PG0026 Is the C-terminal Signal Peptidase of a Novel Secretion System of Porphyromonas gingivalis

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Background: Several virulence factors of Porphyromonas gingivalis have a novel C-terminal signal that directs secretion across the outer membrane.

Results: The predicted catalytic amino acid of PG0026 was essential for the removal of this signal.

Conclusion: PG0026 is a novel C-terminal signal peptidase.

Significance: We have identified a novel signal peptidase of a new type of secretion system.

Protein substrates of a novel secretion system of Porphyromonas gingivalis contain a conserved C-terminal domain (CTD) of ~70–80 amino acid residues that is essential for their secretion and attachment to the cell surface. The CTD itself has not been removed by a novel signal peptidase. More than 10 proteins have been shown to be essential for the proper functioning of the secretion system, and one of these, PG0026, is a predicted cysteine protease that also contains a CTD, suggesting that it may be a secreted component of the secretion system and a candidate for being the CTD signal peptidase. A PG0026 deletion mutant was constructed along with a PG0026C690A targeted mutant encoding an altered catalytic Cys residue. Analysis of clarified culture fluid fractions by SDS-PAGE and mass spectrometry revealed that the CTD was released intact into the surrounding medium in the wild type strain, but not in the PG0026 mutant strains. Western blot experiments revealed that the maturation of a model substrate was stalled at the CTD-removal step specifically in the PG0026 mutants, and whole cell ELISA experiments demonstrated partial secretion of substrates to the cell surface. The CTD was also shown to be accessible at the cell surface in the PG0026 mutants, suggesting that the CTD was secreted but could not be cleaved. The data indicate that PG0026 is responsible for the cleavage of the CTD signal after substrates are secreted across the OM.

Bacteroidetes represent a major bacterial phylum that is prevalent in a diverse range of habitats (1–3) and include species that are of considerable medical, veterinary, and environmental importance (4–7). In the oral cavity, Bacteroidetes species such as Porphyromonas gingivalis and Tannerella forsythia are pathogens associated with chronic periodontitis in humans (8), whereas other Bacteroidetes are prevalent in endodontic infections (9). Chronic periodontitis is an inflammatory disease that results in the destruction of the supporting tissues of the tooth and is a major cause of tooth loss in adults (8). P. gingivalis is a Gram-negative anaerobic bacterium that secretes abundant cysteine proteases called gingipains. The gingipains are Arg-specific (RgpB and RgpA) and Lys-specific (Kgp) cysteine proteinases (10, 11) and are considered to be major virulence factors (11–14). Recently, a novel protein secretion system designated por secretion system (PorSS) has been reported in P. gingivalis that is conserved in most Bacteroidetes species and is linked to gliding motility (15). The known substrates of this secretion system all possess a conserved C-terminal domain (CTD) of ~70–80 amino acids (16) and are referred to herein as CTD proteins.

The gingipains are the most important and best studied CTD proteins of P. gingivalis, and the primary interest in the PorSS to date has been to understand how these CTD proteins are secreted across the outer membrane (OM) and attached to the cell surface. CTD proteins are extensively modified, generating diffuse bands on SDS-PAGE with molecular masses generally 20 kDa higher than that predicted from their sequence (17, 18). In particular, proteolytically cleaved gingipain domains that lack an immediately adjacent CTD also lack this modification (17). Due to the binding of extensively modified CTD proteins to an LPS binding matrix, the modification was proposed to be LPS (19). Furthermore, a monoclonal antibody (mAb-1B5) that recognizes the Manα1-2Manα1-phosphate side chains present in anionic polysaccharide, the polysaccharide portion of A-LPS (20, 21), also recognizes extensively modified CTD proteins (22), suggesting that the modification is A-LPS.

The CTD has been shown to be required for secretion and A-LPS modification because P. gingivalis mutants expressing RgpB lacking the entire CTD (16) or lacking just the last two C-terminal residues (23) accumulated unmodified pro-RgpB in the periplasm. Furthermore, it was recently demonstrated that

2 The abbreviations used are: PorSS, por secretion system; CTD, C-terminal domain; OM, outer membrane; TLCK, Nα-tosyl-L-lysyl chloromethyl ketone hydrochloride; CF, culture fluid; CCF, clarified CF, CCF, filtered clarified culture fluid; Bis-Tris, 2-(bis[2-hydroxyethyl]amino)-2-(hydroxy- methyl)propane-1,3-diol.
the CTD alone was sufficient for the secretion, modification, and cell surface attachment of substrates using GFP-CTD fusions (24). The CTD is not present in mature soluble forms of CTD proteins (25, 26) and has never been identified or detected in membrane-associated (extensively A-LPS modified) forms (17, 18, 24), suggesting that the CTD is the site of extensive A-LPS modification or that it is cleaved as part of the normal secretion and maturation of CTD proteins. However, extensive mutation analysis of potential glycosylation sites within the CTD of RgpB did not reveal any carbohydrate modification sites (27).

Since the first identified component of the PorSS, porT (28), at least 10 other PorSS components have been discovered by characterization of the respective secretion-deficient mutants (15, 29–34). For each mutant, precursor gingipains were found to accumulate in the periplasm or cell medium, whereas extensively modified gingipains were absent. The mutants also exhibited significantly reduced gingipain activities. Despite the characterization of many secretion mutants, little is known about the mechanism of secretion or the component(s) that form the secretion channel. The only protein to have a specific role assigned to date is the LptO protein (PG0027; PorV), which was shown to be required for the O-deacylation of LPS lipid A (33). In the same study, A-LPS was found to co-accumulate in the periplasm of lptO and porT mutants, leading to the proposal that PorSS coordinates the secretion of both CTD proteins and A-LPS to the cell surface. It is proposed that at the cell surface, A-LPS is deacylated by LptO and conjugated to CTD proteins, thus attaching them to the cell surface, producing an electron-dense surface layer (33). Gingipains were positively detected in the surface layer, indicating that this layer was the product of this combined secretion and attachment system (33). Immediately upstream of lptO is porU (PG0026), which was previously demonstrated to be required for gingipain secretion (15) and was also predicted to contain a CTD (16). In this study, we show that PG0026 is predicted to be a cysteine protease of the gingipain family and provide a comprehensive characterization of PG0026 mutants to demonstrate that cleavage of the CTD at the cell surface is dependent on PG0026 containing an active site Cys residue. Hence we conclude that PG0026 is the CTD peptidase responsible for CTD cleavage prior to conjugation to A-LPS.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Culture Conditions**—P. gingivalis strains listed in **supplemental Table S1** were grown on solid medium (TSBHI agar) containing trypticase soy agar (40 g/liter), brain heart infusion (5 g/liter), 5% (v/v) lysed defibrinated horse blood, cysteine hydrochloride (0.5 g/liter), and menadione (5 mg/ml) or in TSBHI broth under anaerobic conditions as described previously (33). The appropriate antibiotic was included in the media to select for the relevant antibiotic marker of the strain but omitted in the final harvested liquid culture.

**DNA Isolation and PCR**—Plasmid DNA was purified using the Wizard® Plus SV miniprep DNA purification system (Promega). Genomic DNA was obtained according to Chen and Kuo (35) from PBS-washed P. gingivalis cells grown on solid medium. Primers used in this study are listed in **supplemental Table S2**. PCR fragments used for cloning were amplified using 0.04 units/µl of Platinum Taq DNA polymerase high fidelity (Invitrogen) in 1× buffer supplied by the manufacturer, 5 mM MgCl2, 0.4 mM dNTP, 0.4 µM or 4 ng/µl each of forward and reverse primers, and 0.6 ng/µl genomic DNA from strain W50. PCR screening of recombinant *Escherichia coli* plasmid clones was performed as above except for 0.03 units/µl GoTaq Flexi DNA polymerase (Promega), 1× Green GoTaq Flexi buffer (Promega), and one colony used as template DNA. *P. gingivalis* mutants were screened using the conditions described above except that 0.04 units/µl BIOTAQ Red DNA polymerase (Bioline), 1× NH4 reaction buffer (Bioline), and 1.5–4 ng/µl genomic DNA were used.

**Construction of PG0026 Deletion Mutants**—Deletion mutants in PG0026 were created by double crossover recombination using the suicide vector pΔPG26-ermF that contains upstream and downstream regions of the PG0026 gene directionally cloned adjacent to an erythromycin resistance cassette (ermF) (**supplemental Fig. S1**). A 660-bp upstream fragment of PG0026 (26U) and a 504-bp downstream internal fragment of PG0026 (26D) were PCR-amplified using the primer pairs 26U-F-SphI + 26U-R-BamHI and 26D-F-PstI + 26D-R-Sacl, respectively. The PCR fragments were A-tailed before separate ligation into pGEM-Teasy. 26U was then excised with BamHI/Sphl and ligated into BamHI/Sphl-digested pAL30 (36), creating plasmid p26U-ermF. Cloned 26D was excised from pGEM-Teasy with PstII/Sacl and ligated into PstII/Sacl-digested p26U-ermF, creating pΔPG26-ermF. Plasmid pΔPG26-ermF was linearized with Scal and electrooporated into *P. gingivalis* as described previously (33).

**Construction of PG0026 Complementation Strain and Targeted PG26C690A Catalytic Mutant**—Introduction of the PG0026 gene into the fimA locus of *P. gingivalis* for complementation purposes was achieved using plasmid pKD713-PG26. The PG0026 gene, including 561 bp upstream of the predicted start codon and 115 bp downstream of the stop codon, was PCR-amplified, digested with PstI, and ligated into PstI-digested pKD713, creating the PG0026 complementation plasmid pKD713-PG26+. The entire PG0026 sequence within the plasmid was verified by DNA sequencing. This plasmid was then linearized with NotI and electrooporated into *P. gingivalis* strain 33277. The resulting selected clones were verified by PCR. One positive clone was then chosen for inactivation of the native PG0026 gene by electroporation using pΔPG26-ermF as above, and the clones with the correct gene inactivated were identified by PCR (**supplemental Fig. S1**). To create the PG0026 catalytic Cys-targeted mutant strain, 33277 PG26C690A, plasmid pKD713-PG26C690A was generated by introducing the specific site-directed mutation using the QuikChange II XL site-directed mutagenesis kit (Stratagene), the primers PG0026_C690A and PG0026_C690A анти, and pKD713-PG26+ as template. The entire PG0026C690A gene in pKD713-PG26C690A was verified by DNA sequencing. The PG0026C690A gene was introduced into the fimA locus of strain 33277 as described above.
Construction of E. coli Recombinant Protein Expression Vectors—The DNA fragment corresponding to amino acid residues 24–408 of the PG0026 coding sequence was amplified by PCR from P. gingivalis W50 genomic DNA using oligonucleotides 26-TagF and 26N-TagR (supplemental Table S2). The amplicon was then cloned into expression vector pET-30 Ek/LIC (Merck) and transformed into NovaBlue GigaSingles™ competent cells (Merck) according to the manufacturer’s manual. The rggp-B-CTD(422) DNA fragment encoding RgpB amino acid residues 651–736 (representing 86 CTD amino acids) was cloned into the pET28 expression vector (Novagen) essentially as described previously (37) except that the His tag codons were incorporated into the forward oligonucleotide primer, rather than originating from the vector sequence. Briefly, oligonucleotide primers CTD422-FOR and CTD422-REV were used to amplify genomic DNA from strain W50 by PCR, and the amplification product was purified and ligated to pGEM-Teasy (Promega). Purified pGEM-Teasy-rggp-B-CTD(422) DNA was digested with Ncol and ligated into Ncol-digested pET28a (Novagen). Ligation products were initially transformed into competent E. coli Alpha-Select cells, and then pET28-rggp-B-CTD(422) was subsequently purified and transformed into the E. coli expression host, BL21 (DE3).

Gingipain and Hemagglutination Activity Assays—The gingipain and hemagglutination activity assays were performed as described previously (33).

LPS Lipid A Analysis—Lipid A was released and extracted from membrane samples and analyzed by MALDI-TOF as described previously (33).

Protein Fractionation—Whole cell lysates were prepared from 10 ml of cell culture grown to a flask OD650 nm of 0.61 as described previously (33) except that 20 mM N-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK, Sigma-Aldrich) was used and cell lysates were passed through a 29-gauge needle 5× and left on ice for 30 min. An equal volume of 4× NuPAGE LDS sample buffer (Invitrogen) containing 20 mM TLCK and 50 mM DTT was added and then incubated at 80 °C for 3 min and stored at −80 °C. The culture fluid (CF) and clarified CF (CCF) were obtained as described previously (33). The CF was also filtered through 0.22 μm before centrifugation at 170,000 × g, 4 °C for 55 min to produce clarified cell culture fluid (CCFF). The periplasm and outer membrane vesicles were prepared as described previously (33). For the preparation of membrane and soluble fractions, washed cells from 45 ml of culture were resuspended in 3 ml of TC50 buffer (50 mM Tris-HCl, pH 8, 50 mM NaCl, 5 mM CaCl2), sonicated (33), and centrifuged at 900 × g, 4 °C for 10 min to remove unbroken cells. The supernatant was then centrifuged at 48,000 × g, 4 °C for 20 min to pellet membranes. The supernatant was saved as the soluble fraction containing cytoplasmic and periplasmic proteins, and the membrane pellet containing both inner and outer membranes was washed with 12 ml of TC50 buffer and finally resuspended in 0.72 ml of TC50 containing 1% (v/v) Triton X-114, 20 mM TLCK, and 10 μl/ml protease inhibitor cocktail (Sigma-Aldrich). The wet weight of the unbroken cell pellet was used to calculate a percentage relative to the wet weight of the whole cell pellet for loading normalization of membrane and soluble fractions relative to whole cell lysates.

SDS-PAGE and Western Blot—Fractionated samples were separated by reducing SDS-PAGE using either MOPS or MES running buffer, and proteins were then transferred to nitrocellulose membranes (Whatman) using a Criterion blotter or Trans-Blot Turbo blotting system (Bio-Rad). The membranes were prestained with Ponceau S to visualize protein transfer, destained in PBS containing 0.05% (v/v) Tween 20 (PBST0.05), and blocked with 5% (w/v) nonfat skim milk powder (Diploma, Fonterra Brands, Melbourne, Australia) in PBST0.05. The membranes were incubated with the appropriate primary mouse antibody followed by goat anti-mouse IgG conjugated to horseradish peroxidase (diluted 1:7000) (Sigma) and detected by chemiluminescence as described previously (26). The following primary mouse antibodies were used. Anti-CPG70 and anti-RgpB were raised to native purified proteins (26). Anti-rLptO (33) and anti-rKgpA1 (37) were raised to recombinant E. coli expressed and His-tagged purified proteins. Anti-rPG26N was raised to the 385-amino acid His-tagged N-terminal domain of PG0026 (from Gln24 to Leu808). Expressed soluble rPG26N was purified by Ni2+ -column affinity and size exclusion chromatography by Protein Express (Department of Biochemistry and Molecular Biology, Monash University, Melbourne, Australia). rPG26N was further purified by SDS-PAGE, transferred to nitrocellulose membrane, and excised before injection into mice according to the methodology described previously (33). Recombinant His-tagged RgpB-CTD(422) protein was purified from soluble E. coli fractions using nickel resin column chromatography following the methodology described previously (37). The purity of the resulting purified rRgpB-CTD(422) was verified using MS analysis before injection into mice. mAb 1B5 was kindly provided by Professor M. A. Curtis (22).

ELISA—Whole cell ELISA using formalin-killed whole cells as coating antigens was performed as described previously (33) except that 1 × 109 cells (50 μl)/well were used, 1:100 diluted goat serum (Sigma) was added to the blocking solution, and 10 mM Na2HPO4, pH 4 (glacial acetic acid), and 2 mM H3O2 was used for the substrate buffer.

In-gel Digestion and Mass Spectrometry—In-gel digestion using trypsin was performed after reduction with DTT and alkylation with iodoacetamide as published previously (38). Tryptic digests were acidified with TFA and analyzed by LC-MS/MS using a Dionex Ultimate 3000 system (Thermo Scientific) coupled to an HCT Ultra ion trap mass spectrometer as described previously (33). Proteins were identified by MS/MS Ion Search using Mascot v 2.2 (Matrix Science) against the P. gingivalis ATCC 33277 database. Search parameters were as follows: database = P. gingivalis, enzyme = trypsin or semi-trypsin, missed cleavages = 1, fixed modifications = carbamidomethyl (Cys), optional modifications = oxidation (Met), MS tolerance = 1.5 Da, MS/MS tolerance = 0.5 Da. When the same protein was identified from multiple gel fractions, the Mascot scores for that protein were summed.

Scanning Electron Microscopy—Square glass coverslips (22 mm) were prepared by smearing a 0.1% solution of polyethyleneimine (PEI) and dried by heating over a flame. Bacterial samples were incubated on PEI-coated glass coverslips for 1 h. Following incubation, the excess sample was drained, and cov-
**RESULTS**

**Identification of the CTD Cleavage Site of CTD Proteins**—To determine that the cleaved CTD was being released into the growth medium, CCF, obtained from wild type *P. gingivalis* strain ATCC 33277, was analyzed by SDS-PAGE, and the region less than 15 kDa was excised, digested with trypsin, and analyzed by LC-MS/MS. CTDs were found to be the major components of the fraction less than 10 kDa with 46 unique CTD peptides identified from 13 different CTD proteins, demonstrating that the CTD is indeed cleaved, and released largely intact into the growth medium (Table 1). The database was searched, allowing for peptide matches with a nontrypptic cleavage (enzyme = “semitryptic”), resulting in the identification of 11 semitryptic peptides (Table 1). All were from the predicted N-terminal region of the CTDs and therefore revealed the likely CTD cleavage sites for these CTD proteins. Examination of the putative cleavage sites indicated a preference toward cleavage C-terminal to a polar or acidic amino acid residue (such as Ser, Thr, Asn, or Asp), and N-terminal to a small amino acid residue (such as Gly, Ser, Ala, or Asp).

Of the proteins previously shown to be involved in the PorSS, we identified using BLAST, Conserved Domains search, and Fugue analyses that an internal domain of PG0026 exhibited sequence and structural (Z score 38.7) similarity to the gingipains (clan C25) of *P. gingivalis* with His^557^ and Cys^590^ being the predicted active site residues (Fig. 1A). As PG0026 contains a predicted CTD, we first investigated whether PG0026 exhibited the properties of typical secreted CTD proteins, namely localization to the OM, extensive modification, and absence of CTD. OM vesicle and membrane fractions were prepared from wild type *P. gingivalis* W50 and analyzed by SDS-PAGE and LC-MS/MS. PG0026 was primarily identified from a sharp band at ~140 kDa both in the membrane fraction (data not shown) and in OM vesicles (Fig. 1B), suggesting an OM location. Moreover, peptides from the CTD and peptides close to the predicted N-terminal signal peptide cleavage site were also identified by MS (Fig. 1A), demonstrating that the major form of PG0026, unlike typical CTD proteins, was not obviously modified and possessed an intact CTD. BLAST searches using full-length PG0026 confirmed that the distribution of this protein is confined to those *Bacteroidetes/Chlorobi* species that encode components of the PorSS, consistent with a role for PG0026 in the secretion system. In particular, the CTD of PG0026 was very well conserved, and although it contained the major CTD motifs common to typical CTD proteins, it was considerably longer and exhibited additional regions of conservation (Fig. 1C). In regard to domains A and B of PG0026 (Fig. 1A), no putative functions were revealed from the bioinformatic searches employed. In strains W83 and ATCC 33277, the PG0026 (PGN_0022) and lptO genes are immediately adjacent to each other and in the same orientation. In fact, in the majority of species studied,

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**TABLE 1**

Identification of cleaved CTDs and their putative cleavage sites

| Protein name | Accession number | Identified semitryptic peptide and cleavage site | Mascot score | CTD peptides |
|--------------|------------------|-----------------------------------------------|--------------|--------------|
| RgpA         | PG2024 PGN_1970  | VDYIPD/GAVDVAQKPTTYTVVGK                      | 42           | 4            |
| RgpB         | PG0506 PGN_1466  | VKVBST/LSTVMDKVPYTVVGK                        | 75           | 4            |
| HBP33        | PG0616 PGN_0659  | DKARP/SSTFQMTAQVKTPYTVVGK                     | 53           | 6            |
| CPG70        | PG2032 PGN_0335  | EVILDO/GVEVAVQGTVLIRPOQGTK                    | 65           | 4            |
| P59 (TapA)   | PG2102 PGN_0152  | QNTHAN/GVEVAVQGMSM                           | 84           | 3            |
| HagA         | PG1837 PGN_1733  | ATLLIT/SLATVMDKVPYTVVGK                      | 27           | 3            |
| PG0654       | PG1374 PGN_0852  | YERGSP/SLAVDAPRTV                            | 63           | 4            |
| PG2172       | PG0123            | YKGGST/SLTN1GLOR                             | 50           | 3            |
| PG2216       | PG1739 PGN_0693  | TDMQCN/SLTDVAVNESLK                         | 63           | 4            |
| Pad          | PG1424 PGN_0898  | Not identified                               | 44           | 2            |
| Pad          | PG1798 PGN_1767  | Not identified                               | 44           | 2            |

*a The semitryptic peptides identified by MS/MS are shown underlined, together with the six amino acid residues immediately N-terminal to the proposed cleavage sites (indicated by a slash).

*b The Mascot score shown is for the underlined semitryptic peptide (shown in the previous column). Scores above 35 were significant according to Mascot (p < 0.05); however, due to the consistent pattern of results, scores above 25 were deemed significant.

**The total number of CTD peptides identified.**

erslips with adhered cells were immersed in 2.5% glutaraldehyde in PBS for 1 h. Coverslips were then rinsed three times in PBS for 10 min each before being dehydrated in increasing concentrations of ethanol consisting of 10, 30, 50, 70, 90, and 100% ethanol in water for 10 min each step. The coverslips were then dried in a Balzers critical point dryer (Balzers Pfeiffer, Balzers, Liechtenstein) and mounted onto 25-mm aluminum stubs with double-sided carbon tabs. The edges of the coverslips were treated with silver liquid, dried, and then gold-coated in an Edwards S150B sputter coater (Edwards High Vacuum, Crawley, West Sussex, UK). The cells on coverslips were imaged with the Philips XL30 field emission scanning electron microscope (Philips, Eindhoven, Netherlands) at a voltage of 2 kV.

**Cryo-electron Microscopy**—Cryo-EM was performed as described previously (33).

**Bioinformatics**—BLAST searches that also incorporated searching of the Conserved Domains database (CD search) were conducted from the National Center for Biotechnology Information (NCBI) website (blast.ncbi.nlm.nih.gov). The multiple alignment of PG0026 homologs was conducted using ClustalW with default settings. The Fugue search web tool (39, 40) was used at the National Institute of Biomedical Innovation (tardis.nibio.go.jp/homstrad). The **PG0026** gene clusters in *Bacteria* were obtained from the Integrated Microbial Genomes website, img.jgi.doe.gov, or from the Oral Pathogen Sequences Databases website, www.oralgen.lanl.gov/oralgen/.

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the PG0026 and lptO genes were found to be linked (supplemental Fig. S2). In a small number of species, these genes were found to be separated by one or more genes.

Construction and Complementation of the PG0026 Mutant and Phenotype Analyses—PG0026 was insertionally inactivated in both W50 and 33277 strain backgrounds by homologous recombination, replacing 2521 bp (840 amino acids) of PG0026 coding sequence from amino acids Ser85 to Val925 with the ermF antibiotic cassette. The disruption of PG0026 in both strains was validated by PCR (supplemental Fig. S1) and Western blot analysis (see below). Both W50 PG0026 and 33277 PG0026 isogenic mutants displayed a nonpigmented colonial phenotype on blood agar (Fig. 2, A and B, and supplemental Fig. S1) but developed a light brown color after more than 14 days of incubation (data not shown). The colonies of the PG0026 mutants were also smaller and never reached the size of their respective WT strains (Fig. 2B). The growth rate of the mutants was slower in liquid medium with a doubling time of \(7–9\) h when compared with \(3\) h for the WT strains. The W50 PG0026 and 33277 PG0026 mutants displayed significantly reduced hemagglutination activity with a decrease of at least 16 HA units relative to WT (Fig. 2C). The 33277 PG0026 and W50 PG0026 mutants produced significantly reduced whole cell gingipain Arg-specific activities of 10% (33277 PG0026, Fig. 3, and...
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W50 PG0026, data not shown) and Lys-specific activities of 19% (33277 PG0026, Fig. 3) and 32% (W50 PG0026, data not shown) relative to their respective parental WT strain whole cell activities.

To verify that the observed PG0026 mutant phenotype was due specifically to mutation of the PG0026 gene, the PG0026 mutation was complemented. Repeated attempts to complement the PG0026 mutation by introduction of a second PG0026 copy into the fimA locus of either W50 PG0026 or 33277 PG0026 mutants using electroporation proved unsuccessful. Therefore an alternative strategy was utilized to obtain an identical PG0026 supplementation genotype. A second PG0026 gene copy was first introduced into the fimA locus of the WT strain using pKD713-PG26/H11001 followed by deletion of the native PG0026 gene. As the electroporation efficiency of pKD713-PG26/H11001 was too low using W50, this strategy could only be employed using strain 33277 where deletion occurred at the native PG0026 locus in ~50% of the resulting clones, whereas the fimA locus copy was deleted in the remaining 50%. One clone with the correct genotype (validated by PCR, supplemental Fig. S1), was chosen as the complementation strain, 33277 PG0026C690A. All 33277 PG0026C690A clones were similar to the PG0026 deletion mutant in that they were not pigmented on blood agar, grew at a slower rate, and produced smaller colonies than the WT (Fig. 2, A and B). The C690A targeted mutant also displayed reduced hemagglutination (Fig. 2C) and gingipain activities that were comparable with that of the deletion mutant (Fig. 3). The data indicate that Cys690 was correctly identified as the catalytic Cys residue and that a proteolytically active PG0026 is necessary for the proper secretion and maturation of the gingipains.

Abnormalities of PG0026 Mutant Cells Revealed by EM Analyses—Analysis of PG0026 and PG26C690A mutants by Gram stain showed that up to 5% of cells displayed an abnormally elongated cell morphology, whereas elongated cells were only infrequently (~0.01%) observed in the WT strains. SEM also showed this elongated morphology of the PG0026 mutants (Fig. 4A), with the most frequent elongated length estimated to range between 1.7 and 5 μm. However, a number of elongated cells of the mutant strain measured up to 30 μm, which was not observed in the WT strain. It was observed by cryo-EM that in contrast to WT W50 cells, W50 PG0026 mutant cells lacked the electron-dense surface layer immediately surrounding the OM, indicating that PG0026 is essential for the biogenesis of this layer (Fig. 4B). W50 PG0026 mutant cells also produced abnormally large vesicles (Fig. 4B) that were similar in size to those of the porT mutant (33). Lipid A analysis of the PG0026 mutant produced a similar lipid A profile to WT, suggesting no involvement of PG0026 in lipid A maturation (supplemental Fig. S3).

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PG0026 Is a C-terminal Signal Peptidase

Accumulation of CTD Proteins in the Periplasm of the PG0026 Mutant—To assess whether PG0026 is required for the secretion of CTD proteins, the periplasm of the PG0026 mutant was obtained and compared with wild type and the lptO mutant by SDS-PAGE. It was found that the profile of the PG0026 mutant was more similar to lptO rather than to WT (Fig. 5A). Identification of the proteins in each gel fraction by LC-MS/MS analysis enabled the identification of 202 nonredundant proteins including 20 CTD proteins and 13 proteins that have been localized to the periplasm in other experiments (see Footnote 3).

The Predicted Catalytic Cys of PG0026 Is Required for the Maturation of CTD Proteins—To better understand the location and processing of selected CTD proteins and components of the secretion system in the PG0026 and lptO mutants, cultures were quantitatively separated into whole cell, membrane, soluble, CF, and CCF fractions and analyzed by Western blot (Fig. 6). A specific band at ~140 kDa in the anti-rPG26N blots was seen in all strains except the PG0026 mutant, verifying the successful deletion of the gene (Fig. 6). The PG0026 complementation strain expressed PG0026 in whole cells to the same level as in WT, and this was evenly partitioned between the membrane and soluble fractions as in the WT. In contrast, PG0026C690A was not as highly expressed as WT PG0026 in whole cells, and the majority of it was found in the soluble fraction with only a small amount in the membrane fraction, indicating that PG0026C690A was only partially secreted when compared with WT. The successful expression of PG0026 at the fimA locus demonstrated that PG0026 is monocistronic and has its own promoter. Although the lptO mutant expressed PG0026 in whole cells at almost WT levels, the majority of the protein was found in the soluble fraction (Fig. 6), consistent with its accumulation in the periplasm (Fig. 5). LptO was found exclusively in the membrane fraction of the WT and PG0026+ strains; however, in both PG0026 and PG0026C690A mutant strains, some LptO was also detected in the soluble fraction.

In the WT and PG0026+ complementation strains, CPG70 and RgpB largely existed as diffuse 90–120- and 65–85-kDa bands, respectively, corresponding to their modified mature forms that were mainly found in the membrane fraction (Fig. 6). Similarly, the bands detected with anti-KgpA1 in these strains included discrete bands at 41 and 43 kDa, most likely corresponding to fully processed KgpA1 and RgpA1, and a diffuse band at ~45–55 kDa corresponding to fully processed and modified RgpA1 (11). Again, these forms were mainly localized to the membrane fraction. In contrast, in the lptO mutant and both PG0026 and PG0026C690A mutants, CPG70, RgpB, and Kgp/RgpA were all incompletely cleaved and processed, and the majority of these partially cleaved forms were found in the soluble fraction (Fig. 6). These CTD proteins were also not diffuse and not modified with A-LPS, which appeared to be the case for most, if not all, CTD proteins in these mutants because the mAb 1B5 Western blot only detected the low molecular mass, nonprotein-linked forms of A-LPS (Fig. 6). Functional PG0026 containing its putative active site Cys residue is therefore required for the proper secretion, maturation, and modification of CTD proteins. Although the mutants mainly produced precursor forms of RgpA and Kgp that were detected with anti-KgpA1, fully processed A1 domains (41 and 43 kDa) appeared to be produced at a low level in the PG0026 mutants and not at all in the lptO mutant, which is consistent with the generation of some Arg- and Lys-specific activities in the PG0026 and PG0026C690A mutants (Fig. 3) and no activity in the lptO mutant (33). However, the diffuse band at ~45–55 kDa, corresponding to fully processed and modified RgpA1, was not detected in PG0026, PG0026C690A, and lptO mutants (Fig. 6).
In the PG0026 mutant, there were four RgpB forms detected in whole cells labeled I (98 kDa), II (82 kDa), III (70 kDa), and IV (56 kDa) in Fig. 6. All four forms possessed the CTD domain, as detected by anti-RgpB-CTD(422), and were progressively truncated from the N terminus. Bands I–III were present in all strains and were mainly found in the soluble fraction, consistent with them being periplasmic proforms of RgpB. In contrast, the RgpB band IV was mainly confined to the PG0026 mutants, where it was strongly detected with anti-rRgpB, but only weakly with anti-RgpB. Unlike bands I–III, this band was relatively intense in the membrane fraction, raising the possibility that this form was secreted. The WT exhibited a weak band IV, presumably because it was rapidly processed further to form mature RgpB, whereas band IV was not detected in the lptO mutant. Taken together, it appears that maturation of RgpB had stalled at the band III stage in the lptO mutant, whereas it had stalled at band IV in the PG0026 mutant. The estimated molecular mass of band IV (56 kDa) is the same as the calculated molecular mass of RgpB with its CTD intact but with the entire prodomain removed, suggesting a block in maturation at the CTD cleavage stage in the PG0026 mutant. In the WT and PG0026 + -complemented strain, most of the CPG70, RgpB, and KgpA1 domains and the mAb 1B5-reactive proteins were cell-associated (Fig. 6). MS analysis of gel segments from the CCF fractions revealed an abundance of cytoplasmic proteins, suggesting that some cell lysis had occurred (data not shown).

The Predicted Catalytic Cys of PG0026 Is Required for CTD Cleavage at the Cell Surface—Formalin-killed whole cells were used as coating antigen in a whole cell ELISA to examine relative cell surface abundances of PG0026 and CTD proteins or
their specific domains between WT and mutant strains (Fig. 7). Anti-rPG26N, in addition to recognizing PG0026, was found to react with other protein bands in Western blots. As a result, the PG0026 mutant produced a high background in the anti-rPG26N ELISA. Assuming that the background was similar in the other strains tested, each of the other strains (33277, PG26\(^\ast\), 33277 PG26C690A, and 33277 lptO) had a detectable amount of PG0026 on the cell surface (Fig. 7). The WT strain appeared to have the highest amount of surface-exposed PG0026, whereas the lptO mutant had the least, consistent with the reduced amount of membrane-associated PG0026 detected by Western blot in the lptO mutant (Fig. 6).

Importantly, a consistent negative result for all other antisera with 33277 lptO confirmed that CTD proteins were not present on the cell surface of this mutant. The ELISA results showed that the cell surface abundances of RgpB, CPG70, and A1 adhesin domains of the PG0026 complementation strain (33277 PG26\(^\ast\)) were similar to the WT strain (Fig. 7), whereas their abundance in both PG0026 mutants was reduced, demonstrating that translocation of CTD proteins across the OM in these mutants was partially active. This partial secretion was consistent with the generation of a small amount of whole cell proteolytic activity in the PG0026 mutant (Fig. 3) but no activity in the lptO mutant (33). The most striking ELISA results were for anti-RgpB-CTD(422), which detected the CTD domain of RgpB on the cell surface of only the PG0026 and PG0026C690A mutants, indicating that the CTD of RgpB was being secreted but not cleaved. This form of RgpB with the CTD intact is con-

![FIGURE 7. Whole cell ELISA analysis of secreted cell surface CTD proteins.](image-url)
FIGURE 8. Cleaved RgpB CTD fragment is only detected in wild type strains and not in PG0026 or iptO mutants. Anti-RgpB-CTD(422) Western blots of TCA-precipitated CCFF (1 ml) and whole cell (WC) (equivalent to 120 µl of culture) fractions as indicated were subjected to reducing SDS-PAGE using 10% NuPAGE Bis-Tris gel ( Invitrogen). Lane 1, 33277; lane 2, 33277 PG26-; lane 3, 33277 PG26C690A; lane 4, 33277 iptO; lane 5, 33277 PG26; and lane 6, W50. All fractions were from cells grown to a sidearm OD_{600\text{nm}} of 0.61 (cuvette OD_{600\text{nm}} reading of 1.8) except W50, which was from a cuvette OD_{600\text{nm}} of 0.4. S, protein molecular weight standard, SeeBlue Plus2 ( Invitrogen).

sistent with the 56-kDa membrane-associated RgpB ( band IV) that was detected by Western blot in the PG0026 and PG0026C690A mutants ( Fig. 6).

To further validate that the cleavage of CTD was blocked in the PG0026 mutant strains, CCFF fractions and whole cell lysates were analyzed by Western blot using MES buffer to optimize the separation of low molecular mass proteins. The transferred proteins were then probed with anti-rRgpB-CTD ( Fig. 8 ). The cleaved CTD (7 kDa) of RgpB was detected strongly in WT CCFF samples but was absent in the iptO mutant and both PG0026 and PG0026C690A mutants as well as all whole cell fractions. The cleaved CTD was also detected in the CCFF of the PG0026 complement strain, further confirming the role of PG0026 in the cleavage of the CTD. In addition to anti-rRgpB-CTD Western blot, MS analysis of the PG0026 mutant was also performed to verify that there were no cleaved CTDs from other CTD proteins. No CTD peptides were identified by MS in the fraction less than 10 kDa, suggesting that none of the CTD proteins had their CTD cleaved.

DISCUSSION

The PorSS is a novel Gram-negative protein secretion system present in the Bacteroidetes phylum (15). Its substrates contain a conserved CTD whose function and fate have been debated for some time. In strain HG66, RgpB is secreted as a discrete 50-kDa C-terminally truncated soluble enzyme. The crystal structure of this soluble form suggested that CTD cleavage occurred somewhere after Ser435 (25). However, in strain W50, where RgpB is mainly found as an extensively carbohydrate-modified form of molecular mass 70–90 kDa, the fate of the CTD was unknown. The apparent absence of the CTD in this form was explained by it being the site of extensive modification because the CTD was the only part of the protein that exhibited significant sequence similarity to other extensively modified CTD proteins (17). We show here for the first time that the CTD itself is not modified, but is removed from CTD proteins upon secretion, with modification presumably occurring at or close to the mature C terminus of the substrate protein. The CTDs appeared to be cleaved in only one place because only a single N-terminal semitryptic peptide was identified for each CTD (Table 1). None of the sites were consistent with Arg- or Lys-gingipain cleavage, pointing to the presence of a specific CTD peptidase. In RgpB, cleavage was found to occur immediately C-terminal to Thr334, producing a slightly shorter RgpB than previously reported (25). This slight discrepancy is likely to be attributed to the different strains used in the studies.

It has been recently shown that the CTD of various CTD proteins is sufficient for the secretion, modification, and cell surface attachment of a fused GFP in P. gingivalis (24). Successive truncations in the CTD of HBP35 identified that just the last 22 amino acids were sufficient to act as a functional signal for secretion and surface attachment of the GFP (24). The last 22 amino acids of the CTD are therefore the primary signal for this secretion and attachment process, and perhaps the region that interacts with the secretion machinery in order for CTD cleavage to occur. A question to consider is whether after directing secretion, the CTD also directs modification, or whether modification is a corollary of CTD cleavage. RgpA and Kgp are each proteolytically processed into a catalytic domain and several adhesin domains; however, only the most C-terminal adhesins (RgpAA and KgpAA) are extensively modified (11, 17), suggesting a proximal relationship between the CTD and the modification. Because the CTD is not itself modified, it is likely that modification is linked to CTD cleavage, with the modification site being located at or very close to the mature C terminus of the substrate protein.

The link between CTD cleavage and modification is further strengthened by the finding that PG0026 did not have its CTD cleaved and was not extensively modified ( Fig. 1B). The presence of a CTD within the PG0026 sequence, its localization to the OM, and its detection on the cell surface by ELISA are consistent with a surface location for this protein. The reason for the lack of CTD cleavage and modification in PG0026 may be attributed to the differences observed in its CTD sequence (Fig. 1C ). The regions of the PG0026 CTD that are conserved in PG0026 homologs (Fig. 1C, highlighted in yellow) but not found in other P. gingivalis CTD proteins are N-terminal to the portion of the CTD that was found to be essential for the secretion and attachment of GFP (24), suggesting that PG0026 may be secreted across the OM normally. The unique features of the PG0026 CTD may then be responsible for blocking its cleavage.

It is not new for a secretion system component to also be a substrate because in the assembly of the type III secretion system needle complex, several components including the needle, tip, and pore must be secreted sequentially through the growing type III secretion system apparatus prior to the secretion of the effector proteins (41–43). In contrast, in the PorSS, it appears that the secretion channel is functional without PG0026 because the mutant was able to secrete some CTD proteins to the cell surface as detected by ELISA ( Fig. 7), and these remained on the surface perhaps only due to retention of their

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CTD. PG0026 may therefore be secreted at the same time as other CTD proteins, using the same CTD signal, but cannot be cleaved due to having unique regions of sequence within the CTD (Fig. 1C). The cleaved CTDs were not detected in whole cells (Fig. 8) but appeared to be abundant in the clarified culture fluid due to the high number of CTD peptides recovered (Table 1), indicating that the form of CTD detected on the cell surface by ELISA was uncleaved and that cleaved CTDs are efficiently released into the culture fluid. The cleaved CTDs were also intact, suggesting they were resistant to gingipain cleavage. Furthermore, their presence in the culture fluid suggests that CTD proteins are secreted across the outer membrane inclusive of their CTD, such that CTD cleavage occurs on the cell surface. This is supported by the immunological detection of the CTD on the cell surface of mutants lacking a functional PG0026 (Fig. 7), indicating that PG0026 is the CTD peptidase. All other data obtained in this study are consistent with this conclusion. Firstly, PG0026 is a predicted gingipain, and site-directed mutagenesis of its predicted active Cys residue to Ala produced a mutant that exhibited essentially the same phenotype as the deletion mutant, including the absence of the electron-dense surface layer, indicating that all of the defects observed in the mutant could be attributed to the loss of its proteolytic capability. Secondly, PG0026 was detected on the cell surface where CTD cleavage must occur (Fig. 7). Thirdly, although partial secretion and partial gingipain activity were observed in the PG0026 mutants, no cleaved CTDs were found (Fig. 8). Fourthly, although RgpB maturation in the lptO mutant appeared to stall in the periplasm with the generation of a 70-kDa isoform that was predicted to contain some residual prosequence, in the PG0026 mutants, maturation stalled at the generation of a membrane-associated 56-kDa isoform, only requiring CTD cleavage and A-LPS modification to complete its maturation (Figs. 6, 7, and 9).

The observation of partial gingipain secretion and activity in the PG0026 mutants indicates a partially functioning secretion system and is in contrast with the lptO mutant, where secretion is blocked before the outer membrane. The mechanism of blockage in the lptO mutant is unclear. LptO may interact physically with the secretion pore, or else assembly of a functional pore may be dependent on the presence of deacylated lipid A in

**FIGURE 9. Model for the secretion and maturation of RgpB.** Step 1, cytoplasmic RgpB<sub>Full-length</sub> is proposed to be transported across the inner membrane by the SEC translocon due to the possession of a leader sequence. Step 2, proforms of RgpB corresponding to bands I–III (Fig. 6) are proteolytically processed and secreted from the periplasm to the cell surface. Step 3, the final step of prodomain removal occurs during secretion or on the cell surface to produce band IV (Fig. 6). Step 4, the CTD is cleaved by PG0026 on the cell surface. PG0026 may interact with LptO and/or other proteins (??). Step 5, RgpB<sub>cat</sub> is modified with A-LPS. Feedback loop 6, there is likely to be a limited capacity to hold CTD proteins on the cell surface prior to their permanent attachment to the OM via A-LPS. When this limit is reached, further secretion from the periplasm would be blocked. In the lptO mutant, step 2 (secretion) is blocked such that band IV is not produced, and neither RgpB nor CTD are detected on the cell surface by ELISA (Fig. 7). In PG0026 mutants, band IV accumulates in the membrane fraction (Fig. 6), and both RgpB and CTD are detected on the cell surface by ELISA (Fig. 7). Accumulation of bands I–III in the periplasm of PG0026 mutants maybe due to the backlog of unprocessed CTD proteins on the cell surface.
the OM produced by LptO. In contrast, in the PG0026 mutants, there is accumulation of immature CTD proteins both in the periplasm (Fig. 6) and on the cell surface (Figs. 6 and 7). The accumulation on the cell surface is presumably a direct consequence of PG0026 mutation, whereas accumulation in the periplasm suggests that there may be a backlog of unprocessed CTD proteins on the cell surface that ultimately block further secretion due to the inability to transfer CTD proteins out of the secretion pore toward PG0026 (Fig. 9). In the lptO mutant, PG0026 is absent from the membrane and is found instead accumulated in the periplasm (Figs. 6 and 7) (33). This means that both LptO and PG0026 are not functioning in the lptO mutant, but because the defect in gingipain secretion and maturation is total in the lptO mutant, LptO must be essential for gingipain secretion, whereas PG0026 is not. Furthermore, mutation of PG0026 had no effect on the deacetylation of lipid A, whereas LptO was found to be essential for lipid A deacetylation (33), indicating that LptO is still functioning in the PG0026 mutant in this respect. Partial CTD protein secretion in the PG0026 mutants is consistent with PG0026 functioning down-stream of secretion. We therefore conclude that PG0026 cleaves the CTD after secretion of the CTD protein (Fig. 9). After secretion of PG0026 through the secretion pore by the same mechanism as other CTD proteins, PG0026 may be anchored on the cell surface via an interaction with LPS or an outer membrane protein. It is interesting in this regard that LptO was found exclusively in the OM fraction of the WT and PG0026− strains; however, in both PG0026 and PG0026C690A mutants, some LptO was also detected in the soluble fraction. Hence this may suggest that the efficient incorporation of LptO into the OM may be dependent on the presence of PG0026, and may imply an interaction between PG0026 and LptO at the cell surface. Alternatively, this observation could be due to the up-regulation in expression of LptO in both PG0026 and PG0026C690A mutants causing a concomitant overload to the normal kinetics of membrane insertion; however, this latter explanation is unlikely as LptO was also up-regulated in the PG0026− complementation strain but did not show any LptO in the soluble fraction (Fig. 6). After CTD cleavage by PG0026, the cleaved CTD dissociates, and A-LPS is conjugated to the new C terminus of the substrate protein, thereby anchoring the mature protein to the cell surface (Fig. 9). The conjugation may be performed by an uncharacterized domain of PG0026 such as domain A or B (Fig. 1A) or by an ancillary component of the PorSS. Other potential functions for domain A and B of PG0026 include cell surface binding and CTD cleavage selectivity.

In conclusion, we propose that the major secretion pathway operating in the Bacteroidetes/Chlorobi phyla involves two signals present in the secretory proteins. The first is an N-terminal signal peptide that directs translocation across the cytoplasmic membrane via the SEC machinery and is then cleaved by signal peptidase I. The second is the CTD signal that directs secretion across the OM via the PorSS machinery and is then cleaved by PG0026 prior to conjugation to A-LPS and surface attachment. To our knowledge, this is the first signal peptidase of Gram-negative bacterial secretion systems shown to cleave a C-terminally located signal.

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