

## Identification and Characterization of the Capsular Polysaccharide (K-Antigen) Locus of *Porphyromonas gingivalis*

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Capsular polysaccharides of gram-negative bacteria play an important role in maintaining the structural integrity of the cell in hostile environments and, because of their diversity within a given species, can act as useful taxonomic aids. In order to characterize the genetic locus for capsule biosynthesis in the oral gram-negative bacterium *Porphyromonas gingivalis*, we analyzed the genome of *P. gingivalis* W83 which revealed two candidate loci at PG0106-PG0120 and PG1135-PG1142 with sufficient coding capacity and appropriate gene functions based on comparisons with capsule-coding loci in other bacteria. Insertion and deletion mutants were prepared at PG0106-PG0120 in *P. gingivalis* W50—a K1 serotype. Deletion of PG0109-PG0118 and PG0116-PG0120 both yielded mutants which no longer reacted with antisera to K1 serotypes. Restriction fragment length polymorphism analysis of the locus in strains representing all six K-antigen serotypes and K<sup>-</sup> strains demonstrated significant variation between serotypes and limited conservation within serotypes. In contrast, PG1135-PG1142 was highly conserved in this collection of strains. Sequence analysis of the capsule locus in strain 381 (K<sup>-</sup> strain) demonstrated synteny with the W83 locus but also significant differences including replacement of PG0109-PG0110 with three unique open reading frames, deletion of PG0112-PG0114, and an internal termination codon within PG0106, each of which could contribute to the absence of capsule expression in this strain. Analysis of the Arg-gingipains in the capsule mutants of strain W50 revealed no significant changes to the glycan modifications of these enzymes, which indicates that the glycosylation apparatus in *P. gingivalis* is independent of the capsule biosynthetic machinery.

The putative virulence determinants of *Porphyromonas gingivalis*, a black-pigmenting, gram-negative oral anaerobe, have received considerable attention because of the association of this bacterium with destructive periodontal disease (45) and, more recently, with systemic conditions such as cardiovascular diseases (33) and preterm low birth weight (38). Factors of *P. gingivalis* considered to be of importance in the pathogenesis of periodontal disease include the major cell surface macromolecules, capsular polysaccharide (CPS) (or K-antigen) and lipopolysaccharide (LPS), and the extracellular/surface cysteine proteases, the Arg- and Lys-gingipains (30).

Macromolecules on the surface of bacteria confer ultrastructural stability and are important for recognition by and interaction with the environment. In pathogenic bacteria, surface macromolecules also form a defensive barrier against the host's immune system. In this regard capsular polysaccharides represent a significant class of surface macromolecules which may contribute to these surface properties in many gram-negative bacteria. In addition, K-antigens are responsible for serospecificity and hence constitute a useful taxonomic tool (51).

The K-antigens of *P. gingivalis* are considered to contribute

significantly to virulence (50). There are at least six different serotypes (K1 to K6) in addition to K<sup>-</sup> strains. The K<sup>-</sup> group is typified by the ability to autoaggregate and adhere to pocket epithelium and other oral bacteria (11, 23, 24, 27, 50). In a clinical study of the immune response to bacterial antigens in adult periodontal patients, a significantly elevated immunoglobulin G (IgG) serum antibody response to purified K1 capsular polysaccharide antigen was reported (43). However, another group showed that antibodies to all K1 to K6 antigens were represented in sera of patients with adult or general early-onset forms of periodontal disease (6).

Animal studies have demonstrated that K<sup>-</sup> strains are significantly less virulent in models of soft tissue destruction (28, 29). More recently, Gonzalez et al. (16) reported that mice immunized with purified capsular polysaccharide from *P. gingivalis* A7436 (K1) (unpublished data) demonstrated elevated immunoglobulins (IgM and IgG) to whole cells of *P. gingivalis* and were protected against alveolar bone loss when challenged with live bacteria (16). Hence there is growing evidence that the capsular polysaccharide of *P. gingivalis* is an important component of the host-pathogen interface in periodontal disease, and the presence and nature of the K-antigen may have a bearing on the outcome of the disease.

The genetics of capsule biosynthesis in *P. gingivalis* and the factors governing serospecificity are largely unknown although sequence analysis of the genome of *P. gingivalis* W83 has revealed four potential loci resembling those involved in glycan

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TABLE 1. List of bacterial strains used

Bacterium	Relevant genotype or serotype	Identification no.	Source
<i>E. coli</i> XL-1 Blue MRF'	DNA cloning/plasmid maintenance		Stratagene
<i>P. gingivalis</i> W50	Wild type (K1)		ATCC 53978
<i>P. gingivalis</i> GPA	Δ PG0116-PG0120::erm		This paper
<i>P. gingivalis</i> GPB	PG0117::erm		This paper
<i>P. gingivalis</i> GPC	Δ PG0109-PG0118::erm		This paper
<i>P. gingivalis</i> W83	K1, sequenced strain <sup>a</sup>		37
<i>P. gingivalis</i> ATCC 33277			
<b><i>P. gingivalis</i> HG91 (381)</b>	K <sup>-</sup>	1	
<i>P. gingivalis</i> HG1694	K <sup>-</sup>	2	
<i>P. gingivalis</i> HG1695	K <sup>-</sup>	3	
<b><i>P. gingivalis</i> HG66 (W83)</b>	K1	4	
<i>P. gingivalis</i> HG1631 (22KN6-12)	K1	5	
<i>P. gingivalis</i> HG1700	K1	6	
<i>P. gingivalis</i> HG1644 (AJW5)	K2 <sup>b</sup>	8	
<i>P. gingivalis</i> HG1706	K2	9	
<b><i>P. gingivalis</i> HG1025 (A7A1-28, ATCC 53977)</b>	K3	10	
<i>P. gingivalis</i> HG1648 (CLN17-6-1)	K3	11	
<i>P. gingivalis</i> HG1701	K3	12	
<b><i>P. gingivalis</i> HG1660 (ATCC 49417)</b>	K4	13	
<i>P. gingivalis</i> HG1702	K4	14	
<i>P. gingivalis</i> HG1703	K4	15	
<b><i>P. gingivalis</i> HG1690</b>	K5	16	
<i>P. gingivalis</i> HG1704	K5	17	
<i>P. gingivalis</i> HG1707	K5	18	
<b><i>P. gingivalis</i> HG1691</b>	K6	19	
<i>P. gingivalis</i> HG1705	K6	20	
<i>P. gingivalis</i> HG1708	K6	21	

<sup>a</sup> All other K-serotypes were from Laine and van Winkelhoff's collection (29) and are presented as "serotype, identification number". Typed strains are in bold.

<sup>b</sup> For technical reasons, this typed member of the K2 serogroup was omitted from the experiments described.

polymer biosynthesis in other bacteria (37). Furthermore, Chen et al. (9) have suggested that PG0106-PG0120 may represent the capsule locus based on analysis of gene variation at this locus using DNA microarray genotyping of *P. gingivalis* 381 (9). Thus the first aim of the current investigation was to use a bioinformatics and mutagenesis strategy to identify the capsule locus in *P. gingivalis* and explore the genetic basis for capsule variation in this organism. The second aim relates to the putative involvement of the K-antigen locus in glycosylation reactions in *P. gingivalis*. Earlier work from this laboratory demonstrated that the Arg-gingipains are variably modified by the addition of glycan chains, and these modifications contribute to the diversity of Arg-gingipain isoforms produced by this organism. In other bacteria, where glycosylation of surface and extracellular proteins has been examined, there is evidence to suggest that the biosynthetic machinery of surface polymer production may on occasion be involved in the generation of glycans which are subsequently attached to surface glycoproteins (15, 47). In the present study, mutants were generated in the K-antigen biosynthesis locus which enabled us to examine its role in the general glycosylation reactions in *P. gingivalis*.

#### MATERIALS AND METHODS

**Bacterial physiology and maintenance.** *P. gingivalis* strains (Table 1) were grown either on blood agar plates containing 5% defibrinated horse blood or brain heart infusion broth supplemented with hemin (5 μg ml<sup>-1</sup>) and menadione (1 μg ml<sup>-1</sup>), in an anaerobic atmosphere of 80% N<sub>2</sub>, 10% H<sub>2</sub>, and 10% CO<sub>2</sub> (29, 34). Clindamycin HCl was added to 5 μg ml<sup>-1</sup> for selection of *ermF*. *Escherichia coli* was grown with aeration in Luria-Bertani broth or solid media (Luria-

Bertani broth containing 1.5% technical agar no. 3). Ampicillin (Na<sup>+</sup> salt; 100 μg ml<sup>-1</sup>) or erythromycin (300 μg ml<sup>-1</sup>) was added to the growth media to select for pUC-derived or *ermAM*-containing plasmids, respectively.

**Assays for Arg- and Lys-gingipain protease activities and hemagglutination.** Arg-X and Lys-X protease activities were measured using *N*-benzoyl-DL-arginine *p*-nitroanilide (DL-BAPNA) and *N*-α-acetyl-L-lysine-*p*-nitroanilide (AcLyspNA), respectively, as substrates in spectrophotometric assays as previously described (41). Units are expressed as change in absorbance at 405 nm per minute at 30°C. Hemagglutination was performed using sheep red blood cells (Invitrogen) washed three times with phosphate-buffered saline (PBS) and brought to 0.8% in the same buffer. Doubling dilutions of 24-h whole bacterial cultures in brain heart infusion were made in a 96-well plate, and an equal volume of red cells was added to each well and allowed to settle for 2 h.

**Fluorescent labeling of proteases with DNS-EGR-CK.** Labeling of Arg-X and Lys-X proteases in culture supernatants of *P. gingivalis* with active-site directed inhibitor DNS-EGR-CK (dansyl-Glu-Gly-Arg-chloromethyl ketone) has been previously described (3).

**SDS-PAGE and Western blotting.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 10% polyacrylamide gels (26). The gels were stained with Alcian blue (the cationic dye binds to polyanionic compounds [22]) or transferred to nitrocellulose membranes and probed with either antisera Rb4 (anti-α catalytic domain of RgpA/B), Rb3158 (anti-β adhesin domain of RgpA), or monoclonal antibody (MAb) 1B5 (anti-glycan). These antibodies are described in detail elsewhere (2, 10). Densitometry was performed using a Syngene imager and GeneTools software.

**Double immunodiffusion (Ouchterlony).** K-serotyping was carried out by double immunodiffusion assay as previously described (27). Heat-stable extracts of *P. gingivalis* were prepared from cells from 1-ml, 48-h grown broth cultures. The cultures were centrifuged for 10 min at 10,000 × g in weighed microcentrifuge tubes. The cell pellets were gently resuspended in PBS (200 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub>, pH 7.4), washed twice, and finally resuspended in PBS to 100 mg/ml. Suspensions were autoclaved at 120°C for 20 min, allowed to cool, and centrifuged as before. The supernatants (15 μl of each) were added to wells cut equidistant from each other in 1% agarose (Sigma, United Kingdom). The

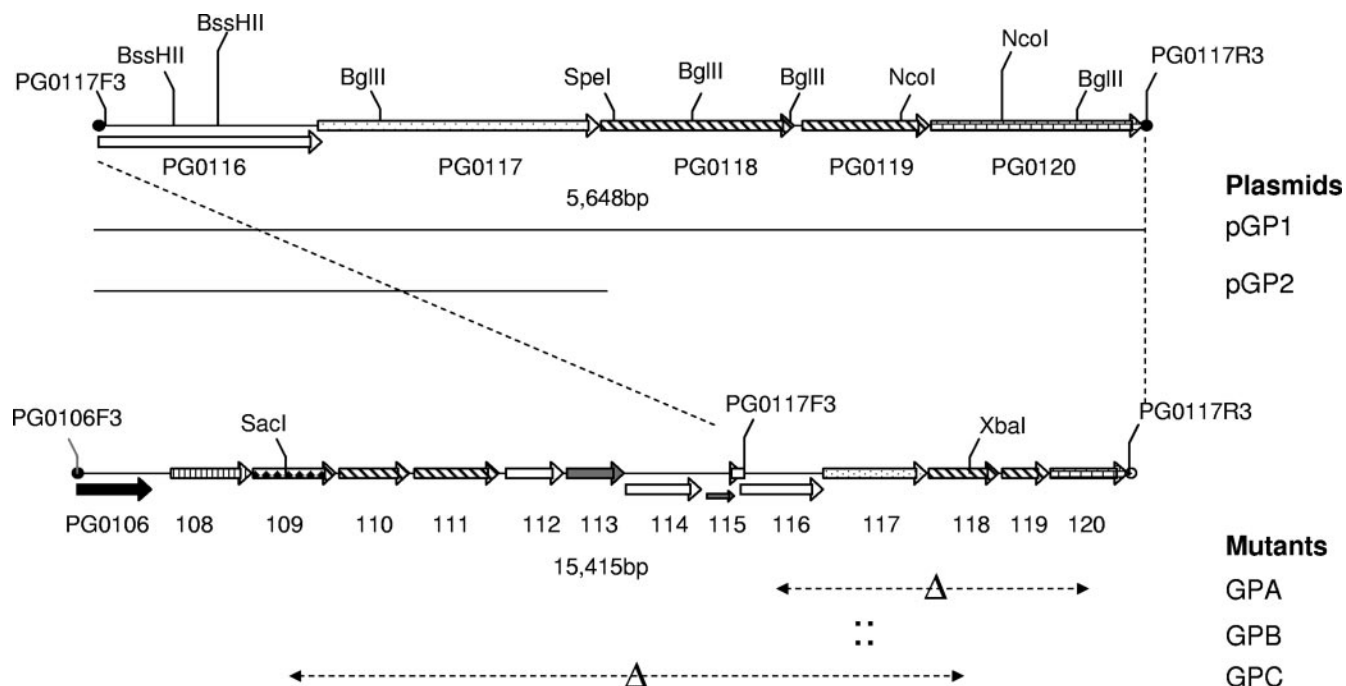


FIG. 1. Genetic organization of the GP (capsule) locus of *P. gingivalis* W83. Rectangles with terminal arrows indicate the position and direction of open reading frames. The open reading frames are coded to represent the nature of the translation products: glycosyl transferases (PG0110, PG0111, PG0118, and PG0119), conserved hypothetical proteins (PG0112, PG0114, and PG0116), a unique protein (PG0109), GlcNAc epimerase (PG0120), acetyltransferase (PG0113), serine acetyltransferase (PG0115), UDP-Glc (GDP-Man) dehydrogenase (PG0108), an undecaprenyl phosphate GlcNAc transferase (PG0106), and a flippase (PG0117). Thin solid lines represent DNA that is harbored in plasmids pGP1 or pGP2. Relevant restriction enzyme sites are shown on the expanded 3' end. PG numbers that include F (forward) or R (reverse) refer to the name and position of PCR primers. Areas within the GP locus that were deleted to generate *P. gingivalis* GPA and GPC are shown with dashed lines and "Δ". The position of insertion of the *erm* cassette to generate the PG0117 mutant is denoted with "∴".

components were allowed to diffuse against *P. gingivalis* W83 (K1) antiserum in the central well for 48 h at 4°C (27).

**DNA manipulations: generation of *P. gingivalis* isogenic mutants.** Plasmids used in this work are listed in Fig. 1. Chromosomal DNA was isolated from 24-h-grown cultures using a Puregene DNA isolation reagent (Flowgen), and all plasmids were purified using ion-exchange chromatography (QIAGEN). Sources of reagents and details of all the methods have been previously described (1, 39). Restriction and modifying enzymes were purchased from New England Biolabs. General manipulation of DNA, restriction and mapping of plasmids, and transformation of *E. coli* were as described by Sambrook et al. (42) and have also been reported in detail elsewhere (2).

Chromosomal DNA from *P. gingivalis* W50 was used as the template for

cloning purposes. Our studies focused mainly on PG0106-PG0120. The genetic organization at this locus, the positions of PCR primer sites, and the mutants constructed at this locus are shown in Fig. 1. Initially, a 5,648-bp region, corresponding to PG0116-PG0120 (TIGR; <http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=gpg>) (37), was amplified using primer pair PG0117F3 (5'-AGAAGGCTTCAAAGGTTGGG-3') and PG0117R3 (5'-GCA TCGAGTAAGAGCATCCG-3') with Reddy Load Extensor mix (buffer 2; AB-gene) (Table 2). The parameters used on a Techne thermal cycler were as follows: initial denaturation for 2 min at 94°C, followed by 25 cycles at 94°C (30 s), 60°C (30 s), and 68°C (6 min), and a final extension at 94°C (8 min). The amplicon was then purified from agarose gel, blunted and phosphorylated (End-IT; Cambio), and cloned into SmaI-restricted and dephosphorylated pUC18not

TABLE 2. Properties of oligonucleotides used

Name	Sequence (5' → 3')	Target <sup>a</sup>	Size (kb)	Source
PG0117F3 PG0117R3	AGAAGGCTTCAAAGGTTGGG GCATCGAGTAAGAGCATCCG	PG0116-PG0120	5.65	This paper
PG0106F3 PG0117R3	ATTTTCAGGGATGGGCAGAAG	PG0106-PG0120	15.41	This paper
ErmFF2 ErmAMR2	TTCGTTTTACGGGTCAGCAC ACTTTGGCGTGTTCATTGC	<i>erm</i>	2.10	This paper
αF1 αR1	AGTGAGCGAACTTCGGAGC GGTATCACTGGGTATAACCTGTCC	α region of <i>rgpA</i>	1.70	4
GalFECF2 Wcaj4ECF3	TTCGTTTTATTGGTTTCCTTG TAGATTGAACTCGCTTTGGG	<i>porR</i> region (PG1135-PG1142)	9.60	15

<sup>a</sup> PG notation refers to The Institute for Genomic Research (TIGR) locus ID of *P. gingivalis* W83 genome.

(18) for transformation into *E. coli* XL-1 Blue MRF'. The resulting plasmid, pGP1, was further manipulated by removal of the SpeI-EcoRI (the latter was derived from the residual multiple cloning site at the 3' end) fragment to generate pGP2. A 2.1-kb SacI-PstI *erm* cassette of pVA2198 encoding macrolide-lincosamide resistance (14) was cloned into pGP1 and pGP2 at the BssHII-NcoI and BglII sites to generate pGPΔE and pGP2E, respectively. The *erm* cassette and the flanking *P. gingivalis* DNA were retrieved by NotI digestion and then electrotransformed into *P. gingivalis* W50 cells to generate mutants GPA (deletion of PG0116-PG0120) and GPB (insertion in PG0117). DNA from these mutants was used as a template in PCRs with the primers above, in permutations with ErmFF2 (5'-TTCGTTTTACGGGTCAGCAC-3') and ErmAMR2 (5'-AC TTTGGCGTGTTCATTGC-3') to confirm orientation and integration of the *erm* cassette. The strategy used to delete PG0109-PG0118 (Fig. 1) was a modification of the above. The 15.4-kb PG0106-PG0120 region was amplified from *P. gingivalis* W50 using the PCR extensor mixture above with primers PG0106F3 (5'-ATTTTCAGGGATGGGCAGAAG-3') and PG0117R3 (above) with initial denaturing at 94°C (2 min), followed by 25 cycles at 92°C, 60°C, and 68°C for 30 s, 30s, and 16 min, respectively, and a final cycle at 92°C for 18 min. The amplicon was purified and restricted with SacI and XbaI, and the two extreme arms (3.0 kb and 2.3 kb) were purified by agarose gel electrophoresis and ligated to a 2.1-kb SacI-XbaI fragment of the *erm* cassette. The ligation mixture was reamplified, and the DNA was used to electrotransform *P. gingivalis* W50 to clindamycin resistance. The selected deletion mutant was designated *P. gingivalis* GPC (Fig. 1).

**Heterogeneity of the GP locus of *P. gingivalis*.** Using the primer pair PG0106F3 and PG0117R3 we attempted to amplify a 15.4-kb GP locus, corresponding to region PG0106-PG0120 of *P. gingivalis* W83, from DNA templates of three members of each of the six K-serotypes of *P. gingivalis* as well as K<sup>-</sup> strains. Purified amplicons were then restricted with BglII, and the profiles were compared by agarose gel electrophoresis. In addition, the 3' end of the GP locus from each of the three representative members of the K-serotypes of *P. gingivalis* was amplified with primer pair PG0117F3 and PG0117R3 (5.6 kb), to target the gene encoding a potential glycan translocase (PG0117) and its flanking regions. All DNA samples from each of the K-serotypes of *P. gingivalis* were also tested with primers for a 1.7-kb catalytic α domain of Arg-gingipain A, *rgpA* (4), as well as a 9.68-kb region encompassing *porR* (PG1138) and *porS* (PG1137) (15).

**DNA sequencing and analysis of results.** Two sets of ethanol-precipitated amplicons from *P. gingivalis* HG91 (381) were used as templates in cycle sequencing using ABI BigDye terminators with primers PG0106F3, PG0117F3, or PG0117R3; the latter two were initially used for HG1703. Purified products were run on an automated capillary DNA sequencer. Subsequent primer walking on both strands ensured a >>5-fold coverage. Analysis of sequences used Genamics Expression (Genamics), NIX, and JEMBOSS suites (7) at The Rosalind Franklin Centre for Genomics Research in addition to the facilities at the National Center for Biotechnology Information.

**Nucleotide sequence accession numbers.** The consensus sequences derived from the above analysis have been submitted to DNA databases under accession numbers AJ969093 (HG91, 381) and AJ969094 (HG1703).

## RESULTS

**Bioinformatics identification of candidate capsule synthesis loci.** A search of the *P. gingivalis* W83 genome (<http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=gpg>) (37) highlighted four potential polysaccharide biosynthesis loci at PG0106-PG0120 (designated the GP locus), PG1135-PG1142 (*porR* locus), PG0435-PG0437, and PG1560-PG1565 based on the presence of genes encoding multiple glycosyl transferases and products involved in polymer export in other bacteria (e.g., putative *wzx* at PG0117 and PG1137). On the grounds that the latter two loci have only limited coding potential, probably insufficient to encode all the biosynthetic machinery required for capsule biosynthesis and export, we concentrated on the GP and *porR* loci in the first instance. Further examination of the GP locus revealed the presence of genes putatively encoding four glycosyl transferases (PG0110, PG0111, PG0118, and PG0119), three conserved hypothetical proteins (PG0112, PG0114, and PG0116), serine acetyltransferase (PG0115), a

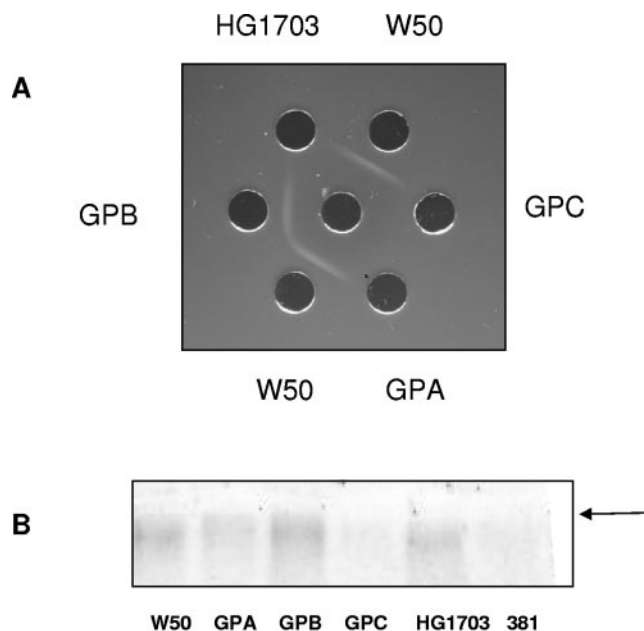


FIG. 2. Analysis of supernatants of autoclaved cells of *P. gingivalis* parent and capsule mutant strains. A. Supernatants from autoclaved cells of *P. gingivalis* grown for 24 h were loaded in the peripheral wells of the agarose, and K1-specific antiserum was placed in the middle well. Samples were allowed to diffuse for 48 h to facilitate precipitin formation. B. The above samples were also run in SDS-PAGE and stained with Alcian blue. The arrow corresponds to the junction between stacking and running gel.

unique protein (PG0109), a GlcNAc epimerase (PG0120), an acetyltransferase (PG0113), a UDP-Glc (GDP-Man) dehydrogenase (PG0108), an undecaprenyl phosphate GlcNAc transferase (PG0106), and a flippase or *Wzx* homologue (PG0117; Fig. 1). Hence we initially targeted the GP locus for mutagenesis and further study.

Since there were no recognizable essential or housekeeping genes within the GP locus, three sets of *P. gingivalis* mutants were generated in this region of the chromosome. For these studies we utilized *P. gingivalis* W50, a strain we have genetically manipulated frequently in previous studies, which appears genetically indistinguishable from the sequenced strain W83. The following mutants were prepared: GPA, a deletion of PG0116-PG0120; GPB, an insertion into the flippase (PG0117); and GPC, a more extensive deletion of PG0109-PG0118 (Fig. 1). Six representatives of each candidate mutant, selected on clindamycin, were screened using PCR analysis of chromosomal DNA to confirm legitimate chromosomal integration of plasmid constructs through double crossover and allelic exchange. In each case, all six candidate mutants were shown to carry the correct insertion/deletion, and one mutant from each of the GPA, GPB, and GPC categories was chosen for more detailed phenotypic analysis.

**K-serotype antigen expression in GP mutants.** Supernatants of autoclaved cells of *P. gingivalis* W50 and GP mutants were examined using double immunodiffusion to determine precipitin formation with K1-specific antiserum (Fig. 2). Extracts of the GPC mutant failed to form a typical K1-antigen precipitin line in this assay. Hence PG0109-PG0118 of the GP locus is

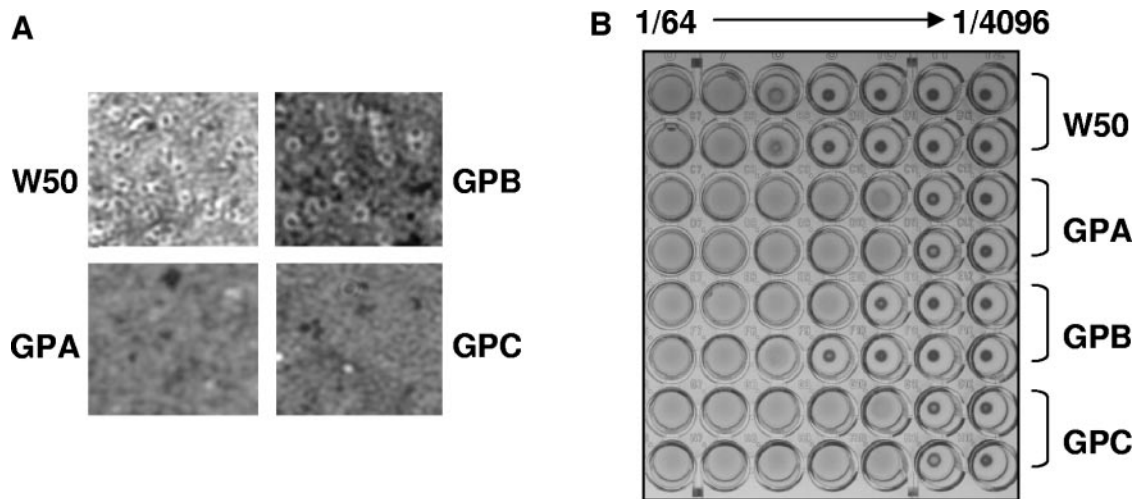


FIG. 3. Phenotypes of *P. gingivalis* W50 and GPA, GPB, and GPC capsule locus mutants. A. Negative staining of cells with India ink and fuchsin stain. W50 and GPB are surrounded by a colorless halo which is absent in GPA and GPC cells. The colorless halo is due to the negatively charged capsule which does not pick up any stain. B. Hemagglutination plate assay using sheep red blood cells (0.8%).

required for K-antigen biosynthesis in *P. gingivalis* W50. Similarly no precipitin was formed by extracts of the GPA mutant with K1 antiserum, indicating that the five contiguous genes at the 3' end of the GP locus (PG0116-PG0120) are essential for K-antigen synthesis (Fig. 2). In contrast, insertional inactivation of PG0117 in GPB had no effect on K-antigen production assessed by this assay. These data were also confirmed by Western blotting of whole-cell proteins after electrophoresis in 10% SDS-PAGE, using K1-specific antiserum. The K-serotype antigen of the parent strain appears as a diffuse band greater than 200 kDa in the separating gel. No immunoreactivity was observed in this region of the blots in the case of either GPC or GPA, whereas GPB was indistinguishable from the parent strain (data not shown). Furthermore, Alcian blue staining of gels following SDS-PAGE of autoclaved cells of *P. gingivalis* (Fig. 2B) showed significant reduction in staining of polyanionic glycan in GPA and GPC as assessed by densitometry (peak height of W50 was 36.21 versus 23.54 for GPA and no detectable peak for GPC). This is analogous to that of the nonencapsulated *P. gingivalis* 381 (HG91) which again showed no detectable peak by densitometry. In contrast, the peak height of Alcian blue staining of GPB was equivalent to that of the parent strain. Together these data provide strong evidence that PG0106-PG0120 represents the serotype K-antigen biosynthetic locus of *P. gingivalis* W50.

Figure 3A shows images of *P. gingivalis* W50, GPA, GPB, and GPC mutant cells negatively stained with India ink and fuchsin taken under light microscopy. Cells of W50 and GPB appear stained with a white halo around them which is due to the negative staining of the capsule, whereas GPA and GPC mutant cells appear stained, but no halo is visible, demonstrating the absence of capsule. These data confirm the earlier immunochemical investigation. In order to assess the influence of loss of capsule expression on the interaction of the mutants with host cells, we next compared their respective hemagglutination titers. A representative plate assay is shown in Fig. 3B. Cells deficient in capsule expression (GPA and GPC) consistently demonstrated a higher end point titer than the parent

strain and the capsule-positive mutant GPB. We next examined the heterogeneity at this locus in different K-serotypes.

**Heterogeneity of the GP locus in K-serotypes of *P. gingivalis*.** DNA from three representatives of each of the six K-serotypes and the K<sup>-</sup> strains of *P. gingivalis* was isolated and used in PCRs with a primer pair (PG0106F3 and PG0117R3) designed to the GP locus of strain W83. Amplicons were produced from 12 of the 20 strains (Fig. 4A). *P. gingivalis* W83 (lane 4) produced an amplicon of the expected size (15.4 kb), but there were significant differences in amplicon length in several of the other PCR-positive strains. For example, the only representative of the K3-serotype (Fig. 4A, lane 12; HG1701) which was PCR positive gave a smaller product (~9 kb). None of the K5-serotype strains were positive with this primer pair (data not shown). Restriction fragment length polymorphism (RFLP) patterns with BglII revealed further variations within serotypes and between serotypes (Fig. 4B). *P. gingivalis* W50 and W83 (K1) gave rise to the predicted 10 bands of >0.3 kb, and the other two strains of this serotype gave nearly identical profiles (lanes 5 and 6). The two PCR-positive representatives of K2 had similar profiles with only four bands of >0.3 kb. As might be predicted, the K<sup>-</sup> strains showed the most serotype variation within this group, all three strains giving entirely different RFLP patterns. Among these K<sup>-</sup> strains, HG1695 (lane 3) had an RFLP pattern which was markedly similar to W83, HG1631, HG1700, and W50, suggesting that this isolate may have originally been a K1-serotype.

Closer examination of the 3' end of the GP locus (PG0116-PG0120; Fig. 4C) indicated that this region may be more heterogeneous than the entire GP locus. In this case, the primer pair amplified the region in only 6 out of 20 strains; 3 of K1 (and W50), 2 of K<sup>-</sup> (381 and HG1695), and 1 of K4 (HG1703). Furthermore, the amplicons from *P. gingivalis* 381 and HG1703 were ~4.50 and ~3.00 kb, respectively, smaller than the 5.65 kb of *P. gingivalis* W50. Thus, some of the genes within this region are either missing completely or are present elsewhere on the chromosomes of these strains. On the basis of this relatively limited study, there is some conservation within se-

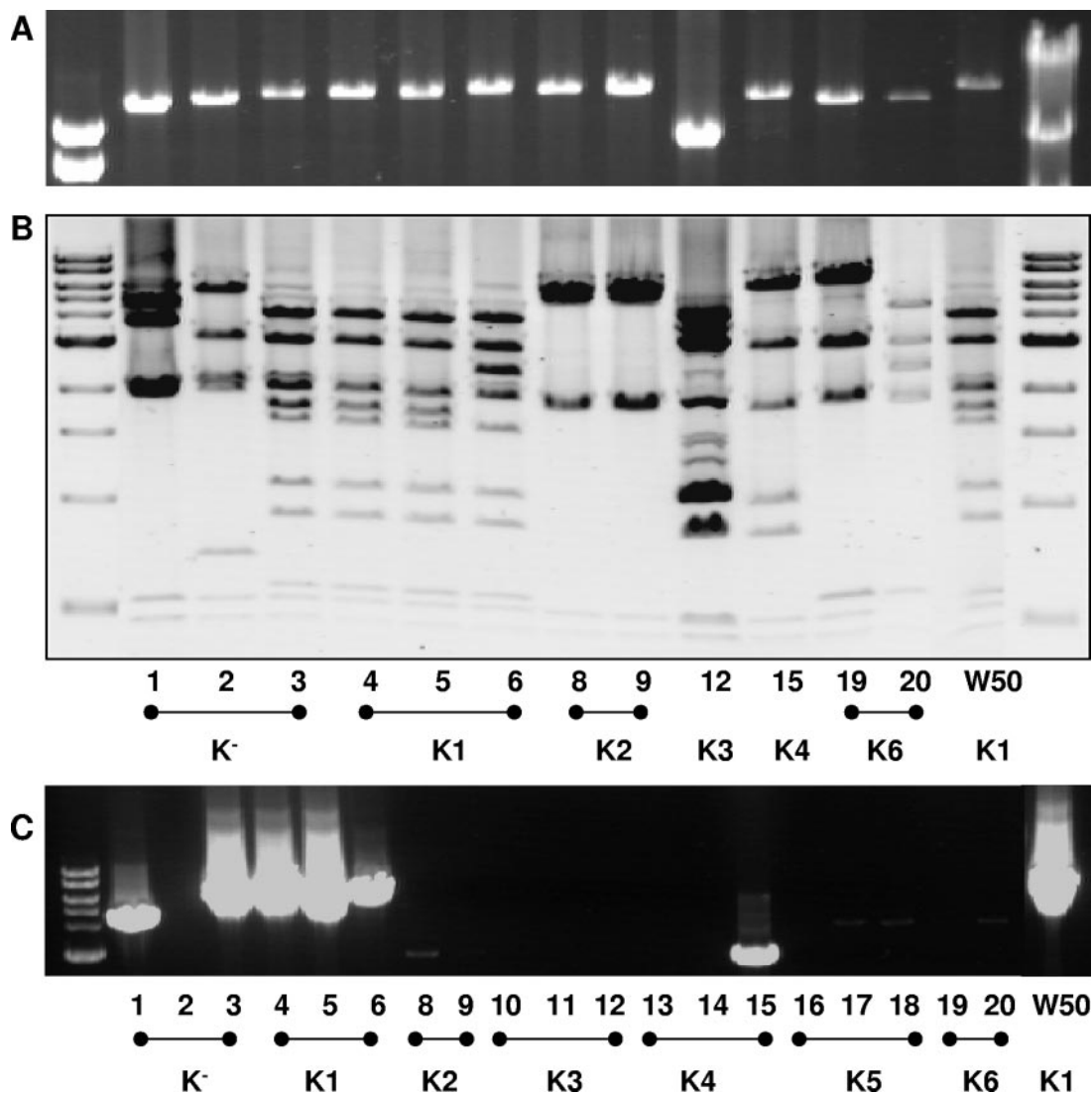


FIG. 4. Restriction fragment length polymorphism among *P. gingivalis* K-serotypes. A. Amplification of the GP (capsular polysaccharide) locus from *P. gingivalis* K-serotyped strains using primers PG0106F3 and PG0117R3 (Fig. 1). Strains used were as follows: for K<sup>-</sup>, 1 is HG91 (381), 2 is HG1694, and 3 is HG1695; for K1, 4 is HG66 (W83), 5 is HG1631, and 6 is HG1700; for K2, 8 is HG1644 and 9 is HG1706; for K3, 12 is HG1701; for K4, 15 is HG1703; and for K6, 19 is HG1691 and 20 is HG1705. An expected fragment of 15,415 bp (corresponding to PG0106-PG0120) was obtained from *P. gingivalis* W50. Note the variation in sizes of the amplicons (~10 to 15.4 kb) from the other strains. B. BglIII digests of the above samples. Products corresponding to 3.972, 2.941, 1.963, 1.718, 1.561, 1.051, 0.872, 0.514, 0.463, and 0.360 kb were expected from *P. gingivalis* W50. C. The 3' region of the GP (capsular) polysaccharide locus (PG0117-PG0120) was amplified from all the K-serotypes of *P. gingivalis*. Details of strains are given in Table 1.

rototypes and considerable variation between serotypes at the GP locus. Both properties are consistent with the suggestion that this region represents the K-antigen capsule locus of this bacterium. As a control for these PCR experiments, we also amplified the 1.7-kb region encoding the  $\alpha$  catalytic domain of Arg-gingipain A protease, *rgpA*. This gene could be amplified from 18 of the 20 strains (data not shown); the other two were negative probably because of minor variations in nucleotide sequences at the primer target sites within the genomes; *rgpA* is known to be highly conserved in clinical and laboratory strains of *P. gingivalis* (4).

The other candidate capsule locus identified by bioinformatics analysis of the *P. gingivalis* genome, at PG1135-PG1142,

appears highly conserved. In 14 of the 20 strains representing five different serotypes as well as K<sup>-</sup> strains, the 9.68-kb region of the genome corresponding to this locus was amplified and restriction digest of these amplicons using ClaI generated nearly identical RFLP patterns (Fig. 5). In a manner similar to the GP locus, no amplicons were obtained with the K5-serotypes with these primer pairs. Furthermore, mutants within this region reacted with K-serotype antigens. Hence, this region of the chromosome is not involved in K-antigen biosynthesis.

**DNA sequence analysis of the capsule locus in *P. gingivalis* 381.** *P. gingivalis* 381 is classified as a K<sup>-</sup> strain on the basis of nonreactivity with K-typing sera and ultrastructural investigations using electron microscopy (50). Nonetheless PCR of

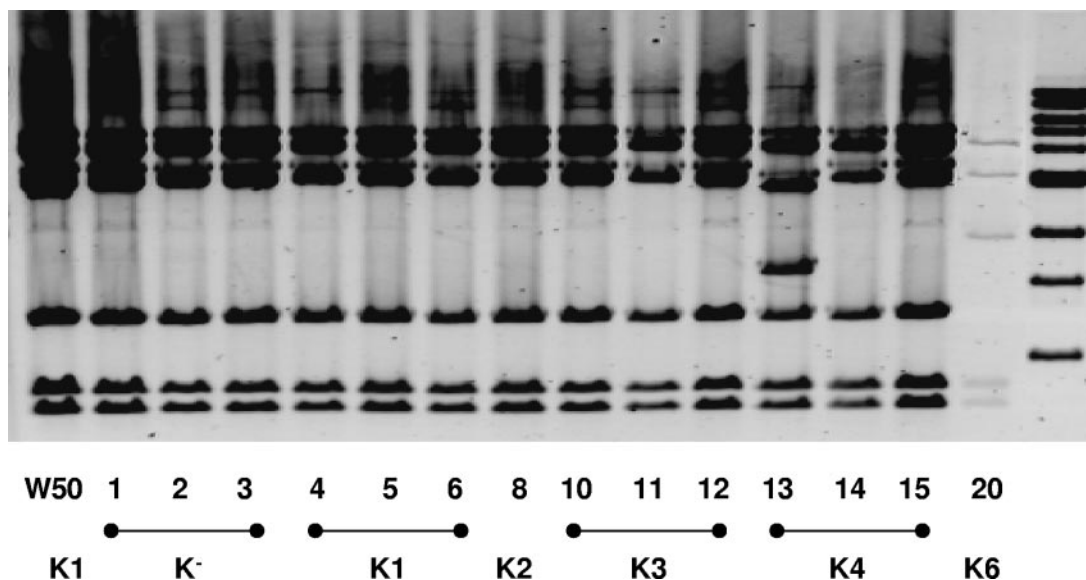


FIG. 5. Analysis of the *porR* locus in different K-serotype strains. The amplicon (9,679 bp) corresponding to the *porR* locus (PG1135-PG1142) was amplified and then restricted with *Cla*I restriction enzyme. Details of strains used are given in Table 1. Expected sizes (from *P. gingivalis* W50) are 4.008, 2.927, 1.196, 0.805, 0.719, and 0.024 kb. The final fragment is outside the limit of the gel. In all cases, the profiles of *P. gingivalis* W50 and W83 were identical. Similarly *P. gingivalis* 33277 was also identical in every respect to HG91 (381). Thus, unlike the capsule (GP) locus, *porR* locus is remarkably well conserved within serotypes and between different K-serotypes.

strain 381 using primers PG0106F3 and PG0117R3 to the capsule locus of strain W83 (Fig. 4A) amplified a product of approximately 13 kb (versus 15.4 kb for strain W83). Some of this difference may be accounted for by alterations to the 3' end of the capsule locus in this strain since amplification of this region using PG0117F3 and PG0117R3 yielded an amplicon of approximately 4.5 kb versus the 5.7-kb product amplified from both W83 and W50. In order to determine the basis for the lack of K-antigen production in strain 381, we sequenced the GP locus corresponding to the amplicon in Fig. 4A (lane 1).

The consensus sequence of 12,884 bp of *P. gingivalis* 381 encodes 12 open reading frames and has features similar to strain W83, including low percent GC (Table 3). Gene order is preserved, however pairwise alignment of the nucleotide sequences demonstrates that at least 50% of the capsule locus of 381 is very different from that of W83 (represented as a dot plot in Fig. 6). The regions of the GP locus of W83 that are apparently absent in 381 encode PG0109-PG0114 and PG0117-PG0118. A more detailed comparison of the capsule loci of strains 381 and W50 is shown in Fig. 7. The translation

products of the genes at the extreme 5' and 3' end of the locus, PG0106 and PG0120, are both present in 381, exhibiting 98.6 and 99% identity, respectively. This was to be expected, as the primer pair PG0106F3 and PG0117R3 used to amplify the locus and initiate DNA sequencing in strain 381 is designed to sequences within these genes. However, PG0106<sub>381</sub> is interrupted as a result of an internal termination codon (TGA: Gly<sup>217</sup> to stop). The PG0109 and PG0110 open reading frames in the W83 capsule locus are replaced by three open reading frames (*orf15*, *orf18*, and *orf24*) in the 381 capsule locus. These three open reading frames are not present elsewhere in the W83 genome. Orf18 is unique to strain 381 although a 95-amino-acid (aa) stretch is 31% identical (e = 0.14; 50% similarity) to a protein from the common frog, *Xenopus laevis*. Orf15 and Orf24 exhibit 38 and 34% identity to putative glycosyl transferases from *Enterococcus faecalis* V583 (group 2) and *Desulfotalea psychrophila* (group 1), respectively. The next downstream open reading frame in 381, *orf10*, may be an allele of PG0111<sub>W83</sub> encoding a protein with marginal identity of 22% over 401 aa.

The next three open reading frames in the W83 sequence (PG0112, PG0113, and PG0114) are missing from the *P. gingivalis* 381 locus. PG1205<sub>381</sub> is homologous (59% identity, 77% similarity) to DNA binding proteins of the histone family; there are at least 10 members in W83, two (PG1205 and PG1497) exhibit 57% identity (77% similarity) to each other over the entire 156 aa. PG0115 and PG0116 are both present in the 381 sequence although PG0116<sub>381</sub> (conserved hypothetical) is truncated by 11 aa at the C terminus. The sequence encoding PG0117 (Wzx, a glycan translocase) is absent from strain 381. PG0118<sub>381</sub> (glycosyl transferase) is only 56% identical over 338 aa to the corresponding translated sequence in W83. In contrast, PG0119 and PG0120 are nearly identical in

TABLE 3. Main features of the capsular polysaccharide loci of *P. gingivalis* W83 (K1) and 381 (K<sup>-</sup>) strains

Feature	Strain	
	381 (K <sup>-</sup> )	W83 (K1)
Size (bp)	12,884	15,415
% GC <sup>a</sup>	40	40
Orfs	12	14
Unique proteins	1	1
Conserved hypotheticals	1	3
Pseudogenes	1	0

<sup>a</sup> The average % GC of the whole genome of W83 is 48.3.

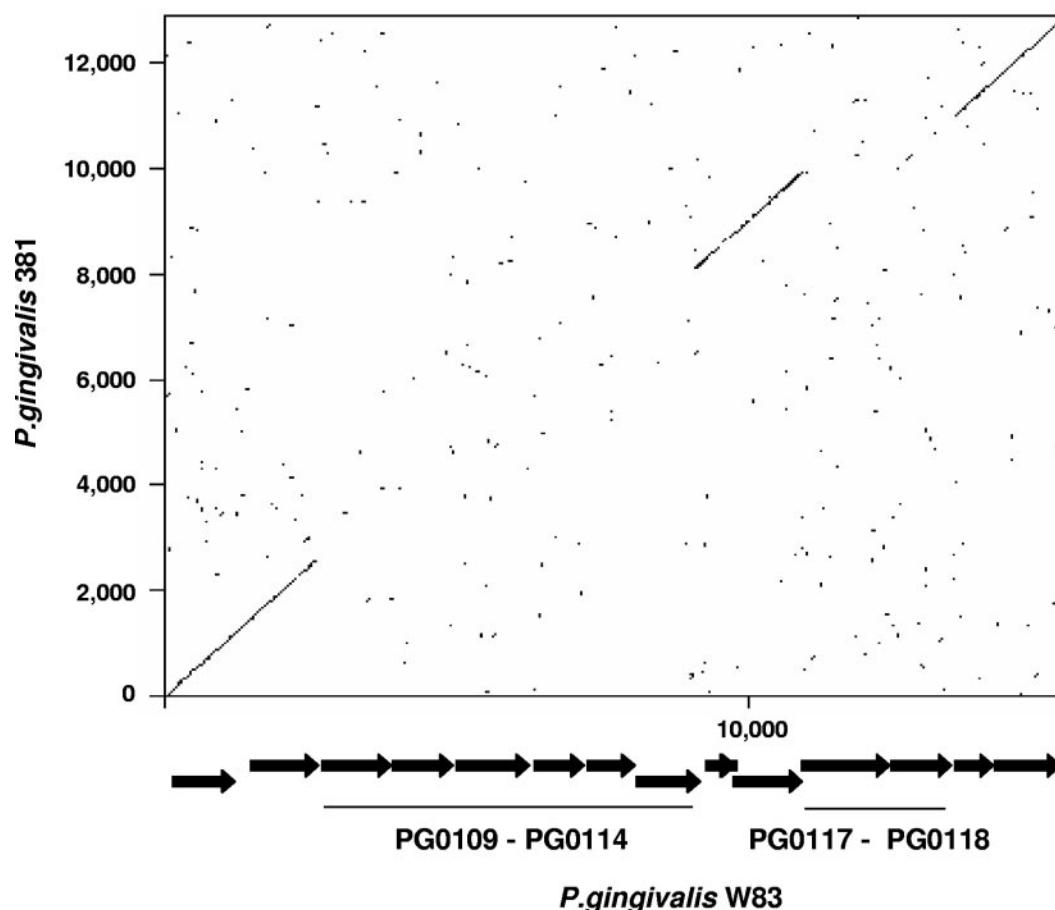


FIG. 6. Comparison of the nucleotide sequences of the capsular polysaccharide loci of *P. gingivalis* W83 (K1) and HG391 (381; K<sup>-</sup>). The nucleotide sequences were compared using DOTPLOT with a scanning window of 10 bases. Areas of identity are represented by diagonal lines; discontinuity corresponds to genes missing or replaced in 381 within the locus.

the two strains. In summary, there are several key differences in the capsule synthesis locus in strain 381 compared to the corresponding region in strain W83. These include an internal stop codon in PG0106, substitutions and deletions throughout PG0109-PG0114, and deletion of PG0117.

Since our investigations indicated that the 3' end of the locus is essential for K-antigen synthesis in strain W50 and to provide further comparative data, we also sequenced the 3-kb product amplified using PG0117F3 and PG0117R3 from *P. gingivalis* HG1703 (K4)—the only other K-antigen-positive strain from which we were able to amplify this region. Comparison of the nucleotide sequence with the corresponding region of W83 (Fig. 7) indicated that, similar to strain 381, PG0116, PG0119, and PG0120 are all conserved in HG1703, PG0116 is truncated by 14 amino acids, and PG0117 is absent. Furthermore, PG0118 is also absent from this strain.

**Analysis of Arg-gingipain glycosylation in capsule mutants of *P. gingivalis*.** The construction of the mutant GPC, in which the K-antigen capsule locus has been deleted from *P. gingivalis* W50, allowed us to determine the requirement of this locus for glycosylation of the Arg-gingipains. Deletion of PG0109-PG0118 had no effect on the total Arg-X and Lys-X activity in GPC (Arg-X, 3.51 units/ml in GPC versus 3.13 units/ml in W50; Lys-X, 1.06 units/ml in GPC versus 0.96 units/ml in W50

[24-h cultures]), and the distribution of these activities between cells and supernatant (data not shown) were indistinguishable between the two strains. Furthermore, Western blotting of whole cells and culture supernatants of the parent and mutant strains using Rb4, which recognizes the RgpA  $\alpha$  catalytic chain, and MAb 1B5, which reacts with the glycan additions to the Arg-gingipains and a repeating unit cell surface polymer, revealed no significant differences (Fig. 8). Arg- and Lys-gingipains in spent culture medium were fluorescently labeled with DNS-EGR-CK, subjected to SDS-PAGE, and viewed under UV light. This allows visualization of the heavily glycosylated membrane isoforms of Arg-gingipains and monomeric RgpA<sub>cat</sub>/catalytic and adhesin domains of HRgpA (RgpA isoform containing both the catalytic [ $\alpha$ ] and adhesin [ $\beta$ ] domains) which comigrate (3). Comparison of the DNS-EGR-CK fluorescently labeled products in the culture supernatants of *P. gingivalis* W50, GPA, GPB, and GPC demonstrated identical amounts of the membrane type isoforms in all four preparations (data not shown).

## DISCUSSION

The first goal of these investigations was to experimentally determine the chromosomal location of the capsule locus of *P.*



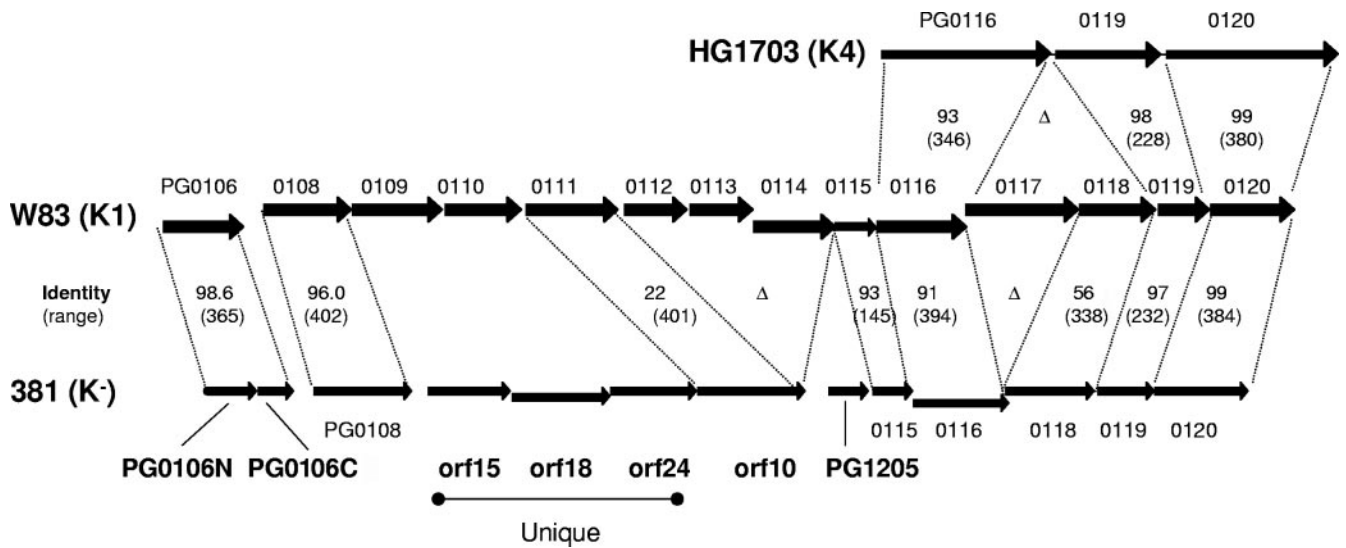


FIG. 7. Comparison of the sequences of the capsular polysaccharide loci of *P. gingivalis* W83 (K1), 381 (K<sup>-</sup>), and HG1703 (K4). Each locus is represented as a black line with Orf's and directions as arrows. The nomenclature used for genes/proteins refers to those already employed for *P. gingivalis* W83; genes/proteins are labeled *orf*/*Orf* when the above nomenclature does not apply. Numbers between loci correspond to identity (from multiple sequence alignments), and the range of amino acids is indicated. "Δ" indicates deletion of *orf* that is limited to the range indicated with dashed lines.

*gingivalis*. Four genetic loci which could theoretically have a role in polysaccharide biosynthesis were identified by Nelson et al. (37) during the annotation of the *P. gingivalis* W83 genome. Subsequently PG0106-PG0120 was suggested by Chen et al. (9) to represent the potential coding region for capsule biosynthesis based on the gene content at this locus and the marked

divergence at this locus in strain 381 (HG91)—a natural K<sup>-</sup> strain. The targeted mutagenesis strategy employed in the current work provides the first experimental evidence to support this suggestion. Deletion of 10 genes at PG0109-PG0118 within this locus generated a mutant, GPC, which no longer synthesizes a K-antigen based on the absence of reactivity of extracts

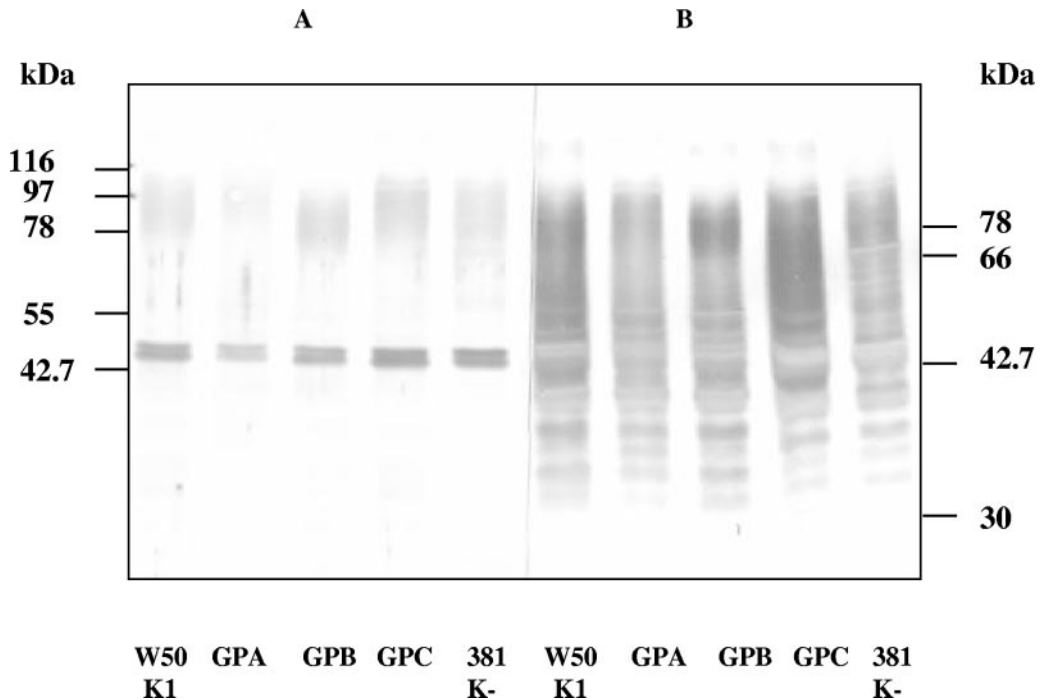


FIG. 8. Analysis of *P. gingivalis* cell pellets by Western blotting with antibodies Rb4 and MAb 1B5. Proteins from *P. gingivalis*, cells grown for 48 h, and the corresponding supernatants were subjected to SDS-PAGE and blotted onto nitrocellulose membranes and probed (A) with antiserum to the catalytic domain of RgpA/B (Rb4) or (B) antibody to the glycan epitope (MAb 1B5).

of autoclaved cells with K-antigen typing serum and a significant reduction in staining of polyanionic glycans in cell extracts with Alcian blue. A similar phenotype was observed in GPA, in which PG0116-PG0120 is deleted. Insertional inactivation of PG0117, a putative flippase involved in export of capsule polysaccharide repeating units, had no effect on recognition by K-antigen typing serum. Similarly, negative staining of *P. gingivalis* W50 and mutant GP cells with India ink and fuchsin showed the presence of a colorless halo around the stained W50 and GPB cells, whereas stained GPA and GPC cells lacked the halo around the cells. The halo is due to the capsule which does not pick up any stain due to its polyanionic character. The GPB mutant cells retain the ability to make capsule. It is possible that there is an alternative gene product(s) which may compensate for the loss of the function of PG0117. For example, PG0117 is paralogous to both PG0912 (47% identity, 67% similarity over 509 amino acids) and topologically to PG1137 (no primary sequence homology), and these protein(s) may fulfil the role of PG0117 in GPB. Alternatively, it is possible that the translocase function is performed by a different gene at this locus in the parent strain (e.g., the open reading frames of unknown function at PG0109, PG0112, PG0114, and PG0116): the Wzx family of proteins shows extensive sequence variation and hence can be difficult to identify on the basis of sequence alone.

The monosaccharide composition of the capsular polysaccharide of *P. gingivalis* ATCC 53978 (W50) was determined to be mannuronic acid, glucuronic acid, galacturonic acid, galactose, and 2-acetamido-2-deoxy-D-glucose (*N*-acetylglucosamine) in a molar ratio of 1:2:1:1:2, respectively (13). Synthesis of mannuronic and glucuronic acid could occur directly from GDP-Man and UDP-Glc by a GDP-Man-dehydrogenase (a key regulatory enzyme in alginate biosynthesis in *Pseudomonas aeruginosa* [49]) and UDP-Glc-dehydrogenase (an enzyme required for virulence in the yeast *Cryptococcus neoformans* [17]), respectively. PG0108 (see legend to Fig. 1) exhibits significant similarity to these NAD<sup>+</sup>-dependent dehydrogenases and could catalyze both sets of reactions. Galacturonic acid could be derived from UDP-GlcA by the action of a UDP-glucuronate-4-epimerase as described for *Arabidopsis* (35) or via a UDP-Galacturonic acid-4-epimerase (*cap1J*) as in *Streptococcus pneumoniae* type 1, which catalyzes the conversion of UDP-GlcA to UDP-GalA (36). PG1560 and PG0347 show 43% similarity over 352 amino acids and 40% similarity over 364 amino acids, respectively, to UDP-galacturonic acid-4-epimerase from *S. pneumoniae* type 1, so galacturonic acid may be synthesized by this mechanism in *P. gingivalis*. Synthesis of UDP-GlcNAc could be directed by genes elsewhere on the chromosome or UDP-GalNAc could be converted to UDP-GlcNAc by the action of the GlcNAc-epimerase, GalE, or the epimerase at PG0120. The synthesis of the K-antigen could originate from ligation of GlcNAc to the lipid carrier undecaprenyl pyrophosphate (catalyzed by PG0106). Subsequent polymerization of sugars could then be catalyzed by glycosyl transferases (PG0110, PG0111, PG0118, and PG0119), followed by translocation (PG0117) to achieve deposition of CPS on the surface of *P. gingivalis*. Thus, in *P. gingivalis* GPA, initiation of synthesis could have been halted, whereas in *P. gingivalis* GPC, polymerization and perhaps transport/translocation of the K-antigen polymer may be defective.

To date six serotypes (K1 to K6) and K<sup>-</sup> of *P. gingivalis* are recognized (27, 29). It was only possible to amplify a defined region of the K-antigen locus in 60% of the strains, and no amplicon was obtained from the three representatives of the K5-serotype. The RFLP patterns of these amplicons indicated significant heterogeneity between different serotypes, as would be predicted for a locus encoding the biosynthetic machinery for different carbohydrate polymers. DNA sequence variation at this locus was more pronounced at the 3' end; PCR products could be amplified from only 15% of the *P. gingivalis* strains, and the significant size variation of these products suggested loss of open reading frames. These observations are supported by recent and unpublished whole-genome DNA microarray data which indicate the absence of genes from this region in the genome of *P. gingivalis* 381 (HG91, K<sup>-</sup>), HG1660 (ATCC 49417, K4), HG1025 (A7A1-28, K3), and HG1691 (K6). Having now definitively identified the K-antigen locus in *P. gingivalis*, it may be possible to develop a PCR-based methodology for serotyping which takes advantage of the genetic heterogeneity of the locus and circumvents the requirement for bacterial growth, cell extractions, and typing sera. Similar approaches have been adopted for the serotype assignment of a number of bacteria including *S. pneumoniae*, *Haemophilus influenzae*, *Neisseria meningitidis*, and *Campylobacter jejuni* (19–21, 25, 31, 32) ([http://www.sanger.ac.uk/Projects/S\\_pneumoniae/CPS/](http://www.sanger.ac.uk/Projects/S_pneumoniae/CPS/)).

Analysis of the DNA sequences of the entire K-antigen locus of *P. gingivalis* 381 provided some insights into the ancestry of this strain and the absence of K-antigen expression. While the locus of 381 is syntenic with that of W83 and demonstrated similarity based on RFLP analysis of this region with K1 strains, there are significant differences. PG0106<sub>381</sub> is probably nonfunctional while PG0109 and PG0110 have been replaced with Orf15, Orf18, and Orf24. The counterparts of the glycosyltransferases at PG0111 and PG0118 in 381, if functionally equivalent, are likely to exhibit different specificities as the sequences, though within the same group, are extremely diverse. In *P. gingivalis* GPB (PG0117), the inactivated flippase did not have a major impact on the phenotype of the mutant. However, in 381, in addition to the absence of PG0117, there is truncation of PG0116<sub>381</sub>. Furthermore, PG0112, PG0113, and PG0114 are not found in 381 although a signature in PG1205<sub>381</sub> is present in PG0114<sub>W83</sub>. The presence of PG1205 at the 3' end of the locus may also have a negative effect on K-antigen expression. Although the transcriptional organization of the locus in strain 381 has not been established, in other organisms capsule genes are frequently cotranscribed, and it is possible that insertion of PG1205 has disrupted transcription at the 3' end of the locus in this strain.

Recently, a link has been established between the biosynthesis of glycosylated surface and extracellular protein virulence determinants and cell surface polysaccharides in other bacteria. In *P. aeruginosa* 1244, it has been reported that the pilin is covalently modified with a trisaccharide and serine [ $\alpha$ -5*N*- $\beta$ -OHC (4)7*N*f*m*Pse-(2 $\rightarrow$ 4)- $\beta$ -Xyl-(1 $\rightarrow$ 3)- $\beta$ -FucNAc-(1 $\rightarrow$ 3)- $\beta$ -Ser] which is identical in structure to the repeating unit of the O-polysaccharide of LPS of this bacterium (8, 12). Inactivation of *wbpM* and *wbpL*, genes involved in the initial steps of O-antigen biosynthesis in *P. aeruginosa*, leads to loss of both the LPS O-antigen and also the pilin glycosylation in this organism. Other examples where a single biosynthetic gene

product is used by different polysaccharide synthesis pathways include GalE in *C. jejuni*—an epimerase which catalyzes the interconversion of Glc to Gal and GlcNAc to GalNAc. Inactivation of GalE in this organism leads to truncation of the lipo-oligosaccharide, loss of capsular polysaccharide, and disruption of N-linked glycosylation. Similarly, *galE* inactivation in *N. meningitidis* causes truncation of a trisaccharide moiety which is covalently attached to the pilin of this organism and disruption of the outer core of the lipo-oligosaccharide (46). The net influence of glycan modifications on the properties of the resultant macromolecules remains to be determined, although there is some indication that these additions may represent a means of antigenic variation (5, 48).

We have previously shown that the Arg-gingipains of *P. gingivalis* are members of this growing family of bacterial glycoproteins (3, 10, 15, 39–41, 44). The carbohydrate modifications to these enzymes appear structurally diverse and may be linked to the protein chain through a variety of different sugar residues (40), but there is currently no information on the biosynthetic machinery involved in their synthesis. However, a monoclonal antibody, MAb 1B5, which recognizes glycan additions to the Arg-gingipains also cross-reacts with a cell surface polymer of *P. gingivalis*. Hence an additional goal of the present investigation was to determine whether the locus responsible for capsule biosynthesis in *P. gingivalis* is also involved in the glycosylation of these proteases.

Deletion of the K-antigen locus and the resultant loss of K-antigen expression had no discernible effect on the glycosylation of the Arg-gingipains. The heavily modified membrane type RgpA had an identical migration pattern on SDS-PAGE, there were no effects on Arg-X protease activity, the distribution of the activity between cells and supernatant was unaltered, and the mutants in K-antigen expression retained reactivity with MAb 1B5, which reacts with a glycan epitope on some isoforms of these enzymes and a cell-associated carbohydrate polymer. Hence it appears that the maturation pathway of the Arg-gingipains which leads to a highly variable degree of glycan modification is independent of K-antigen biosynthesis.

The contribution of the K-serotype antigen to the overall biology of *P. gingivalis* and its role in the persistence of this organism during chronic infections of the periodontal tissue are under continuing investigation. The increased binding capacity of nonencapsulated *P. gingivalis* strains to other bacteria and host cell surfaces referred to previously (11, 23, 24) may reflect an increased exposure of bacterial surface adhesins in these capsule-negative strains. Similarly, we observed an increase in hemagglutination titers in the capsule mutants in the present study. It may therefore be beneficial to the organism under certain circumstances or stages of the infection to have reduced expression of capsule to facilitate adherence.

In other bacteria the serotype capsule frequently plays an important function in evasion of the immune response, in particular complement killing and phagocytosis, which are both important features of the inflammatory response in periodontal disease. Analysis of the mechanism of evasion of serum killing by *P. gingivalis*, which will be described in a separate communication, suggests that the presence of the K-antigen is not a requirement for serum resistance in this bacterium. Instead, resistance is related to the production of

an alternative cell surface anionic polymer. However, given the evidence from animal studies that the possession of the K-antigen of *P. gingivalis* is required for maximal expression of virulence, the mutants generated in the present report should prove useful in delineating the *in vivo* role of this polysaccharide.

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