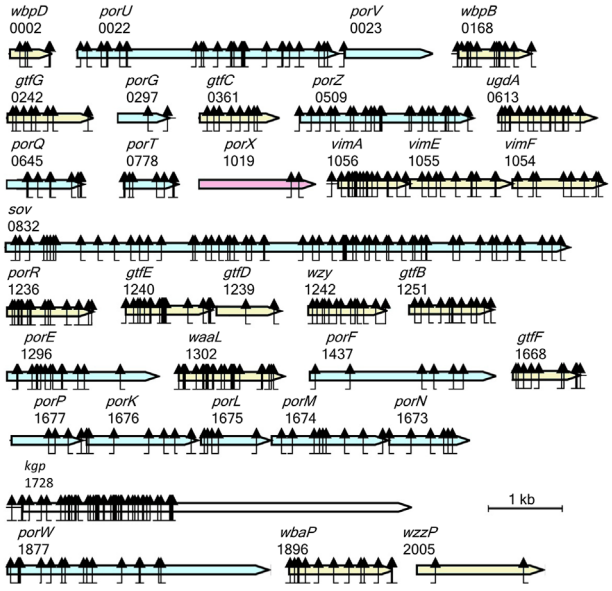

FIGURE 1 The *ermF*-ITR insertion sites in the T9SS-related genes of non-pigmented Tn mutants. Black arrows indicate the *ermF*-ITR insertion sites. PGN numbers of CDS of *P. gingivalis* ATCC 33277 are indicated with their gene names. Genes in blue, yellow and pink encode the T9SS components, proteins for A-LPS biosynthesis and regulator protein for T9SS component expression, respectively.

3.2 | Characterization of PGN_0297 and *gppX* mutants

We constructed PGN_0297 and *gppX* gene deletion mutants from *P. gingivalis* ATCC 33277. The PGN_0297 mutant (KDP1031) was completely devoid of colony pigmentation on the blood agar plate (Figure 2). KDP1031 had also lost hemagglutination and gingipain activities of the cells and culture supernatants (Figure 3). Plasmid-mediated complementation of the PGN_0297 gene (KDP1033) restored the colony pigmentation and hemagglutination. Gingipain activities of KDP1033 were recovered to 40–100% of that of wild type. These results clearly demonstrated that the PGN_0297 gene encodes an essential component of the T9SS and was tentatively designated *porG*.

The *gppX* (KDP1030) mutant constructed here formed black-pigmented colonies on the blood agar plates and

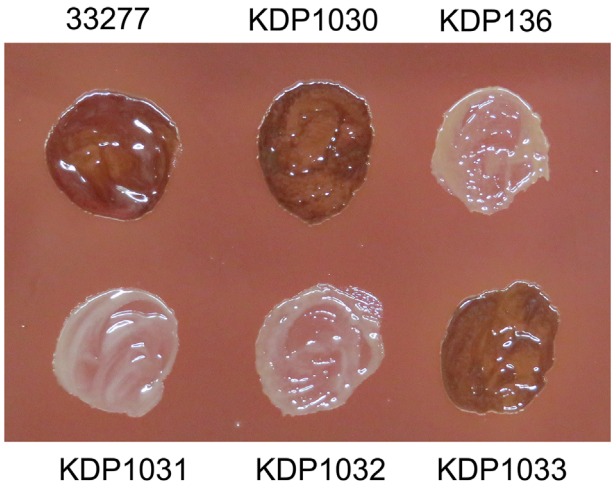

FIGURE 2 Pigmentation of *P. gingivalis* strains. *P. gingivalis* cells were anaerobically cultured on the blood agar plates for 3 days. Strain: 33277, wild type; KDP1030, Δ*gppX*::*ermF*; KDP136, *kgp*::*cat rgpA*::*ermF rgpB*::*tetQ*; KDP1031, Δ*porG*::*ermF*; KDP1032, Δ*porG*::*ermF*/pTIO-*tetQ*; KDP1033, Δ*porG*::*ermF*/pTIO-*tetQ*-*porG*$^{+}$.

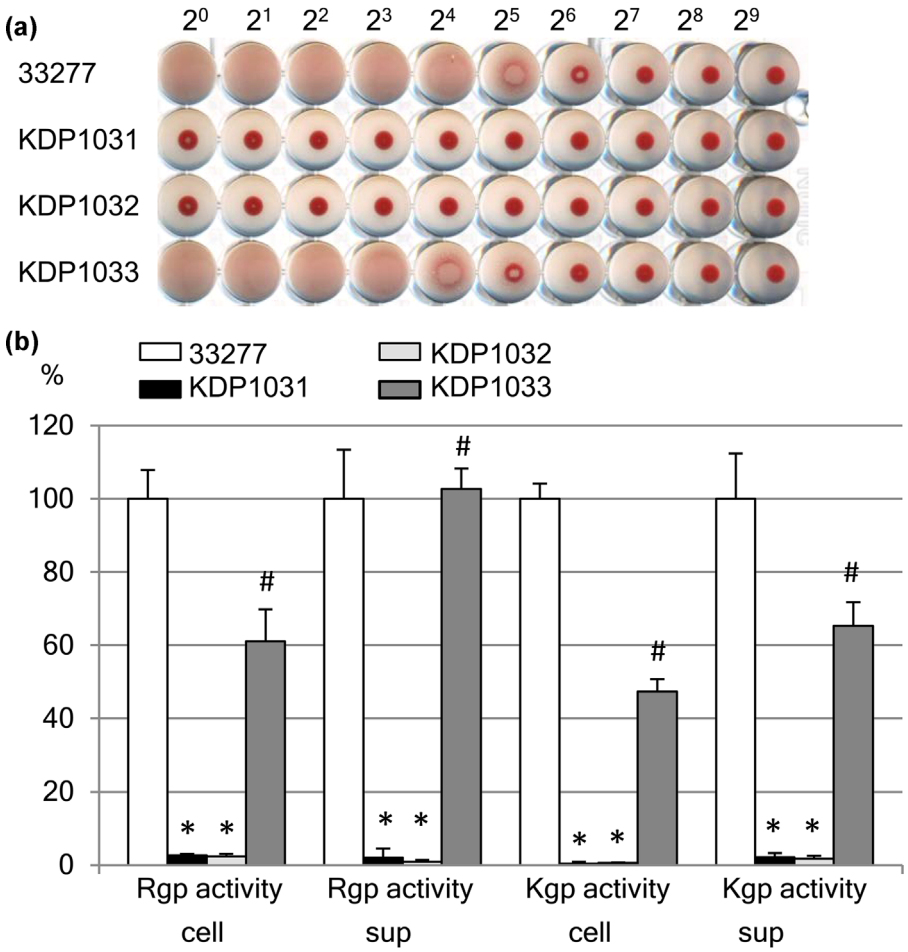

FIGURE 3 Hemagglutination and gingipain activities of *P. gingivalis* strains. (a) Hemagglutination of *P. gingivalis* strains. Twofold serial dilutions of each strain were mixed with 1% sheep red blood cells and stored for 3 hr at room temperature. (b) Rgp and Kgp activities of cells (cell) and vesicle-containing culture supernatants (sup) of *P. gingivalis* strains. The means of each protease activity, with ATCC 33277 (wild type) activity regarded as 100%. The bars are expressed as the means + standard deviation, for triplicate samples from one of the two independent experiments. Statistically significant differences ($p < 0.01$) were seen in comparison with 33277 (*) or KDP1031 (*ΔporG*::*ermF*) (#). Strain: 33277, wild type; KDP1030, *ΔgppX*::*ermF*; KDP1031, *ΔporG*::*ermF*; KDP1032, *ΔporG*::*ermF*/pTIO-*tetQ*; KDP1033, *ΔporG*::*ermF*/pTIO-*tetQ-porG*$^+$.

produced gingipains similar to the wild type (Figure 2). We also constructed an insertion-type mutant of *gppX* and found that the insertion mutant showed no defective phenotype in the T9SS (data not shown).

4 | DISCUSSION

The T9SS was first found in the major periodontal pathogen, *P. gingivalis*; it plays a crucial role in the secretion of proteinaceous virulence factors such as gingipain proteases in the organism [47]. The T9SS has been studied to elucidate its structure and precise secretion mechanism [48–50]; however, the entire structure has not been determined to date. Many genes have been characterized with respect to their relationship to the T9SS. We identified *porR* [16] and *porT* [17] as T9SS-related genes. After that, many other genes have been reported to be T9SS-related genes [2,18–24]. However, the comprehensive isolation of the T9SS-related genes from *P. gingivalis* has not been successfully performed. In this study, using an exhaustive mariner Tn library and Tn-seq analysis, we identified 702 insertion sites in non-pigmented Tn mutants (Tables 1 and 2). The total number of genes with Tn insertion was 55:34 of these genes, including *kgp*, were already reported to be related to colony pigmentation and the remaining 21 genes were newly found. However, further deletion mutant analysis of the newly found 21 genes revealed no relationship to colony pigmentation. Pigmentation-related genes characterized so far can be classified into three types: (i) expression of Kgp protease; (ii) the T9SS components; and (iii) attachment of T9SS cargo proteins to A-LPS. Among the genes listed in Table 1, Kgp protease is encoded by *kgp*; T9SS components are encoded by *porE*, *porF*, *porG*, *porK*, *porL*, *porM*, *porN*, *porP*, *porQ*, *porT*, *porU*, *porV*, *porW*, *porZ* and *sov*; and biosynthesis of A-LPS involves the products encoded by *gtfB*, *gtfC*, *gtfD*, *gtfE*, *gtfF*, *gtfG*, *rfa*, *porR*/

wbpE, *udgA/wbpA*, *vimA*, *vimE*, *vimF*, *waaL*, *wbaP*, *wbpB*, *wbpD*, *wzx*, *wzy* and *wzzP*. The genes *porX*, *porY* and *sigP* are involved in the regulation of T9SS component genes. *omp17* may be involved in translocation of PorU to the outer membrane and its full function [21]. The results showing that approximately 1.6% Tn mutants had no pigmentation and the fact that the *P. gingivalis* ATCC 33277 genome has 2090 CDS suggests that the genome has 33.4 pigmentation-related genes, which is very close to the number of pigmentation-related genes isolated in this study (34 genes). This suggested that other pigmentation-related genes may not be further obtained by the experimental procedure using the *P. gingivalis* ATCC 33277 genome in this study.

In this study, we strictly examined Tn insertion mutants for colony pigmentation and excluded the mutants that showed delayed pigmentation from further analysis. This may be the reason why *omp17*, *porY*, *rfa*, *sigP* and *wzx* mutants were not detected in the non-pigmented Tn mutants found in this study. The *gppX* gene product is annotated as a two-component system: GppX has two putative transmembrane regions, followed by a sensor histidine kinase domain and a response regulator domain. In contrast to the previous study [45], we found in this study that both the deletion and insertion mutants of *gppX* showed colony pigmentation and gingipain production similar to the wild type, indicating that *gppX* is not directly involved, at least in the T9SS of strain ATCC 33277.

Among the genes listed in Table 1, only *porG* (encoding PGN_0297) has not been examined for its importance in the T9SS by any mutant study. *porG* is highly conserved in *P. gingivalis* strains (>97.5% amino acid identity) and its homologous genes are present in the phylogenetically-related bacteria, *Parabacteroides distasonis*, *Tannerella forsythia*, *Porphyromonas asaccharolytica*, *Prevotella ruminicola* and *Prevotella intermedia*, which possess the T9SS-related genes. In this study, we constructed the *porG* deletion mutant and found that the mutant showed deficiency in the T9SS, which was confirmed by the complementation study. PorG was predicted to be an outer membrane β-barrel protein by Pfam database [32]. This protein was reported to co-purify with the large PorK/N ring complex and directly interact with the PorK and PorN proteins, through their putative periplasmic loop [32]. Then, PorG was suggested to have a role in assembly and/or stability of the PorK/N complex. We demonstrated that PorG is an essential component of the T9SS in this study. Further study of PorG interaction with the PorK/N complex may elucidate the T9SS secretion machinery.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest regarding this article.

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