

C-Terminal Domain Residues Important for Secretion and Attachment of RgpB in *Porphyromonas gingivalis*[∇]

Nada Slakeski, Christine A. Seers, Kaiting Ng, Caroline Moore, Steven M. Cleal, Paul D. Veith, Alvin W. Lo, and Eric C. Reynolds*

Cooperative Research Centre for Oral Health, Melbourne Dental School and Bio21 Institute, The University of Melbourne, Melbourne, Australia

Received 1 July 2010/Accepted 11 October 2010

Porphyromonas gingivalis, a periodontal pathogen, expresses a group of surface proteins with a common C-terminal domain (CTD) that are exported by a novel secretion system to the surface, where they are covalently attached. Using RgpB as a model CTD protein, we have produced a series of site-directed mutations in the CTD sequence at conserved residues and at residues that may be modified and, hence, surface attached. The mutant RgpB proteins were expressed in a *P. gingivalis* host lacking functional RgpB and RgpA Arg-specific proteases. The RgpB mutants produced were Y674F, Y674F Y718F, T675Q S679Q T682Q T684Q, T693Q, F695A, D696A, N698A, G699P, G716P, T724Q, T728Q T730Q, and K732Q and a protein with a deletion of residues 692 to 702 (Δ 692-702). The mutants were characterized for cell-associated Arg-specific protease activity and for cellular distribution using anti-Rgp antibodies and Western blotting of culture fractions. All the mutants exhibited cell-associated Arg-specific activity similar to that of the positive control except for the D696A and Δ 692-702 mutants. For all mutants, except D696A and Δ 692-702, the RgpB proteins were found modified and attached to the cell surface, which was the same profile found in the positive-control strain. Only trace amounts of the precursor form of the Δ 692-702 mutant were detected in the outer membrane, with none detected in the periplasm or culture fluid although cell transcript levels were normal. The results suggest that residues 692 to 702 of the CTD, in particular, residue D696, have an important role in the attachment of RgpB at the cell surface and that without attachment secretion does not occur.

Porphyromonas gingivalis is an anaerobic bacterium found in subgingival dental plaque and has been implicated as a major pathogen in the initiation and progression of chronic periodontitis (3). There is evidence to suggest that individuals with periodontitis may be more susceptible to cardiovascular diseases (11), and the disease in pregnant women has been linked to preterm birth and low birth weight of their infants (8, 21). The cysteine proteinases, called gingipains, are major virulence factors of *P. gingivalis* and include the Arg-gingipains (RgpA and RgpB derived from the genes *rgpA* and *rgpB*, respectively) which are specific for hydrolysis of arginyl peptide bonds, and Lys-gingipain (Kgp derived from *kgp*), which is specific for hydrolysis of lysyl peptide bonds (5, 36). Two protein forms are derived from *rgpB*: a soluble discrete enzyme RgpB and a cell surface-attached form which represents a posttranslationally modified form of the protein. The surface-attached form of RgpB contains heterogeneously modified isoforms that migrate as a diffuse band of 70 to 90 kDa on SDS-PAGE (20, 40) and has been suggested to contain up to 30% carbohydrate (4, 5). In contrast, the soluble RgpB that has a truncated C-terminal domain (CTD) migrates as a discrete band of 50 kDa (10).

The outer membrane proteome of *P. gingivalis* has been characterized (35, 40), and a common C-terminal domain of approximately 70 to 80 amino acyl residues in length was iden-

tified in 34 *P. gingivalis* proteins (35). These CTD sequences were aligned to reveal a consensus sequence that can be divided into five regions (A to E) (Fig. 1) (35) despite little other sequence similarity. These proteins were described as the C-terminal domain sequence-related family (CTD family). Alignment of the CTD of these *P. gingivalis* proteins with the RgpB CTD revealed that there are several well-conserved amino acid residues, with just two fully conserved residues (equivalent to G699 and G716 of RgpB) (Fig. 1) (35). Genetic manipulation of RgpB to remove the CTD resulted in accumulation of the precursor form within the periplasmic space and loss of posttranslational modification, surface attachment, and function of the enzyme (35), suggesting that the CTD is important for RgpB export through the outer membrane and that modification was associated with functional protein export.

More recently, it has been demonstrated that truncation of the last two RgpB residues (VK) from the C terminus was sufficient to prevent transit of the RgpB precursor form out of the periplasm (19). Notably, however, VK is not a conserved residue pair at the C termini of many other CTD family proteins, occurring only in the CTD of the closely related HagA and Kgp (Fig. 1). The recombinant RgpB precursor polypeptide lacking VK was inactive and lacked the posttranslational modification seen in wild-type surface-attached RgpB (19). Again, these results show that modification is associated with functional export of RgpB.

Monoclonal antibody (MAb) 1B5 has been shown to recognize modified (surface-attached) RgpB but not the C-terminally truncated soluble form of the protein found in the culture supernatant, which lacks the CTD (5, 6). Recently, Paramonov

* Corresponding author. Mailing address: Melbourne Dental School, The University of Melbourne, 720 Swanston Street, Melbourne, Victoria 3010, Australia. Phone: 613 9341 1547. Fax: 61 3 9341 1596. E-mail: e.reynolds@unimelb.edu.au.

[∇] Published ahead of print on 22 October 2010.

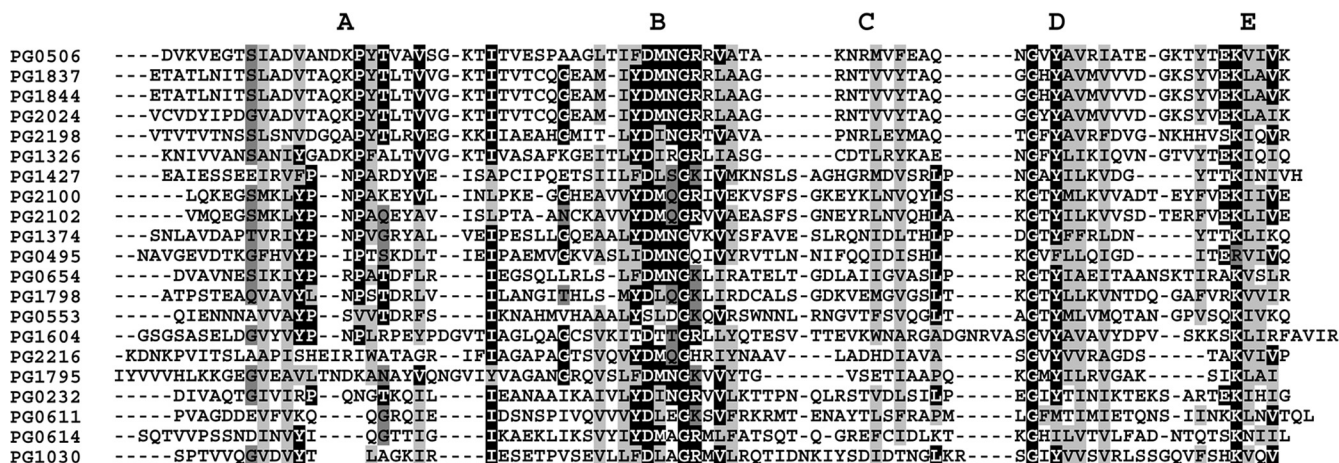


FIG. 1. Alignment of the C termini of experimentally identified *P. gingivalis* proteins with homology to the RgpB C terminus. The sequences are described using TIGR annotation, with PG0506 representing RgpB. The CTD is divided into five regions designated with the letters A to E above the alignment. The black shading indicates identical residues (>40% identity), light gray shading indicates nonpolar substitutions (A, V, F, P, M, I, L, W, and Y), and dark gray shading indicates polar (S, T, Y, H, C, N, G, and Q), acidic (D and E), or basic (R and K) substitutions. Residues that do not have >40% identity but have >70% conservative substitutions are also shaded.

et al. (25) identified the MAb 1B5 epitope as a phosphorylated branched mannan (Man α 1-2Man α 1-phosphate), which is also a component of a novel anionic lipopolysaccharide (A-LPS) of *P. gingivalis*. The basic structure of LPS is lipid A covalently linked to a core oligosaccharide, which is further linked to a polysaccharide comprising repeating units. Two different polysaccharides have been characterized in *P. gingivalis* LPS, designated O-LPS and A-LPS. O-LPS contains O-polysaccharide (O-antigen) tetrasaccharide repeating units (24, 26), whereas A-LPS contains anionic polysaccharide (APS) repeating units of phosphorylated branched mannan (25, 29). The epitope recognized by MAb 1B5 was localized to the Man α 1-2Man α 1-phosphate side chain in APS and is not present in O-LPS or capsule polysaccharide (25).

The increase in the molecular weight (MW) and diffuse nature of the modified RgpB protein on SDS-PAGE gels is consistent with a glycolipid modification such as LPS. This, together with MAb 1B5 recognizing A-LPS, has led to the suggestion that the CTD of RgpB is essential not only for outer membrane export but also for covalent attachment to the cell surface A-LPS anchor (27). Interestingly, MAb 1B5 also binds to other CTD-containing proteins, namely, RgpA_{A4} (one of four related RgpA adhesins), P59 (PG2102), and P27 (PG1795), indicating that these proteins also have Man α 1-2Man α 1-phosphate-associated modifications (40). Hence, based on this reactivity with MAb 1B5, it has been proposed that the CTD proteins are covalently attached to A-LPS on the cell surface through a modified CTD (27). Peptide mass fingerprinting (PMF) analyses of the mature forms of these proteins has not identified any peptide from the CTDs (35, 40), which may be because the modification blocks identification and/or because the CTD, upon secretion, is cleaved either when or before the protein is covalently attached to A-LPS. The CTD is also present in proteins from a range of bacteria in the *Bacteroidetes* phylum, and the proteins that have been characterized are also attached to the cell surface (9, 39, 41). These results suggest that the CTD is a component of a unique

protein secretion and cell surface attachment system in a range of pathogenic bacteria.

To further investigate the function of specific residues of the CTD in protein export and surface attachment through potential sites of O- and N-linked modification, we have created a series of site-directed mutants and a motif B (Fig. 1) deletion mutant within the RgpB CTD and investigated the cellular distribution of these recombinant RgpB (rRgpB) mutant proteins. We show that although substitution of most residues had no effect on rRgpB activity, a D696A mutation and a motif B (residues 692 to 702) deletion mutation abolished secretion and surface attachment of the protein.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. Bacterial strains and plasmids used in this study are listed in Table 1. *P. gingivalis* strains were grown as described previously (35). *P. gingivalis* allele exchange mutants were selected on horse blood agar (HBA; Oxoid, Basingstoke, United Kingdom) plates in the presence of 10 μ g ml⁻¹ erythromycin and 10 μ g ml⁻¹ chloramphenicol. Subsequently, brain heart infusion (BHI; Oxoid) broth cultures were supplemented with 5 μ g ml⁻¹ erythromycin and 5 μ g ml⁻¹ chloramphenicol. *Escherichia coli* strains were grown aerobically at 37°C on Luria-Bertani (LB) agar or in LB broth supplemented with 100 μ g ml⁻¹ ampicillin.

Electroporation of *P. gingivalis*. Electroporation-competent *P. gingivalis* cells were prepared as follows. An overnight-grown starter culture (1.5 ml) of *P. gingivalis* was inoculated into 200 ml of BHI broth and incubated overnight to an optical density at 650 nm (OD₆₅₀) of 0.3 to 0.7. Cells were then centrifuged (at 8,000 \times g for 20 min at 4°C), and the pellet was washed in 200 ml of ice-cold electroporation buffer (EP buffer; 10% [vol/vol] glycerol, 1 mM MgCl₂). The cells were then centrifuged (at 8,000 \times g for 20 min at 4°C), and the pellet was suspended in 400 μ l of ice-cold EP buffer. Cells (80 μ l) were aliquoted to cold microcentrifuge tubes, and 300 to 800 ng of linearized plasmid was added; the mixture was then incubated on ice for 5 min before being transferred into a 0.1-cm gap cuvette (*E. coli* pulser cuvette; Bio-Rad Laboratories Inc., CA) and electroporated at 1.8 kV, with a capacitance of 25 μ F and resistance of 200 Ω . One ml of BHI broth containing 5 μ g ml⁻¹ hemin and 0.5 mg ml⁻¹ cysteine was added, and cells were incubated overnight anaerobically before selection of transformants on HBA plates containing 10 μ g ml⁻¹ erythromycin or 10 μ g ml⁻¹ erythromycin and 5 μ g ml⁻¹ chloramphenicol, as appropriate, with anaerobic incubation at 37°C for up to 10 days.

TABLE 1. Bacterial strains and plasmids

Plasmid or strain	Description ^a	Reference or source
Plasmids		
pAL30	<i>ermF</i> ligated into pGem-TEasy	7
pBH1.1	pGem-TEasy: <i>rgpB</i> (nt 493-3166)	20
pUC <i>tetQ</i>	pUC18:: <i>tetQ</i>	This study
pJY674F	pBH1.1- <i>rgpB</i> (Y674F)	This study
pJY674Y718F	pBH1.1- <i>rgpB</i> (Y674F Y718F)	This study
pJD696A	pBH1.1- <i>rgpB</i> (D696A)	This study
pKTG699P	pBH1.1- <i>rgpB</i> (G699P)	This study
pKTG716P	pBH1.1- <i>rgpB</i> (G716P)	This study
pKTK732Q	pBH1.1- <i>rgpB</i> (K732A)	This study
pKTT728QT730Q	pBH1.1- <i>rgpB</i> (T728Q T730Q)	This study
pKTTSTT	pBH1.1- <i>rgpB</i> (T675Q S679Q T682Q T684Q)	This study
pKTT724Q	pBH1.1- <i>rgpB</i> (T724Q)	This study
pKTERM1	pUC <i>tetQ</i> :: <i>ermF</i>	This study
pKTG6	pKTERM1:: <i>rgpB</i> (G699P)	This study
pKTG7	pKTERM1:: <i>rgpB</i> (G716P)	This study
pKTK1	pKTERM1:: <i>rgpB</i> (K732Q)	This study
pKTTT1	pKTERM1:: <i>rgpB</i> (T728Q T730Q)	This study
pKTB1	pKTERM1:: <i>rgpB</i> (nt 493-3166)	This study
pKTY1	pKTERM1:: <i>rgpB</i> (Y674F)	This study
pKTD1	pKTERM1:: <i>rgpB</i> (D696A)	This study
pKTTY	pKTERM1:: <i>rgpB</i> (Y674F Y718F)	This study
pKTTSTT	pKTERM1:: <i>rgpB</i> (T675Q S679Q T682Q T684Q)	This study
pKTT	pKTERM1:: <i>rgpB</i> (T724Q)	This study
pCM1	pGem-TEasy: <i>rgpB</i> (nt 493-2961)-SfoI	This study
pCM2	pCM1:: <i>rgpB</i> (nt 3015-3166)	This study
pCM3	pKTERM1:: <i>rgpB</i> Δ(692-702)	This study
pCMT693Q	pKTERM1:: <i>rgpB</i> (T693Q)	This study
pCMF695A	pKTERM1:: <i>rgpB</i> (F695A)	This study
pCMN698A	pKTERM1:: <i>rgpB</i> (N698A)	This study
pAL34	934-bp 5-prime fragment of <i>pg0553</i> ligated between <i>ApaI</i> and <i>AatII</i> sites of pAL30	This study
pAL34.1	1,122-bp 3-prime fragment of <i>pg0553</i> ligated between <i>SpeI</i> and <i>SalI</i> sites of pAL34	This study
<i>P. gingivalis</i> strains		
W50AB	W50 <i>rgpA</i> :: <i>cat</i> <i>rgpB</i> :: <i>tetQ</i> ; Cm ^r Tc ^r	40
W50AB-Pos	W50 <i>rgpA</i> :: <i>cat</i> <i>rgpB</i> :: <i>tetQ</i> :: <i>rgpB</i> ; Cm ^r Em ^r	This study
W50AB-Neg	W50 <i>rgpA</i> :: <i>cat</i> <i>rgpB</i> :: <i>tetQ</i> :: <i>ermF</i> ; Cm ^r Em ^r	This study
W50AB-G699P	W50 <i>rgpA</i> :: <i>cat</i> <i>rgpB</i> :: <i>tetQ</i> :: <i>ermF</i> - <i>rgpB</i> (G699P); Cm ^r Em ^r	This study
W50AB-G716P	W50 <i>rgpA</i> :: <i>cat</i> <i>rgpB</i> :: <i>tetQ</i> :: <i>ermF</i> - <i>rgpB</i> (G716P); Cm ^r Em ^r	This study
W50AB-K732Q	W50 <i>rgpA</i> :: <i>cat</i> <i>rgpB</i> :: <i>tetQ</i> :: <i>ermF</i> - <i>rgpB</i> (K732P); Cm ^r Em ^r	This study
W50AB-T728T730Q	W50 <i>rgpA</i> :: <i>cat</i> <i>rgpB</i> :: <i>tetQ</i> :: <i>ermF</i> - <i>rgpB</i> (T728Q T730Q); Cm ^r Em ^r	This study
W50AB-D696A	W50 <i>rgpA</i> :: <i>cat</i> <i>rgpB</i> :: <i>tetQ</i> :: <i>ermF</i> - <i>rgpB</i> (D696A); Cm ^r Em ^r	This study
W50AB-Y674F	W50 <i>rgpA</i> :: <i>cat</i> <i>rgpB</i> :: <i>tetQ</i> :: <i>ermF</i> - <i>rgpB</i> (Y674F); Cm ^r Em ^r	This study
W50AB-Y674Y718F	W50 <i>rgpA</i> :: <i>cat</i> <i>rgpB</i> :: <i>tetQ</i> :: <i>ermF</i> - <i>rgpB</i> (Y674F Y718F); Cm ^r Em ^r	This study
W50AB-T724Q	W50 <i>rgpA</i> :: <i>cat</i> <i>rgpB</i> :: <i>tetQ</i> :: <i>ermF</i> - <i>rgpB</i> (T724Q); Cm ^r Em ^r	This study
W50AB-TSST	W50 <i>rgpA</i> :: <i>cat</i> <i>rgpB</i> :: <i>tetQ</i> :: <i>ermF</i> - <i>rgpB</i> (T675Q S679Q T682Q T684Q); Cm ^r Em ^r	This study
W50AB-T693Q	W50 <i>rgpA</i> :: <i>cat</i> <i>rgpB</i> :: <i>tetQ</i> :: <i>ermF</i> - <i>rgpB</i> (T693Q); Cm ^r Em ^r	This study
W50AB-F695A	W50 <i>rgpA</i> :: <i>cat</i> <i>rgpB</i> :: <i>tetQ</i> :: <i>ermF</i> - <i>rgpB</i> (F695A); Cm ^r Em ^r	This study
W50AB-N698A	W50 <i>rgpA</i> :: <i>cat</i> <i>rgpB</i> :: <i>tetQ</i> :: <i>ermF</i> - <i>rgpB</i> (N698A); Cm ^r Em ^r	This study
W50AB-Δ692-702	W50 <i>rgpA</i> :: <i>cat</i> <i>rgpB</i> :: <i>tetQ</i> :: <i>ermF</i> - <i>rgpB</i> Δ(692-702); Cm ^r Em ^r	This study
ECR179	<i>E. coli</i> JM109, pAL34	This study
ECR179.1	<i>E. coli</i> JM109, pAL34.1	This study
ECR190	<i>P. gingivalis</i> W50 <i>pg0553</i> :: <i>ermF</i>	This study

^a Tc^r, tetracycline resistance; Cm^r, chloramphenicol resistance; Em^r, erythromycin resistance. Nucleotides (nt) are numbered according to GenBank accession number AF007124.

Construction of *P. gingivalis* RgpB CTD site-directed mutants. The various plasmids produced in the course of this work are described in Table 1, the amino acids within the RgpB CTD that were subject to mutation or deletion are shown in Fig. 2, and an overview of the recombination strategy is shown in Fig. 3.

P. gingivalis W50AB (40) has *rgpA* and *rgpB* inactivated by the insertion of *cat* (chloramphenicol resistance cassette) and *tetQ* (tetracycline resistance cassette), respectively. The *tetQ* cassette was selected as the homologous recombination target for the insertion of recombinant *rgpB* into the *P. gingivalis* W50AB chromosome. The recombination cassette to be used, pKTERM1, was generated as follows: *tetQ* was excised from pNJR12 (16) using *EcoICRI* digestion and ligated into the *SmaI* site of pUC18, generating plasmid pUC*tetQ*. The *ermF* gene

(erythromycin resistance) was excised from pAL30 (7) using *EcoICRI* and *SacII*, blunt ends were generated using T4 DNA polymerase, and the insert was ligated into *EcoRV*-digested pUC*tetQ*. The resultant recombinant plasmid in which *ermF* was in the same orientation as *tetQ* was designated pKTERM1.

A QuikChange II site-directed mutagenesis kit (Stratagene) was employed to produce a series of plasmids encoding rRgpBs with CTD mutations, using pBH1.1 as a template and the various primers listed in Table 2. The encoded mutations were the following: Y674F, Y674F Y718F, T675Q S679Q T682Q T684Q (termed TSST), T693Q, F695A, D696A, N698A, G699P, G716P, T724Q, T728Q T730Q, K732Q, and K732Q (Fig. 2). Each mutated *rgpB* gene was fully sequenced to confirm that only the desired mutations were generated. A 2.7-kbp

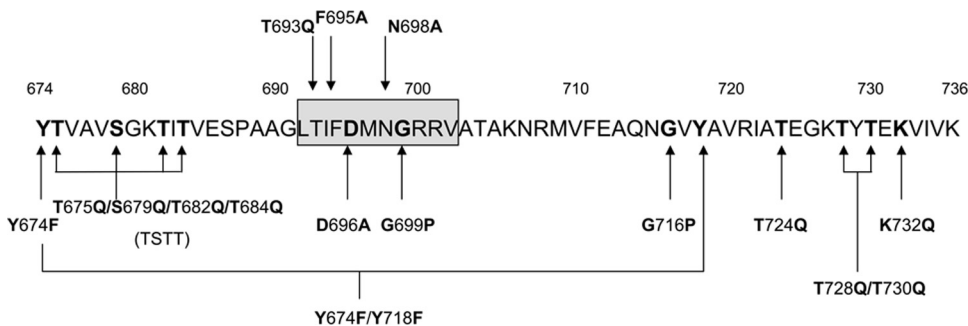


FIG. 2. Site-directed and deletion mutagenesis map of the RgpB CTD. Arrows point to the sites of mutation, with the amino acid changes indicated. Double and quadruple mutations are indicated by joined arrows. Numbering is from the *rgpB* primary translation. Amino acids 692 to 702 (boxed) were deleted in the W50AB-Δ692–702 mutant.

fragment containing *rgpB* was excised from each mutated plasmid and pBH1.1 using NotI digestion and ligated to NotI-digested pKTERM1 (Table 1; Fig. 3). Recombinant plasmids (Table 1) in which the *rgpB* genes were in the reverse orientation to *tetQ* and *ermF* were used to transform *P. gingivalis*. The cassettes were introduced into *P. gingivalis* W50AB by electroporation for allele exchange with *tetQ* (schematically represented in Fig. 3) with transformants selected on HBA plates supplemented with erythromycin and chloramphenicol. The positive control (W50AB-Pos) had *rgpB* of wild-type sequence and *ermF* inserted into *tetQ*, while the negative control (W50AB-Neg) had only *ermF* inserted into *tetQ*. All *P. gingivalis* allele exchanges were confirmed by testing for tetracycline sensitivity and by extensive PCR analysis. Only those constructs in which the orientation of *rgpB* was opposite to that of *ermF* were used for further analyses in order to eliminate the possibility of differential polar effects on *rgpB* expression.

Construction of *P. gingivalis* RgpB(Δ692–702) deletion mutant. Nucleotides encoding amino acids (aa) 692 to 702 (LTIFDMNGRRV) (Fig. 2) were deleted from *rgpB* using the following stepwise strategy. The primers dmngRRevCM and M13 universal forward (Table 2) were used to amplify a 2.7-kbp region of pBH1.1, containing *rgpB* nucleotides 493 to 2981 in the GenBank entry AF007124, and the amplicon was ligated to pGEM-TEasy (Promega). An SfoI restriction site was engineered into the construct at the 3' junction of the *rgpB* codons and the multiple-cloning site using site-directed mutagenesis (as previously described) with dmngRForSfoI and dmngRRevSfoI to produce pCM1. Nucleotides 3015 to 3166 of *rgpB* (encoding 34 C-terminal amino acids and 3'

flanking regions) were then amplified as part of a 250-bp amplicon by PCR using pBH1.1 template and oligonucleotides dmngRForCM and M13 universal reverse. The PCR amplicon was treated with T4 DNA polymerase and then digested with NsiI and ligated to SfoI-NsiI-digested pCM1, producing pCM2. The mutated *rgpB* gene was then excised from pCM2 using AatII and NsiI digestion and ligated to AatII/NsiI-digested pKTERM1, producing pCM3. Following nucleotide sequencing to confirm integrity, pCM3 DNA was electroporated into *P. gingivalis*, with recombinants isolated as outlined above.

Construction of *P. gingivalis* W50 PG0553 isogenic mutant. To make the *pg0553* inactivation cassette, a 934-nucleotide 5' region of *pg0553* of *P. gingivalis* W50 was PCR amplified using *Pfu* DNA polymerase (Promega, Madison, WI) and primer pair PG0553-AatII-For (TCGATGACGTCGTTGCCACGGAAGATGGCC) and PG0553-ApaI-Rev (CAAATCGGGCCCGCAGTCAGGACTGCTGC). The amplicon was digested with AatII and ApaI and then ligated 3' to *ermF* in AatII- and ApaI-digested pAL30 (7) to produce pAL34 (Table 1). A 1,122-nucleotide 3' region of *pg0553* was then PCR amplified using PG0553-SalI-For2 (AATTGAGTTCGACACCATGTTCGGCCTGCCA) and PG0553-SpeI-Rev2 (AACGATACTAGTCCTCTGCGCTATCGTGTCT) digested with SalI and SpeI and ligated 5' to *ermF* in SalI-SpeI-digested pAL34 to produce pAL34.1 (Table 1). Plasmid pAL34.1 was linearized with ScaI and transformed into *P. gingivalis* strain W50 with recombinants selected on HBA plates supplemented with 10 μg ml⁻¹ erythromycin. Confirmation of DNA integration was performed by PCR using the primer pairs PG0553KO-For (GCAGTCAGGACTGGCTGCC) and PG0553KO-Rev (CGGAGGGAGTTGTACCTTC). This created the *P. gingivalis* W50 PG0553 isogenic mutant (ECR190) (Table 1).

***P. gingivalis* cell fraction preparation.** *P. gingivalis* cells grown in BHI broth (40 ml) were harvested at an OD₆₅₀ of 0.61 (cell density of 2.5 × 10⁹ cells per ml) by centrifugation (8,000 × g for 20 min at 4°C). The supernatant was retained, and the pellet was suspended in 4 ml of TC150 buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 5 mM CaCl₂, 20 mM cysteine-HCl). An aliquot of the cells (2 ml) was centrifuged, and the pellet was collected, suspended in 200 μl of TC150 buffer in the presence of 10 mM Nα-*p*-tosyl-L-lysine-chloromethyl ketone (TLCK; Sigma), and stored at -70°C for later use in Western immunoassays. Culture supernatants were subjected to ultracentrifugation (300,000 × g for 30 min) to pellet outer membrane vesicles, and the clarified supernatant was retained. For Western blotting the proteins in 2 ml of supernatant were precipitated using 10% (vol/vol) trichloroacetic acid (TCA) (35) and suspended in 15 μl of loading dye. *P. gingivalis* Sarkosyl-insoluble outer membranes and TCA-precipitated periplasmic protein fractions were prepared as previously described (35).

Arginine amidolytic activity assay. Whole cells (10⁸ in 20 μl) plus 340 μl of TC150 buffer or 360 μl of vesicle-free supernatant were assayed for Arg-X amidolytic activity using *N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA; Sigma) as previously described (35) using a temperature-controlled Cary 50 Bio UV-visible spectrophotometer (Varian Australia Pty Ltd., Victoria, Australia). BAPNA assays were performed on triplicate samples from three separate cultures, and activity is expressed as μmol of BAPNA/min/10¹¹ cells.

Western blot analyses. Proteins of whole cells, outer membrane preparations, concentrated culture supernatant (5 μl), and periplasmic preparations were subjected to SDS-PAGE using NuPAGE Novex Bis-Tris gels (Invitrogen Corporation) and 1× NuPAGE morpholinepropanesulfonic acid (MOPS)-SDS running buffer, following the manufacturer's instructions, with the SeeBlue Plus2 prestained protein standard (Invitrogen Corporation). Gels were stained using Coomassie blue R250 (Bio-Rad, Hercules, CA) or SYPRO Ruby (Molecular

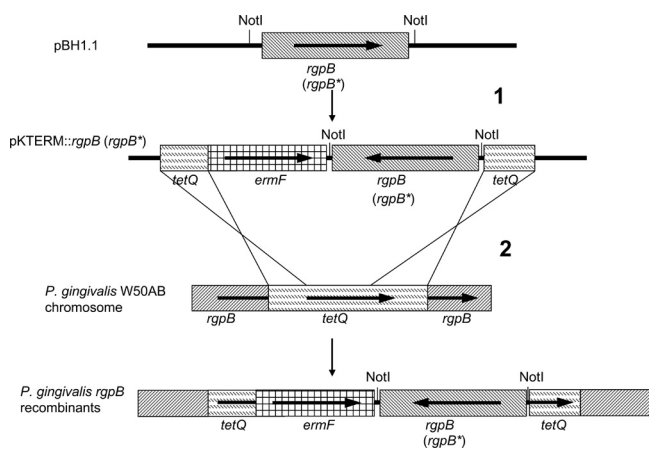


FIG. 3. Introduction of recombinant *rgpB* into *P. gingivalis* by allele exchange. In step 1, wild-type sequence (*rgpB*) or mutated *rgpB* (*rgpB*^{*}) was excised from pBH1.1-based plasmids by NotI digestion and ligated into the NotI site of pKTERM1. In step 2, the cassette was linearized and electroporated into *P. gingivalis* W50AB to allow double-crossover recombination events. Horizontal arrows indicate open reading frames and their orientations. The name of each gene is indicated below the arrow. *tetQ*, tetracycline resistance-encoding gene; *ermF*, erythromycin resistance gene; *rgpB*, arginine gingipain B gene.

TABLE 2. Oligonucleotide primers used to construct CTD mutation(s) in *rgpB*^a

Primer name	Sequence (5'→3')	Primer location (nt) ^b	Description
M13 universal forward	TGTAACACGACGGCCAGT		
M13 universal reverse	CAGGAAACAGCTATGACC		
dmngrForSfoI	GTCTGCTGCCGGCGCCGAGAGCTCCCAACGCG	2968–2978	<i>rgpB</i> (nt 2986–2978)-SfoI-pGemTEasy (nt 103–118)
dmngrRevSfoI	CGCGTTGGGAGCTCTCGGCGCCGGCAGCAGGAC	2978–2968	Reverse complement of dmngrForSfoI
dmngrRevCM	CCCAGCAGCAGGACTTTCTAC	2981–2961	<i>rgpB</i> (nt 2981–2961)
dmngrForCM	GCTACTGCTAAAAACCGCATGGTATTC	3015–3041	<i>rgpB</i> (nt 3015–3041)
Gly699Profor	CTGACGATCTTCGATATGAACCCCCGTCGTGTAGCTAC	2982–3019	RgpB with Gly699 → Pro
Gly699Prorev	GTAGCTACACGACGGGGTTCATATCGAAGATCGTCAG	3019–2982	Reverse complement of Gly699Profor
Gly716Profor	GAAGCACAAAACCCTGTGTATGCCGTTCG	3042–3070	RgpB with Gly716 → Pro
Gly716Prorev	CGAACGGCATAACAGGGTTTTGTGCTTC	3070–3042	Reverse complement of Gly716Profor
HSMUT2for	ATCGCTCAAGAAGGCAAGCAGTATCAAGAAGTTATAGTG	3072–3113	RgpB with Thr728 → Gln and Thr730 → Gln
HSMUT2rev	CACTATAACCTTTTCTTGATACTGCTTGCCTTCTTGAGCGAT	3113–3072	Reverse complement of HSMUT2for
HSMUT3for	GGCAAGACGTATACAGAACAGGTTATAGTGAAGTAATTC	3084–3122	RgpB with Lys732 → Gln
HSMUT3rev	GAATTACTTCACTATAACCTGTTCTGTATACGCTCTTGCC	3122–3084	Reverse complement of HSMUT3for
S679T684for	CTGTAGCTGTACAAGTAAGACGATACAAGTAGAAAAGTCC TGCTG	2932–2976	RgpB with Ser679 → Gln and Thr684 → Gln
S679T684rev	CAGCAGGACTTTCTACTTGTATCGTCTTACCTTGTACAGCT ACAG	2976–2932	Reverse complement of S679T684for
T675T682for	GATAAGCCTTATCAAGTAGCTGTACAAGTAAGCAGATAC AAGTAGAAAAGTC	2919–2970	RgpB with Thr675 → Gln and Thr682 → Gln
T675T682rev	GACTTTCTACTTGTATCTGCTTACCTTGTACAGCTACTTGTAT AAGGCTTATC	2970–2919	Reverse complement of T675T682for
T724Q-kt-for	GCCGTTTCGATCGCTCAAGAAGGCAAGACGTATAC	3063–3097	RgpB with Thr724 → Gln
T724Q-kt-rev	GTATACGCTTTGCTTCTTGAGCGATGCGAACGGC	3097–3063	Reverse complement of T724Q-kt-for
RgpBD696A-for	GGCTGACGATCTTCGCTATGAACGGCCGTCG	2980–3010	RgpB with Asp696 → Ala
RgpBD696A-rev	CGACGGCCGTTTCATAGCGAAGATCGTCAGCC	3010–2980	Reverse complement of RgpBD696A-for
RgpBY718F-for	CACAAAACGGCGTGTTCGCGTTCGCATCGC	3046–3076	RgpB with Tyr718 → Phe
RgpBY718F-rev	GCGATGCGAACGGCAAACACGCCGTTTTGTG	3076–3046	Reverse complement of RgpBY718F-for
RgpBY674F-for	CGTAGCCAATGATAAGCCTTTACTGTAGCTGTATCAGG	2909–2947	RgpB with Tyr674 → Phe
RgpBY674F-rev	CCTGATACAGCTACAGTAAAAGGCTTATCATTGGCTACG	2947–2909	Reverse complement of RgpBY674F-for
RgpBT693Qfor	CCTGCTGCCGGGCTGCAAATCTTCGATATG	2970–2999	RgpB with Thr693 → Gln
RgpBT693Qrev	CATATCGAAGATTTGCAGCCCGGACGACG	2999–2970	Reverse complement of RgpBT693Qfor
RgpBF695Afor	CCGGGCTGACGATCGCAGATATGAACGGCCG	2977–3007	RgpB with Phe695 → Ala
RgpBF695Arev	CGGCCGTTTCATATCTGCGATCGTCAGCCCGG	3007–2977	Reverse complement of RgpBF695Afor
RgpBN698Afor	CGATCTTCGATATGGCCGGCCGTCGTGTAGC	2986–3016	RgpB with Asn698 → Ala
RgpBN698Arev	GCTACACGACGGCCGCATATCGAAGATCG	3016–2986	Reverse complement of RgpBN698Afor

^a All the primers were purchased from GeneWorks, Pty. Ltd., Australia.

^b Primer alignment location in *rgpB* of *P. gingivalis* W50 according to the *rgpB* sequence of GenBank accession number AF007124. nt, nucleotides.

Probes Inc., Eugene, OR), or proteins were electrotransferred onto nitrocellulose membranes. Membranes were blocked and probed with anti-rRgpA_{cat} (where RgpA_{cat} indicates the catalytic domain of RgpA) mouse antibodies (anti-Rgp antibodies) diluted 1 in 500 in TNT buffer (25 mM Tris-HCl [pH 7.5], 500 mM NaCl, 0.1% [vol/vol] Triton X-100) as described previously (35). The immunoreactive proteins were detected using SuperSignal West Pico substrate chemiluminescence (Pierce Biotechnology, Inc., Rockford, U. S. A.) and a Las 3000 instrument (Fujifilm) for image capture.

Protein identification. Proteins were identified in SDS-PAGE gels using in-gel digestion and mass spectrometry (MS) using matrix-assisted laser desorption ionization–two-stage time of flight mass spectrometry (MALDI-TOF/TOF MS) as described previously (18). MS spectra were used to search a *P. gingivalis*

protein sequence database for protein identification, as described previously (40).

Statistical analysis. Arg-specific proteolytic activities as measured using BApNA as a substrate were statistically analyzed using one-way analysis of variance (ANOVA) with a Scheffe *a posteriori* multiple comparison using an SPSS statistical software program (version 17; Chicago, IL).

RESULTS

Generation of site-directed mutations of the RgpB CTD. To investigate the possible role of specific residues of the CTD of

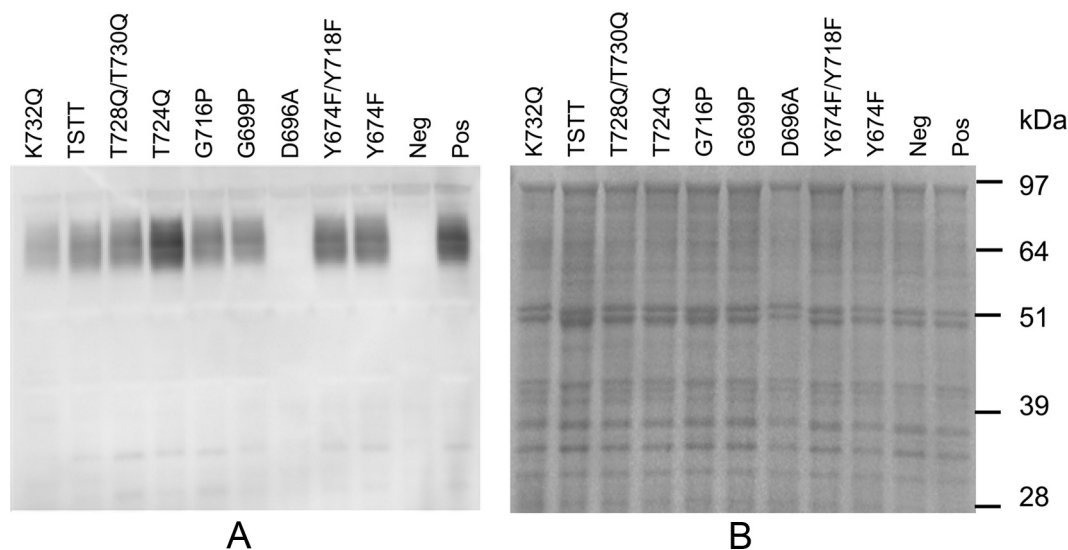


FIG. 4. Western blot analyses of outer membrane proteins of *P. gingivalis* allele exchange mutants probed with anti-Rgp. (A) Western blot. (B) The corresponding SDS-PAGE gel stained with Coomassie blue. Pos, positive control (wild-type sequence RgpB); Neg, negative control. Other recombinant RgpB proteins are indicated by their mutations.

RgpB in the secretion and cell surface attachment of the protein, residues within the RgpB CTD were mutated by site-directed mutagenesis or deletion, and the rRgpBs produced in *P. gingivalis* were characterized for Arg-X amidolytic activity and by Western blotting using anti-Rgp antibodies. Thirteen rRgpBs with CTD mutations were produced: Y674F, Y674F Y718F, T675Q S679Q T682Q T684Q (termed TSTT), T693Q, F695A, D696A, N698A, G699P, G716P, T724Q, T728Q T730Q, and K732Q and a mutant with a deletion of residues 692 to 702 ($\Delta 692-702$) (Fig. 2). RgpB residues Y674 and Y718 are reasonably conserved in the CTD sequences and, hence, were mutated to the structurally conservative phenylalanine to investigate whether they are involved in O-linked modification. The eight Ser/Thr residues were mutated in four separate mutants. Ser/Thr residues of the N-terminal cluster were targeted in the same mutant in RgpB(T675Q S679Q T682Q T684Q), and the other Thr residues were mutated in RgpB(T693Q), RgpB(T724Q), and RgpB(T728Q T730Q) in order to investigate whether these residues are potential sites for O-linked modification.

Alignment of the RgpB CTD with CTD sequences of other CTD family proteins revealed that RgpB D696, G699, G716, and K732 are highly conserved residues (Fig. 1) (35), and thus these were also targeted for mutation in this study. D696 was mutated to alanine, and K732 was mutated to glutamine to test the role of residue polarity and charge, respectively, on secretion and attachment. The conserved RgpB G699 and G716 residues may play a role in the conformational stability of the CTD; therefore, these residues were mutated to proline in an attempt to perturb CTD structure.

Analysis of RgpB mutants. (i) Arginine amidolytic activity. No Arg-X amidolytic activity could be detected in clarified, unconcentrated culture supernatants of any of the strains, including the positive control (W50AB-Pos) expressing the wild-type RgpB enzyme. In contrast, all strains exhibited measurable whole-cell Arg-X amidolytic activity except for the D696A

and $\Delta 692-702$ mutants, where no activity could be detected. The whole-cell Arg-X amidolytic activity of the W50 positive control (W50AB-Pos) was $4.7 \pm 0.3 \mu\text{mol of BApNA}/\text{min}/10^{11}$ cells, and the whole-cell activities of the mutants Y674F, Y674F Y718F, T675Q S679Q T682Q T684Q, T693Q, F695A, N698A, G699P, G716P, T724Q, T728Q T730Q, and K732Q were not significantly different from the activity of W50AB-Pos.

(ii) Cellular distribution and modification of RgpB mutants. To assess the cellular distribution and modification of the CTD-mutated RgpB proteins, outer membrane, periplasmic protein fractions, clarified supernatant, and whole-cell lysates of the recombinant strains were subjected to SDS-PAGE and Western blot analyses using anti-Rgp antibodies. Western blot analysis of outer membrane fractions of all RgpB CTD mutant strains using the anti-Rgp antibodies showed that the characteristic diffuse band at 70 to 90 kDa, corresponding to modified RgpB (cell surface-attached isoform), was readily observed in all RgpB CTD mutant strains except for the motif B deletion mutant RgpB($\Delta 692-702$), the RgpB(D696A) mutant, and the negative-control strain W50AB-Neg (Fig. 4, 5, and 6). In mutant $\Delta 692-702$ the Western blot of the outer membrane fraction revealed a trace amount of a single band of 80 kDa, corresponding to the size of the unprocessed precursor form of the mutated RgpB containing the prodomain, catalytic domain, and mutated CTD (Fig. 5). Repeated Western blotting of outer membrane preparations of the Y674F, Y674F Y718F, T675Q S679Q T682Q T684Q, T693Q, F695A, N698A, G699P, G716P, T724Q, T728Q T730Q, and K732Q mutants and the positive control (W50AB-Pos) indicated no significant differences in the intensities of the 70- to 90-kDa (cell surface attached) RgpB isoform. Western blots of clarified, concentrated supernatant proteins showed similar profiles, with all strains except the $\Delta 692-702$, D696A, and W50AB-Neg strains producing trace amounts of a 50-kDa fully processed form of RgpB in the clarified and concentrated culture supernatant (Fig. 5, 6, and 7). The preparations used for Fig. 5 and 6 were

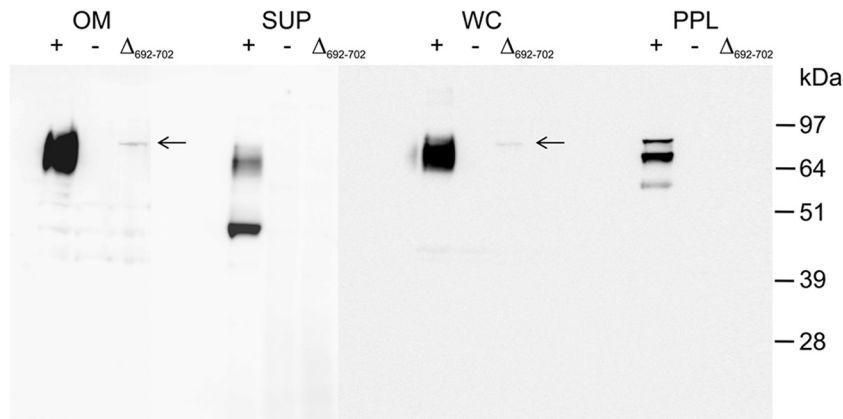


FIG. 5. Western blot analyses of protein fractions of the *P. gingivalis* RgpB(Δ 692-702) mutant probed with anti-Rgp. +, positive control; -, negative control. Fractions probed with anti-Rgp are indicated as follows: OM, outer membrane; SUP, supernatant; WC, whole cell; PPL, periplasmic. The arrows indicate the faint 80-kDa band corresponding to precursor RgpB.

contaminated with membrane vesicles so that the membrane-attached isoform (70- to 90-kDa band) was also present in the culture supernatant samples. Repeated Western blotting showed no significant differences between the intensities of the 50-kDa RgpB isoform of the positive control and that of any of the mutants except for Δ 692-702 and D696A. Western blots of periplasmic-enriched protein fractions showed no accumulation of mutated RgpB in the periplasm of the Δ 692-702 mutant (Fig. 5) or D696A mutant (data not shown).

In the protein profiles of the culture supernatants of the negative control and the Δ 692-702 and D696A mutants, a prominent band of approximately 63 kDa appeared that was not present in any of the other strains tested that contained RgpB active enzyme (Fig. 7A; also data not shown). These bands were excised from the gel, digested with trypsin, and analyzed by MALDI-TOF/TOF mass spectrometry. Peptide mass fingerprinting (PMF) indicated that the major protein present in the bands from the three strains was PG0553 (Table 3). This assignment was verified by tandem MS (MS/MS) of

four of the observed peptides (Table 3). One of these peptides (m/z 1,494) could not be identified using the default parameters; however, it matched the peptide QISFGGEPLSFSSR with the N-terminal glutamine converted to pyroglutamine. This glutamine is the predicted N terminus of the protein after removal of the signal peptide. Mass spectrometric analysis of this protein revealed that it was a C-terminally truncated form of PG0553 (a predicted endopeptidase) (Table 3).

To investigate the link between PG0553 and RgpB processing and activity, a *P. gingivalis* W50 *pg0553* isogenic mutant was constructed. The disruption of *pg0553* was confirmed using PCR (data not shown). The Arg-X amidolytic cell surface activity of the *P. gingivalis* W50 *pg0553* mutant (ECR190) was not significantly different from that of the W50 wild type. Furthermore, RgpB was present as the cell-attached 70- to 90-kDa isoform in the PG0553 mutant, indicating that the PG0553 protein was not essential for secretion and attachment of RgpB.

Western blotting was repeated for all culture/cell fractions of the Δ 692-702 and D696A mutants, including whole cell, inner and outer membrane preparations, and periplasmic and culture fluid fractions; however, RgpB could not be detected in any fraction. To eliminate the possibility of a secondary mutation elsewhere in the cell causing the Δ 692-702 and D696A phenotype, a second mutant strain for each was generated by transformation of the background strain W50AB once again with pCM3 and pJD696A, respectively. The phenotype of depressed Rgp(Δ 692-702) and RgpB(D696A) production was reproduced in the second mutants (data not shown), and so the *rgpB*(Δ 692-702) and *rgpB*(D696A) recombinant loci were amplified by PCR and subjected to DNA sequencing, whereupon no errors in nucleotide sequences were detected. To address the possibility that the reduced RgpB(Δ 692-702) and RgpB(D696A) production could result from reduced gene transcription, the expression levels of *rgpB*(Δ 692-702) and *rgpB*(D696A) relative to the control wild-type *rgpB* gene were determined. Real-time reverse transcription-PCR of total RNA preparations showed no significant difference between the levels of wild-type *rgpB* positive-control transcript and *rgpB*(Δ 692-702) and *rgpB*(D696A) transcripts (results not

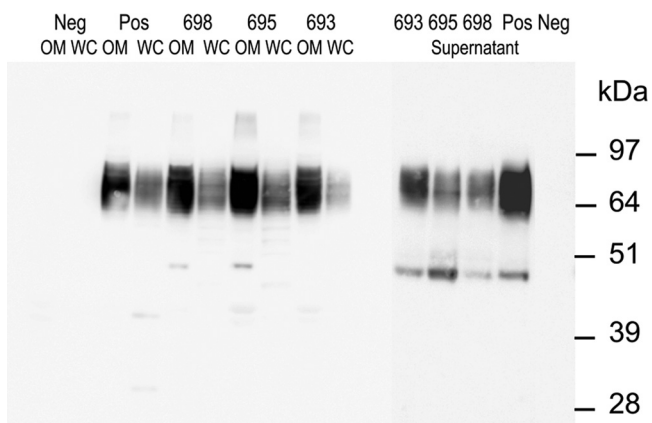


FIG. 6. Western blot analyses of protein fractions of *P. gingivalis* allele exchange mutants probed with anti-Rgp. Neg and Pos are the negative and positive controls, respectively. Fractions probed with anti-Rgp are indicated as follows: 698, RgpB(N698A); 695, RgpB(F695A); 693, RgpB(T693Q); OM, outer membrane; WC, whole cell.

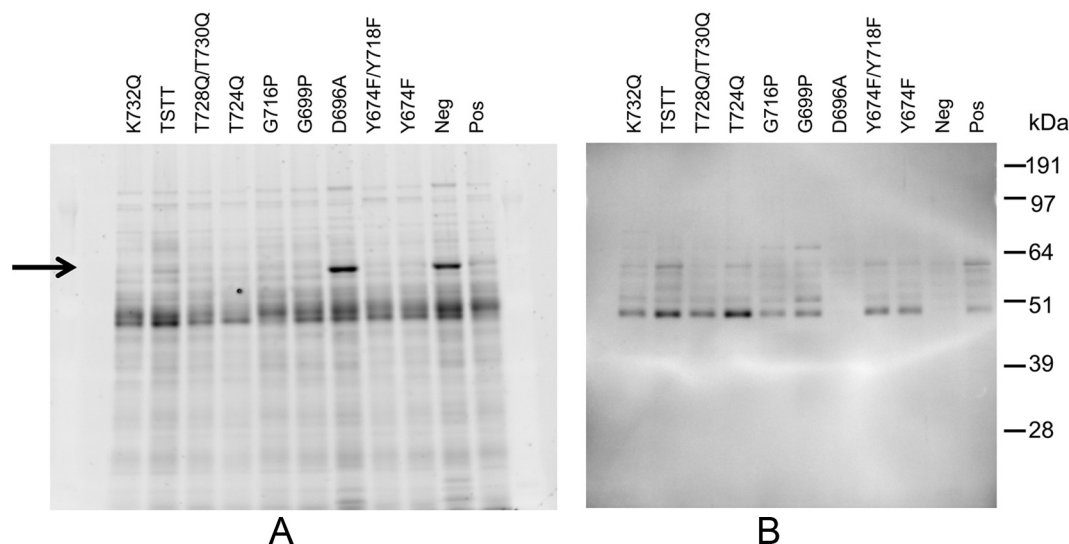


FIG. 7. Western blot analyses of clarified and concentrated supernatant proteins of *P. gingivalis* allele exchange mutants probed with anti-Rgp. (A) The SDS-PAGE gel stained with Coomassie blue. (B) The corresponding Western blot. Pos, positive control (wild-type sequence RgpB); Neg, negative control. Other recombinant Rgp proteins are indicated by their mutations. The arrow indicates the abundant PG0553 which is found in the D696A and negative control.

shown). There was no transcript detected in the RgpB negative-control strain, W50AB-Neg.

DISCUSSION

In the present study, S679, T675, T682, T684, T693, N698, T724, T728, T730, Y674, and Y718 residues of the RgpB CTD were selected to screen as potential site(s) of modification and membrane attachment. Whole-cell enzyme activities and Western blot analyses of RgpB with the TSTT mutation, RgpB(T724Q), RgpB(Y674F), RgpB(Y693F), and RgpB(Y674F Y718F) in outer membrane fractions showed the diffuse band of 70 to 90 kDa, as found in the positive control, indicating surface attachment of the mutated proteins. The lack of obvious change in the attachment profiles of these mutated RgpBs suggests that these residues are not essential for secretion or surface attachment of the protein. Whole-cell enzyme activities and Western blotting of whole-cell, outer membrane, periplasm, and supernatant fractions of RgpBs mutated at the conserved residues G699, G716, and K732 also indicated that the protein was attached

to the cell surface. G699 and G716 were mutated to prolines in an attempt to perturb the structure of the conserved CTD regions B and D, respectively. In light of the results, the proline substitution was not sufficient to abolish the function of the CTD with respect to secretion and attachment. It was considered possible that perturbation of the structure of RgpB CTD may affect the processing/folding of the protein and, therefore, specific activity of the secreted RgpB. Mikolajczyk et al. (17) have demonstrated that sequential autolytic processing of the prodomain and CTD is required for full specific activity of RgpB, such that changes to the CTD sequence may affect this sequential autolytic processing. However, in this current study, substitution of Pro for conserved Gly residues of the CTD did not affect secretion, attachment, or activity of RgpB.

In a separate study of the RgpB CTD, the last 13 residues of the RgpB CTD were truncated in five separate mutants, and the last five residues were individually mutated to alanines to investigate the function of the CTD (19). The only common residue that was targeted in that study (19) and in the current study was the conserved K732 (K503 in the mature RgpB). In the current study K732 was targeted to investigate the importance of this charged residue located only five amino acids from the C terminus of the CTD. We postulated that the positive charge of the lysine in close proximity to the C terminus may interact with the negatively charged membrane for export of RgpB to the surface. The importance of charged residues involved in sorting of bacterial extracellular proteins can be seen with the Gram-positive sortases (34). Sortases are bacterial enzymes responsible for the covalent attachment of secreted proteins to the cell wall in Gram-positive bacteria. Proteins destined for sortase-mediated attachment contain an N-terminal Sec leader and a C-terminal cell wall sorting signal (CWS) that is characterized by an LPXTG motif, a hydrophobic region, and C-terminal positive charge (34). Sortase cleaves

TABLE 3. Mass spectrometric analysis data identifying PG0553

Sample/peptide ^a	Experiment ^b	Mascot score	Expect value
W50AB-Neg	PMF	73	1.1E-04
W50AB-D696A	PMF	81	1.9E-05
²⁸ QISFGGEPLSFSSR ⁴¹	MS/MS	60	5.5E-05
⁵⁶ LTPDFNPEDLIAQSR ⁷⁰	MS/MS	85	1.2E-08
¹¹⁹ AITLYYDAFNIPEGGR ¹³⁴	MS/MS	71	2.3E-07
³²⁹ SDGLLLQLNDEVPLR ³⁴³	MS/MS	52	1.8E-05

^a The identities of the four peptides were independently confirmed by MS/MS using the negative-control sample. All peptides matched to within 50 ppm of the calculated mass.

^b PMF experiments were conducted on the 63-kDa bands excised from the blot shown in Fig. 7A.

the CWS motif between the threonine-glycine bond and subsequently catalyzes the transpeptidation of the threonine carbonyl to an amine of a pentaglycine cross-bridge, tethering the C terminus of the protein to the bacterial cell wall (2). Mutational analysis of the two positive residues positioned only a few residues from the C terminus of the CWS in staphylococcal protein A showed that this positive charge is required for retention of the polypeptide within the secretory pathway (34).

In the current study, the conserved RgpB Lys732 was mutated to glutamine, which is the most structurally similar uncharged residue to lysine, to remove charge but maintain integrity of the polar structure. The RgpB(K732Q) mutant exhibited Arg-specific amidolytic activity and a Western blot profile similar to that of the positive-control strain, with the presence of the modified 70- to 90-kDa RgpB in outer membrane fractions. These results differ from those observed previously (19) with an RgpB(K732A) mutant, where mutation of Lys732 to an Ala resulted in an accumulation of two distinct bands of approximately 65 and 52 kDa in the periplasm, in addition to the modified 70- to 90-kDa form of RgpB in the outer membrane fraction, albeit at lower intensity than that of the positive control (19). Further, the K732A mutant showed smaller amounts of active enzyme (54% compared to the positive control). These results of smaller amounts of attached RgpB suggest a problem with secretion in this mutant (19). Alanine is a small amino acid with aliphatic hydrophobic properties, which differ significantly from lysine, which has a large side chain and positive charge. Collectively, the results indicate that the positive charge of K732 is not essential for export or attachment of RgpB to the outer membrane; however, it appears that the lysine, possibly because of its polarity and/or the bulk of its side chain, may be important for proper recognition by the secretion system.

The most significant change in RgpB surface attachment in the site-directed mutants was observed with the D696A mutation, where the conserved Asp696 was mutated to Ala. Western blot analyses revealed that Rgp(D696A) could not be detected in whole cells or in any fraction (periplasm, inner membrane, and outer membrane) of the cell or supernatant. As the levels of *rgpB* transcript in W50-D696A and W50AB-Pos did not differ, and as the RgpB(D696A) precursor could not be detected in the periplasm or culture fluid of the mutant, these findings suggest that the protein may be blocked in the secretion apparatus, which results in the downregulation of translation and/or the upregulation of proteolysis.

Removal of the RgpB CTD motif B that contains Asp696 resulted in a trace amount of unprocessed precursor being detected in the outer membrane fraction of the bacterium but no detection of the protein in any other fraction, including the periplasm and culture fluid. The trace levels and the precursor form of the protein are consistent with the lack of enzyme activity in any fraction (whole cells, supernatant, clarified supernatant, and periplasm) of the mutant. As transcription was also not affected in this mutant, it appears that translation of the RgpB(Δ 692–702) protein again may have been downregulated and/or that proteolysis was upregulated due to the protein being trapped in the outer membrane secretory apparatus. In both the Asp696Ala mutant and the motif B deletion mutant, RgpB(Δ 692–702), a 63-kDa protein was substantially increased in abundance, and analysis of this protein by mass

spectrometry indicated that it was the predicted endopeptidase PG0553. The high level of abundance of this protein, together with the possible downregulated translation of the mutated RgpB proteins (Asp696 and motif B), may account for the small amount of RgpB protein that could be detected in any fractions of these mutants relative to that of the wild type.

To investigate a possible link between the PG0553 proteinase and cell surface processing of RgpB, an isogenic mutant of *P. gingivalis* W50 lacking PG0553 was created. The cell surface Arg-X amidolytic activity of the *pg0553* mutant was not significantly different from that of the control. Further, the cell surface-attached isoform (70 to 90 kDa) of RgpB was present in the *pg0553* mutant, suggesting that PG0553 does not have an essential role in the processing of RgpB. As PG0553 was also found substantially increased in abundance in the negative-control strain W50AB-Neg not expressing either RgpA or RgpB, as well as in the D696A and Δ 692–702 mutants, it is possible that the high abundance of PG0553 (a cell surface/culture fluid protease) may be related to the lack of the RgpA and RgpB proteases.

The Δ 692–702 deletion mutant and the D696A mutant phenotypes were different from those of the RgpB mutants lacking the entire CTD (35) or the last 2 to 13 C-terminal residues of the CTD (19) as the latter mutated RgpB proteins accumulated in the periplasm. This can be explained as the CTD truncated mutants (19, 35) would not be recognized by the outer membrane secretion system since they lacked the signal or complete signal for outer member secretion and, hence, accumulated in the periplasm. The Δ 692–702 deletion mutant and the D696A mutant still contained the signal for outer membrane secretion but were unable to be modified (attached) and, hence, became trapped in the secretion system. These results suggest that regions D plus E (Fig. 1) involving the last 22 residues of the RgpB CTD act as the recognition signal for the CTD secretion system, whereas regions B plus C, involving D696, in motif B are the attachment recognition sequence. The results further suggest that secretion and attachment of RgpB are coordinated.

With the exception of Asp696, none of the site-directed mutations performed in this study, including others in motif B, significantly affected enzyme presentation on the surface of the bacterium, and so it seems likely that none of these residues are sites of modification and therefore surface attachment. It can be concluded, therefore, that Asp696 of motif B is important for CTD function. The most likely role of Asp696 and motif B is in recognition by a protein of the outer membrane secretion system that cleaves the CTD and/or covalently attaches the CTD onto a sugar moiety of its membrane anchor A-LPS to form the membrane-modified isoform of the protein. Hence, when Asp696 is missing, the CTD protein cannot be processed and becomes trapped in the outer membrane secretion system. Asp696 does not appear to be essential as a small number of CTD proteins have a Ser, Thr, or Asn residue in its place. This suggests that a small polar residue will fulfill the role of Asp696 in motif B. For example, PG0553, the endopeptidase upregulated in mutants not expressing Rgp enzyme activity, is a CTD protein with a Ser residue in place of RgpB's Asp696 (Fig. 1). Interestingly, PG0553 was found predominantly as a C-terminally truncated isoform in the culture supernatant of the Rgp mutant strains not expressing Arg-X

activity. The presence of PG0553 and RgpB in the culture supernatant without the CTD sequence suggests that cleavage of the attached CTD sequence may be the mechanism for release of the surface-attached protein into the culture fluid. As the entire CTD sequence is missing in the culture supernatant isoforms, it is tempting to speculate that the cleavage site of the CTD for ultimate release of the fully processed protein into the culture fluid may involve region A (Fig. 1).

Localization of proteins to the surface of the cell of Gram-negative bacteria requires export of the proteins across the inner membrane, the periplasm, and the outer membrane. Six types of outer membrane protein export systems have been identified that fall into two main categories, those dependent on the Sec translocon in the inner membrane and those independent of Sec (1, 15). Proteins that are Sec dependent have N-terminal sequences that serve as targets for SecB or the signal recognition particle (SRP) that function to target the nascent polypeptide chain to the Sec translocon in the inner membrane (28, 38). After inner membrane transit, passage of Sec-dependent proteins into or across the outer membrane involves transport complexes of the type II system (30a), the chaperone-usher pathway (33), or the type V system, where the polypeptide facilitates its own export by insertion of the carboxyl domain into the outer membrane to form a β -barrel pore in a mechanism referred to as autotransport (13). *P. gingivalis* has no proteins with sequence similarity to these known Sec-dependent, outer membrane export system proteins (42). Therefore, it appears that the CTD family proteins are exported across the outer membrane by a new type of transport system.

The *P. gingivalis* proteins PorT (32), PG27 (14), and Sov (30), which have been demonstrated to be required for the secretion of RgpB, RgpA, and Kgp, share the same bacterial species distribution as the CTD and appear to be part of a novel *Bacteroidetes*-specific secretion system. Recently, Sato et al. (31) reported a list of 55 proteins that share this same species distribution and demonstrated the involvement of some, including PorK, PorL, PorM, PorN, PorP, PorW, and PorU (PG26) in RgpB, RgpA, and Kgp secretion. The system was designated the Por secretion system (PorSS), and some of the proteins of the system are orthologues of gliding motility proteins of *Flavobacterium johnsoniae*, thus providing a link between the novel protein translocation system and a motility apparatus in the *Bacteroidetes* phylum (31).

Recently, we have shown in *P. gingivalis* isogenic mutants lacking PG27 or PorT that both CTD proteins and A-LPS accumulate in the periplasm, indicating that secretion and attachment of the CTD protein to the A-LPS anchor are coordinated (Y.-Y. Chen, B. Peng, M. D. Glew, et al., submitted for publication). The results of the current study with RgpB also suggest that secretion and attachment are coordinated. This coordination could be achieved by the secretion of the CTD protein and translocation of the A-LPS being linked through the covalent attachment of the CTD or cleaved CTD to the A-LPS anchor. Given the range and extent of the mutations performed in this study, in particular all the conserved residues of the CTD, the results suggest that the Ser/Thr/Tyr/Asn residues of the RgpB CTD domain are not the sites of modification and surface attachment. It seems likely that the CTD acts as a site of recognition by a *P. gingivalis* processing enzyme(s),

possibly a novel sortase-like enzyme that cleaves the CTD sequence and attaches the C-terminal carbonyl to a sugar amine of A-LPS. Although sortase activity was initially ascribed to Gram-positive microorganisms only, more recently, sortase homologues have been identified in Gram-negative bacteria (12, 22, 23). Organisms can contain several sortase-like proteins in their genomes (23), and the level of sequence identity is low for orthologous proteins (<25%), making identification difficult (23). Further research is required to identify the protein(s) involved in the coordinated secretion and cell surface attachment of the CTD proteins. In conclusion, the results of the current study show that the CTD motif B containing Asp696 is involved in the cell surface attachment of RgpB in *P. gingivalis* and that without attachment secretion of the protein does not occur.

ACKNOWLEDGMENTS

We acknowledge James Edward Fielding and Brigitte Hoffmann for their technical support.

This study is supported by National Health and Medical Research Council grant number 3500427 and the Cooperative Research Centre for Oral Health Science.

REFERENCES

- Albers, S. V., Z. Szabo, and A. J. Driessen. 2006. Protein secretion in the *Archaea*: multiple paths towards a unique cell surface. *Nat. Rev. Microbiol.* 4:537–547.
- Bentley, M. L., H. Gaweska, J. M. Kielec, and D. G. McCafferty. 2007. Engineering the substrate specificity of *Staphylococcus aureus* sortase A. The $\beta 6/\beta 7$ loop from SrtB confers NPQTN recognition to SrtA. *J. Biol. Chem.* 282:6571–6581.
- Christersson, C. E., R. G. Dunford, P. O. Glantz, and R. E. Baier. 1989. Effect of critical surface tension on retention of oral microorganisms. *Scand. J. Dent. Res.* 97:247–256.
- Curtis, M. A., J. Aduse-Opoku, and M. Rangarajan. 2001. Cysteine proteases of *Porphyromonas gingivalis*. *Crit. Rev. Oral Biol. Med.* 12:192–216.
- Curtis, M. A., H. K. Kuramitsu, M. Lantz, F. L. Macrina, K. Nakayama, J. Potempa, E. C. Reynolds, and J. Aduse-Opoku. 1999. Molecular genetics and nomenclature of proteases of *Porphyromonas gingivalis*. *J. Periodontol. Res.* 34:464–472.
- Curtis, M. A., A. Thickett, J. M. Slaney, M. Rangarajan, J. Aduse-Opoku, P. Shepherd, N. Paramonov, and E. F. Hounsell. 1999. Variable carbohydrate modifications to the catalytic chains of the RgpA and RgpB proteases of *Porphyromonas gingivalis* W50. *Infect. Immun.* 67:3816–3823.
- Dashper, S. G., C. S. Ang, P. D. Veith, H. L. Mitchell, A. W. Lo, C. A. Seers, K. A. Walsh, N. Slakeski, D. Chen, J. P. Lissel, C. A. Butler, N. M. O'Brien-Simpson, I. G. Barr, and E. C. Reynolds. 2009. Response of *Porphyromonas gingivalis* to heme limitation in continuous culture. *J. Bacteriol.* 191:1044–1055.
- Dortbudak, O., R. Eberhardt, M. Ulm, and G. R. Persson. 2005. Periodontitis, a marker of risk in pregnancy for preterm birth. *J. Clin. Periodontol.* 32:45–52.
- Duchaud, E., M. Boussaha, V. Loux, J. F. Bernardet, C. Michel, B. Kerouault, S. Mondot, P. Nicolas, R. Bossy, C. Caron, P. Bessieres, J. F. Gibrat, S. Claverol, F. Dumetz, M. Le Henaff, and A. Benmansour. 2007. Complete genome sequence of the fish pathogen *Flavobacterium psychrophilum*. *Nat. Biotechnol.* 25:763–769.
- Eichinger, A., H. G. Beisel, U. Jacob, R. Huber, F. J. Medrano, A. Banbula, J. Potempa, J. Travis, and W. Bode. 1999. Crystal structure of gingipain R: an Arg-specific bacterial cysteine proteinase with a caspase-like fold. *EMBO J.* 18:5453–5462.
- Geismar, K., K. Stoltze, B. Sigurd, F. Gynzelberg, and P. Holmstrup. 2006. Periodontal disease and coronary heart disease. *J. Periodontol.* 77:1547–1554.
- Haft, D. H., I. T. Paulsen, N. Ward, and J. D. Selengut. 2006. Exopolysaccharide-associated protein sorting in environmental organisms: the PEP-CTERM/EpsH system. Application of a novel phylogenetic profiling heuristic. *BMC Biol.* 4:29.
- Henderson, I. R., F. Navarro-Garcia, M. Desvaux, R. C. Fernandez, and D. Ala'Aldeen. 2004. Type V protein secretion pathway: the autotransporter story. *Microbiol. Mol. Biol. Rev.* 68:692–744.
- Ishiguro, I., K. Saiki, and K. Konishi. 2009. PG27 is a novel membrane protein essential for a *Porphyromonas gingivalis* protease secretion system. *FEMS Microbiol. Lett.* 292:261–267.

15. Kostakioti, M., C. L. Newman, D. G. Thanassi, and C. Stathopoulos. 2005. Mechanisms of protein export across the bacterial outer membrane. *J. Bacteriol.* **187**:4306–4314.
16. Maley, J., N. B. Shoemaker, and I. S. Roberts. 1992. The introduction of colonic-Bacteroides shuttle plasmids into *Porphyromonas gingivalis*: identification of a putative *P. gingivalis* insertion-sequence element. *FEMS Microbiol. Lett.* **72**:75–81.
17. Mikolajczyk, J., K. M. Boatright, H. R. Stennicke, T. Nazif, J. Potempa, M. Bogoy, and G. S. Salvesen. 2003. Sequential autolytic processing activates the zymogen of Arg-gingipain. *J. Biol. Chem.* **278**:10458–10464.
18. Ngo, L. H., P. D. Veith, Y. Y. Chen, D. Chen, I. B. Darby, and E. C. Reynolds. 2010. Mass spectrometric analyses of peptides and proteins in human gingival crevicular fluid. *J. Proteome Res.* **9**:1683–1693.
19. Nguyen, K. A., J. Travis, and J. Potempa. 2007. Does the importance of the C-terminal residues in the maturation of RgpB from *Porphyromonas gingivalis* reveal a novel mechanism for protein export in a subgroup of gram-negative bacteria? *J. Bacteriol.* **189**:833–843.
20. O'Brien-Simpson, N. M., R. A. Paolini, B. Hoffmann, N. Slakeski, S. G. Dashper, and E. C. Reynolds. 2001. Role of RgpA, RgpB, and Kgp proteinases in virulence of *Porphyromonas gingivalis* W50 in a murine lesion model. *Infect. Immun.* **69**:7527–7534.
21. Offenbacher, S., V. Katz, G. Fertik, J. Collins, D. Boyd, G. Maynor, R. McKaig, and J. Beck. 1996. Periodontal infection as a possible risk factor for preterm low birth weight. *J. Periodontol.* **67**:1103–1113.
22. Pallen, M. J., R. R. Chaudhuri, and I. R. Henderson. 2003. Genomic analysis of secretion systems. *Curr. Opin. Microbiol.* **6**:519–527.
23. Pallen, M. J., A. C. Lam, M. Antonio, and K. Dunbar. 2001. An embarrassment of sortases—a richness of substrates? *Trends Microbiol.* **9**:97–102.
24. Paramonov, N., D. Bailey, M. Rangarajan, A. Hashim, G. Kelly, M. A. Curtis, and E. F. Hounsell. 2001. Structural analysis of the polysaccharide from the lipopolysaccharide of *Porphyromonas gingivalis* strain W50. *Eur. J. Biochem.* **268**:4698–4707.
25. Paramonov, N., M. Rangarajan, A. Hashim, A. Gallagher, J. Aduse-Opoku, J. M. Slaney, E. Hounsell, and M. A. Curtis. 2005. Structural analysis of a novel anionic polysaccharide from *Porphyromonas gingivalis* strain W50 related to Arg-gingipain glycans. *Mol. Microbiol.* **58**:847–863.
26. Paramonov, N. A., J. Aduse-Opoku, A. Hashim, M. Rangarajan, and M. A. Curtis. 2009. Structural analysis of the core region of O-lipopolysaccharide of *Porphyromonas gingivalis* from mutants defective in O-antigen ligase and O-antigen polymerase. *J. Bacteriol.* **191**:5272–5282.
27. Pathirana, R. D., N. M. O'Brien-Simpson, and E. C. Reynolds. 2010. Host immune responses to *Porphyromonas gingivalis* antigens. *Periodontol.* **2000** **52**:218–237.
28. Pohlschroder, M., W. A. Prinz, E. Hartmann, and J. Beckwith. 1997. Protein translocation in the three domains of life: variations on a theme. *Cell* **91**:563–566.
29. Rangarajan, M., J. Aduse-Opoku, N. Paramonov, A. Hashim, N. Bostanci, O. P. Fraser, E. Tarelli, and M. A. Curtis. 2008. Identification of a second lipopolysaccharide in *Porphyromonas gingivalis* W50. *J. Bacteriol.* **190**:2920–2932.
30. Saiki, K., and K. Konishi. 2007. Identification of a *Porphyromonas gingivalis* novel protein Sov required for the secretion of gingipains. *Microbiol. Immunol.* **51**:483–491.
- 30a. Sandkvist, M. 2001. Biology of type II secretion. *Mol. Microbiol.* **40**:271–283.
31. Sato, K., M. Naito, H. Yukitake, H. Hiraoka, M. Shoji, M. J. McBride, R. G. Rhodes, and K. Nakayama. 2010. A protein secretion system linked to bacteroidete gliding motility and pathogenesis. *Proc. Natl. Acad. Sci. U. S. A.* **107**:276–281.
32. Sato, K., E. Sakai, P. D. Veith, M. Shoji, Y. Kikuchi, H. Yukitake, N. Ohara, M. Naito, K. Okamoto, E. C. Reynolds, and K. Nakayama. 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.
33. Sauer, F. G., K. Futterer, J. S. Pinkner, K. W. Dodson, S. J. Hultgren, and G. Waksman. 1999. Structural basis of chaperone function and pilus biogenesis. *Science* **285**:1058–1061.
34. Schneewind, O., D. Mihaylova-Petkov, and P. Model. 1993. Cell wall sorting signals in surface proteins of gram-positive bacteria. *EMBO J.* **12**:4803–4811.
35. Seers, C. A., N. Slakeski, P. D. Veith, T. Nikolof, Y. Y. Chen, S. G. Dashper, and E. C. Reynolds. 2006. The RgpB C-terminal domain has a role in attachment of RgpB to the outer membrane and belongs to a novel C-terminal-domain family found in *Porphyromonas gingivalis*. *J. Bacteriol.* **188**:6376–6386.
36. Slakeski, N., P. S. Bhogal, N. M. O'Brien-Simpson, and E. C. Reynolds. 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.
37. Reference deleted.
38. Veenendaal, A. K., C. van der Does, and A. J. Driessen. 2004. The protein-conducting channel SecYEG. *Biochim. Biophys. Acta* **1694**:81–95.
39. Veith, P. D., N. M. O'Brien-Simpson, Y. Tan, D. C. Djatmiko, S. G. Dashper, and E. C. Reynolds. 2009. Outer membrane proteome and antigens of *Tannerella forsythia*. *J. Proteome Res.* **8**:4279–4292.
40. Veith, P. D., G. H. Talbo, N. Slakeski, S. G. Dashper, C. Moore, R. A. Paolini, and E. C. Reynolds. 2002. Major outer membrane proteins and proteolytic processing of RgpA and Kgp of *Porphyromonas gingivalis* W50. *Biochem. J.* **363**:105–115.
41. Xie, G., D. C. Bruce, J. F. Challacombe, O. Chertkov, J. C. Dettler, P. Gilna, C. S. Han, S. Lucas, M. Misra, G. L. Myers, P. Richardson, R. Tapia, N. Thayer, L. S. Thompson, T. S. Brettin, B. Henrissat, D. B. Wilson, and M. J. McBride. 2007. Genome sequence of the cellulolytic gliding bacterium *Cytophaga hutchinsonii*. *Appl. Environ. Microbiol.* **73**:3536–3546.
42. Yen, M.-R., C. R. Peabody, S. M. Partovi, Y. Zhai, Y.-H. Tseng, and M. H. S. Saier, Jr. 2002. Protein-translocating outer membrane porins of Gram-negative bacteria. *Biochim. Biophys. Acta* **1562**:6–31.