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

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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 Tn 4351 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.

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

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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 (fimbrijn) 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 microorganism, 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 μg ml⁻¹; chloramphenicol (Cm), 20 μg ml⁻¹; erythromycin (Em), 10 μg ml⁻¹; and tetracycline (Tc), 0.7 μg ml⁻¹.

**Construction of plasmids and bacterial strains.** For nucleotide sequencing of chromosomal DNA regions in the vicinity of Tn4351 insertion sites, PstI 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 PstI site of plu4K10II (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 NotI plus BamHI and BamHI plus KpnI, respectively, and both digested DNAs were ligated with plu4K10II DNA double-digested with NotI plus KpnI, resulting in pRF1. The 2.2 kb BamHI–BglII eDNA cartridge of pKD399 (Shoji et al., 2004) was inserted into the BamHI site of pRF1 to yield pRF2 (rfa-2::[ermF ermAM]). P. gingivalis 33277 was then transformed with NotI-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 PstI and inserted into the BamHI site of pKD703 (Shoji et al., 2004) containing the tetQ DNA block of pKD375 (Shi et al., 1999) at the BamHI site, resulting in pRF3 (fimA::[rfa⁺ tetQ]). Strain Prfa1 was then transformed with NotI-linearized DNA of pRF3 to yield strain Prfa2 (rfa-2::[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 CN0613F/CN0613R and cloned into plasmid pCR4-TOPO (Invitrogen). The resulting plasmid was digested with Aval and then treated with the Klenow fragment and ligated with the BglII linker to yield pUG1. The BglII–BglII eDNA cartridge was inserted into the BglII site of pUG1, resulting in pUG2 (ugdA2::[ermF ermAM]). Strain 33277 was transformed with NotI-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 BamHI, and inserted into the BamHI site of pKD713, resulting in pUG3 (fimA::[ugd⁺ tetQ]). Strain Pugd1 was then transformed with NotI-linearized DNA of pUG3 to yield strain Pugd2 (ugdA2::[ermF ermAM] fimA::[ugd⁺ 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 SalI plus PstI and inserted into the SalI–PstI region of pBluescript II SK(−) to yield pKD856. The C-terminal domain DNA (0.4 kb) of rrgB 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 rrgB was PCR-amplified from 33277 chromosomal DNA using the primer pair BCF/F and inserted into the corresponding region of pKD856 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 KM/F and KM/R. The amplified DNA was digested with XhoI plus BgII and inserted into the corresponding region of pKD856 to yield pKD859. Next, the Aval–Aval region (0.3 kb) of pKD859 was replaced by a 2.6 kb Aval 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 (kkg'–rrgB'–myc). The KpnI–NotI DNA fragment of pKD860 containing the kgp'–rrgB'–myc chimera gene DNA was treated with T4 DNA polymerase and ligated with the BamHI–digested and Klenow-treated pKD713 DNA (Kikuchi et al., 2005), resulting in pKD861 (fimA::[kkg'–rrgB'–myc tetQ]). For construction of P. gingivalis strains possessing the kgp'–rrgB'–myc chimera gene, KDP129 (kkg) and KDP351 (kpg porT) were transformed with NotI–linearized pKD861 DNA to yield KDP380 (kkg fimA::[kkg'–rrgB'–myc tetQ]) and KDP381 (kpg porT fimA::[kkg'–rrgB'–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 N1255F/N1255R. The amplified DNA was digested with XhoI plus HindIII and inserted into the corresponding region of pKD856 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 BamHI–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 BsrIII-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 erm–ermAM DNA cartridge (2.2 kb) obtained from pKD399 by BamHI 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 (OD$_{600}$ 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 10000 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$^\text{-}$p-tosyl-L-hisine 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-Lyzer 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 10000 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 100000 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 MgCl$_2$ 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 EView 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 (GPKpN; Sigma) and N-2-benzoyl-DL-Arg-p-nitroanilide (BApkN; Sigma) in 50 mM Tris/HCl (pH 8.5) containing 5 mM i-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 kkg'–rgpB'–myc chimera gene product (sKgp-RgpB-Myc) was purified using EView 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 EView 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 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 µl per well of a 10 µg 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 µl 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 µl peroxide ABTS [2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)] substrate was added. The binding activities were assessed by measuring A₄₅₀ 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, CDVYTDHGDLNVPRM, was conjugated to keyhole limpet haemocyanin, purchased from Sigma Genosis. 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.

*PstI* 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

**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, CDVYTDHGDLNVPRM, was conjugated to keyhole limpet haemocyanin, purchased from Sigma Genosis. Anti-Hgp44 antiserum was obtained as described previously (Sato et al., 2005).

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
constructed mutants with disruption of udgA and rfa by gene-directed mutagenesis. We first constructed suicide vector plasmids containing the disrupted rfa (plasmid pRF2) and udgA (plasmid pUG2). Introduction of NotI-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 udgA 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-9 myc chimaera genes were introduced into the fimA locus of strain Prfa1, resulting in strains Prfa2 (rfa fimA::rfa+) and Prfa3 (rfa fimA::rfa-9 myc), respectively. The udgA+ gene was introduced into the fimA locus of strain Pugd1, resulting in strain Pugd2 (udgA fimA::udgA+). 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 udgA mutant showed almost the same haemagglutination as that of the wild-type strain. These results demonstrated that the rfa and udgA 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 udgA 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 udgA 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 udgA 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 polyproteins 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 (udgA), 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. 2. Physical maps around the rfa and udgA gene regions. Triangles indicate the Tn4351 insertion sites of strains Tn6-5, Tn7-1, Tn7-3 and Tn10-4. P, PstI.](image)
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,

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**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 OD900 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 (fimA::fimA'); 8, Prfa1 (rfa); 9, 33277 (wild-type); 10, Prfa3 (fimA::fimA').

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**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 (fimA::fimA'); 5, Prfa3 (rfa fimA::fimA'); 6, Pugd1 (ugdA).
SDDPEKVPFV, corresponding to that encoded by the propeptide DNA region of the \textit{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 \textit{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 \textit{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 \textit{rfa} mutant and that all of the protein molecules were secreted into the extracellular milieu. Immunoblot analysis with mAb 1B5 revealed that the \textit{rfa} mutant was defective in production of anionic polysaccharides and glycan additions to Rgp. In the \textit{ugdA} mutant, immunoblot profiles with anti-Kgp and anti-Hgp44 were almost the same as those of the wild-type parent strain. The \textit{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 \textit{rfa’-’myc} chimera gene in which \textit{rfa} and \textit{myc-tag} were fused. Strain Prfa3 (\textit{rfa fimA::rfa’-’myc}), in which the chimera gene was introduced into the \textit{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. 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).

(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 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
purified an LPS fraction from the \( \text{rf} \) deletion mutant, but could not perform ELISA analysis due to hydrophobicity of the purified LPS. Besides the \( \text{rf} \) deletion mutant Prfa1, we constructed an \( \text{rf} \) insertion mutant (Prfa4), which had replacement of an internal region of the \( \text{rf} \) 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 \( \mu \)-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 kgpB 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 against some isoforms of Rgp and cell-associated carbohydrate of 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 AccIII 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.

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