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**REVIEW（JAOB/Rising Members Award）**

# Por Secretion System of *Porphyromonas gingivalis*

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**Abstract**：The virulence factors of pathogenic bacteria are major secretory proteins that are directly linked to their pathogenicity. These secretory proteins are translocated across the membranes of bacterial cells by translocase nanomachines, which consists of integral membrane proteins. The periodontal pathogen, *Porphyromonas gingivalis*, secretes trypsin-like proteases（gingipains）either as a large complex on the bacterial cell surface or into the extracellular milieu. Gingipains are important virulence factors, because they degrade host proteins. They are responsible for the processing/maturation of other *P. gingivalis* virulence factors. At least six types of translocase nanomachines have been found in Gram-negative bacteria；however, *P. gingivalis* does not have genes homologous to those coding these secretion systems in the bacterial genome and not much is known about the mechanism of gingipain secretion. In this study, the proteins responsible for gingipain secretion, *i.e.*, PorK, PorL, PorM, PorN, and PorW, were identified by comparative genome analysis and genetic experiments. We named the gingipain secretion system the Por secretion system（PorSS）. Genes encoding PorSS proteins are conserved among a group of bacteria including periodontal pathogens such as *Tannellera forsythia* and *Prevotella intermedia* in the phylum *Bacteroidetes*. In addition, homologous genes are involved in gliding motility and chitinase secretion in *Flavobacterium johnsoniae*, another member of the phylum *Bacteroidetes*. Two other genes, *porX* and *porY*, encoding the regulatory factors of PorSS gene expression were identified at the same time. The expression of the *porT*, *porK*, *porL*, *porM*, and *porN* genes was downregulated in PorX- or PorY-defective mutants. PorSS and its regulatory system appear to be associated with the pathogenicity of various bacteria in the phylum *Bacteroidetes*.

## Introduction

The protein secretion systems of bacteria have been investigated extensively, because secretion of the virulence factors of pathogenic bacteria is directly linked to their pathogenicity.

In Gram-negative bacteria, secreted proteins are translocated across the inner and outer membranes by a translocase nanomachines consisting of integral membrane proteins. Six secretion systems, types Ⅰ—Ⅵ, have been identified in Gram-negative bacteria[1)]. These are divided broadly into two main groups, a two-step system including types Ⅱ and Ⅴ, and a one-step system including types Ⅰ, Ⅲ, Ⅳ, and Ⅵ（Fig. 1）. In the two-step system, secreted proteins are first translocated from the cytoplasm to the periplasm through the Sec or Twin-arginine（Tat）system. The Sec system is involved in the transport of both

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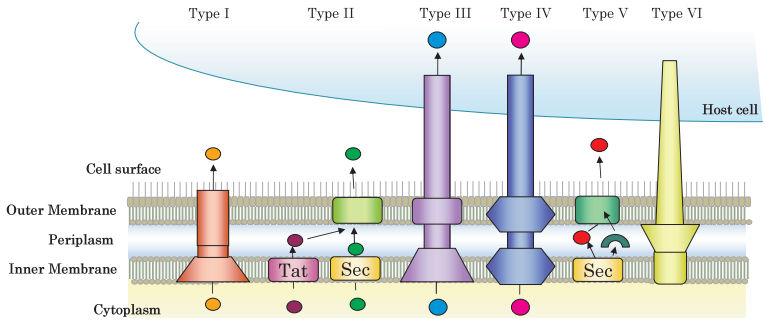


**Fig. 1** Schematic models of type Ⅰ—Ⅵ secretion systems in Gram-negative bacteria

In the type Ⅰ secretion system, secreted proteins are transported from the cytoplasm to the extracellular milieu in a single step. In the type Ⅱ secretion system, secreted proteins are transported from the cytoplasm to the extracellular milieu in a two-step process. Initially, proteins are transported through the inner membrane by the Sec machinery and are then translocated through the outer membrane by the type Ⅱ secretion system. In the type Ⅲ secretion system, secreted proteins are transported from the cytoplasm to the host cell.

secreted and inner membrane proteins in an unfolded conformation. On the other hand, the Tat system is involved in the transport of proteins in a folded conformation. After cleavage of a signal peptide by signal peptidase, the secreted protein is released into the periplasm and then translocated across the outer membrane to the extracellular environment through one of the secretion systems. In the type Ⅱ secretion system, the precursor secreted proteins are translocated across the inner membrane by the Sec translocon or the Tat pathway. After cleavage of signal peptide by a signal peptidase, the secreted proteins are translocated across the outer membrane by the type Ⅱ secretion system, which consists of multicomponent machines spanning inner and outer membranes. The type Ⅱ secretion system is involved in the secretion of virulence factors in many Gram-negative bacteria, such as aerolysin in *Aeromonas hydrophila*, the heat-labile toxin in enterotoxigenic *Escherichia coli*, and cholera toxin in Vibrio cholerae. The type Ⅴ secretion system：The autotransporter and the two-partner secretion systems are grouped within the type Ⅴ secretion system due to the similarities between these systems. The translocator and passenger domains are encoded by a single gene in the autotransporter system, but are encoded by two separate genes in the two-partner secretion system. After these precursor proteins have been transported across the inner membrane through the Sec translocon, the translocator domains are transported across the outer membrane using their passenger domain. In the one-step system, the type Ⅰ, Ⅲ, Ⅳ, and Ⅵ secretion systems form complexes across the two membranes and allow both direct export of the virulence factors from the cytoplasm to the extracellular milieu or injection of effector proteins into the cytoplasm of the host cells (Fig. 1). The type Ⅰ secretion system of Gram-negative bacteria consists of three proteins：the ABC transporter protein, the oligomeric membrane fusion protein, and the outer membrane protein. This simple system is associated with, for example, the secretion of HlyA (hemolysin A) from *E. coli*, CyaA (Pore forming/adenylate cyclase) from *Bordetella pertussis*, and HasA (Haemophore) from *Serratia maecescens*. The type Ⅲ secretion system, which consists of a membrane-embedded basal body, needle, and tip structure, are conserved in both flagellar and virulence-associated secretion systems[2].

Pathogenic bacteria including *Salmonell typhimurium*, enteropathogenic, and enterohaemorrhagic *E. coli*, inject effector proteins directly into their host cells using the type Ⅲ secretion system. The type Ⅳ secretion system is found in both Gram-positive and Gram-negative bacteria and is associated with the conjugative transfer of plasmid DNA or transposons, DNA uptake from and release into the extracellular milieu, and the transport of effector molecules into target cells[3]. Conjugative transfer contributes to the emergence of antibiotic-resistant bacteria. Pathogenic bacteria such as *Helicobacter pylori* and *Legionella pneumophila* use the type Ⅳ secretion system. The type Ⅵ secretion system was recently identified and its mechanism has not yet been elucidated. The type Ⅵ secretion system is usually encoded by a cluster of 12—25 genes within a pathogenicity island in Gram-negative bacteria, such as *Pseudomonas aeruginosa* and *Salmonella typhimurium*[4]. The function and characterization of the components of the type Ⅵ system remain to be elucidated.

These secretion systems are not, however, present in periodontal pathogens of the *Bacteroidetes* phylum, such as *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Prevotella intermedia*. *P. gingivals*, a principal pathogen of periodontal disease, has many virulence factors, including proteinases, fimbriae, and hemagglutinins. Most of the proteolytic activities of the extracellular and cell surface proteinases are derived from a group of cysteine proteinases named gingipains. In addition to the degradation of host proteins, gingipains are responsible for the processing/maturation of *P. gingivalis* virulence factors, including hemagglutinins and fimbriae[5]. Gingipains are encoded by three separate genes in the *P. gingivalis* genome, *kgp* for the Lys-specific cysteine proteinase, and *rgpA* and *rgpB* for two Arg-specific cysteine proteinases[6—8]. They are synthesized in the cytoplasm as precursor proteins with a signal peptide, a pro-region, a proteinase domain, hemagglutinin domain, and a C-terminal domain. These molecules are then translocated across the inner membrane, periplasm, and outer membrane. The transported gingipains are then located on the bacterial cell surface as large complexes or secreted into the extracellular milieu (Fig. 2A). Because *P. gingivalis* has no homology with the gene clusters of known secretion systems, the mechanism of gingipain secretion is not well understood.

A *porT* mutant, which was constructed by transposon mutagenesis in a previous study, accumulates gingipain precursor forms in the periplasmic space and shows no gingipain activity. In the *porT* mutant, gingipain, TapA protein[9], and Hbp35[10] are not secreted at the cell surface. These proteins contain the C-terminal domain, which has conserved DxxG and GxY motifs as well as charged residues at the C-terminal end[11]. In addition, these proteins immunoreact with a monoclonal antibody, 1B5, which is immunoreactive to a cell surface anionic polysaccharide (APS) and to the glycan addition of gingipains. It is possible that these outer membrane proteins, which contain the conserved C-terminal domain, are secreted by a previously unknown secretion system.

## *P. gingivalis* Genes Involved in Colonial Black Pigmentation on Blood Agar

*P. gingivalis* forms characteristic black-pigmented colonies on blood agar plates due to the accumulation of $\mu$-oxo heme dimers on the cell surface (Fig. 2B). Rgp activity is crucial for converting oxyhaemoglobin to methaemoglobin, which is more susceptible to Kgp degradation for the eventual release of iron (Ⅲ) protoporphyrin Ⅸ and the production of $\mu$-oxo heme dimers; therefore, the *kgp* and *rgpA rgpB kgp* mutants form less and non-pigmented colonies, respectively, caused by defects in the biosynthesis of $\mu$-oxo heme dimers. Because the black pigmentation on blood agar is associated with gingipain activity at the cell surface, the pigmentation-related genes characterized so far can be classified as three types of genes: those which are involved in gene expression, surface attachment of gingipain-adhesin complexes, and membrane transportation.

Gene expression: The *kgp* and *rgpA rgpB kgp* mutants form less and non-pigmented colonies.

Surface attachment of gingipain-adhesin complexes: Kgp and Rgp are present as cell surface gingipain-adhesin complexes consisting of Rgp and

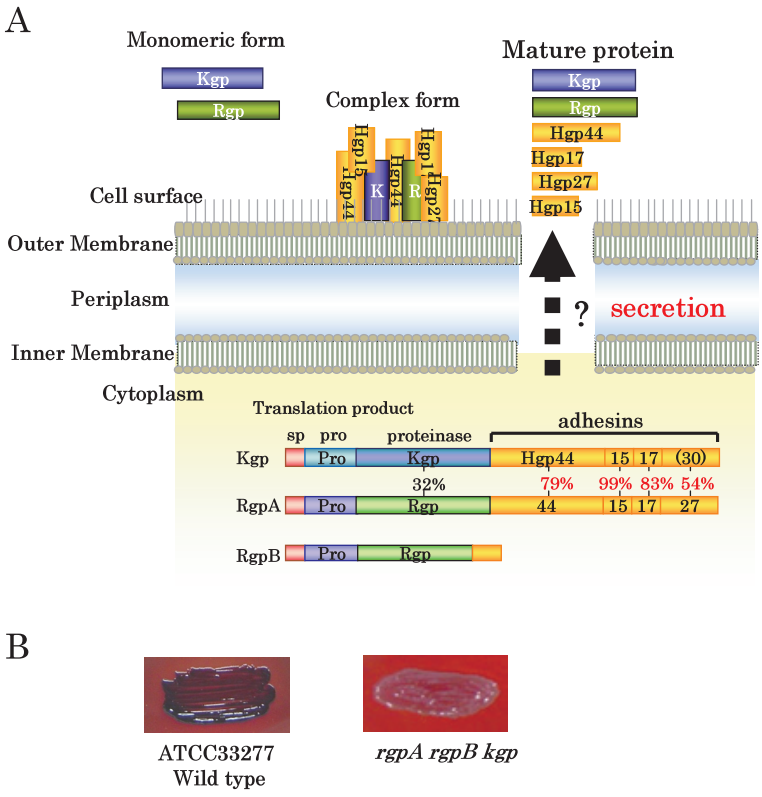


**Fig. 2** Gingipain secretion

A：Schematic representation of subcellular localization of gingipains in *P. gingivalis*. Gingipains are synthesized in the cytoplasm as precursor proteins having an N-terminal signal peptide, pro region, protease domain, and C-terminal domain. They are then translocated across the inner membrane, periplasm, and outer membrane. Transported gingipains are either located on the bacterial cell surface as a large complex or secreted into the extracellular milieu. B：Colony pigmentation. *P. gingivalis* cells were grown on blood agar plates at 37℃ under anaerobic conditions.

Kgp proteinases, which are encoded by *rgpA*, *rgpB*, and *kgp*, as well as adhesins, which are encoded by *rgpA*, *kgp*, and *hagA*. These gingipain-adhesin complexes are anchored by cell surface polysaccharides. The *porR* (related to the biosynthesis of APS)[12], *vimA*[13], *vimE*, *vimF* (putative glycosyltransferase)[14], *rfa* (related to the biosynthesis of lipopolysaccharides (LPS) and APS)[15], *gtfB* (glycosyltransferase)[16], and PG1051 (putative O-antigen ligase)[17] genes were identified as being associated with the biosynthesis of LPS and/or cell surface polysaccharides that can function as anchorage points for gingipain-adhesin complexes. Gingipain activity was detected in the supernatant but not in intact cells.

Membrane transportation：The three genes, *porT*, *sov*, and *PG27*, mutants of which exhibit no pigmentation, are involved in membrane transportation of gingipain-adhesin complexes[18—20]. High molecular weight precursor forms of Rgp, Kgp, and adhesions are accumulated in the periplasmic space.

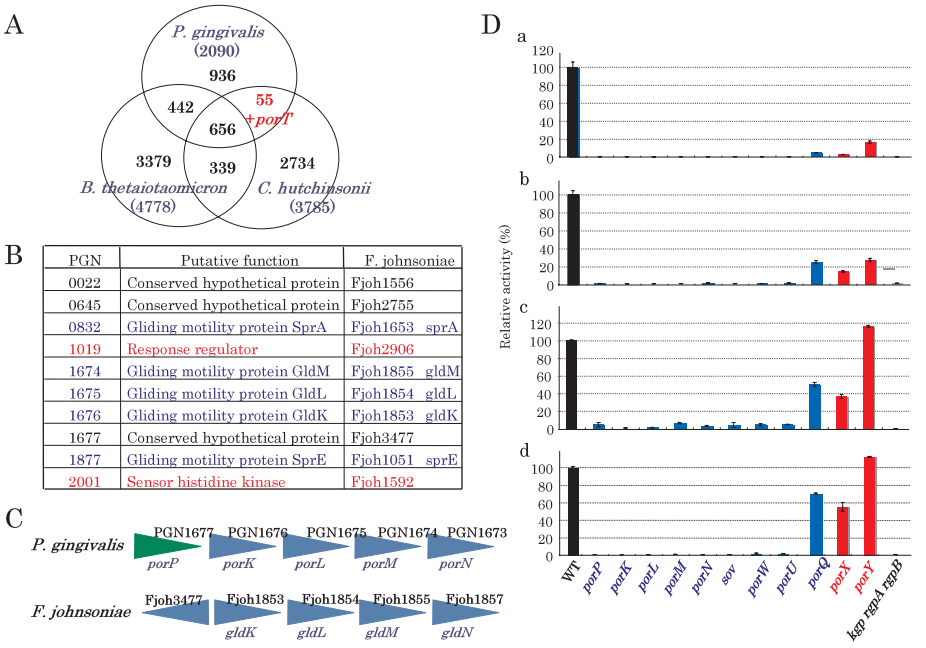


**Fig. 3** Identification of new genes responsible for Rgp and Kgp activities

A：Venn diagram analysis of genes of *P. gingivalis*, *C. hutchinsonii*, and *B. thetaiotaomicron*. The pairwise reciprocal best hits were calculated among *P. gingivalis* ATCC 33277, *B. thetaiotaomicron* VPI-5482, and *C. hutchinsonii* ATCC 33406. Homologous genes were identified by BLASTP searches using E-value threshold $\leq 10^{-10}$. Orthologous genes were defined as reciprocal best hits. B：Genes involved in gingipain secretion by comparative genome analysis. C：*porP—N* gene clusters of *P. gingivalis*. Homologous genes of *F. johnsoniae* are indicated at the bottom. D：Rgp and Kgp activities in intact cells and in culture supernatants. (a) Kgp activities of whole cells, (b) Kgp activities of culture supernatants, (c) Rgp activities of whole cells, and (d) Rgp activities of culture supernatants. *P. gingivalis* cells were grown in enriched brain heart infusion medium at 37℃ for 24 h under anaerobic conditions. Kgp and Rgp activities were determined using the synthetic substrates t-butyl-oxycarbonyl-L-valyl-L-leucyl-L-lysine-4-methyl-7-coumarylamide (Boc-Val-Leu-Lys-MCA) and carbobenzoxy-L-phenyl-L-arginine-4-methyl-7-coumarylamide (Z-Phe-Arg-MCA) in 20 mM sodium phosphate buffer (pH 7.5) containing 5 mM cysteine in a total volume of 1 mL. One unit of enzyme activity was defined as the amount of enzyme required to release 1 nmol of 7-amino-4-methylcoumarin under the assay conditions. Kgp and Rgp activities are indicated at units per mL of cell suspensions or culture supernatants. All cultures had similar cell densities of approximately 1.0 absorbance at $OD_{600\ nm}$.

## *P. gingivalis* Genes Involved in Gingipain Secretion

In a previous study, a non-pigmented mutant was isolated by transposon mutagenesis. The non-pigmented mutant *porT* accumulates the high molecular weight precursor forms of gingipains in the periplasmic space, and therefore shows no gingipain activity in either intact cells or the supernatant[19]. PorT pro-

tein is required for gingipain secretion to the cell surface in *P. gingivalis*.

Gingipains have the Sec signal peptide at their N-termini. Typical Sec-dependent secretion systems are composed of several proteins, the genes which form a secretion cluster on the chromosome. To identify possible additional components of the gingipain secretion system, we performed Venn diagram analysis of CDSs (Fig. 3A). The *porT* orthologs have not been identified outside the *Bacteroidetes* phylum. They have been identified in the genomes of *Cytophaga hutchinsonii* ATCC 33406 and *Flavobacterium johnsoniae* but not in those of colon bacteria, *Bacteroides fragilis* and *Bacteroides thetaiotaomicron,* which are more closely related to *P. gingivalis*[21,22]. In addition to *porT*, 55 homologous genes were detected in both *P. gingivalis* and *C. hutchinsonii* but not in *B. thetaiotaomicron* (Fig. 3A)[23—25]. Mutant studies revealed that 11 of the 55 genes were responsible for the black pigmentation on blood agar. We designated them *porP* (PGN1677), *porK* (PGN1676), *porL* (PGN1675), *porM* (PGN1674), *porN* (PGN1673), *porQ* (PGN0645), *porU* (PGN0022), *porW* (PGN1877), *porX* (PGN1019), and *porY* (PGN2001). These mutants showed no or decreased gingipain activity and the high-molecular-weight precursor forms of gingipains were found to accumulate in the mutant cells.

Five of these genes, *porK*, *porL*, *porM*, *sov*, and *porW*, are homologous to the *F. johnsoniae* gliding motility genes, *gldK*, *gldL*, *gldM*, *sprA*, and *sprE*, respectively[21,22]. Sov (PGN_0832) was reported to be involved in the secretion of gingipains and the *sov* mutant showed decreased activities of Rgp and Kgp in both whole cells and supernatants[26]. Two other genes, *porX* and *porY*, are similar to genes encoding the response regulatory protein kinase and histidine sensor kinase of the two-component system, respectively (Fig. 3B).

## Subcellular Localization of the PorK—PorN Proteins

*porP*, *porK*, *porL*, *porM*, and *porN* are located on the *P. gingivalis* chromosome as a gene cluster. PorK includes a bacterial lipoprotein-specific signal sequence, whereas PorP, PorM, and PorN include typical signal sequences at their N-termini. *P. gingivalis* cell lysates were fractionated into the cytoplasm/periplasm, inner membrane, and outer membrane fractions by the Triton X-100 method and then subjected to SDS-PAGE and immunoblot analysis. PorL and PorM were mainly located in the inner membrane, while PorK and PorN were found in the outer membrane. To investigate whether PorK—N proteins form a protein complex, we performed blue-native gel electrophoresis, immunoblot, and LC-MS analyses. PorK and PorN were mainly detected at a molecular mass of more than 1,200 kDa, while PorL and PorM were detected at 1,100 kDa. These results suggest that PorK, PorL, PorM, and PorN form at least two protein complexes.

## *F. johnsoniae sprT* Mutants Are Defective in Gliding Motility

*F. johnsoniae* cells glide at a speed of approximately 5—10 nm/s over wet glass surfaces. The mechanism of this gliding is unknown but it is not connected with flagellar motility, type Ⅳ pilus-mediated twitching motility, or mycoplasma gliding motility[27]. Previous studies have revealed that 16 genes (*gldA*, *gldB*, *gldD*, *gldF*, *gldH*, *gldI*, *gldJ*, *gldK*, *gldL*, *gldM*, *gldN*, *sprA*, *sprB*, *sprC*, *sprD*, and *sprE*) are involved in gliding motility and chitinase activity in *F. johnsoniae*[21,22,27,28]. The *porT* ortholog (*sprT*)-defective mutant showed no gliding motility on 1.5% solid agar (Fig. 4A). The cell surface adhesin-SprB protein of *F. johnsoniae*, which is involved in the formation of swarming colonies on solid agar, is not secreted at the cell surface in the *sprT* mutant[27]. A recent study[29] showed that *sprF* has some similarity to *porP* of *P. gingivalis*, mutants of which produced no swarming colonies on agar. The SprB protein is produced but not transported to the cell surface of the *sprF* mutant, as in the *sprT* mutant. The PorSS in *F. johnsoniae* is needed for the assembly of SprB and the other cell surface components of the motility machinery.

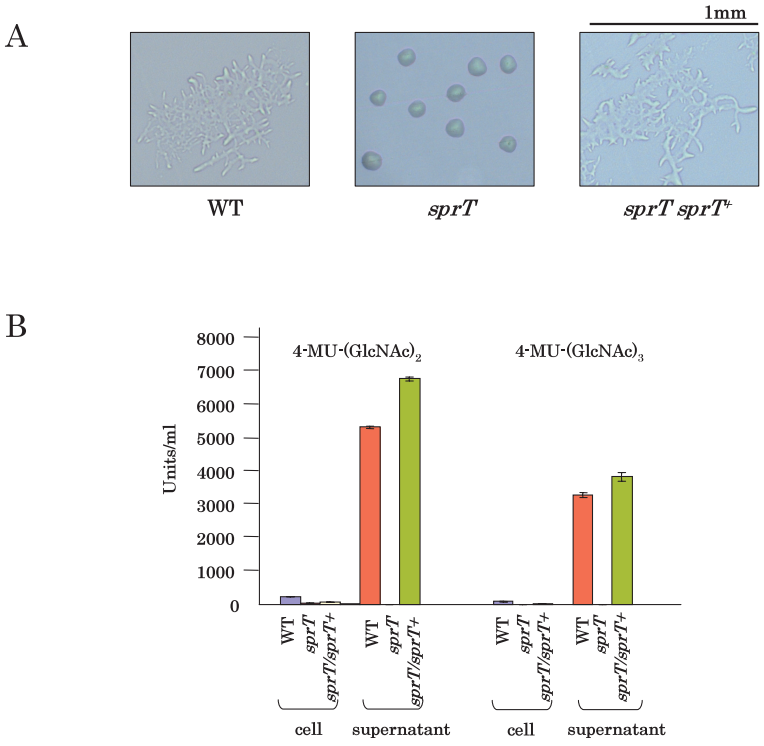


**Fig. 4** Effect of *F. johnsoniae sprT* on gliding motility and extracellular chitinase activity

A：Colony morphology of wild-type, mutant, and complemented strains. *F. johnsoniae* cells were grown on PY2 agar at 25℃. B：Chitinase activity was determined with the synthetic substrates 4-methylumbelliferyl, N-acetyl-β-D-glucosaminide (4-MU-GlcNAc)；4-methylumbelliferyl, N,N′-diacetyl-β-D-chitobioside [4-MU-$(GlcNAc)_2$]；and 4-methylumbelliferyl β-D-N,N′, N′′-triacetylchitotrioside [4-MU-$(GlcNAc)_3$] using a chitinase assay kit (Chitinase Assay Kit, Fluorimetric；Sigma) according to the manufacturer's instructions. Enzyme assays were performed in triplicate. One unit of enzyme activity was defined as the amount of enzyme that is required to release 1 mmol of 4-methylumbelliferone per min. Chitinase activity is indicated as units per mL of cell suspensions or culture supernatants.

### *F. johnsoniae sprT* Mutant Is Defective in Extracellular Chitinase Activity

Gliding motility-defective mutants showed very low chitinase activity[21]. To examine whether this was the result of failed chitinase secretion, the chitinase activities of *sprT* mutant, *sprT*-complemented, and wild-type strains were determined with three substrates：4-methylumbelliferyl, N-acetyl-β-D-glucosaminide；4-methylumbelliferyl, N,N′-diacetyl-β-D-chitobioside；4-methylumbelliferyl β-D-N,N′,N′′-triacetylchitotrioside. In the *sprT* mutant, chitinase activity was not detected in either the bacterial cells or culture supernatants, in contrast to the results of *sprT*-complemented and wild-type strains (Fig. 4B). Compared to the culture supernatant proteins, the protein band corresponding to chitinase (Fj_4555) was

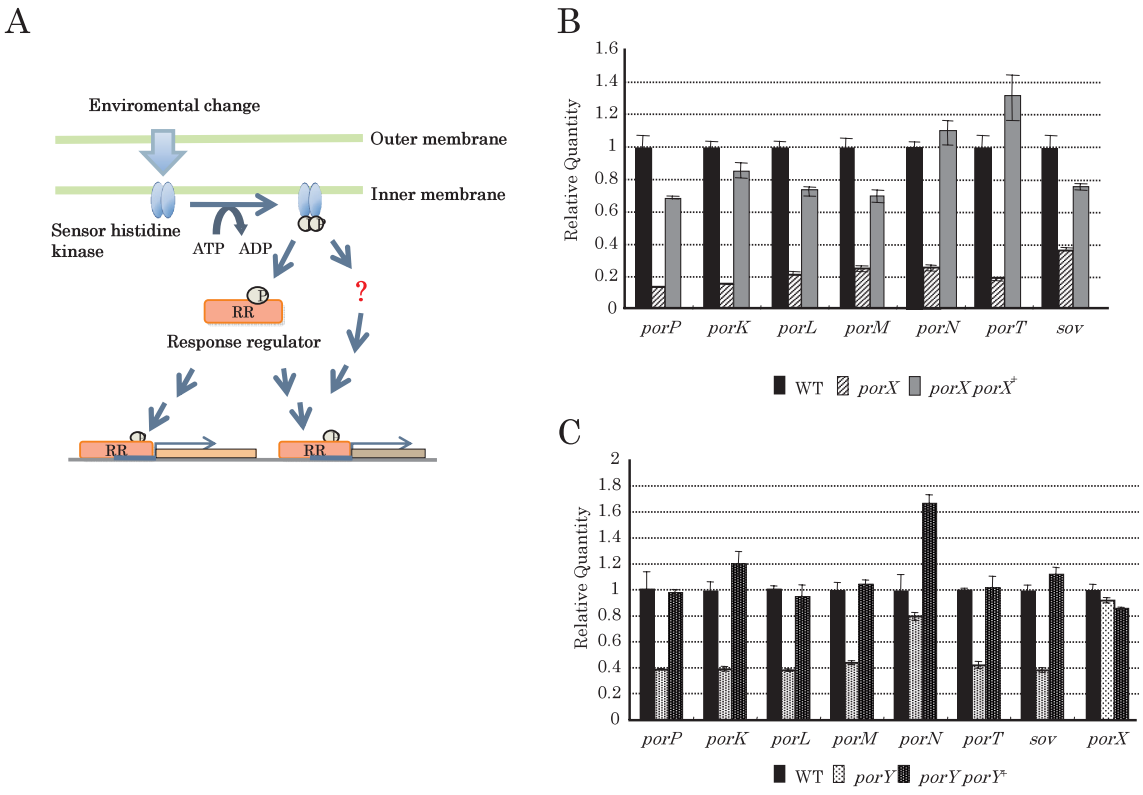


**Fig. 5** Real-time RT-PCR analysis of PorSS-related genes in *porX* and *porY* mutant
A：Schematic representation of the bacterial two-component system. Signal transduction consists of the sensor histidine kinase and response regulator. B：Real-time RT-PCR analysis of gene expression of *porP*, *porK*, *porL*, *porM*, *porN*, *porT*, and *sov* in the wild-type, *porX*, and *porX porX*$^{+}$ (complemented) strains. For the quantification of gene expression by real-time RT-PCR, total RNA was isolated from *P. gingivalis* cells grown to mid-exponential phase (approximately 1.0 absorbance at $OD_{600\,nm}$). The expression level of each targeted gene was normalized to that of the 16S rRNA gene, which was used as a reference. All PCR reactions were carried out in triplicate. C：Real-time RT-PCR analysis of gene expression of *porP*, *porK*, *porL*, *porM*, *porN*, *porT*, *sov*, and *porX* in the wild-type, *porY*, and *porY porY*$^{+}$ (complemented) strains.

found in *sprT*-complemented and wild-type strains but not in the *sprT* mutant by using SDS-PAGE and a peptide mass fingerprint mass spectrometer. These results show that PorSS functions as a protein secretion system in *F. johnsoniae*.

## PorX and PorY Regulate Gene Expression of Por Secretion System

To sense and respond to environmental changes, stresses, and intracellular communication, bacteria have various signal transduction systems. The two-component system, which consists of histidine kinase and response regulator protein, is one of the most prevalent signal transduction systems among bacteria. A sensor histidine kinase molecule catalyzes its own autophosphorylation, and then transfers the phosphate to a response regulator molecule. Finally, the phosphorylated response regulator activates promoters of the target genes (Fig. 5A).

PorX and PorY are similar to a response regulator and a sensor histidine kinase, respectively. The non-pigmented *porX* mutant accumulated high-molecular-weight precursor forms of Kgp in the cell lysates, and

compared to wild-type cells they showed lower gingipain activity. In the weakly pigmented *porY* mutant, mature and high-molecular-weight proteins immunoreactive to anti-Kgp were found in the cell lysate and showed approximately 20% Kgp activity of that shown by wild-type cells (Figs. 3D and 5B). In these mutants, the 180-kDa protein immunoreactive to anti-Kgp was found in the whole-cell lysate. Tiling microarray analysis, which was performed to identify the target genes of *porX*, revealed that 20 genes, including *porT*, *sov*, *porK*, *porL*, *porM*, *porN*, and *porP*, were downregulated in the *porX* deletion mutant. Downregulation of these genes was also found in the *porY* mutant. This downregulated gene expression was confirmed by RT-PCR (Fig. 5B and 5C).

## Conclusion

The novel protein secretion system PorSS appear to be present among a group of bacteria, including periodontal pathogens such as *Tannellera forsythia* and *Prevotella intermedia*, and fish pathogens such as *F. psychrophilum* and *Flavobacterium columnare* in the phylum *Bacteroidetes*. PorSS is involved in the virulence of *P. gingivalis* because a number of proteins secreted by PorSS are virulence factors such as gingipains. PorSS is also responsible for gliding motility and chitin utilization of *F. johnsoniae*. Suppression of PorSS may be a good approach to control periodontal pathogens and others.

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