Construction and characterization of a nonpigmented mutant of *Porphyromonas gingivalis*: cell surface polysaccharide as an anchorage for gingipains

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A nonpigmented mutant of *Porphyromonas gingivalis* was constructed by using transposon mutagenesis. The mutant possessed the transposon DNA at the novel gene *porR*. Gene targeted mutagenesis revealed that *porR* was responsible for pigmentation. The *porR* gene shared similarities with genes of the *degT* family, the products of which are now considered to be transaminases involved in biosynthesis of sugar portions of cell-surface polysaccharides and aminoglycosides. The *porR* mutant showed a pleiotropic phenotype: delayed maturation of fimbrillin, preferential presence of Rgp and Kgp proteinases in culture supernatants, and no haemagglutination. The *porR* mutant had altered phenol extractable polysaccharide compared to the *porR* sibling strain. A mAb, 1B5, that reacts with sugar portions of *P. gingivalis* cell surface polysaccharide and membrane-type Rgp proteinase showed no reaction with the cell lysates of the *porR* mutant. These results indicate that *porR* is involved in biosynthesis of cell surface polysaccharide that may function as an anchorage for Rgp, Kgp, haemagglutinins and the haemoglobin receptor protein.

Keywords: cell surface polysaccharide, cysteine proteinases, colonial pigmentation, haemagglutination, fimbrillin maturation

INTRODUCTION

*Porphyromonas gingivalis*, an oral anaerobic bacterium, has been implicated as an important aetiological agent of adult periodontal disease (Haffajee & Socransky, 1994). This micro-organism requires haem, haemoglobin or transferrin as an iron source for its growth (Gibbons & MacDonald, 1960; Shah et al., 1979; Brochu et al., 2001). It produces black-pigmented colonies on laked blood agar and the black pigment is found to be μ-oxo dimers of haem (iron protoporphyrin IX) (Smallley et al., 1998). We suggested in our previous study (Nakayama et al., 1998) that it has a unique iron acquisition system from erythrocytes, in which arginine-specific cysteine proteinase (Arg-gingipain, Rgp), lysine-specific cysteine proteinase (Lys-gingipain, Kgp), haemoglobin receptor protein (HbR) and haemagglutinins (HAs) are involved as a complex. Interestingly, these proteins are intragenerically encoded by *rgpA*, *kgp* and *hagA* (Pavloff et al., 1995; Okamoto et al., 1996; Han et al., 1996). In this novel iron acquisition mechanism, *P. gingivalis* cells may adhere to erythrocytes by the HA proteins of the complex. Then, the proteinases may...
extensively digest surface proteins of erythrocytes, resulting in the release of haemoglobin. The released haemoglobin may be stored on the surfaces as the incorporated into the cells and used as nutrients. Surplus to yield haem and peptides that can be constructed by site-directed mutagenesis exhibited less and no pigmentation, respectively, which demonstrates resulting in the release of haemoglobin. The released extensively digest surface proteins of erythrocytes, (Shi et al., 1999, 2000; Curtis et al., 1996; Booth & Lehner, 1997; Kelly et al., 1997; Shibata et al., 1999), but a precise mechanism of the iron acquisition and storage system is still unknown.

In early studies of *P. gingivalis*, pigment-less variants that were spontaneously isolated showed a weak ability to produce Rgp and Kgp, indicating that these proteinases or their genes might be responsible for accumulation of the μ-oxo dimers on the cell surfaces (McKee et al., 1988; Shah et al., 1989). We found that the kgp mutant and the rgpA rgpB kgp triple mutant constructed by site-directed mutagenesis exhibited less and no pigmentation, respectively, which demonstrates the involvement of these gene products in haem storage on the cell surfaces (Shi et al., 1999; Okamoto et al., 1998).

Transposon mutagenesis has been applied to isolation of pigment-less mutants of *P. gingivalis* by several researchers (Genco et al., 1995a, b; Chen et al., 2000; Simpson et al., 1999). Chen et al. (2000) isolated nonpigmented mutants that had the transposon Tn4351 DNA within kgp. In addition, Simpson et al. (1999) found that a nonpigmented mutant has the insertion sequence element IS1126 at the promoter locus of kgp. These results confirmed the involvement of kgp in pigmentation. Recently, non-kgp mutations causing nonpigmentation have been found (Chen et al., 2000; Abaibou et al., 2001). Chen et al. (2000) found that Tn4351 was inserted into a putative glucosyl (rhamnosyl) transferase-encoding gene in several nonpigmented mutants and Abaibou et al. (2001) found that the gene vma located downstream of recA is responsible for pigmentation.

In this study, we isolated a nonpigmented mutant that had a transposon insertion within the novel gene porR. The porR gene had homology with genes encoding transaminase involved in biosynthesis of sugar portions of LPSs and aminoglycosides. A porR mutant constructed by targeted mutagenesis with a suicide plasmid also exhibited nonpigmentation. We found that the mutant showed a defect of polysaccharide biosynthesis and altered distribution of Rgp, Kgp, HA and HbR proteins in *P. gingivalis* cell fractions.

**METHODS**

**Transpositional mutagenesis and gene-directed mutagenesis.** Transposon Tn4351 was introduced into *P. gingivalis* ATCC 33277 essentially according to the method of Hoover & Yoshimura (1994). Plasmid R751::Tn4351Ω4 (Shoemaker et al., 1986) carries two copies of Tn4351 containing the Em′ gene (*ermF*) and can be transferred from *Escherichia coli* to *P. gingivalis*. However, this plasmid is unable to replicate in *P. gingivalis* cells. Therefore, Em′ transconjugants obtained by mating of *E. coli* containing R751::Tn4351Ω4 and *P. gingivalis* should be generated by transposition of Tn4351 from R751:: Tn4351Ω4 to the *P. gingivalis* chromosome. *P. gingivalis* was grown anaerobically in enriched brain heart infusion (BHI) broth (Shi et al., 1999) at 37 °C to an OD,abs of 0.4 and *E. coli* HB101 containing R751::Tn4351Ω4 was grown aerobically in L broth at 37 °C to an OD,abs of 0.2. *P. gingivalis* ATCC 33277 culture (20 ml) was mixed with the *E. coli* culture (4 ml), centrifuged and resuspended in 2 ml enriched BHI broth. The cell suspension (0.1 ml) was spotted on a blood agar plate, incubated aerobically at 37 °C for 1 h and anaerobically for 15 h. The growing cells on the plate were harvested, resuspended in 1 ml enriched BHI broth, spread on blood agar plates containing 200 μg gentamicin ml⁻¹ (to kill *E. coli* donor cells) and 10 μg erythromycin ml⁻¹, and incubated anaerobically at 37 °C for 7 days.

Gene-directed mutagenesis using a derivative of the suicide vector plasmid pKDCMZ (Nakayama, 1994) was essentially the same as described above except that *E. coli* DH5α harbouring R751 and pKD282, a derivative of pKDCMZ, was used as the donor strain.

**Plasmid construction.** A Psfl DNA fragment (6.8 kb) containing Tn4351 DNA of the chromosome of KDP105, a nonpigmented mutant isolated in this study, was cloned into a unique Psfl site of pUC18, resulting in pKD267. The largest EcoRI fragment of pKD267 was self-ligated to yield pKD278 that contained one flanking region adheiring to the transposition site of Tn4351. The NruI region (137 bp) of pKD278 was replaced by a kanamycin-resistance gene cartridge (1.3 kb) of pUC4K, resulting in pKD281. Then, an EcoRI–Psfl fragment (20 kb) of pKD281 was inserted into a unique Smal site of suicide vector plasmid pKDCMZ, giving rise to pKD282.

**Preparation of *P. gingivalis* cell fractions.** *P. gingivalis* cultures in 40 ml enriched BHI broth at various growth phases were centrifuged at 10000 g at 4 °C for 30 min. The supernatants of the cultures after the centrifugation were then subjected to ultracentrifugation at 10000 g at 4 °C for 2 h to separate into the fractions of culture supernatants and vesicles. The bacterial cell pellets were dissolved in 5 ml 10 mM Tris/HCl (pH 7.5) containing Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK), leupeptin and EDTA at 0, 1 and 5 mM, respectively and disrupted by sonication to yield the crude cell extracts. The crude cell extracts were centrifuged at 10000 g for 1 h at 4 °C. The supernatants were saved and ultracentrifuged at 10000 g for 2 h at 4 °C, resulting in the membrane fraction. The vesicle and membrane fractions were directly dissolved in Laemmli buffer (Laemmli, 1970) and subjected to SDS-PAGE (10% gel). The cell extracts and culture supernatants were diluted or concentrated, and mixed with the Laemmli buffer.

**Enzymic assays.** Lys-X and Arg-X specific cysteine proteinase activities were determined by use of the synthetic substrates N-p-tosyl-Gly-Pro-Lys-x acid (GPkpNA; Sigma) and N-α-benzoyl-DL-Arg-p-nitroanilide (BApNA; Sigma), respectively. In brief, various volumes of the cell lysates and supernatants of the culture were added to a reaction mixture (1 ml) containing 0.25 mM GPkpNA, 5 mM L-cysteine and 20 mM phosphate buffer (pH 7.5) for Kgp, and a reaction mixture (1 ml) containing 0.5 mM BApNA, 10 mM L-cysteine, 10 mM CaCl₂, 100 mM Tris/HCl (pH 8.0) for Rgp. The reaction mixtures were incubated at 40 °C for Kgp and 30 °C for Rgp. After samples were added, the A₄₅₀ was continuously measured on a spectrophotometer. Proteinase activities in cell
extracts and culture supernatants were determined by increase in absorbance per min per ml culture.

**Northern blot analysis.** Total RNA was extracted from *P. gingivalis* cells grown to mid-exponential phase (OD$_{600}$ 0.3) using an RNA purification kit (Qiagen RNaseasy). Five micrograms of RNA were electrophoresed in 1·2% agarose gel and then transferred to a nylon membrane (Hybond-N, Amersham Pharmacia Biotech) according to the method described by Sambrook et al. (1989). The antisense mRNA probes specific for the 0·5 kb BstXI (Tyr$^{183}$)-SpbI (Met$^{183}$) region of rgpB and the 0·5 kb AccI (Thr$^{346}$)-EcoRI (Gln$^{166}$) region of kgp were constructed by using the pSPUTK plasmid (Stratagene). The RNA probes were labelled with digoxigenin using the DIG RNA labelling kit (Roche). Northern blot hybridization and detection were carried out according to the manufacturer’s recommendation.

**RT-PCR.** This was done essentially according to the method of Matsuo et al. (1995). Oligonucleotides 5′-ATGCTACTCTGCTTTCTCCA-3′ and 5′-TTGTAGAAGCCGGTTGAAATGTT-3′ were used for detection of porR expression.

**Gel electrophoresis and immunoblot analysis.** SDS-PAGE was performed essentially according to the method of Laemml (1970) except that the sample buffer contained TLCK, leupeptin and EDTA at 0·1, 1 and 5 mM, respectively. For immunoblotting, proteins on SDS gels were electrophoretically transferred to nitrocellulose membranes using a semidry blotting system (Pharmacia). The blotted membranes were immunostained with anti-RgpB antibody (Kadowaki et al., 1998), anti-HbR antiserum (Nakayama et al., 1998), anti-ambrellin antiserum (Nakayama et al., 1996), mAb 61BG1.3 (Shi et al., 1999) or mAb1B5 (Curtis et al., 1999), and signals were detected using an ECL detection system (Pharmacia).

**Purification of cell surface polysaccharide.** This was done by the phenol/water method (Westphal & Jann, 1965). Visualization of the cell surface polysaccharide preparation on SDS-polyacrylamide gels was done according to Tsai & Frasch (1982).

**Haemagglutination assay.** Cultures (24 h) of *P. gingivalis* strains in enriched BHI broth were centrifuged, washed with PBS and resuspended in PBS. The bacterial suspensions were then diluted in a two-fold series with PBS. A 100 μl aliquot of each dilution was mixed with an equal volume of sheep erythrocyte suspension (2·5% in PBS) and incubated in a round-bottom microtitre plate at room temperature for 3 h. The haemagglutination titre was determined as the last dilution exhibiting full agglutination.

**Other methods.** Electrotransformation and Southern blotting were done as described previously (Nakayama et al., 1995).

**RESULTS**

**Isolation of a nonpigmented mutant of *P. gingivalis* by transposon mutagenesis, and molecular cloning of the transposon insertion region of its chromosome**

Several nonpigmented colonies appeared among Em$^{r}$ transconjugant colonies after mating between *E. coli* HB101 containing R751::Tn4351 and *P. gingivalis* ATCC 33277. *P. gingivalis* KDP105 was randomly chosen from the transconjugants and characterized. Southern blot hybridization analysis revealed that the KDP105 chromosome contained a single Tn4351 insertion. A PstI fragment (6·8 kb) containing the entire Tn4351 DNA on the chromosome of KDP105 was cloned by using the method of marker (Tc$^{r}$ on Tn4351 DNA) rescue. Sequencing of one of the flanking regions revealed that there was one ORF truncated by the transposon insertion in the region.

**Construction of a porR mutant by gene-directed mutagenesis**

To determine whether nonpigmentation of KDP105 was attributable to the disrupted ORF, we constructed a mutant with disruption of the ORF. We introduced the Km$^{r}$ gene cartridge into the Nrrl region within the ORF and constructed a suicide vector plasmid containing the disruption (pKD282). Introduction of pKD282 into *P. gingivalis* ATCC 33277 by mobilization produced a number of Em$^{r}$ transconjugants. Southern blot hybridization analysis with chromosomal DNA of 10 Em$^{r}$ transconjugants revealed that the chromosomal DNA from all of the transconjugants examined possessed full-length plasmid pKD282 DNA inserted at the ORF region. The transconjugants were classified into three types with respect to the location of the Km$^{r}$ gene cartridge (Fig. 1). Of these, the transconjugants with chromosomal structures I and II could be generated by
reciprocal recombination with a single crossover event between the homologous DNA regions of the chromosome and the plasmid. However, the generation of structure III would require non-reciprocal recombination. This type of transconjugant was also obtained in our previous studies (Nakayama, 1994, 1997). Representative transconjugants exhibiting structures II and III were designated KDP108 and KDP107, respectively. Colonial pigmentation was determined by anaerobic growth of these strains on TS plates containing defibrinated and laked sheep blood. KDP107 showed no pigmentation, while KDP108 showed black pigmentation. This result demonstrated that the ORF was responsible for colonial pigmentation of \textit{P. gingivalis} since both strains had the same chromosomal structure except for the ORF (Fig. 1). The 5’ portion of the ORF was cloned from the chromosome of KDP107 by the method of marker (Km\textsuperscript{r}) rescue. Combined with the nucleotide sequence of the whole ORF (Fig. 1), we named the ORF \textit{porR} (regulation of porphyrin accumulation on the cell surface). We determined the \textit{porR} expression of KDP107 and KDP108 by RT-PCR, with the result that KDP107 showed no expression of \textit{porR}, while KDP108 did show expression (data not shown).

**Similarity of \textit{porR} with the genes involved in biosynthesis of sugar portions of LPS and aminoglycosides**

The \textit{porR} ORF is 1152 bp, encoding a protein with a predicted molecular mass of 42263 Da. No apparent amino-terminal signal sequence was detected; therefore, a cytoplasmic location was expected. As shown in Fig. 2, the PorR protein is similar to the products of the \textit{bplC} gene (Allen & Maskell, 1996) in \textit{Bordetella pertussis}, the \textit{wlaK} gene (Fry et al., 1998) of \textit{Campylobacter jejuni} and the \textit{spsC} gene (Glaser et al., 1993) of \textit{Bacillus subtilis}. These gene products are involved in the biosynthesis of sequence of the whole ORF (Fig. 1). We named the ORF \textit{porR} (regulation of porphyrin accumulation on the cell surface). We determined the \textit{porR} expression of KDP107 and KDP108 by RT-PCR, with the result that KDP107 showed no expression of \textit{porR}, while KDP108 did show expression (data not shown).

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Characterization of the \textit{porR} mutant

(1) \textbf{Rgp and Kgp activities.} In a previous study (Okamoto \textit{et al.}, 1998), we found that the \textit{kgp} mutant showed reduced pigmentation. We determined Rgp and Kgp activities of cell extracts and culture supernatants of the \textit{porR} mutant and the \textit{porR}+ sibling strain at various growth phases. The \textit{porR} mutant grew as well as the \textit{porR}+ sibling strain in enriched BHI broth. As shown in Fig. 3, the \textit{porR} mutant did not express Rgp activity in early growth phase, but after prolonged incubation Rgp activity strongly appeared in the culture supernatants, while the activity remained very weak in the cell extracts. The \textit{porR} mutant showed a similar result in Kgp activity (Fig. 4).

(2) \textbf{Northern blot hybridization analysis using \textit{rgp} and \textit{kgp} probes.} Total RNA was isolated from early-exponential-phase cultures of the \textit{porR} mutants and the wild-type strains. Northern blot hybridization analysis using a common \textit{rgp} probe RNA revealed that \textit{rgpA} and \textit{rgpB} of the \textit{porR} mutants were transcribed as well as those of the wild-type parent and \textit{porR}+ sibling strains (data not shown). The \textit{kgp} gene of the \textit{porR} mutants was also transcribed in the same amounts as that of the wild-type strains. These results indicate that the \textit{porR} mutation did not affect the gene expression of \textit{rgpA}, \textit{rgpB} or \textit{kgp} at the transcriptional level.

(3) \textbf{Immunoblot analyses using anti-Rgp/Kgp antiserum, anti-HA mAb and anti-HbR antiserum.} Membrane fractions of the \textit{porR} and \textit{porR}+ strains grown in enriched BHI broth for 13, 17 and 21 h were subjected to SDS-PAGE and immunoblot analysis using anti-Rgp/Kgp antiserum (Fig. 5). The \textit{porR} mutant had much smaller amounts of 50 kDa Rgp (RgpA and RgpB), and 55 kDa Kgp than the \textit{porR}+ strain. Moreover, membrane-type Rgp (mt-RgpA and mt-RgpB) which was observed in the membrane fraction of the \textit{porR}+ strain as diffuse heterogeneous bands of 70–90 kDa, was not seen in the \textit{porR} mutant. Cell extracts, vesicle fractions and culture supernatants of the \textit{porR} mutant grown in enriched BHI broth for 48 h were subjected to SDS-PAGE and immunoblot analysis (Fig. 6). In immunoblot analysis using mAb 61BG1.3, which reacts with HA domains (HGP44 and HGP17), HA proteins with low molecular masses were observed in the culture supernatant of the \textit{porR} mutant, whereas its cell extracts showed only immunoreacted protein bands with high molecular masses. On the other hand, the mature (low molecular mass) forms of HA proteins were seen in the
2 of Rgp expression might affect fimbrillin maturation, whereas the analysis using anti-HbR antiserum, HbR was present in vesicle fractions but in the culture supernatants. (Kadowaki et al., 1998). To determine whether the delay of Rgp expression might affect fimbrillin maturation, whole-cell extracts of the porR and porR+ strains that had been grown in enriched BHI broth for 13, 17, 21 and 36 h were subjected to SDS-PAGE and immunoblot analysis using anti-fimbrillin antiserum (Fig. 7). The porR+ strain showed a mature form of fimbrillin (43 kDa) without its precursor forms at all the time points. On the other hand, the porR mutant showed a precursor form of fimbrillin (43-5 kDa) as well as mature fimbrillin (43 kDa) until 21 h incubation. However, 36 h after the start of incubation, the porR mutant produced only mature fimbrillin, which was consistent with the increase of Rgp activity in the strain.

(5) Cell surface polysaccharide and haemagglutination of the porR mutant. Cell surface polysaccharide was extracted from P. gingivalis cells using the phenol/water method. Compared with the porR+ sibling strain, the porR mutant had small amounts of LPS and associated polysaccharide (Fig. 8). In a previous study, we found that the HA domains derived from rgpA, kgp and bagA were responsible for haemagglutination of P. gingivalis. Since KDP107 produced no mature HA domain proteins, we determined whether the porR mutant cells agglutinated sheep erythrocytes. The haemagglutination titre of the porR mutant was less than 1, whereas the titres of the porR+ strains KDP108 and ATCC 33277 were 64, indicating that porR was responsible for haemagglutination.

(6) Immunoblot analysis using mAb 1B5. Recently, Curtis et al. (1999) have isolated a mAb (1B5) that reacts with mt-Rgp and LPS preparations. This mAb seems to react with carbohydrates since periodate treatment abolished the reaction. The porR mutant showed no mt-Rgp and deficiency in LPS or another surface polysaccharide, suggesting that the porR gene product may be involved in the biosynthesis of a common component between sugar portions of mt-Rgp and cell surface polysaccharide. Therefore, we went on to determine presence of mAb 1B5-reactive products in the porR mutant. The cell extracts and culture supernatants of the wild-type strains. The vesicle fractions showed the same protein profiles as those of the cell extracts in both of the porR and porR+ strains (data not shown). In the immunoblot analysis using anti-HbR antiserum, HbR was present in all the fractions of the wild-type strains, whereas the porR mutant showed HbR not in the cell extracts or vesicle fractions but in the culture supernatants.

(4) Fimbrillin maturation. Fimbrillin, a subunit protein of fimbriae, is produced as a precursor form possessing 46 additional amino acids at the amino terminus and then processed by Rgp and Kgp to generate mature fimbrillin (Kadowaki et al., 1998). To determine whether the delay of Rgp expression might affect fimbrillin maturation,
extracts of the porR mutant showed no reaction with mAb 1B5 (Fig. 9).

**DISCUSSION**

The pleiotropic phenotype of the porR mutant apparently indicates that PorR may have a regulatory function. Indeed, PorR shows similarities to DegT, a *B. stearothermophilus* protein that seems to possess features characteristic of two-component regulatory proteins (Takagi et al., 1990). However, proteins homologous to PorR and DegT that have been found in many micro-organisms appear to be involved in biosynthesis of sugar portions of LPS and aminoglycosides. Thorson et al. (1993) suggested that these proteins provide a catalytic rather than a regulatory role, in which they function as transaminases within their respective pathways. In this study, we found that the porR mutant had altered phenol-extractable polysaccharide compared to the wild-type strain. Since the phenol/water method (Westphal & Jann, 1965) that we used for purification of polysaccharides in this study can extract both capsular polysaccharide and LPS from this organism (Farquharson et al., 2000), we cannot determine which (or both) polysaccharide the porR mutant is deficient in. Curtis et al. (1999) found that mt-Rgp is shown to be differentially modified by the post-translational addition of carbohydrate and that mAb 1B5, which can react with mt-Rgp, has the ability to react with LPS of *P. gingivalis*. Immunoblot analyses using anti-Rgp/Kgp antiserum and mAb 1B5 revealed that the porR mutant had neither mt-Rgp nor mAb 1B5-reactive substances.

Chen et al. (2000) isolated nonpigmented mutants that had a transposon insertion at a gene with homology to a glycosyl (rhamnosyl) transferase, an enzyme involved in the synthesis of O-antigen side chains of LPS. The mutants also showed reduced levels of Rgp and haemagglutinin activities. Interestingly, the rhamnosyl transferase gene was located approximately 1-5 kb upstream of *porR*. Although there has been no direct evidence to show the relationship with respect to gene expression between *porR* and the rhamnosyl transferase gene, these findings imply that both of the putative rhamnosyl transferase gene and *porR* may be involved in the same biosynthesis pathway.

Very recently, another nonpigmented mutant has been found in the study concerning *P. gingivalis* recA. A mutation of the *vimA* gene located downstream of *recA* was responsible for deficiency in pigmentation and haemagglutination of this mutant (Abai-Bou et al., 2001). Moreover, Rgp and Kgp were mostly found in particle-free medium of the *vimA* mutant. Cell suspension and vesicles of the mutant showed very little gingipain activity. These properties of the *vimA* mutant are very similar to those of the porR mutant. We constructed a *vimA* mutant using gene replacement with *vimA*: Em<sup>r</sup> and found that the *vimA* mutant produced no mAb 1B5-reactive substances (unpublished data). These findings indicate that *vimA* may also be involved in biosynthesis of the saccharide portions of mt-Rgp and cell surface polysaccharide.

Rgp and Kgp were produced but not retained on the cell surfaces of the porR mutant and most of the gingipains were found in the culture supernatants. Moreover, the mature forms of HA and HbR proteins were also released into the culture supernatants, leading to no haemagglutination of the mutant cells. These results indicate that the porR mutant is incapable of retaining those mature proteins derived from rgp and kgp genes on the cell surfaces. A defect of the cell surface polysaccharide in the mutant may cause this incapability since levels of major outer-membrane proteins are drastically decreased in *Salmonella typhimurium* E. coli and *Shigella flexneri* mutants synthesizing very defective LPS such as the deep rough mutants (Koplow & Goldfine, 1974; Ames et al., 1974; Sandlin et al., 1995).

Potempa et al. (1995) found that distribution of Rgp and Kgp activities in *P. gingivalis* cell fractions are different among *P. gingivalis* strains. Strain H66 produces Rgp and Kgp mostly in particle-free culture supernatants, whereas strains ATCC 33277 and W50 produce them in all the fractions (particle-free culture supernatants, vesicles, membrane fractions and membrane-free cell extracts). The difference in Rgp and Kgp distribution among *P. gingivalis* strains might be related to the degree of production or maturation of cell surface polysaccharide.

In our previous studies (Kadowaki et al., 1998; Nakayama et al., 1996) we found that FimA fimbrillin remained in a precursor form in the Rgp-null mutant and that prefimbirillin expressed in *E. coli* was converted to the mature fimbrillin *in vitro* when incubated with purified Rgp, but its conversion was suppressed by potent Rgp inhibitors. The porR mutant cells in early exponential growth...
phase produced a precursor form of FimA fimbrillin and after prolonged incubation, the precursor was converted into a mature form of FimA fimbrillin. The molecular mass of the FimA precursor was 43.5 kDa, 0.5 kDa larger than that of the mature FimA fimbrillin. This indicates that the precursor has about five additional residues compared to the mature FimA, which is consistent with our recent finding that the precursor form of FimA accumulated on the cell surface of the rggA rggB kgp mutant had six additional residues (TSNSNK) at the amino terminus compared to the mature FimA (unpublished data). Fimbrillin maturation of the porR mutant appears to be synchronized with the increase of Rgp/Kgp activity. The present results strongly suggest that conversion of prefimbrillin to mature fimbrillin in vivo is dependent on Rgp/Kgp activity.

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