molecular oral microbiology

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Identification and characterization of novel glycoproteins involved in growth and biofilm formation by *Porphyromonas gingivalis*

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SUMMARY

Porphyromonas gingivalis **has been implicated as a major pathogen associated with chronic periodontitis. To extend our knowledge of post-translational protein glycosylation in *P. gingivalis*, a proteomic analysis involving two-dimensional polyacrylamide gel electrophoresis combined with carbohydrate staining and mass spectrometry was performed. Four novel glycoproteins, PGN0743, PGN0876, PGN1513 and PGN0729, in *P. gingivalis* ATCC 33277 were identified. These four identified glycoproteins possess a range of biochemical activities and cellular localization. PGN0743 contains a sequence motif identifying it as a FKBP-type cis-trans isomerase, which has activity usually associated with chaperone functions. PGN0876 and PGN1513 contain tetratricopeptide repeat domains that mediate protein–protein interactions. PGN0729 encodes the outer membrane protein 41 precursor, which was previously identified as Pgm6, and is homologous to the OmpA protein in *Escherichia coli.* Several different types of glycoprotein were identified, suggesting that *P. gingivalis* possesses a general mechanism for protein glycosylation. PGN0743-deficient and PGN0876-deficient mutants were constructed to examine the role(s) of the two identified glycoproteins. Both mutants showed a decreased growth rate under nutrient-limited conditions and reduced biofilm formation activity. These results suggest that the novel glycoproteins PGN0743 and PGN0876 play an important role in the growth and colonization of *P. gingivalis*.**

INTRODUCTION

Porphyromonas gingivalis has been implicated as a major pathogen associated with chronic periodontitis (Lamont & Jenkinson, 1998). The bacterium expresses a number of potential virulence factors, including lipopolysaccharide, gingipains and fimbriae (Holt & Ebersole, 2005). Gingipains, composed of arginine-specific (RgpA and RgpB) and lysine-specific (Kgp) proteases, are thought to be involved in a wide range of pathological and physiological processes (Imamura *et al.*, 2003). *P. gingivalis* has at least two types of fimbriae, termed FimA and Mfa1, which are distinguished by the sizes of the major fimbrilin subunits (Yoshimura *et al.*, 2009). FimA fimbriae are adhesins, which mediate the colonization

of oral surfaces and the invasion of host cells (Amano *et al.*, 2004). Although the role of Mfa1 fimbriae is less well understood, recent studies show that they are necessary for synergistic biofilm formation by *P. gingivalis* and *Streptococcus gordonii* (Park *et al.*, 2005).

Although eukaryotic organisms possess mechanisms responsible for the *N*-glycosylation and *O*-glycosylation of almost half of their cellular proteins, protein glycosylation is thought to be rare in bacteria. Glycosylation in bacteria is usually limited to several abundant polymeric surface proteins, such as flagellins, pilins and S-layer proteins (Schäffer *et al.*, 2001; Benz & Schmidt, 2002; Fletcher *et al.*, 2007). The only well-characterized general glycosylation mechanism in bacteria is that in *Campylobacter jejuni* and related species, in which glycans are N-linked to asparagine residues (Szymanski *et al.*, 1999; Wacker *et al.*, 2002). A recent study identified a general *O*-glycosylation system in *Bacteroides fragilis* that seems to be central to its physiology (Fletcher *et al.*, 2009). *P. gingivalis* belongs to the same phylum (*Cytophaga-Flavobacterium-Bacteroides*) as *B. fragilis*; however, few cell surface glycoproteins have been reported for *P. gingivalis*. Rgps are glycosylated, with their carbohydrate domains containing phosphorylated branched mannans that can contribute to the anchoring of Rgps to the outer membrane (Paramonov *et al.*, 2005). The OMP85 protein, an outer membrane protein expressed by *P. gingivalis*, is glycosylated (Nakao *et al.*, 2008). Another study demonstrated that the native Mfa1 fimbria is actually a glycoprotein that contains fucose, mannose, *N*-acetylglucosamine, and *N*-acetylgalactosamine (Zeituni *et al.*, 2010).

The aim of the present study was to perform a comprehensive analysis of glycosylated proteins expressed by *P. gingivalis* to further our knowledge of the post-translational modifications occurring in this bacterium. We identified four glycoproteins with different predicted functions: PGN0743 (a probable FKBP peptidyl proryl cis-trans isomerase), PGN0876 [a tetratricopeptide repeat (TPR) domain protein], PGN1513 (a hypothetical protein) and PGN0729 (an outer membrane protein 41 precursor). The present study also used isogenic mutants to show that PGN0743 and PGN0876 are involved in biofilm formation and growth.

METHODS

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are shown in Table 1. All *P. gingivalis* strains were maintained on Brucella HK agar (Kyokuto Pharmaceutical Industrial, Tokyo, Japan) supplemented with 5% [volume/volume (v/v)] laked rabbit blood, 2.5 μg ml^{-1} haemin, 5 μg ml^{-1} menadione and 0.1 μg ml^{-1} dithiothreitol (DTT), plus appropriate antibiotics at 37°C for 7 days under anaerobic conditions [10% (v/v) CO_2, 10% (v/v) H_2 and 80% (v/v) N_2]. Erythromycin (final concentration, 10 μg ml^{-1}) or chloramphenicol (5 μg ml^{-1}) was added to the medium when necessary. The *P. gingivalis* was cultured in sTSB liquid medium [trypticase soy broth supplemented with 0.25% (weight/volume; w/v) yeast extract, 2.5 μg ml^{-1} haemin, 5 μg ml^{-1} menadione and 0.1 μg ml^{-1} DTT]. For the growth experiments Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% (w/v) bovine serum albumin (BSA; fraction V; Wako, Osaka, Japan) was also used as previously described (Nagano *et al.*, 2007). *Escherichia coli* were grown in Luria–Bertani medium supplemented, when necessary, with 50 μg ml^{-1} ampicillin, 50 μg ml^{-1} kanamycin or 200 μg ml^{-1} erythromycin.

DNA manipulations

Restriction endonucleases, DNA ligase and related enzymes were purchased from Takara (Otsu, Japan) or New England Biolabs (Ipswich, MA). The oligonucleotides used for polymerase chain reaction (PCR; Table 2) were synthesized by Sigma Genosys (Ishikari, Japan). Standard PCR experiments were performed using a high-fidelity DNA polymerase, Pyrobest (Takara), in a PCR Thermal Cycler Dice™ (Takara).

Construction of mutants

All mutants used in this study were derivatives of *P. gingivalis* ATCC 33277. An erythromycin resistance gene cassette (*ermF-ermAM*) was inserted into the gene of interest (Fletcher *et al.*, 1997). To construct the PGN0743-deficient mutant, the internal sequence of *pgn0743* was amplified by PCR with a

Table 1 Bacterial strains and plasmids

Strain or plasmid	Genotype or relevant characteristics	Source or reference
Porphyromonas gingivalis		
ATCC 33277	Wild-type, type strain	ATCC
PGN0743::erm	PGN0743-insertional mutant from ATCC 33277, Em^r	This study
PGN0876::erm	PGN0876-insertional mutant from ATCC 33277, Em^r	This study
JI-1	FimA-deletion mutant from ATCC 33277, Cm^r	Hasegawa *et al.* (2009)
Escherichia coli		
DH5α	As chemically competent cells	Takara
TOP10	As chemically competent cells	Invitrogen
Plasmids		
pCR-Blunt II-TOPO	Cloning vector, Km^r	Invitrogen
pVA2198	Plasmid use for a drug cassette, *ermF-ermAM*, Em^r	Fletcher *et al.* (1997)
pCR-TOPO PGN0743	pCR-Blunt II-TOPO containing 1.3-kb *pgn0743* region, Km^r	This study
pCR-TOPO PGN0876	pCR-Blunt II-TOPO containing 2.2-kb *pgn0876* region, Km^r	This study
pCR-TOPO PGN0743::erm	*ermF-ermAM* was inserted into *Sca*I site of pCR-PGN0743, Km^r Em^r	This study
pCR-TOPO PGN0876::erm	*ermF-ermAM* was inserted into *Bgl*II site of pCR-PGN0876, Km^r Em^r	This study

Cm^r, chloramphenicol resistance; Em^r, erythromycin resistance; Km^r, kanamycin resistance; ATCC, American Type Culture Collection.

high-fidelity, blunt-end-producing DNA polymerase (Pyrobest, Takara) with primers PGN0743F and PGN0743R (Table 2), whose sequences were designed based on the *P. gingivalis* ATCC 33277 genome sequence database (Naito *et al.*, 2008) (http://www.ncbi.nlm.
nih.gov/pubmed/). The amplified 1.3-kbp fragment was ligated into a pCR-BluntII-TOPO plasmid vector (Invitrogen, Carlsbad, CA), and the resulting plasmid, named pCR-TOPO PGN0743, was introduced into host *E. coli* TOP10 cells (Invitrogen). *ermF-ermAM*, in which *ermF* (expressed in *Porphyromonas* and *Bacteroides* spp.) and *ermAM* (expressed in *E. coli*) were present, was excised from plasmid pVA2198 by digestion with *Pvu*II and inserted into the *Sca*I site of *pgn0743*. The fragment containing *ermF-ermAM* was linearized with *BstE*I and *Nco*I, and *P. gingivalis* ATCC 33277 was mutated by transformation with the linearized DNA. The PGN0876 disruption mutant was constructed in a similar manner. In brief, the internal sequence of *pgn0876* was amplified by PCR with primers PGN0876F and PGN0876R (Table 2). The 2.2-kbp PCR product was ligated into a pCR-BluntII-TOPO plasmid vector and the resulting plasmid, named pCR-TOPO PGN0876, was introduced into host *E. coli* TOP10. The erythromycin cassette was inserted into the *Bgl*II site in the *pgn0876* fragment. After the fragment containing *ermF-ermAM* was linearized with *Nco*I and *Nru*I, it was used to mutate *P. gingivalis* ATCC 33277. The specific gene disruptions were confirmed by PCR and Southern blotting (data not shown).

Table 2 Primers used in this study

Primer	Sequence (5′–3′)
PGN0743F	GGGCCCGAAACCCACCCTTTCGG
PGN0743R	GCGGGACATTTCGAACTGATTGG
PGN0876F	GGTTCGTCCTTCGGGTCGTACAG
PGN0876R	GCTCGGCAAGCCATTTACTGGTC

P. gingivalis cell fractionation and sample preparation

All fractionation procedures were performed at 4°C. Bacterial cells were collected by centrifugation at 10,000 ***g*** for 20 min. Cells were washed gently and then resuspended in 10 mM phosphate-buffered saline (PBS, pH 7.4) containing protease inhibitors (0.1 mM *N*-α-*p*-tosyl-L-lysine chloromethyl ketone, 0.2 mM phenylmethylsulphonyl fluoride and 0.1 mM leupeptin) to prevent proteolytic degradation. Whole-cell samples for two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) were prepared by treating washed cells with 10% trichloroacetic acid (TCA) for 20 min on ice. The TCA-treated cells were recovered by centrifugation at 20,000 ***g*** for 20 min and washed twice with diethyl ether to remove the TCA. After being dried at room temperature, the resulting material was used as whole-cell proteins. For preparation of the soluble fraction, the cells were disrupted using a sonicator (Bioruptor UCD-200T; Cosmo Bio, Tokyo,

Japan) using repeated 30-s bursts at 30-s intervals for 15 min. After unbroken cells were removed by centrifugation at 1,000 ***g*** for 10 min, the supernatant was used as the whole-cell lysate. The whole-cell lysate was subjected to ultracentrifugation at 100,000 ***g*** for 60 min in a Beckman fixed-angle rotor (TLA-100.2; Palo Alto, CA) to separate the sediment from the supernatant (Murakami *et al.*, 2002). The supernatant was used as soluble fraction for 2D-PAGE.

2D-PAGE

Isoelectric focusing in the first dimension was carried out in an Ettan IPGphor II equipped with a cup-loading manifold (GE Healthcare UK Ltd., Buckinghamshire, England) as described previously (Masuda *et al.*, 2006). The whole-cell and soluble fraction samples were dissolved in solubilization solution {7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulphonic acid [CHAPS], 1 mM ethylenediamine tetraacetic acid [EDTA], 40 mM Tris base, and 0.2% tributyl phosphine}. The dissolved samples were applied to Immobiline DryStrips (pH 4–7 and pH 6–11; 13 cm; GE Healthcare UK Ltd) swollen with a rehydration solution [7 M urea, 2 M thiourea, 4% CHAPS, 0.5% IPG buffer (pH 4–7 and pH 6–11; GE Healthcare UK Ltd), 1 mM EDTA, 50 mM DTT, and bromophenol blue]. Isoelectric focusing was initially conducted at 100 V for 1 h, then at 500 V gradually increasing to 5,000 V for 3 h, and then at 5,000 V for 6 h. After isoelectric focusing, the strips were equilibrated in buffer [50 mM Tris–HCl, pH 8.8, 6 M urea, 30% glycerol, 2% sodium dodecyl sulphate (SDS) and bromophenol blue] containing 1% DTT followed by incubation in the same buffer (except that DTT was replaced by 2.5% iodoacetamide). The equilibrated strips were layered on top of 12% polyacrylamide gels using molten agarose. The SDS–PAGE was then performed in the second dimension (Masuda *et al.*, 2006) and the gels were stained with ProQ Emerald 300 (Invitrogen) and Coomassie brilliant blue R-250 (CBB).

Protein analysis by mass spectrometry

After 2D-PAGE, protein spots were analysed using matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) as described previously (Masuda *et al.*, 2006; Abe *et al.*, 2011). After in-gel tryptic digestion, peptides were extracted, concentrated and analysed using a 4800 MALDI TOF/TOF Analyzer (Applied Biosystems, Foster City, CA). Proteins were identified from the MS peaks using the MS-Fit peptide mass fingerprinting methods in the Mascot program (http://www.matrixscience.com/).

Detection of glycosylated proteins

To detect glycosylations in *P. gingivalis*, samples were run on 2D-PAGE and carbohydrates were stained using the ProQ Emerald 300 glycoprotein stain kit (Invitrogen) according to the manufacturer's instructions. Briefly, gels were incubated in 100 ml of a 50% methanol/5% acetic acid fixative solution for 1 h followed by incubation in fresh fixative solution overnight to completely remove residual SDS. The gel was then washed three times in 3% acetic acid for 15 min each time, and the glycans were oxidized by incubation in 1% periodic acid/3% acetic acid for 1 h. The gel was washed four times for 15 min each in 3% acetic acid to remove any residual periodate. The gel was then incubated in ProQ Emerald 300 dye solution for 120 min followed by three incubations in 3% acetic acid (each for 15 min). The green fluorescence signal from the ProQ Emerald 300 dye was visualized under UV illumination at 300 nm. The CandyCane glycoprotein molecular weight standards (Invitrogen) provided in the ProQ Emerald glycoprotein stain kit were used as positive controls for staining.

Saliva

Saliva was collected in a sterile centrifuge tube on ice from healthy donors and pooled, as described previously (Palmer *et al.*, 2001; Kuboniwa *et al.*, 2009). DTT was added to a 2.5 mM final concentration, then the saliva was gently stirred on ice for 10 min and centrifuged at 3,000 ***g*** for 20 min at 4°C. The clarified saliva supernatant was decanted, 3 volumes of distilled water was added, and the 25% saliva was filtered through a 0.22-μm pore size filter and frozen in 40-ml aliquots. Immediately before use, the sterile saliva was thawed at 37°C; the slight precipitate was pelleted at 1,430 ***g*** for 5 min, and the clear 25% saliva supernatant was used in experiments.

P. gingivalis biofilm formation

Biofilm formation by *P. gingivalis* was quantified using a microtitre plate assay (Merritt *et al.*, 2005) specifically adapted for *P. gingivalis* (Chen *et al.*, 2002; Kuboniwa *et al.*, 2009). Briefly, overnight cultures of *P. gingivalis* were diluted 1/20 in diluted sTSB medium (sTSB/PBS ratio, 1 : 2). Aliquots (200 μl) of the diluted samples were anaerobically incubated in the 25% saliva-coated wells of a flat-bottom 96-well polystyrene plate (BD, Franklin Lakes, NJ) for 24 h at 37°C. A sample (100 μl) of each cell culture was measured for optical density at 600 nm (OD_{600}) to assess planktonic bacterial growth. After discarding the planktonic bacterial cells in the wells, bacterial cells bound to the wells were gently washed three times with 10 mM PBS (pH 7.4), air dried, and then stained with 200 μl of 0.5% (w/v) crystal violet for 15 min. After washing twice with 10 mM PBS (pH 7.4) and then with sterile water to remove excess dye, the cell-bound dye was eluted using 200 μl of 99% ethanol. Biofilm formation was quantified by measuring the OD_{595}. Biofilms formed on polystyrene dishes were also evaluated by microscopic observation. Briefly, overnight cultures of *P. gingivalis* were diluted 1/20 in sTSB. Aliquots (5 ml) of the diluted samples were anaerobically incubated in the 25% saliva-coated dish (60 mm in diameter, Nunc, Tokyo, Japan) for 24 h at 37°C. The biofilms formed on each sheet were washed twice with 10 mM PBS (pH 7.4), stained for 15 min with 0.5% (w/v) crystal violet, and rinsed twice with sterile water. Biofilms were observed under an optical microscope (Olympus BX41; Tokyo, Japan). JI-1, a mutant deficient in *fimA* (Hasegawa *et al.*, 2009), was included as a negative control for biofilm formation.

Statistical analysis

Data were evaluated by analysis of variance and the Dunnett's multiple comparisons test ($P < 0.01$).

RESULTS

Proteomic analysis of whole-cell proteins

We investigated the proteomic profile of whole-cell proteins separated by 2D-PAGE before a comprehensive analysis of novel glycoproteins in *P. gingivalis*. Although proteomic analyses of *P. gingivalis* whole-cell proteins using 2D-PAGE have been reported (Masuda *et al.*, 2006; Yoshimura *et al.*, 2008), these studies have covered only a limited number of the *P. gingivalis* proteins. In the present study, whole-cell proteins from *P. gingivalis* were separated by 2D-PAGE in the pI 4–7 and pI 6–11 ranges, resulting in a reproducible pattern of spots (see Fig. S1). A Mascot search of the obtained peptide mass fingerprint spectra identified the proteins in 260 spots in the pI 4–7 range and in 39 spots in the pI 6–11 range (see Fig. S1 and Table S1). This proteome map was fundamental to the glycoprotein analysis of *P. gingivalis* ATCC 33277 described below. Four candidate glycoproteins (described below) were identified from the 299 spots (see Fig. S1A,B and Table S1).

Identification of glycoproteins in *P. gingivalis*

The proteomic approach is applicable to a comprehensive analysis of novel glycoproteins because 2D-PAGE combined with ProQ Emerald staining can analyse multiple proteins in a single gel with high sensitivity and reproducibility. Whole-cell proteins from *P. gingivalis* were separated by 2D-PAGE in the pI 4–7 and pI 6–11 ranges, resulting in a reproducible pattern of spots (Fig. 1A,C). To identify specific glycoproteins, 2D-PAGE gels were stained with the ProQ Emerald stain, which is a general stain for carbohydrates (Fig. 1B,D). Analysis of glycoproteins in the pI 4–7 range revealed three major spots with strong fluorescence intensity and masses of 35 kDa (Spot No. 1), 42 kDa (Spot No. 2) and 30 kDa (Spot No. 3). The 35-kDa, 42-kDa and 30-kDa proteins were identified as PGN0876, PGN1513 and PGN0743, respectively, by a MASCOT search of the obtained peptide mass fingerprint spectra (Table 3). The 41-kDa protein (Spot No. 4) appeared in the pI 6–11 range and was identified as PGN0729 (Table 3). The four identified glycoproteins had different biochemical activities. PGN0876 contains tetratricopeptide repeat (TPR) domains that mediate protein–protein interactions, and PGN0743 possesses a sequence motif identifying it as the FKBP-type peptidyl proryl cis-trans isomerase (PPIase), FkpA, whose activity is usually associated with chaperone functions (Duguay & Silhavy, 2004). PGN1513 was identified as a hypothetical protein. The search

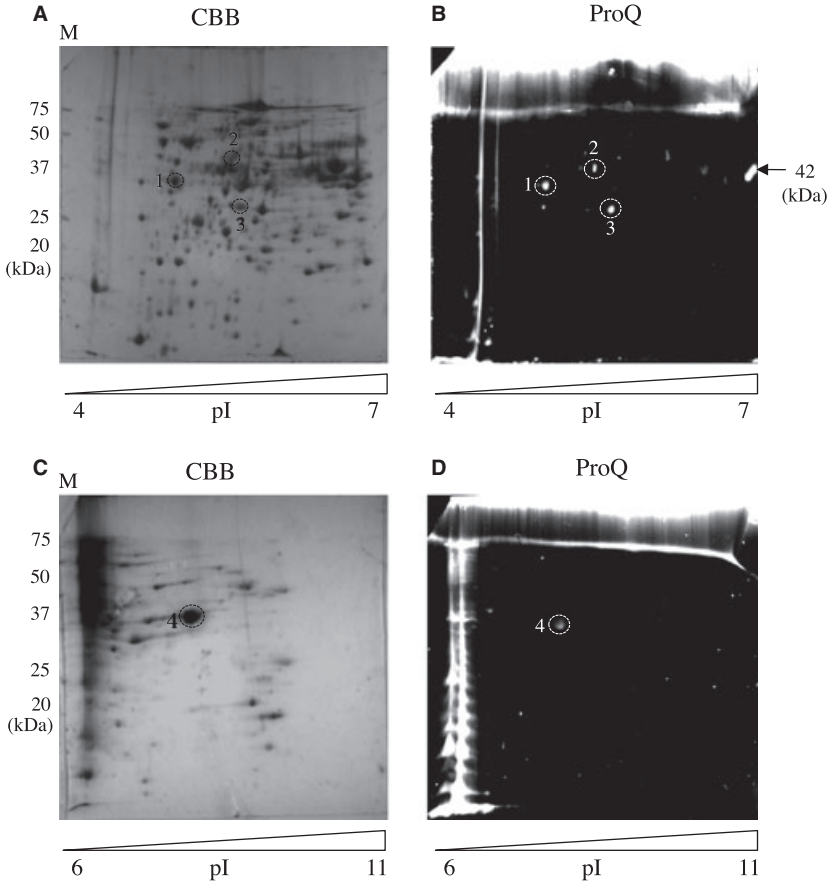

Figure 1 Coomassie brilliant Blue (CBB) -stained and ProQ Emerald-stained two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) gels containing *P. gingivalis* whole-cell proteins. Whole-cell proteins were separated by 2D-PAGE in the ranges pI 4–7 (A, B) and pI 6–11 (C, D). The gels were first stained with ProQ Emerald to identify glycoproteins (right panel) and then stained with CBB to identify proteins (left panel). The arrow indicates the glycoprotein marker (CandyCane). The number of circles refers to the glycoproteins shown in Table 3. pI = isoelectric point, with pH increasing from left to right across the gel.

Table 3 *P. gingivalis* glycoproteins detected by ProQ Emerald staining

Spot no.[1]	CDS no.[2]	Predicted mass (kDa)	Subcellular location	Annotation
1, 5, 6, 7	PGN0876	46	Periplasm	TPR domain protein
2, 8, 9, 10	PGN1513	51	Periplasm	Conserved hypothetical protein
3, 11	PGN0743	28	Periplasm	Probable FKBP-type peptidyl-prolyl cis-trans isomerase FkpA
4	PGN0729	43	Outer membrane	Outer membrane protein 41 precursor

[1]Spot number refers to the protein spots in Figs 1–3.
[2]Protein coding sequence (CDS) number of *P. gingivalis* ATCC 33277 in the genome database (Naito *et al.*, 2008).

domain annotation program [SMART (http://smart.embl-heidelberg.de/)] revealed that PGN1513 contains three TPR domains located on residues 43–73, 287–320 and 324–357. PGN0729 encodes the outer membrane protein 41 precursor that we previously identified as Pgm6, which is homologous to the OmpA protein in *E. coli* (Murakami *et al.*, 2002). Therefore, *P. gingivalis* possess glycosylated proteins with a range of different functions and they are distinct from typical bacterial glycoproteins, such as gingipains and Mfa1 fimbriae, as previously reported (Paramonov *et al.*, 2005; Zeituni *et al.*, 2010). Next, the LipoP program (Juncker *et al.*, 2003) was used to predict the localization of the identified glycopro-

teins. PGN0876, PGN0743 and PGN1513 were predicted to have signal peptides and type I signal peptidase cleavage sites, but lacked trans-membrane helical regions, suggesting that they are likely to be present in the periplasm. Consistent with this prediction, PGN0876, PGN0743 and PGN1513 were detected in the soluble fraction in the pI 4–7 and 6–11 ranges (Fig. 2B and Table 3). Therefore, these three proteins are likely to be located in the periplasm or cytoplasm. However, PGN0729 was not detected in the soluble fraction in the pI 6–11 range (data not shown), suggesting that PGN0729 (Pgm6) is likely to be located at the cell membrane. Indeed, our previous reports show that Pgm6/7 localizes to the outer membrane as heterotrimers (Murakami *et al.*, 2002; Nagano *et al.*, 2005).

PGN0743- and PGN0876-deficient mutants

We next focused on PGN0743, PGN0876 and PGN1513, because they appear to be unique among soluble *P. gingivalis* glycoproteins. Insertion mutants were constructed to determine the roles of PGN0743, PGN0876 and PGN1513. We successfully constructed PGN0743- (PGN0743::erm) and PGN0876-deficient mutants (PGN0876::erm); however, we could not obtain a PGN1513-deficient mutant. The reason for this is unclear: PGN1513 may be an essential gene, or there may have been a technical problem during mutagenesis. To confirm the absence of PGN0743 and PGN0876 protein expression, 2D-PAGE was performed using whole-cell proteins derived from PGN0743::erm and PGN0876::erm. The protein spots observed for wild-type PGN0743 and PGN0876 in CBB-stained gels (Fig. 3A) were not present in PGN0743::erm (Fig. 3C, spot 3) and PGN0876::erm (Fig. 3E, spot 1). Fluorescent spots No. 3 and No. 1 completely disappeared from gels stained with ProQ Emerald shown in Fig. 3D, F. These observations provide evidence that the PGN0743 and PGN0876 proteins are glycosylated, and transcribed from the *pgn0743* and *pgn0876* genes, respectively.

Growth defects in PGN0743::erm and PGN0876::erm under nutrient-limited conditions

The growth rates of both mutants in sTSB, or DMEM containing 1% BSA, under anaerobic conditions at 37°C were compared with those of the wild type. The experiments were repeated at least twice, and typical results are shown in Fig. 4. In sTSB, the 'normal' medium for this organism, the growth rates of PGN0743::erm and PGN0876::erm were comparable with that of the wild type, and all of them reached the stationary phase within 18 h (Fig. 4A). However, in DMEM containing 1% BSA, PGN0876::erm showed growth defects (Fig. 4B). PGN0743 mutants showed slightly slower growth rates compared with the wild-type strain. The wild type reached the stationary phase at 18 h, similar to its growth rate in sTSB; however, PGN0743::erm reached the same level at about 30 h

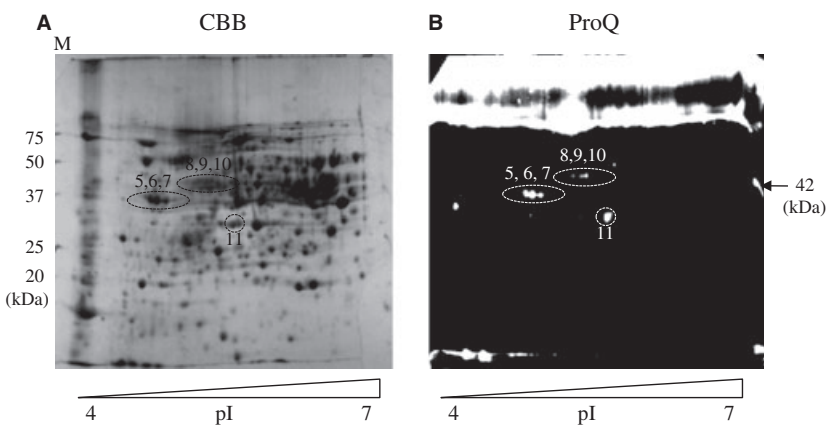

Figure 2 Coomassie brilliant Blue (CBB) -stained and ProQ Emerald-stained two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) gels containing *P. gingivalis* soluble fractions. Soluble fractions separated in the range pI 4–7 were stained with CBB (A) and with ProQ Emerald (B). pI = isoelectric point, with pH increasing from left to right across the gel. The arrow indicates the glycoprotein marker (CandyCane). The number of circles refers to the glycoproteins shown in Table 3.

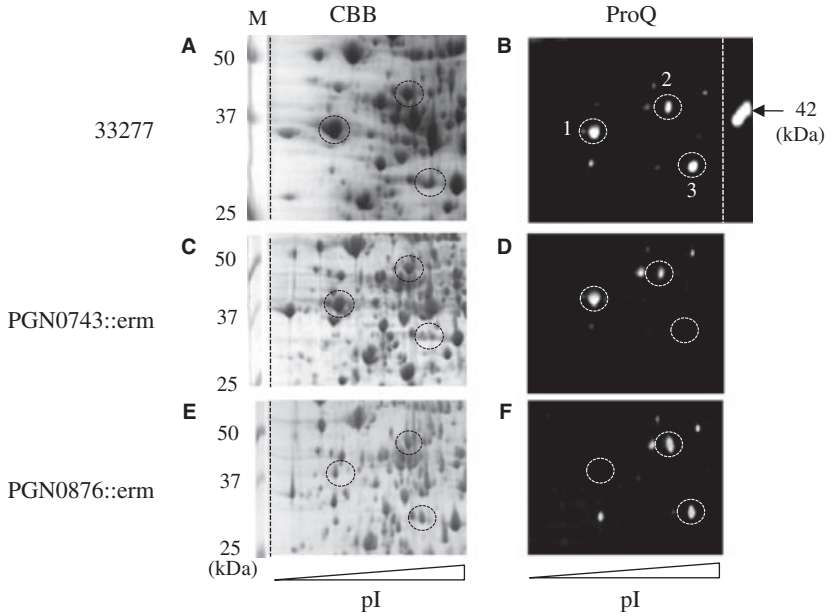

Figure 3 Comparison of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) gel patterns for whole-cell proteins between *P. gingivalis* ATCC 33277 (A, B), PGN0743::erm (C, D), and PGN0876::erm (E, F). Whole-cell proteins were separated by 2D-PAGE in the range pI 4–7. The gels were first stained with ProQ Emerald for glycoprotein analysis (B, D and F) and then with Coomassie Brilliant Blue for protein analysis (A, C and E). The symbols are the same as in Fig. 1.

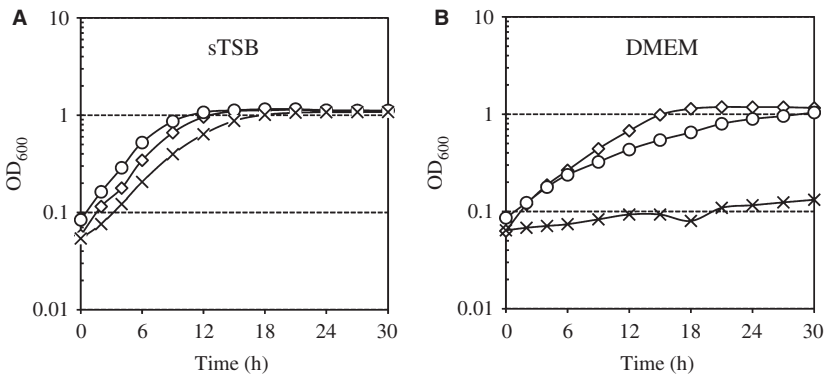

Figure 4 Growth curves for the glycoprotein mutants. *P. gingivalis* ATCC 33277 (◇), PGN0743::erm (○), and PGN0876::erm (×) were cultured in supplemented trypticase soy broth (sTSB; left panel, A) or Dulbecco's modified Eagle's medium supplemented with 1% (w/v) bovine serum albumin (right panel, B). Each medium was inoculated with an early-stationary-phase culture in sTSB at a 1 : 20 ratio. The result is representative of three replicates.

(Fig. 4B). This suggests that PGN0743 and PGN0876 are not essential for growth in rich media under laboratory conditions, but may be required for growth under nutrient-limited conditions or for growth *in vivo*.

Decreased biofilm formation of PGN0743::erm and PGN0876::erm

Biofilm formation by PGN0743::erm and PGN0876::erm was compared with that by the wild-type strain. The levels of biofilm formation on the flat bottoms of 96-well plates were estimated by measuring OD_{595} after staining the bacteria with crystal violet (Fig. 5A). Biofilm formation by PGN0743::erm and PGN0876::erm was significantly decreased compared with that by the wild-type strain, and both mutants formed a similar amount of biofilm compared with JI-1 (Δ*fimA*; used as a negative control). By contrast, the amount of mutant planktonic cells was not significantly different from wild-type strain (data not shown).

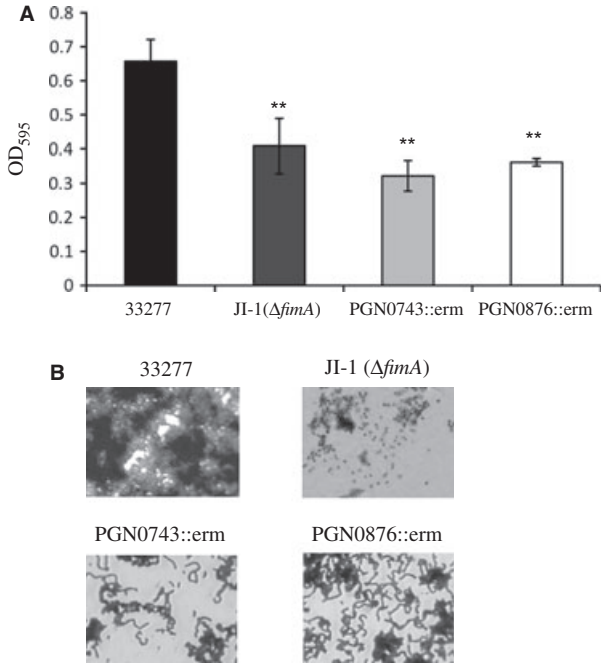

Figure 5 Biofilm formation by the wild type, glycoprotein mutants and the JI-1 (Δ*fimA*) negative control. The amount of biofilm was quantified by measuring the optical density at 595 nm (OD_{595}) followed by crystal violet staining and ethanol elution (A). Data represent the mean ± SD ($n = 3$). $^{**}P < 0.01$ (Dunnett's test). Biofilm structures stained with crystal violet were visualized under a microscope (B). Bars = 10 μm.

Microscopic observation revealed that wild-type cells formed a large amount of biofilm, whereas PGN0743::erm and PGN0876::erm cells produced much less, reflecting the results obtained using crystal violet in the biofilm assay (Fig. 5B). These results showed that PGN0743 and PGN0876 play a role in biofilm formation. Because autoaggregation plays an important role in initial biofilm formation (Kolenbrander *et al.*, 2006), we evaluated the level of autoaggregation in the mutants. The level of PGN0743::erm and PGN0876::erm autoaggregation was similar to that in the wild-type (data not shown). These results suggest that other mechanisms are involved in biofilm formation.

DISCUSSION

Comprehensive glycoproteomic analyses have been reported for gram-negative bacteria such as *C. jejuni* (Young *et al.*, 2002), *Neisseria gonorrhoeae* (Vik *et al.*, 2009), *B. fragilis* (Fletcher *et al.*, 2009), and *Helicobacter pylori* (Hopf *et al.*, 2011); however, this type of study has not been conducted in *P. gingivalis*. To improve our knowledge of post-translational glycosylations in *P. gingivalis*, whole-cell proteins were separated by 2D-PAGE combined with ProQ Emerald staining and mass spectrometry analysis (Figs 1 and 2 and Table 3). We identified four glycoproteins: PGN0743 (a probable FKBP PPIase), PGN0876 (a TPR domain protein), PGN1513 (a hypothetical protein), and PGN0729 (an outer membrane protein 41 precursor) in *P. gingivalis.* Because these four glycoproteins showed diverse biochemical activities, it was assumed that *P. gingivalis* possesses a general mechanism of protein glycosylation. To our knowledge, this is the first study showing that homologues of FKBP PPIase and TPR domain proteins are glycosylated in *P. gingivalis*. We also identified growth defects (Fig. 4B) and a reduction of biofilm formation by PGN0743-deficient and PGN0876-deficient mutants (Fig. 5A,B). Since FimA fimbriae are responsible for bacterial attachment and initiation of colonization (Yoshimura *et al.*, 2009), we next examined FimA expression in both mutants by Western blotting with anti-FimA antiserum. FimA expression by PGN0743::erm and PGN0876::erm was intact and indistinguishable from that in the wild type (data not shown). Therefore, the novel glycoproteins, PGN0743 and PGN0876, play an important role in survival and colonization by *P. gingivalis*.

The ProQ Emerald 300 glycoprotein stain, which reacts with periodate-oxidized carbohydrate groups (Steinberg *et al.*, 2001), is about 50 times more sensitive for glycosylations than the periodic acid-Schiff stain base method using acidic fuchsin dye. Using this highly sensitive method, we detected four distinct spots in whole-cell fractions from *P. gingivalis* ATCC 33277. Interestingly, all four proteins appear to be novel glycoproteins in *P. gingivalis*. Although previously reported cell surface glycoproteins, such as Rgps and Mfa1, were detected as proteins in CBB-stained gels (see Fig. S1A and Table S1), they were not detected in ProQ Emerald-stained gels. There are two possible explanations for this: first, Rgps and Mfa1 may be glycosylated at low levels and, second, the ProQ Emerald stain may show specificity for certain sugar chains. Nevertheless, this staining method will be a powerful tool for further glycoprotein research in *P. gingivalis*. Studies are currently underway to obtain detailed information regarding the cell

surface proteins that are glycosylated in *P. gingivalis* ATCC 33277.

PGN0743 has a sequence motif identifying it as a FKBP PPIase, and shows high homology with glycosylated peptidyl-prolylisomerase NGO1225 from *N. gonorrhoeae* (E-value 2×10^{-42}) (Børud *et al.*, 2010). The present study showed that PGN0743::erm was viable under laboratory conditions, although it grew more slowly than the wild-type strain. It is known that the putative PPIase family in the yeast *Saccharomyces cerevisiae* is essential for growth (Hani *et al.*, 1995). On the other hand, PPIases are not essential for growth of *E. coli* under laboratory conditions, because bacteria carrying mutations in PPIases were still viable (Justice *et al.*, 2005). PPIase has activity that is usually associated with periplasmic chaperone functions (Duguay & Silhavy, 2004); therefore, surface proteins involved in the acquisition of carbon energy sources, such as specific channels, porins and proteases, in *P. gingivalis* may be affected. BLAST-P analysis also showed that PGN0743 shares considerable homology with the FKBP PPIase, GldI, in *Flavobacterium johnsoniae* (E-value 1.64×10^{-5}), which has similar secretion machinery for outer membrane proteins to *P. gingivalis* (Sato *et al.*, 2010). GldI is involved in the folding of the cell envelope protein components of the gliding motility machinery in *F. johnsoniae* (McBride & Braun, 2004). Accordingly, PGN0743 may stabilize protein folding and/or chaperone membrane proteins in *P. gingivalis*.

Conserved domain analysis of PGN0876 using SMART showed that the protein contains three TPR motifs located on residues 135–168, 252–285 and 286–389. The TPRs comprise 34-amino acid repeat motifs that have been identified in a wide range of proteins of diverse biological function and are implicated in protein–protein interactions (D'Andrea & Regan, 2003). Bacterial TPR proteins have a range of functions, including gene regulation, chaperone activity, gliding motility and virulence (Wattiau *et al.*, 1994; Core & Perego, 2003; Broms *et al.*, 2006; Newton *et al.*, 2007; Scott *et al.*, 2008). The glycosylated TPR domain protein, BF2494, from *B. fragilis* shows high homology with PGN0876 (Fletcher *et al.*, 2009, 2011). Previously, we found that PGN0876 (a TPR domain protein) was induced in *P. gingivalis* ATCC 33277 cells cultivated in nutrient-limited medium using a chemostat culture system (Masuda *et al.*, 2006). It was reported that a TprA protein in *P. gingivalis* W83, corresponding to PGN0876, was upregulated under stress conditions when bacterial cells were aerated (Okano *et al.*, 2006), or when cells were placed in a subcutaneous chamber in a mouse (Yoshimura *et al.*, 2008). In the present study, the PGN0876-deficient mutant showed reduced growth under nutrient-limited conditions and also showed reduced biofilm formation activity. Because gingipains, major cell surface proteins, are thought to be involved in a wide range of pathological and physiological processes (Imamura *et al.*, 2003), we examined Rgp and Kgp activities in the PGN0876::erm. The Rgp and Kgp activities were decreased compared with those of the wild type (data not shown). These observations suggest that PGN0876 affects the function of gingipains. Studies were underway to define more clearly the relation between PGN0876 and gingipains by using the complemented-strain.

PGN1513 was identified as a hypothetical protein in the *P. gingivalis* ATCC 33277 genome database. Conserved domain analysis by SMART showed that PGN1513 contained three TPR motifs located on residues 43–73, 287–320 and 324–357. Therefore, PGN1513 is also a TPR protein and may play a role in protein–protein interactions, similar to PGN0876.

In the present study, PGN0729 was identified as an outer membrane protein 41 precursor in the pI 4–7 and 6–11 ranges (see Fig. S1 and Table S1). Our previous report identified the major outer membrane protein, Pgm6 (corresponding to PGN0729) of *P. gingivalis* ATCC 33277, which shares homology with the *E. coli* OmpA protein (Murakami *et al.*, 2002). Amino acid sequence analysis of PGN0729 also revealed significant similarities with orthologous OmpA-like protein genes from *Bacteroidetes*/*Flavobacterium*. Recent studies have identified the *Flavobacterium psychrophilum* OmpA protein as a glycoprotein (Merle *et al.*, 2003; Dumetz *et al.*, 2007).

In summary, this study identified novel glycoproteins in *P. gingivalis*. PGN0876 and PGN0743 are involved not only in growth under nutrient-limited conditions, but also in biofilm formation. Recent studies report that the TPR domain protein and PPIases in oral bacterial species are involved in virulence (Kondo *et al.*, 2010; Reffuveille *et al.*, 2012). Further investigations into the *in vivo* function of PGN0876 and PGN0743 are underway. We believe that these glycoproteins may play an important role in the expression of virulence associated with periodontal infections.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Figure S1. Two-dimensional polyacrylamide gel electrophoresis patterns displayed by whole-cell proteins from *Porphyromonas gingivalis.* Whole-cell proteins were separated in the pI 4–7 (A) and pI 6–11 (B) ranges and stained with Coomassie Brilliant Blue. In total, 299 spots were identified and numbered (see Table S1).

Table S1. Protein spots identified by two-dimensional polyacrylamide gel electrophoresis and matrix-assisted laser desorption ionization–time-of-flight mass spectrometry.

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