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 \( \mathcal{A} \mathcal{B} \mathcal{C} \mathcal{D} \mathcal{E} \mathcal{F} \mathcal{G} \), have been identified in Gram-negative bacteria. These are divided broadly into two main groups, a two-step system including types \( \mathcal{A} \mathcal{B} \mathcal{C} \mathcal{D} \mathcal{E} \mathcal{F} \), and a one-step system including types \( \mathcal{A} \mathcal{B} \mathcal{C} \mathcal{D} \mathcal{E} \mathcal{F} \mathcal{G} \mathcal{H} \mathcal{I} \). 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 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. 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).

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### Key words

- periodontal pathogen
- Bacteroidetes phylum
- proteinase

### 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*.

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### 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...
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 marcescens. 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.  

**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.
Pathogenic bacteria including *Salmonella 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. 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*. The 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. gingivalis*, 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 hemagglutinin and fimbriae. 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. 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, and Hbp35 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. In addition, these proteins immunoreact with a monoclonal antibody, 1B5, which is immuno-reactive 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 μ-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 μ-oxo heme dimers; therefore, the *kgp* and *rgpA* *rgpB* mutants form less and non-pigmented colonies, respectively, caused by defects in the biosynthesis of μ-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* 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
Kgp proteinases, which are encoded by *rgpA*, *rgpB*, and *kgp*, as well as adhesins, which are encoded by *rgpA*, *kgp*, and *hagA*. These gingipain–adhesive 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–adhesive 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–adhesive complexes\(^{18–20}\). High molecular weight precursor forms of Rgp, Kgp, and adhesions are accumulated in the periplasmic space.
In a previous study, a non-pigmented mutant was isolated by transposon mutagenesis. The non-pigmented mutant \(\text{porT}\) 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 por*T* 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 por*T*, 55 homologous genes were detected in both *P. gingivalis* and *C. hutchinsonii* but not in *B. thetaiotaomicron* (Fig. 3A).[21–25] Mutant studies revealed that 11 of the 55 genes were responsible for the black pigmentation on blood agar. We designated them por*P* (PGN1677), por*K* (PGN1676), por*L* (PGN1675), por*M* (PGN1674), por*N* (PGN1673), por*Q* (PGN0645), por*U* (PGN0022), por*W* (PGN1877), por*X* (PGN1019), and por*Y* (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, por*K*, por*L*, por*M*, sov, and por*W*, are homologous to the *F. johnsoniae* gliding motility genes, gld*K*, gld*L*, gld*M*, spr*A*, and spr*E*, 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, por*X* and por*Y*, 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 Por*K*—Por*N* Proteins**

por*P*, por*K*, por*L*, por*M*, and por*N* are located on the *P. gingivalis* chromosome as a gene cluster. Por*K* includes a bacterial lipoprotein-specific signal sequence, whereas Por*P*, Por*M*, and Por*N* 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. Por*L* and Por*M* were mainly located in the inner membrane, while Por*K* and Por*N* were found in the outer membrane. To investigate whether Por*K*—N proteins form a protein complex, we performed blue-native gel electrophoresis, immunoblot, and LC–MS analyses. Por*K* and Por*N* were mainly detected at a molecular mass of more than 1,200 kDa, while Por*L* and Por*M* were detected at 1,100 kDa. These results suggest that Por*K*, Por*L*, Por*M*, and Por*N* form at least two protein complexes.

**F. johnsoniae spr*T* 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 (gld*A*, gld*B*, gld*D*, gld*F*, gld*H*, gld*L*, gld*J*, gld*K*, gld*M*, gld*N*, spr*A*, spr*B*, spr*C*, spr*D*, and spr*E*) are involved in gliding motility and chitinase activity in *F. johnsoniae*.[21,22,27,28] The por*T* ortholog (spr*T*)-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 spr*T* mutant.[27] A recent study[29] showed that spr*F* has some similarity to por*P* 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 spr*F* mutant, as in the spr*T* mutant. The PorSS in *F. johnsoniae* is needed for the assembly of SprB and the other cell surface components of the motility machinery.
**F. johnsoniae** *sprT* Mutant Is Defective in Extracellular Chitinase Activity

Gliding motility-defective mutants showed very low chitinase activity. 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
A.

Environmental change

Outer membrane

Inner membrane

Sensor histidine kinase

ATP

ADP

Response regulator

B.

Relative Quantity

0

0.2

0.4

0.6

0.8

1.0

1.2

1.4

1.6

porP porK porL porM porN porT sob

WT porX porX porY

C.

Relative Quantity

0

0.2

0.4

0.6

0.8

1.0

1.2

1.4

1.6

porP porK porL porM porN porT sob porX

WT porY porY porY

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 sob 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 OD600 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, sob, and porX in the wild-type, porY, and porY porY+ (complemented) strains.

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

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.
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 appears to be present among a group of bacteria, including periodontal pathogens such as Tannemullera 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 Porphyromonas 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.

References

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