Lipopolysaccharide biosynthesis-related genes are required for colony pigmentation of *Porphyromonas gingivalis*

Keiko Sato,^{1†} Nobuo Kido,² Yukitaka Murakami,¹ Charles I. Hoover,^{1,3} Koji Nakayama⁴ and Fuminobu Yoshimura¹

Correspondence

Keiko Sato

satou@nagasaki-u.ac.jp

¹Department of Microbiology, School of Dentistry, Aichi-Gakuin University, 1-100 Kusumoto-cho, Chikusa-ku, Nagoya, Aichi 464-8650, Japan

²Division of Plant Growth Physiology, Nagoya University Graduate School of Biological Sciences, Furou-cho, Chikusa-ku, Nagoya, Aichi 464-8602, Japan

³University of California, San Francisco, CA 94143-0512, USA

⁴Division of Microbiology and Oral Infection, Department of Molecular Microbiology and Immunology, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki, Nagasaki 852-8588, Japan

The periodontopathic bacterium *Porphyromonas gingivalis* forms pigmented colonies when incubated on blood agar plates as a result of accumulation of μ -oxo haem dimer on the cell surface. Gingipain–adhesin complexes are responsible for production of μ -oxo haem dimer from haemoglobin. Non-pigmented mutants (Tn6-5, Tn7-1, Tn7-3 and Tn10-4) were isolated from *P. gingivalis* by Tn4351 transposon mutagenesis [Hoover & Yoshimura (1994), *FEMS Microbiol Lett* **124**, 43–48]. In this study, we found that the Tn6-5, Tn7-1 and Tn7-3 mutants carried Tn4351 DNA in a gene homologous to the *ugdA* gene encoding UDP-glucose 6-dehydrogenase, a gene encoding a putative group 1 family glycosyltransferase and a gene homologous to the *rfa* gene encoding ADP heptose-LPS heptosyltransferase, respectively. The Tn10-4 mutant carried Tn4351 DNA at the same position as that for Tn7-1. Gingipain activities associated with cells of the Tn7-3 mutant (*rfa*) were very weak, whereas gingipain activities were detected in the culture supernatants. Immunoblot and mass spectrometry analyses also revealed that gingipains, including their precursor forms, were present in the culture supernatants. A lipopolysaccharide (LPS) fraction of the *rfa* deletion mutant did not show the ladder pattern that was usually seen for the LPS of the wild-type *P. gingivalis*. A recombinant chimera gingipain was able to bind to an LPS fraction of the wild-type *P. gingivalis* in a dose-dependent manner. These results suggest that the *rfa* gene product is associated with biosynthesis of LPS and/or cell-surface polysaccharides that can function as an anchorage for gingipain–adhesin complexes.

Received 18 October 2008

Revised 24 December 2008

Accepted 6 January 2009

INTRODUCTION

Periodontal disease, the major cause of tooth loss in the general populations of industrial nations, is a chronic inflammatory disease of the periodontium (Neely *et al.*, 2005; Oliver *et al.*, 1998). The obligately anaerobic

Gram-negative bacterium *Porphyromonas gingivalis*, a major causative agent of chronic periodontitis, possesses several potential virulence factors for periodontopathogenicity (Holt *et al.*, 1999; Lamont & Jenkinson, 2000). Among these factors, proteolytic enzymes are of special importance, since some of them have the ability to destroy periodontal tissue directly or indirectly (Andrian *et al.*, 2007; Potempa *et al.*, 2000). The micro-organism produces large amounts of lysine-specific (Lys-gingipain, Kgp) and arginine-specific (Arg-gingipain, Rgp) cysteine proteinases on the cell surface and in the extracellular milieu (Bhogal *et al.*, 1997; Potempa *et al.*, 1995; Rangarajan *et al.*, 1997; Slakeski *et al.*, 1998). These proteinases are secreted into the extracellular milieu or located on the cell surface as complexes non-covalently

[†]Present address: Division of Microbiology and Oral Infection, Department of Molecular Microbiology and Immunology, Nagasaki University Graduate School of Biomedical Sciences, 1-7-1 Sakamoto, Nagasaki, Nagasaki 852-8588, Japan.

Abbreviation: ABTS, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid).

Supplementary tables of strains and primers, and details of the construction of the *rfa* insertion mutant, including a supplementary figure, are available with the online version of this paper.

associated with adhesin domain proteins. A 660 kDa cell-associated gingipain complex, composed of catalytic domains and a haemagglutinin domain such as Hgp44, is associated with lipopolysaccharide (LPS) (Takii *et al.*, 2005).

Rgp and Kgp can degrade collagens (type I and type IV), major components of periodontal connective tissue, and extracellular matrix proteins such as fibronectin and laminin (Kadowaki *et al.*, 2000), suggesting that these proteinases contribute directly to destruction of host tissue *in vivo*. Rgp and Kgp have the ability to disrupt host defence mechanisms by degrading and inactivating immunoglobulins, such as IgG, IgA and secretory IgA, and cytokines such as TNF α and IL-6 (Kadowaki *et al.*, 2000). Moreover, Rgp and Kgp contribute to the development and maintenance of inflammation in the periodontium through activation of the kallikrein-kinin cascade (Imamura *et al.*, 1994), dysregulation of the complement cascade (Wingrove *et al.*, 1992) and dysregulation of the coagulation cascade (Imamura *et al.*, 1997). In addition, Rgp contributes to processing/maturation of a subunit protein (fimbriin) of FimA fimbriae, which are responsible for adherence to periodontal tissue (Njoroge *et al.*, 1997).

The adhesion domain proteins also play important roles in virulence of *P. gingivalis*. Cells of *P. gingivalis* have the abilities to agglutinate erythrocytes and to aggregate platelets (Naito *et al.*, 2006; Sakai *et al.*, 2007). Hgp44 adhesin has a haemagglutinating activity and is one of essential requirements for *P. gingivalis* cell-induced platelet aggregation (Naito *et al.*, 2006). Also, gingipains have been identified as activators of the protease-activated receptors (PARs), implicated in platelet aggregation (Lourbakos *et al.*, 2001). Hgp15 (HbR) adhesin has the ability to bind to haem and haemoglobin and contributes to haem acquisition (Nakayama *et al.*, 1998). Hgp15 adhesin, which is a major protein in culture supernatants of the micro-organism, can suppress *in vitro* osteoclast formation from bone-marrow macrophages (Fujimura *et al.*, 2006).

Although Rgp, Kgp and adhesins, which are encoded by *rgpA*, *rgpB*, *kgp* and *hagA*, play various important roles in virulence of *P. gingivalis*, the mechanisms of transport of these proteins across the two membranes (inner and outer membranes) and adherence to the bacterial cell surface are still unknown. Previous studies have shown a link between colony pigmentation on blood agar plates and Rgp and Kgp activities in *P. gingivalis* cells (Okamoto *et al.*, 1998; Shi *et al.*, 1999). A Kgp-null mutant exhibits reduced pigmentation and a Kgp/Rgp-null mutant shows no pigmentation (Okamoto *et al.*, 1998; Shi *et al.*, 1999). Transposon mutagenesis has been applied to isolation of non-pigmented mutants of *P. gingivalis* in order to characterize the mechanisms of surface expression of Rgp and Kgp (Chen *et al.*, 2000; Hoover *et al.*, 1992; Sato *et al.*, 2005; Shoji *et al.*, 2002). Previously, we isolated five non-pigmented mutants of *P. gingivalis* by transposon mutagenesis (Hoover & Yoshimura, 1994). In this study, we

determined the genes that were responsible for non-pigmentation of these mutants. The genes were found to encode putative ADP heptose-LPS heptosyltransferase (PGN_1255), UDP-glucose 6-dehydrogenase (PGN_0613) and a group 1 family glycosyltransferase (PGN_1251).

METHODS

Strains and culture conditions. All *P. gingivalis* strains used in this study are shown in Supplementary Table S1, available with the online version of this paper. *P. gingivalis* cells were grown anaerobically (10 % CO₂, 10 % H₂, 80 % N₂) in enriched brain heart infusion (BHI) medium and on enriched tryptic soy agar (Nakayama *et al.*, 1995). For a blood agar plate, defibrinated laked rabbit blood was added to enriched tryptic soy agar at a concentration of 5 %. For selection and maintenance of antibiotic-resistant strains, antibiotics were added to the medium at the following concentrations: ampicillin, 50 μ g ml⁻¹; chloramphenicol (Cm), 20 μ g ml⁻¹; erythromycin (Em), 10 μ g ml⁻¹; and tetracycline (Tc), 0.7 μ g ml⁻¹.

Construction of plasmids and bacterial strains. For nucleotide sequencing of chromosomal DNA regions in the vicinity of Tn4351 insertion sites, *Pst*I DNA fragments containing Tn4351 DNA in the chromosomal DNA of *P. gingivalis* strains Tn6-5, Tn7-1, Tn7-3 and Tn10-4 were cloned into the *Pst*I site of pBluescript II SK(-).

A *P. gingivalis* PGN_1255-encoding gene (*rfa*) deletion mutant was constructed as follows. DNA regions upstream and downstream of *rfa* were PCR-amplified from the chromosomal DNA of wild-type strain 33277 using the primer pair N1255UF/N1255UR and the primer pair N1255DF/N1255DR, respectively. (Primers used in this study are listed in Supplementary Table S2.) The amplified DNAs upstream and downstream of *rfa* were double-digested with *Not*I plus *Bam*HI and *Bam*HI plus *Kpn*I, respectively, and both digested DNAs were ligated with pBluescript II SK(-) DNA double-digested with *Not*I plus *Kpn*I, resulting in pRF1. The 2.2 kb *Bam*HI-*Bgl*II *erm* DNA cartridge of pKD399 (Shoji *et al.*, 2004) was inserted into the *Bam*HI site of pRF1 to yield pRF2 (*rfa*-2::[*ermF ermAM*]). *P. gingivalis* 33277 was then transformed with *Not*I-linearized pRF2 DNA to yield strain Prfa1.

An *rfa*⁺-complementing strain of Prfa1 was constructed as follows. The *rfa* region was PCR-amplified from 33277 chromosomal DNA using the primer pair CN1255F/CN1255R, digested with *Bam*HI, and inserted into the *Bam*HI site of pKD713, a derivative of pKD703 (Shoji *et al.*, 2004) containing the *tetQ* DNA block of pKD375 (Shi *et al.*, 1999) at the *Bam*HI site, resulting in pRF3 (*fimA*::[*rfa*⁺ *tetQ*]). Strain Prfa1 was then transformed with *Not*I-linearized DNA of pRF3 to yield strain Prfa2 (*rfa*::[*ermF ermAM*] *fimA*::[*rfa*⁺ *tetQ*]).

A *P. gingivalis* PGN_0613-encoding gene (*ugdA*) insertion mutant was constructed as follows. The *ugdA* region of DNA was PCR-amplified from 33277 chromosomal DNA using the primer pair DN0613F/DN0613R and cloned into plasmid pCR4-TOPO (Invitrogen). The resulting plasmid was digested with *Ava*I and then treated with the Klenow fragment and ligated with the *Bgl*II linker to yield pUG1. The *Bam*HI-*Bgl*II *erm* DNA cartridge was inserted into the *Bgl*II site of pUG1, resulting in pUG2 (*ugdA*2::[*ermF ermAM*]). Strain 33277 was transformed with *Not*I-linearized pUG2 DNA to yield strain Pugd1.

A *ugdA*⁺-complementing strain of Pugd1 was constructed as follows. The *ugdA* region was PCR-amplified from 33277 chromosomal DNA using the primer pair CN0613F/CN0613R, digested with *Bam*HI, and inserted into the *Bam*HI site of pKD713, resulting in pUG3 (*fimA*::[*ugdA*⁺ *tetQ*]). Strain Pugd1 was then transformed with *Not*I-linearized DNA of pUG3 to yield strain Pugd2 (*ugdA*2::[*ermF ermAM*] *fimA*::[*ugdA*⁺ *tetQ*]).

Strains KDP380 and KDP381 were constructed as follows. For construction of a *kgp*'-*rgpB*'-*myc* chimera gene, the *myc*-tag DNA region was PCR-amplified from the pBAD/Myc-His A plasmid (Invitrogen) using the primer pair BADF/BADR. The amplified DNA was digested with *Sall* plus *PstI* and inserted into the *Sall*-*PstI* region of pBluescript II SK(-) to yield pKD856. The C-terminal domain DNA (0.4 kb) of *rgpB* was PCR-amplified from 33277 chromosomal DNA using the primer pair BCF/BCR, digested with *HindIII* plus *BglII*, and inserted into the corresponding region of pKD856, resulting in pKD857. A DNA region (0.2 kb) containing a transcriptional terminator downstream of *rgpB* was PCR-amplified from 33277 chromosomal DNA using the primer pair BTF/BTR. The amplified DNA was digested with *PstI* plus *NotI* and inserted into the corresponding region of pKD857 to yield pKD858. The *kgp* DNA region was introduced into pKD858 by two steps. First, the middle region of the *kgp* gene was PCR-amplified from 33277 chromosomal DNA using the primer pair KMF/KMR. The amplified DNA was digested with *XhoI* plus *BglII* and inserted into the corresponding region of pKD858 to yield pKD859. Next, the *AvaI*-*AvaI* region (0.3 kb) of pKD859 was replaced by a 2.6 kb *AvaI* DNA fragment encoding the signal peptide, propeptide and part of the mature proteinase portion of the *kgp* gene that was isolated from pNKV (Okamoto *et al.*, 1996) to yield pKD860 (*kgp*'-*rgpB*'-*myc*). The *KpnI*-*NotI* DNA fragment of pKD860 containing the *kgp*'-*rgpB*'-*myc* chimera gene DNA was treated with T4 DNA polymerase and ligated with the *Bam*HI-digested and Klenow-treated pKD713 DNA (Kikuchi *et al.*, 2005), resulting in pKD861 (*fimA*::[*kgp*'-*rgpB*'-*myc tetQ*]). For construction of *P. gingivalis* strains possessing the *kgp*'-*rgpB*'-*myc* chimera gene, KDP129 (*kgp*) and KDP351 (*kgp porT*) were transformed with *NotI*-linearized pKD861 DNA to yield KDP380 (*kgp fimA*::[*kgp*'-*rgpB*'-*myc tetQ*]) and KDP381 (*kgp porT fimA*::[*kgp*'-*rgpB*'-*myc tetQ*]), respectively.

P. gingivalis strain Prfa3 was constructed as follows. For construction of a *rfa*'-*myc* chimera gene, the *rfa* region including upstream sequence was PCR-amplified from 33277 chromosomal DNA using the primer pair N1255MF/N1255MR. The amplified DNA was digested with *XhoI* plus *HindIII* and inserted into the corresponding region of pKD858 to yield pRF4. The *KpnI*-*NotI* DNA fragment of pRF4 containing the *rfa*'-*myc* chimera gene DNA was treated with T4 DNA polymerase and then inserted into the *Bam*HI-digested and Klenow-treated fragment of pKD713 (Kikuchi *et al.*, 2005), resulting in pRF5 (*fimA*::[*rfa*'-*myc tetQ*]). For construction of a *P. gingivalis* strain possessing the *rfa*'-*myc* chimera gene, strain Prfa1 (*rfa*) was transformed with *Bss*HII-linearized pRF5 DNA to yield strain Prfa3 (*rfa rfa*'-*myc*).

DNA probes and Southern blot hybridization. Southern blot hybridization was performed as described previously (Kikuchi *et al.*, 2005). The *rfa* region was PCR-amplified from 33277 chromosomal DNA using the primer pair P1255F/P1255R. The *ermF*-*ermAM* DNA cartridge (2.2 kb) obtained from pKD399 by *Bam*HI plus *BglII* double digestion was labelled with the AlkPhos Direct system for chemiluminescence (Amersham Pharmacia). Southern blot hybridization was performed by a standard method using a nylon membrane and hybridized products were developed with CDP-star detection reagent (Amersham Pharmacia).

Haemagglutination assay. Overnight cultures of *P. gingivalis* strains in enriched BHI medium were centrifuged, washed with PBS, and resuspended in PBS (OD₆₀₀ 1.0). The bacterial suspensions were then diluted in a twofold series and applied to the wells of a microtitre plate from left to right. A 100 µl aliquot of each suspension was mixed with an equal volume of sheep erythrocyte suspension (1%, v/v, in PBS) and incubated in a round-bottom microtitre plate at room temperature for 3 h.

Preparation of *P. gingivalis* cell fractions. *P. gingivalis* overnight cultures in enriched BHI broth were centrifuged at 10 000 g for 10 min at 4 °C. Solid ammonium sulfate was added to the supernatant to 50% (w/v) saturation. After centrifugation for 15 min, the pellets were dissolved in 10 mM HEPES (pH 7.4) containing *N*^ε-*p*-tosyl-L-lysine chloromethyl ketone (TLCK), leupeptin and EDTA at 0.1, 1 and 5 mM, respectively, dialysed overnight against 10 mM HEPES (pH 7.4) in a Slide-A-Lyser dialysis cassette (3500 MWCO) (Pierce) and subjected to SDS-PAGE followed by immunoblotting.

Subcellular fractionation. *P. gingivalis* cells from a 1000 ml culture were harvested by centrifugation at 10 000 g for 30 min at 4 °C and resuspended with 40 ml PBS containing 0.1 mM TLCK, 0.1 mM leupeptin and 0.5 mM EDTA. The cells were disrupted in a French pressure cell at 100 MPa by two passes. The remaining intact bacterial cells were removed by centrifugation at 2400 g for 10 min, and the supernatant was subjected to ultracentrifugation at 100 000 g for 60 min to separate the cytoplasm/periplasm fraction (supernatant) and the total membrane fraction (pellet), and then the pellet was treated with 1% (v/v) Triton X-100 in PBS containing 20 mM MgCl₂ for 30 min at 20 °C. The outer-membrane fraction was recovered as a precipitate by ultracentrifugation at 100 000 g for 60 min at 4 °C. The supernatant was obtained as the inner-membrane fraction. To determine the subcellular localization of the Rfa protein, the cytoplasm/periplasm, inner-membrane and outer-membrane fractions of strain Prfa3 were incubated for 60 min at 4 °C with EZview Red Anti c-Myc Affinity Gel (Sigma) and the gel was washed with PBS three times. The washed gel was then collected by centrifugation at 8200 g for 5 min and resuspended with SDS sample buffer. The sample was boiled for 5 min and then subjected to SDS-PAGE and immunoblot analysis with anti-c-Myc antibody (Sigma).

Enzyme assays. Kgp and Rgp activities were determined using the synthetic substrates *N*-*p*-tosyl-Gly-Pro-Lys-*p*-nitroanilide (GPKpNA; Sigma) and *N*- α -benzoyl-DL-Arg-*p*-nitroanilide (BAPNA; Sigma) in 50 mM Tris/HCl (pH 8.5) containing 5 mM L-cysteine in a total volume of 1 ml. After incubation at 37 °C for 10 min, the reaction was terminated by adding 0.2 ml 50% (v/v) acetic acid, and the released *p*-nitroanilide was measured at 405 nm.

Purification of sKgp-RgpB-Myc. The *kgp*'-*rgpB*'-*myc* chimera gene product (sKgp-RgpB-Myc) was purified using EZview Red Anti c-Myc Affinity Gel. Briefly, *P. gingivalis* KDP381 cells (50 ml culture) were solubilized with 4 ml Bugbuster reagent (Novagen) and slowly rocked for 20 min at room temperature. A supernatant was obtained by centrifugation at 5000 g for 15 min at 4 °C and then incubated for 60 min at 4 °C with EZview Red Anti c-Myc Affinity Gel. The resin was packed into a column and washed with 30 ml lysis buffer (150 mM NaCl, 1% Triton X-100, 20 mM HEPES, pH 7.5). The recombinant Kgp-RgpB-Myc protein (Kgp-RgpB-Myc) was eluted with RIPA buffer containing 100 µg c-Myc peptide ml⁻¹.

Mass spectrometry. The gel plugs were subjected to the following steps: washing with 50% (v/v) acetonitrile, washing with 100% acetonitrile, reduction with 10 mM DTT, alkylation with 55 mM iodoacetamide, washing/dehydration with 50% (v/v) acetonitrile, and digestion for 10 h with 10 µg trypsin ml⁻¹. Resulting peptides were extracted from the gel plug with 0.1% (v/v) trifluoroacetic acid/50% (v/v) acetonitrile and concentrated using C-18 Zip-tips (Millipore). Digests were spotted on a MALDI target using α -cyano-4-hydroxycinnamic acid as a matrix. Spectra were acquired on a 4800 MALDI TOF/TOF Analyser (Applied Biosystems). MS/MS spectra were acquired automatically.

LPS analysis. LPS of *P. gingivalis* was prepared as described previously (Darveau *et al.*, 1983), separated on a 15% SDS-PAGE gel

containing 4 M urea and 16 % Novex Tricine gel (Invitrogen), and then visualized by silver staining.

ELISA-based LPS binding assay. A polystyrene microtitre plate (96-well, Maxi-sorp; Nalge Nunc) was first coated with 100 μ l per well of a 10 μ g ml⁻¹ solution of LPS from *P. gingivalis* 33277 in PBS. The plate was sealed and incubated overnight at room temperature. The wells were aspirated, washed four times with 300 μ l wash solution (PBS containing 0.05 %, v/v, Tween 20), and blocked with wash solution containing 2 % (w/v) BSA at 4 °C overnight. After removing the blocking solution, increasing concentrations of Kgp-RgpB-Myc were allowed to interact with bound LPS at room temperature for 2 h. The wells were aspirated and washed three times with wash solution. Kgp-RgpB-Myc bound to LPS was detected by incubation with rabbit anti-gingipain antibody followed by incubation with goat anti-rabbit antibody conjugated with horseradish peroxidase (Bio-Rad). Incubation with each antibody was performed for 2 h at room temperature. In the final step, 100 μ l peroxide ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] substrate was added. The binding activities were assessed by measuring A_{450} values with a microplate reader (model 680; Bio-Rad). All assays were carried out in triplicate and the standard errors were determined.

Preparation of anti-Kgp and anti-Hgp44 antisera. Anti-Kgp antiserum was obtained as described previously (Takii *et al.*, 2005). Polyclonal rabbit anti-Kgp antiserum against a peptide derived from the amino acid sequence (D²²⁹ to M²⁴⁴) of Kgp with an N-terminal cysteine residue, CDVYTDHGDLYNTPVRM, was conjugated to keyhole limpet haemocyanin, purchased from Sigma Genosys. Anti-Hgp44 antiserum was obtained as described previously (Sato *et al.*, 2005).

RESULTS

Identification of a gene disrupted by transposon insertion

Chromosomal DNA of the insertion mutants (Tn6-5, Tn7-1, Tn7-3, and Tn10-4) was digested with various restriction enzymes and subjected to Southern blot hybridization analysis with the *ermF* gene probe, which indicated that the chromosomal DNA contained a single Tn4351 insertion (Fig. 1). The hybridized bands detected in Tn7-1 and Tn10-4 showed the same pattern in each restriction digestion, suggesting that these mutants contained Tn4351 insertion either in the same gene or in closely linked genes.

*Pst*I fragments (9.8, 11, 11 and 11 kb) of the chromosomal DNA of Tn6-5, Tn7-1, Tn7-3 and Tn10-4, respectively, were isolated, ligated with a vector plasmid, and cloned by the marker-rescue method with Tc^r on Tn4351 DNA. Sequencing of the cloned DNA fragments revealed that the insertion sites were located 162, 421 and 661 bp downstream of the first nucleotide residues of the initiation codons of PGN_0613, PGN_1251 and PGN_1255 in strains Tn6-5, Tn7-1 and Tn7-3, respectively (Naito *et al.*, 2008) (Fig. 2). BLAST search analysis suggested that the PGN_0613 gene was homologous to the *ugdA* gene encoding UDP-glucose 6-dehydrogenase, that the PGN_1251 gene encoded a putative group 1 family glycosyltransferase and that the PGN_1255 gene was homologous to the *rfa* gene encoding ADP heptose-LPS heptosyltransferase. The

Fig. 1. Southern blot analysis of Tn4351-transposed non-pigmented mutants. (a) Schematic structure of Tn4351. (b) Southern blot hybridization with Tn4351. Genomic DNA from strains Tn6-5, Tn7-1, Tn7-3 and Tn10-4 was digested with *Bam*HI (lane 1), *Pst*I (lane 2) and *Sma*I (lane 3), and subjected to Southern blot analysis with probe DNA specific to the erythromycin-resistance marker present on Tn4351. (c) Further Southern blot hybridization with Tn4351. Genomic DNA from strains Tn7-1, Tn7-3 and Tn10-4 was digested with *Hind*III (lane 1) and *Pst*I/*Hind*III (lane 2), and subjected to Southern blot analysis with the probe DNA.

PGN_0613 and PGN_1255 genes were thus designated *ugdA* and *rfa*, respectively (Fig. 2). The transposon insertion sites of strains Tn7-1 and Tn10-4 were revealed to be the same by sequencing PCR-amplified DNA around the PGN_1251 gene region.

Construction of *rfa* and *ugdA* mutants by gene-directed mutagenesis

To investigate whether the non-pigmentation of Tn6-5 and Tn7-3 was attributable to *ugdA* and *rfa*, respectively, we

Fig. 2. Physical maps around the *rfa* and *ugdA* gene regions. Triangles indicate the Tn4351 insertion sites of strains Tn6-5, Tn7-1, Tn7-3 and Tn10-4. P, *Pst*I.

constructed mutants with disruption of *ugdA* and *rfa* by gene-directed mutagenesis. We first constructed suicide vector plasmids containing the disrupted *rfa* (plasmid pRF2) and *ugdA* (plasmid pUG2). Introduction of *Not*I-linearized pRF2 and pUG2 DNA into *P. gingivalis* 33277 cells by electroporation produced a number of *Em*^r transformants. The resulting *rfa* mutant (Prfa1) and *ugdA* mutant (Pugd1) could grow in enriched BHI medium as well as the wild-type parent strain, but the two mutants exhibited no pigmentation on blood agar plates (Fig. 3a, b). The *rfa*⁺ and *rfa*'-'myc chimera genes were introduced into the *fimA* locus of strain Prfa1, resulting in strains Prfa2 (*rfa fimA*::*rfa*⁺) and Prfa3 (*rfa fimA*::*rfa*'-'myc), respectively. The *ugdA*⁺ gene was introduced into the *fimA* locus of strain Pugd1, resulting in strain Pugd2 (*ugdA fimA*::*ugdA*⁺). Strains Prfa2, Prfa3 and Pugd2 showed colony pigmentation (Fig. 3a). The *rfa* mutant Prfa1 showed no haemagglutination, while the complemented strains as well as the wild-type strain did show haemagglutination (Fig. 3c). The *ugdA* mutant showed almost the same haemagglutination as that of the wild-type strain. These results demonstrated that the *rfa* and *ugdA* genes were responsible for colony pigmentation and that the *rfa* gene contributed to cell-induced haemagglutination of *P. gingivalis*.

Kgp and Rgp activities of *rfa* and *ugdA* mutants

Since our previous studies indicated that colony pigmentation on blood agar plates is associated with the presence of processed/matured Kgp and Rgp proteinases on the cell surface (Okamoto *et al.*, 1998; Sato *et al.*, 2005; Shi *et al.*, 1999; Shoji *et al.*, 2002), we determined Kgp and Rgp activities in the intact cells and culture supernatants of the

rfa and *ugdA* mutants (Fig. 4). The *rfa* mutant (Prfa1) showed no detectable Kgp and Rgp activities in the intact cells, but these activities were detected in the culture supernatants. Prfa2 (*rfa fimA*::*rfa*⁺) showed almost the same Kgp and Rgp activities in the intact cells and culture supernatants as those of 33277. The culture supernatants of the *ugdA* mutant (Pugd1) had Kgp activity levels three times higher than those of 33277 and had almost the same Rgp activities as those of 33277. The intact cells of the mutant had relatively strong Kgp and Rgp activities.

Immunoblot analysis using anti-Kgp, anti-Hgp44 and 1B5 antibodies

The *kgp* and *rgpA* genes, which are 5193 and 5118 bp, respectively, encode polypeptides that consist of four segments: signal peptide, propeptide, proteinase and adhesin domains. The C-terminal adhesin domains also have four subdomains (Hgp44/A1, Hgp15/HbR/A2, Hgp17/A3 and Hgp27/A4) that are involved in haemagglutination and haemoglobin binding (Sakai *et al.*, 2007). The *rgpB* gene (2208 bp) has signal peptide, propeptide and proteinase domains but lacks most of the adhesin domain. Cells and culture supernatants of Prfa1 (*rfa*), Pugd1 (*ugdA*), KDP136 (*kgp rgpA rgpB*) and 33277 (wild-type) were subjected to immunoblot analyses with anti-Kgp, anti-Hgp44 and 1B5 antibodies (Rangarajan *et al.*, 2008) (Fig. 5). Monoclonal antibody (mAb) 1B5 reacts with anionic polysaccharides on the cell surface and glycan additions to Rgp (Curtis *et al.*, 1999). In the wild-type strain, a protein band with a molecular mass of 50 kDa immunoreactive to anti-Kgp was found in both whole cells and culture supernatant. However, in the *rfa* mutant, the 50 kDa protein was found in culture supernatants but was

Fig. 3. Properties of the *rfa* and *ugdA* mutants. (a) Colony pigmentation. (b) Growth curve in enriched BHI medium. An overnight culture was diluted 20-fold with enriched BHI medium and incubated anaerobically at 37 °C. ●, 33277 (wild-type); ■, Prfa1 (*rfa*); ▲, Pugd1 (*ugdA*). (c) Haemagglutination. *P. gingivalis* cells were grown in enriched BHI medium, washed with PBS, and resuspended in PBS at an OD₆₀₀ of 1.0. The suspension and its dilutions in a twofold series were applied to the wells of a microtitre plate from left to right and mixed with sheep erythrocyte suspension. Rows: 1, KDP136 (*kgp rgpA rgpB*); 2, 33277 (wild-type); 3, Prfa1 (*rfa*); 4, Pugd1 (*ugdA*); 5, KDP381 (*kgp porT fimA::[kgp'-'rgpB'-'myc]*); 6, KDP380 (*kgp fimA::[kgp'-'rgpB'-'myc]*); 7, Prfa2 (*rfa fimA::rfa⁺*); 8, Prfa1 (*rfa*); 9, 33277 (wild-type); 10, Prfa3 (*rfa fimA::[rfa'-'myc]*).

not detected in whole cells. The culture supernatants of the *rfa* mutant contained a major 59 kDa protein that was present at much lower levels in those of the wild-type

strain. Since the 59 kDa protein reacted with anti-Kgp antibody, this protein was subjected to N-terminal amino acid sequencing, which revealed a sequence of ten residues,

Fig. 4. Kgp and Rgp activities of the *rfa* and *ugdA* mutants. *P. gingivalis* cells were anaerobically grown in enriched BHI medium at 37 °C for 24 h. Kgp and Rgp activities of the culture supernatants (sup) and the intact cells (cell) were measured. Bars: 1, 33277 (wild-type); 2, KDP136 (*kgp rgpA rgpB*); 3, Prfa1 (*rfa*); 4, Prfa2 (*rfa fimA::rfa⁺*); 5, Prfa3 (*rfa rfa'-'myc*); 6, Pugd1 (*ugdA*).

Fig. 5. Immunoblot analysis. (a) Cell lysates of *P. gingivalis* strains were subjected to SDS-PAGE and stained with Coomassie brilliant blue (CBB). Cells of strains KDP136 (*kgp rgpA rgpB*) (lane 1), Prfa1 (*rfa*) (lane 2), Pugd1 (*ugdA*) (lane 3) and 33277 (wild-type) (lane 4) were lysed and subjected to SDS-PAGE and immunoblot analysis with anti-Kgp, anti-Hgp44 and 1B5 antibodies. (b) Culture supernatants of Prfa1 (*rfa*) (lane 1), 33277 (wild-type) (lane 2), and Pugd1 (*ugdA*) (lane 3) were subjected to SDS-PAGE and immunoblot analysis with anti-Kgp, anti-Hgp44 and 1B5 antibodies.

SDDPEKVPFV, corresponding to that encoded by the propeptide DNA region of the *kgp* gene. In the wild-type strain, 10, 38 and 44 kDa proteins and smear protein bands with molecular masses of more than 80 kDa, which reacted with anti-Hgp44, were found in the cell lysates (Sato *et al.*, 2005), whereas only anti-Hgp44-immunoreactive smear protein bands with molecular masses of more than 80 kDa were detected in cell lysates of the *rfa* mutant. These results indicated that processed/matured protein molecules such as 50 kDa Kgp and 44 kDa Hgp44 were not attached to the cell surface of the *rfa* mutant and that all of the protein molecules were secreted into the extracellular milieu. Immunoblot analysis with mAb 1B5 revealed that the *rfa* mutant was defective in production of anionic polysaccharides and glycan additions to Rgp. In the *ugdA* mutant, immunoblot profiles with anti-Kgp and anti-Hgp44 were almost the same as those of the wild-type parent strain. The

ugdA mutant also showed no mAb 1B5-immunoreactive molecules.

Subcellular localization of Rfa

To determine the subcellular localization of the Rfa protein, we constructed a *rfa*'-'*myc* chimera gene in which *rfa* and *myc*-tag were fused. Strain Prfa3 (*rfa fimA::rfa*'-'*myc*), in which the chimera gene was introduced into the *fimA* gene locus of strain Prfa1, showed the same colony pigmentation, haemagglutination and gingipain activities as those of the wild-type parent strain (Figs 3 and 4). Cell lysates of Prfa3 were fractionated into cytoplasm/periplasm, inner-membrane and outer-membrane fractions. Each fraction was incubated with EZview resin and then washed with PBS. The washed resin was analysed by SDS-PAGE followed by immunoblot analysis with anti-Myc

Fig. 6. Subcellular localization of the Rfa'-Myc protein. Cell lysates of strain Prfa3 were fractionated to cytoplasm/periplasm, inner-membrane and outer-membrane fractions and the fractions were subjected to SDS-PAGE and stained with Coomassie brilliant blue (CBB). The Rfa'-Myc protein was isolated from each fraction by using the anti c-Myc affinity gel and subjected to SDS-PAGE and immunoblot analysis with anti-Myc antibody. Lanes: M, molecular mass markers; 1, cytoplasm/periplasm fraction; 2, inner-membrane fraction; 3, outer-membrane fraction.

(Fig. 6). A protein band with a molecular mass of 35 kDa immunoreactive to anti-Myc was found only in the inner-membrane fraction, suggesting that Rfa is an inner-membrane protein.

Analysis of LPS fractions of *rfa* and *ugdA* mutants by SDS-PAGE

The LPS profiles of the *rfa* and *ugdA* mutants and the wild-type parent strain were analysed by SDS-PAGE and Tricine-PAGE (Fig. 7). LPS of the wild-type parental strain (33277) exhibited a typical ladder of LPSs with low molecular masses to high molecular masses, while the *rfa* mutant (Prfa1) showed only the presence of a core molecule of LPS. The *ugdA* mutant (Pugd1) exhibited a ladder of LPSs, but it differed from that of the wild-type in containing fewer high-molecular-mass LPSs and more low-molecular-mass LPSs.

Ability of Kgp-RgpB-Myc protein to bind to *P. gingivalis* LPS

We attempted to determine whether LPS of *P. gingivalis* has the ability to bind to gingipain-related proteins such as Kgp and Hgp44. For easy purification of Kgp, we constructed a *kgp'*-*rgpB'*-*myc* chimera gene consisting of the signal peptide, propeptide and proteinase domains of *kgp*, the C-terminal portion of *rgpB*, and a *myc* tag (Fig. 8a). We then obtained strains KDP380 (*kgp*-2::cat

Fig. 7. Analysis of LPS of the *rfa* and *ugdA* mutants. LPS fractions were extracted from strains 33277, Prfa1 and Pugd1 as previously described (Darveau *et al.*, 1983) and subjected to SDS-PAGE (a) and SDS-Tricine-PAGE (b). Lanes: 1, 33277 (wild-type); 2, Prfa1 (*rfa*); 3, Pugd1 (*ugdA*); 4, Prfa2 (*rfa rfa*⁺); 5, Prfa3 (*rfa rfa*⁺-*myc*).

fimA::[*kgp'*-*rgpB'*-*myc tetQ*]) and KDP381 (*kgp*-2::cat *porT*::[*ermF ermAM*] *fimA*::[*kgp'*-*rgpB'*-*myc tetQ*]) by introduction of the *kgp'*-*rgpB'*-*myc* chimera gene DNA into the *fimA* loci of strains KDP129 (*kgp*) and KDP351 (*kgp porT*), respectively. KDP380 showed the same colony pigmentation on blood agar plates and haemagglutination as those of the wild-type strain, suggesting that the *kgp'*-*rgpB'*-*myc* chimera gene is functional *in vivo* (Fig. 8b, c). The intact cells and culture supernatants of KDP380 had about 40 % and 18 %, respectively, of the Kgp activities of the wild-type strain 33277 and almost the same Rgp activities as those of the wild-type strain (Fig. 8d). KDP381 showed no colony pigmentation or haemagglutination, indicating that expression of the *kgp'*-*rgpB'*-*myc* chimera gene as well as the wild-type *kgp* gene depends on the *porT* gene, the product of which may be involved in the membrane transport system for gingipain gene-related products. The *kgp'*-*rgpB'*-*myc* chimera gene product was purified as described in Methods (Fig. 8e). Purified proteins with molecular masses of 78 and 95 kDa were confirmed by MALDI-TOF mass analysis to be the gene products (data not shown). The purified proteins were subjected to LPS binding analysis (Fig. 8f). An LPS fraction that was purified from the wild-type strain 33277 was coated onto 96-well plates. A twofold series of Kgp-RgpB-Myc proteins was allowed to interact with the immobilized LPS. The ELISA-based binding assay showed that the Kgp-RgpB-Myc proteins were able to bind to *P. gingivalis* 33277 LPS in a dose-dependent manner (Fig. 8f). As a control, we

Fig. 8. Dose-dependent binding of the recombinant Kgp-RgpB-Myc chimera protein to LPS. (a) Schematic structure of the *kgp'*-*rgpB'*-*myc* chimera gene. (b) Colony pigmentation. 1, 33277 (wild-type); 2, KDP380 (*kgp fimA* :: [*kgp'*-*rgpB'*-*myc*]); 3, KDP381 (*kgp porT fimA* :: [*kgp'*-*rgpB'*-*myc*]). (c) Haemagglutination. Rows: 1, KDP136 (*kgp rgpA rgpB*); 2, 33277; 3, KDP381; 4, KDP380. (d) Kgp and Rgp activities of KDP380. Kgp and Rgp activities of the intact cells (black bars) and culture supernatants (white bars) of KDP380 were measured. (e) Purification of the recombinant Kgp-RgpB-Myc chimera protein. The Kgp-RgpB-Myc chimera protein was subjected to SDS-PAGE and stained with CBB. (f) ELISA-based LPS binding assay. LPS fractions from strains 33277 (wild-type) (bars 2, 4, 6) and Prfa4 (*rfa* insertion mutant) (bars 1, 3, 5) were coated overnight onto 96-well plates. The recombinant Kgp-RgpB-Myc chimera proteins were allowed to interact with the immobilized LPS at various concentrations. The amount of the recombinant proteins bound was determined by rabbit anti-gingipain antibody and quantified by ABTS substrate. Bars: 1 and 2, 0.18 μ g; 3 and 4, 0.36 μ g; 5 and 6, 0.72 μ g of the recombinant Kgp-RgpB-Myc chimera protein per well.

purified an LPS fraction from the *rfa* deletion mutant, but could not perform ELISA analysis due to hydrophobicity of the purified LPS. Besides the *rfa* deletion mutant Prfa1, we constructed an *rfa* insertion mutant (Prfa4), which had replacement of an internal region of the *rfa* gene by the Em resistance DNA cartridge (for details, see Supplementary Fig. S1a, b, available with the online version of this paper). Colony pigmentation, haemagglutination, Kgp and Rgp activities and 1B5 antibody reactivity of Prfa4 resembled those of Prfa1 (Fig. S1c-f), but the LPS fraction of strain Prfa4 showed a ladder pattern with a phase different from that of the wild-type strain (Fig. S1g). In contrast to the wild-type LPS, the

Kgp-RgpB-Myc proteins showed no dose-dependent binding to Prfa4 LPS (Fig. 8f).

DISCUSSION

P. gingivalis wild-type strains form black-pigmented colonies on blood agar plates as a result of accumulation of μ -oxo haem dimer on the cell surface. Since colony pigmentation on blood agar plates has been shown to be linked with haemagglutination, Kgp/Rgp activity and other virulence factors, pigment-less mutants have been isolated and characterized by several researchers (Chen *et al.*, 2000; Hoover & Yoshimura, 1994; Shi *et al.*, 1999). A number of

studies have suggested that colony pigmentation is associated with the presence of gingipain–adhesin complexes on the cell surface (Bhogal *et al.*, 1997; Farquharson *et al.*, 2000; Takii *et al.*, 2005).

Pigmentation-related genes characterized thus far can be classified into three types: gene expression, membrane transportation and surface attachment of gingipain–adhesin complexes. Gingipain–adhesin complexes comprise Rgp and Kgp proteinases encoded by *rgpA*, *rgpB* and *kgp*, and adhesins encoded by *rgpA*, *kgp* and *hagA*. *kgp* and *rgpA* *rgpB* *kgp* mutants form less- and non-pigmented colonies, respectively, whereas an *rgpA* *rgpB* mutant forms pigmented colonies (Okamoto *et al.*, 1998; Shi *et al.*, 1999). A recent study (Smalley *et al.*, 2007) revealed that Rgp activity is crucial for converting oxyhaemoglobin into the methaemoglobin form, which is rendered more susceptible to Kgp degradation for the eventual release of iron(III) protoporphyrin IX and production of μ -oxo haem dimer.

The two genes *porT* and *sov*, mutants of which exhibit non-pigmentation, have been reported to be involved in membrane transportation of gingipain–adhesin complexes. High-molecular-mass precursor forms of Rgp, Kgp and adhesins are accumulated in the periplasmic space of *porT* and *sov* mutants (Saiki & Konishi, 2007; Sato *et al.*, 2005). The PorT protein is associated with the inner membrane.

Extracellular polysaccharide formation and glycosylation of gingipain–adhesin complexes are required for attachment of gingipain–adhesin complexes to the cell surface. *porR*, *vimA*, *vimE* and *vimF*, mutants of which lose colony pigmentation, appear to be involved in the formation of extracellular polysaccharide and glycan additions of gingipain–adhesin complexes, based on the lack of immunoreactivity to mAb 1B5 shown by the mutants (Shoji *et al.*, 2002; Vanterpool *et al.*, 2005, 2006). Also, Chen *et al.* (2000) isolated a nonpigmented mutant, with a transposon insertion in a gene homologous to a glycosyl (rhamnosyl)transferase-encoding gene, that showed reduced levels of Rgp activity and haemagglutination.

In the present study, we identified the insertion sites of transposon Tn4351 of four non-pigmented mutants isolated in a previous study (Hoover & Yoshimura, 1994) and found that the PGN_0613, PGN_1251 and PGN_1255 genes are involved in colony pigmentation. A 39 kDa protein encoded by PGN_1255 showed similarity with ADP-heptose heptosyltransferase II of other Gram-negative bacteria. In *Salmonella typhimurium* and *Escherichia coli*, two genes encoding heptosyltransferase I and heptosyltransferase II, which are involved in transfer of heptose from ADP-heptose to the inner core of LPS, have been found in their genomes, but only the PGN_1255 gene (*rfa*) has been found as a gene encoding heptosyltransferase in the *P. gingivalis* genome by homology search analysis (Naito *et al.*, 2008). SDS-PAGE analysis of LPS purified from the *P. gingivalis* *rfa* deletion mutant showed no ladder formation and the presence of a truncated core oligosaccharide like those of *E. coli* and *S. typhimurium* ADP-heptose

heptosyltransferase-deficient mutants, suggesting that the *P. gingivalis* Rfa, which is located in the inner membrane, is responsible for synthesis of the inner core region of LPS. The LPS fraction of the *rfa* insertion mutant Prfa4 showed a ladder pattern with a phase different from that of the wild-type strain but with the same interval, suggesting that the mutant has a defect in the core region of LPS. In strain Prfa4, the 2.2 kb *erm* DNA cartridge is inserted into the *AclII* site, which is located 540 bp downstream of the first nucleotide residue of the initiation codon of the *rfa* gene. It is possible that the *rfa* insertion mutant expresses a truncated Rfa enzyme that affects the biosynthesis of the inner core region of LPS. Both deletion and insertion mutants of *rfa* showed no immunoreactivity to mAb 1B5. This mAb is immunoreactive to a cell-surface anionic polysaccharide (APS) and glycan additions of gingipain. Very recently, Rangarajan *et al.* (2008) found by chemical analysis of APS that *P. gingivalis* synthesizes two distinct LPS macromolecules containing different glycan repeating units: O-LPS (with O-antigen tetrasaccharide repeating units) and A-LPS (with APS repeating units). Defects in LPS biosynthesis and mAb 1B5 reactivity of the *rfa* mutants suggest that the *rfa* gene product is responsible for O-LPS and A-LPS formation.

A 46 kDa protein encoded by the PGN_0613 gene has a sequence similarity with UDP-glucose dehydrogenase found in other bacteria. UDP-glucose dehydrogenase oxidizes UDP-glucose directly to UDP-glucuronic acid (UDP-GlcA) (Lin *et al.*, 1995). UDP-GlcA is then converted into UDP-galacturonic acid (UDP-GalA) by the UDP-glucuronic acid epimerase (*lpsL*) in *Sinorhizobium meliloti* (Keating *et al.*, 2002). UDP-GlcA is also converted into UDP-galacturonic acid by the UDP-glucuronate epimerase in *Arabidopsis* (Molhoj *et al.*, 2004). A UDP-glucose dehydrogenase mutant (*exo5*) of *Rhizobium leguminosarum* is defective in GlcA- and GalA-containing polysaccharides such as extracellular polysaccharide (EPS), capsular polysaccharide (CPS) and LPS (Laus *et al.*, 2004). Farquharson *et al.* (2000) showed that CPS (or K-antigen) with gel-like viscoelastic properties in *P. gingivalis* strain ATCC 53978 (W50) contains GlcA and Gal. It is not clear whether extracellular polysaccharides of *P. gingivalis* strain 33277, known as a natural K[−] strain, contain GlcA or GalA. The PGN_0613 mutant (*ugdA*) constructed by gene-directed mutagenesis showed grey pigmentation, but this mutant had Rgp and Kgp proteinases on the cell surface, clearly different from other non-pigmented mutants. The *ugdA* mutant had almost the same cell-bound Rgp and Kgp activities and extracellular Rgp activity as those of the wild-type parent and had a four times higher level of extracellular Kgp activity than that of the wild-type parent. Analysis of LPS by silver-stained SDS-PAGE showed that high-molecular-mass bands were decreased and low-molecular-mass bands were increased in the *ugdA* mutant LPS compared to those in the wild-type LPS. The *ugdA* mutant showed no bands immunoreactive with mAb 1B5, which is specific for a glycan epitope on some isoforms of Rgp and cell-associated carbohydrate

polymers. These results indicate that *ugdA* affects a repeating unit structure of cell-associated carbohydrate polymers. Why does the *ugdA* mutant form grey-pigmented colonies on blood agar plates although Rgp and Kgp proteinases are located at the cell surface of the mutant? One possible hypothesis is that haem extracted from haemoglobin by Rgp and Kgp proteinases cannot bind to the altered repeating unit structure of surface polysaccharides in the *ugdA* mutant LPS.

We also found in this study that recombinant Kgp-RgpB-Myc proteins were able to bind to the LPS fraction of the *P. gingivalis* wild-type strain *in vitro*. The mechanism of the anchoring of gingipain–adhesin complexes to the cell surface has not been elucidated; however, surface polysaccharides appear to be involved in the anchoring mechanism. Although the results of this study suggest that gingipain–adhesin complexes can bind non-covalently to the cell surface, further study is needed to clarify the anchoring mechanism.

ACKNOWLEDGEMENTS

We thank Michael Curtis for antibody mAb 1B5. This work was supported by Grants-in-Aid for Scientific Research (15591958 to Y.M. and 15591957 to F.Y.) from the Japan Society for the Promotion of Science (JSPS) and the AGU High-Tech Research Center Project from The Ministry of Education, Culture, Sports, Science and Technology, Japan.

REFERENCES

- Andrian, E., Mostefaoui, Y., Rouabhia, M. & Grenier, D. (2007). Regulation of matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases by *Porphyromonas gingivalis* in an engineered human oral mucosa model. *J Cell Physiol* **211**, 56–62.
- Bhagal, P. S., Slakeski, N. & Reynolds, E. C. (1997). A cell-associated protein complex of *Porphyromonas gingivalis* W50 composed of Arg- and Lys-specific cysteine proteinases and adhesins. *Microbiology* **143**, 2485–2495.
- Chen, T., Dong, H., Yong, R. & Duncan, M. J. (2000). Pleiotropic pigmentation mutants of *Porphyromonas gingivalis*. *Microb Pathog* **28**, 235–247.
- Curtis, M. A., Thickett, A., Slaney, J. M., Rangarajan, M., Aduse-Opoku, J., Shepherd, P., Paramonov, N. & Hounsell, E. F. (1999). Variable carbohydrate modifications to the catalytic chains of the RgpA and RgpB proteases of *Porphyromonas gingivalis* W50. *Infect Immun* **67**, 3816–3823.
- Darveau, R. P., MacIntyre, S., Buckley, J. T. & Hancock, R. E. (1983). Purification and reconstitution in lipid bilayer membranes of an outer membrane, pore-forming protein of *Aeromonas salmonicida*. *J Bacteriol* **156**, 1006–1011.
- Farquharson, S. I., Germaine, G. R. & Gray, G. R. (2000). Isolation and characterization of the cell-surface polysaccharides of *Porphyromonas gingivalis* ATCC 53978. *Oral Microbiol Immunol* **15**, 151–157.
- Fujimura, Y., Hotokezaka, H., Ohara, N., Naito, M., Sakai, E., Yoshimura, M., Narita, Y., Kitaura, H., Yoshida, N. & Nakayama, K. (2006). The hemoglobin receptor protein of *Porphyromonas gingivalis* inhibits receptor activator NF- κ B ligand-induced osteoclastogenesis from bone marrow macrophages. *Infect Immun* **74**, 2544–2551.
- Holt, S. C., Kesavalu, L., Walker, S. & Genco, C. A. (1999). Virulence factors of *Porphyromonas gingivalis*. *Periodontol* **20**, 168–238.
- Hoover, C. I. & Yoshimura, F. (1994). Transposon-induced pigment-deficient mutants of *Porphyromonas gingivalis*. *FEMS Microbiol Lett* **124**, 43–48.
- Hoover, C. I., Abarbarchuk, E., Ng, C. Y. & Felton, J. R. (1992). Transposition of Tn4351 in *Porphyromonas gingivalis*. *Plasmid* **27**, 246–250.
- Imamura, T., Pike, R. N., Potempa, J. & Travis, J. (1994). Pathogenesis of periodontitis: a major arginine-specific cysteine proteinase from *Porphyromonas gingivalis* induces vascular permeability enhancement through activation of the kallikrein/kinin pathway. *J Clin Invest* **94**, 361–367.
- Imamura, T., Potempa, J., Tanase, S. & Travis, J. (1997). Activation of blood coagulation factor X by arginine-specific cysteine proteinases (gingipain-Rs) from *Porphyromonas gingivalis*. *J Biol Chem* **272**, 16062–16067.
- Kadowaki, T., Nakayama, K., Okamoto, K., Abe, N., Baba, A., Shi, Y., Ratnayake, D. B. & Yamamoto, K. (2000). *Porphyromonas gingivalis* proteinases as virulence determinants in progression of periodontal diseases. *J Biochem* **128**, 153–159.
- Keating, D. H., Willits, M. G. & Long, S. R. (2002). A *Sinorhizobium meliloti* lipopolysaccharide mutant altered in cell surface sulfation. *J Bacteriol* **184**, 6681–6689.
- Kikuchi, Y., Ohara, N., Sato, K., Yoshimura, M., Yukitake, H., Sakai, E., Shoji, M., Naito, M. & Nakayama, K. (2005). Novel stationary-phase-upregulated protein of *Porphyromonas gingivalis* influences production of superoxide dismutase, thiol peroxidase and thioredoxin. *Microbiology* **151**, 841–853.
- Lamont, R. J. & Jenkinson, H. F. (2000). Subgingival colonization by *Porphyromonas gingivalis*. *Oral Microbiol Immunol* **15**, 341–349.
- Laus, M. C., Logman, T. J., Van Brussel, A. A., Carlson, R. W., Azadi, P., Gao, M. Y. & Kijne, J. W. (2004). Involvement of *exo5* in production of surface polysaccharides in *Rhizobium leguminosarum* and its role in nodulation of *Vicia sativa* subsp. *nigra*. *J Bacteriol* **186**, 6617–6625.
- Lin, C. S., Lin, N. T., Yang, B. Y., Weng, S. F. & Tseng, Y. H. (1995). Nucleotide sequence and expression of UDP-glucose dehydrogenase gene required for the synthesis of xanthan gum in *Xanthomonas campestris*. *Biochem Biophys Res Commun* **207**, 223–230.
- Lourbakos, A., Potempa, J., Travis, J., D'Andrea, M. R., Andrade-Gordon, P., Santulli, R., Mackie, E. J. & Pike, R. N. (2001). Arginine-specific protease from *Porphyromonas gingivalis* activates protease-activated receptors on human oral epithelial cells and induces interleukin-6 secretion. *Infect Immun* **69**, 5121–5130.
- Molhoj, M., Verma, R. & Reiter, W. D. (2004). The biosynthesis of D-galacturonate in plants. Functional cloning and characterization of a membrane-anchored UDP-D-glucuronate 4-epimerase from *Arabidopsis*. *Plant Physiol* **135**, 1221–1230.
- Naito, M., Sakai, E., Shi, Y., Ideguchi, H., Shoji, M., Ohara, N., Yamamoto, K. & Nakayama, K. (2006). *Porphyromonas gingivalis*-induced platelet aggregation in plasma depends on Hgp44 adhesin but not Rgp proteinase. *Mol Microbiol* **59**, 152–167.
- Naito, M., Hirakawa, H., Yamashita, A., Ohara, N., Shoji, M., Yukitake, H., Nakayama, K., Toh, H., Yoshimura, F. & other authors (2008). Determination of the genome sequence of *Porphyromonas gingivalis* strain ATCC33277 and genomic comparison with strain W83 revealed extensive genome rearrangements in *P. gingivalis*. *DNA Res* **15**, 215–225.
- Nakayama, K., Kadowaki, T., Okamoto, K. & Yamamoto, K. (1995). Construction and characterization of arginine-specific cysteine proteinase (Arg-gingipain)-deficient mutants of *Porphyromonas*

- gingivalis*. Evidence for significant contribution of Arg-gingipain to virulence. *J Biol Chem* **270**, 23619–23626.
- Nakayama, K., Ratnayake, D. B., Tsukuba, T., Kadowaki, T., Yamamoto, K. & Fujimura, S. (1998). Haemoglobin receptor protein is intragenically encoded by the cysteine proteinase-encoding genes and the haemagglutinin-encoding gene of *Porphyromonas gingivalis*. *Mol Microbiol* **27**, 51–61.
- Neely, A. L., Holford, T. R., Löe, H., Anerud, A. & Boysen, H. (2005). The natural history of periodontal disease in humans: risk factors for tooth loss in caries-free subjects receiving no oral health care. *J Clin Periodontol* **32**, 984–993.
- Njoroge, T., Genco, R. J., Sojar, H. T., Hamada, N. & Genco, C. A. (1997). A role for fimbriae in *Porphyromonas gingivalis* invasion of oral epithelial cells. *Infect Immun* **65**, 1980–1984.
- Okamoto, K., Kadowaki, T., Nakayama, K. & Yamamoto, K. (1996). Cloning and sequencing of the gene encoding a novel lysine-specific cysteine proteinase (Lys-gingipain) in *Porphyromonas gingivalis*: structural relationship with the arginine-specific cysteine proteinase (Arg-gingipain). *J Biochem* **120**, 398–406.
- Okamoto, K., Nakayama, K., Kadowaki, T., Abe, N., Ratnayake, D. B. & Yamamoto, K. (1998). Involvement of a lysine-specific cysteine proteinase in hemoglobin adsorption and heme accumulation by *Porphyromonas gingivalis*. *J Biol Chem* **273**, 21225–21231.
- Oliver, R. C., Brown, L. J. & Loe, H. (1998). Periodontal diseases in the United States population. *J Periodontol* **69**, 269–278.
- Potempa, J., Pavloff, N. & Travis, J. (1995). *Porphyromonas gingivalis*: a proteinase/gene accounting audit. *Trends Microbiol* **3**, 430–434.
- Potempa, J., Banbula, A. & Travis, J. (2000). Role of bacterial proteinases in matrix destruction and modulation of host responses. *Periodontol 2000* **24**, 153–192.
- Rangarajan, M., Aduse-Opoku, J., Slaney, J. M., Young, K. A. & Curtis, M. A. (1997). The prpR1 and prR2 arginine-specific protease genes of *Porphyromonas gingivalis* W50 produce five biochemically distinct enzymes. *Mol Microbiol* **23**, 955–965.
- Rangarajan, M., Aduse-Opoku, J., Paramonov, N., Hashim, A., Bostanci, N., Fraser, O., Tarelli, E. & Curtis, M. (2008). Identification of a second lipopolysaccharide in *Porphyromonas gingivalis* W50. *J Bacteriol* **190**, 2920–2932.
- Saiki, K. & Konishi, K. (2007). Identification of a *Porphyromonas gingivalis* novel protein Sov required for the secretion of gingipains. *Microbiol Immunol* **51**, 483–491.
- Sakai, E., Naito, M., Sato, K., Hotokezaka, H., Kadowaki, T., Kamaguchi, A., Yamamoto, K., Okamoto, K. & Nakayama, K. (2007). Construction of recombinant hemagglutinin derived from the gingipain-encoding gene of *Porphyromonas gingivalis*, identification of its target protein on erythrocytes, and inhibition of hemagglutination by an interdomain regional peptide. *J Bacteriol* **189**, 3977–3986.
- Sato, K., Sakai, E., Veith, P. D., Shoji, M., Kikuchi, Y., Yukitake, H., Ohara, N., Naito, M., Okamoto, K. & other authors (2005). Identification of a new membrane-associated protein that influences transport/maturation of gingipains and adhesins of *Porphyromonas gingivalis*. *J Biol Chem* **280**, 8668–8677.
- Shi, Y., Ratnayake, D. B., Okamoto, K., Abe, N., Yamamoto, K. & Nakayama, K. (1999). Genetic analyses of proteolysis, hemoglobin binding, and hemagglutination of *Porphyromonas gingivalis*. Construction of mutants with a combination of *rgpA*, *rgpB*, *kgp*, and *hagA*. *J Biol Chem* **274**, 17955–17960.
- Shoji, M., Ratnayake, D. B., Shi, Y., Kadowaki, T., Yamamoto, K., Yoshimura, F., Akamine, A., Curtis, M. A. & Nakayama, K. (2002). Construction and characterization of a nonpigmented mutant of *Porphyromonas gingivalis*: cell surface polysaccharide as an anchorage for gingipains. *Microbiology* **148**, 1183–1191.
- Shoji, M., Naito, M., Yukitake, H., Sato, K., Sakai, E., Ohara, N. & Nakayama, K. (2004). The major structural components of two cell surface filaments of *Porphyromonas gingivalis* are matured through lipoprotein precursors. *Mol Microbiol* **52**, 1513–1525.
- Slakeski, N., Bhogal, P. S., O'Brien-Simpson, N. M. & Reynolds, E. C. (1998). Characterization of a second cell-associated Arg-specific cysteine proteinase of *Porphyromonas gingivalis* and identification of an adhesin-binding motif involved in association of the prtR and prtK proteinases and adhesins into large complexes. *Microbiology* **144**, 1583–1592.
- Smalley, J. W., Birss, A. J., Szmigielski, B. & Potempa, J. (2007). Sequential action of R- and K-specific gingipains of *Porphyromonas gingivalis* in the generation of the haem-containing pigment from oxyhaemoglobin. *Arch Biochem Biophys* **465**, 44–49.
- Takii, R., Kadowaki, T., Baba, A., Tsukuba, T. & Yamamoto, K. (2005). A functional virulence complex composed of gingipains, adhesins, and lipopolysaccharide shows high affinity to host cells and matrix proteins and escapes recognition by host immune systems. *Infect Immun* **73**, 883–893.
- Vanterpool, E., Roy, F. & Fletcher, H. M. (2005). Inactivation of *vimF*, a putative glycosyltransferase gene downstream of *vimE*, alters glycosylation and activation of the gingipains in *Porphyromonas gingivalis* W83. *Infect Immun* **73**, 3971–3982.
- Vanterpool, E., Roy, F., Zhan, W., Sheets, S. M., Sangberg, L. & Fletcher, H. M. (2006). VimA is part of the maturation pathway for the major gingipains of *Porphyromonas gingivalis* W83. *Microbiology* **152**, 3383–3389.
- Wingrove, J. A., Discipio, R. G., Potempa, J. & Travis, J. (1992). Activation of complement components C3 and C5 by a cysteine proteinase (gingipain-1) from *Porphyromonas gingivalis*. *J Biol Chem* **267**, 18902–18907.

Edited by: P. Kolenbrander