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Mechanisms of Resistance of *Porphyromonas gingivalis* to Killing by Serum Complement

Jennifer M. Slaney,[1] Alexandra Gallagher,[1] Joseph Aduse-Opoku,[1] Keith Pell,[2] and Michael A. Curtis[1]*

MRC Molecular Pathogenesis Group, Centre for Infectious Disease,[1] and Neuroscience Centre,[2] Institute of Cell and Molecular Science, Barts and the London, Queen Mary's School of Medicine and Dentistry, London, United Kingdom

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The complement system plays an important role in the host defense against infection, and the formation of the terminal complement complex on the bacterial surface has been shown to be particularly important in killing of gram-negative bacteria. The gram-negative periodontal pathogen *Porphyromonas gingivalis* is resistant to complement killing, and possible mechanisms suggested for this resistance include protease production and capsule formation. In this study, *P. gingivalis* Arg- and Lys-gingipain deletion mutants and polysaccharide synthesis deletion mutants have been used to investigate these hypotheses. When Arg- and Lys-gingipain protease mutants were incubated in 20% normal human serum, deposition of complement components on the cell surface was significantly increased compared to that for the wild-type organism. However, despite the increased deposition, the protease mutants maintained resistance to killing and their viability was equal to that seen with heat-inactivated serum. Similar data were obtained when the wild-type organism was treated with gingipain protease inhibitors. K-antigen expression mutants were also resistant to killing. However, mutants which no longer synthesized a surface anionic polysaccharide (APS) (a phosphorylated branched mannan) were extremely sensitive to serum killing. These mutants lack the organized dense glycan surface layer present on the parent strain on the basis of electron microscopy. We conclude that the production of APS at the surface of *P. gingivalis* rather than Arg- and Lys-gingipain synthesis is the principal mechanism of serum resistance in *P. gingivalis*.

Porphyromonas gingivalis has been implicated as one of the principal bacterial agents of periodontal disease by culture (35) and by detection of specific antibody in patients' serum (11, 23). Putative virulence factors of the organism include the production of capsular material (11, 21, 40) and the synthesis of proteolytic enzymes able to cleave immunoglobulins and complement components, both of which may facilitate survival of the host's inflammatory response (9).

The complement system plays an important role in the host defense against infection, and the formation of the terminal complement complex (TCC) on the bacterial surface has been shown to be particularly important in killing of gram-negative bacteria (39). Resistance to serum killing has been identified as a virulence trait in pathogenic bacteria including *Bordetella pertussis* (12), *Salmonella enterica* serovar Typhimurium and *Yersinia enterocolitica* (18). Resistance of strains of *P. gingivalis* to killing by pooled human sera has been demonstrated by Sundqvist and Johansson (37), whose study concluded that although antibodies to all strains tested were present in pooled serum, there was no correlation between sensitivity to killing by serum and antibody levels. Others (9, 25) have shown various sensitivities between strains and also demonstrated different levels of killing by sera from different patients. Okuda et al. (25) concluded that all strains activate complement through both the classical and alternative pathways but that no killing occurs in the absence of antibody.

Subsequently there have been several studies to clarify the interaction between *P. gingivalis* and components of the complement system. Degradation of human serum proteins, including complement factors C3 and C5, has been demonstrated by immunological methods and suggested as a means to explain the high pathogenic potential of *P. gingivalis* (36). Schenkein suggested that degradation may be dependent on the trypsin-like protease activity of *P. gingivalis* (32). This study demonstrated that *P. gingivalis* proteases are not likely to destroy fluid-phase complement components at the concentrations present in gingival crevicular fluid. However, analysis of complement activation in the fluid phase may not reflect the status of complement proteins bound to the bacterial cell surface. A later study demonstrated that *P. gingivalis* W83 failed to accumulate ^{125}I-C3 on the cell surface following opsonization with serum due to cell-associated proteolytic activity, and deposition was increased following treatment with a cysteine protease inhibitor, Nα-*p*-tosyl-L-lysine chloromethyl ketone (TLCK) (33). In contrast, Wingrove et al. (41) suggested that the degradation of C3 and C5 by *P. gingivalis* might lead to generation of active fragments, and they were able to demonstrate the generation of a C5a-like fragment which is biologically active for neutrophil activation. Recently Grenier et al. (17) used *P. gingivalis* ATCC 33277 and mutants deficient in Arg- and Lys-gingipains to demonstrate that resistance to serum bactericidal activity was dependent on these enzymes, although earlier work by this group had suggested that proteases may not be solely responsible for resistance. Grenier and Belanger (16) evaluated the effect of *P. gingivalis* outer

* Corresponding author. Mailing address: MRC Molecular Pathogenesis Group, Institute of Cell and Molecular Science, Barts and The London, Queen Mary's School of Medicine and Dentistry, 4 Newark Street, London E1 2AT, United Kingdom. Phone: 44 (0) 207882 2300. Fax: 44 (0) 207882 2181. E-mail: m.a.curtis@qmul.ac.uk.

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TABLE 1. Strains used in this work

Strain	Genotype	Source or reference
P. melaninogenica 25845		ATCC
P. gingivalis W50		P. D. Marsh, Porton Down, Salisbury, United Kingdom
P. gingivalis W83		H. Werner, Tubingen, Germany
P. gingivalis 23A3		D. Mayrand, Quebec, Canada
P. gingivalis WpH35		W. P. Holbrook, Reykjavic, Iceland
P. gingivalis IET3-10		
P. gingivalis 22KN-6-12		R. Nakamura, Tokushima, Japan
P. gingivalis LB13D-3		D. Mayrand
P. gingivalis HG1241		A. J. van Winkelhoff, Amsterdam, The Netherlands
P. gingivalis 381		S. S. Socransky, Boston, Mass.
Mutants of *P. gingivalis* W50		
W50 BE1		A. S. McKee et al., 1988 (22)
W50 GPA	ΔPG0116-PG0120::*erm*	Aduse-Opoku et al., 2006 (2)
W50 GPB	PG0117::*erm*	Aduse-Opoku et al., 2006 (2)
W50 GPC	DPG0109-PG0118::*erm*	Aduse-Opoku et al., 2006 (2)
W50 E8	*rgpA*α::*tetQ rgpB*α::*erm*	Aduse-Opoku et al., 2000 (1)
W50 K1A	*kgp*α::*erm*	Aduse-Opoku et al., 2000 (1)
W50 PorR	*porR*::*erm*	Gallagher et al., 2003 (14)
W50 PorS	*porS*::*erm*	Gallagher et al., 2003 (14)
W50 WbpB	*wbpB*::*erm*	Gallagher et al., 2003 (14)
W50 BEG	ΔPG1135-PG1141::*erm*	Gallagher et al., 2003 (14)

membrane vesicles on the bactericidal activity of human serum for other oral pathogens and concluded that a heat-stable lipopolysaccharide (LPS) component was involved in addition to the heat-labile proteolytic enzyme(s).

In order for lysis of bacteria to occur, the terminal complement complex, C5b-9, must be assembled on the cell surface and the membrane attack complex must be inserted into the membrane. The aim of the present study was therefore to investigate whether the complement pathway is capable of causing deposition of TCCs on the bacterial surface of *P. gingivalis*, whether deposition correlates with killing of the organism, and whether a lack of bacterial protease activity or changes in cell surface polysaccharides affect these processes.

MATERIALS AND METHODS

Bacteria and growth conditions. Strains of *Prevotella melaninogenica* and *Porphyromonas gingivalis* used in this study are described in Table 1. *P. gingivalis* W50 BE1 is a spontaneous pleiotropic mutant derived from chemostat continuous culture of W50 (22), which is nonpigmented, nonhemagglutinating, and deficient in enzyme activity. The production of *P. gingivalis* W50 mutants deficient in both Arg-gingipains A and B (*rgpA rgpB*) and in Lys-gingipain (*kgp*) was described previously (1). These mutants were prepared by allelic exchange mutagenesis following insertional inactivation in vitro with an *erm* cassette. The production of surface polysaccharide phenotype mutants was led by analysis of the *P. gingivalis* W83 genome. In brief, the genome (TIGR [http://www.tigr.org/]) was interrogated for coding regions with similarity to polysaccharide synthesis loci in other organisms. The first region extends from PG0106 to PG0120 on the *P. gingivalis* genome (TIGR) as shown in Fig. 1. This region encodes homologues of glycosyltransferase proteins in *Bacteroides thetaiotaomicron* (PG0106), *Clostridium thermocellum* (PG0110), and *Streptococcus thermophilus* (PG0118), CapK, a putative capsular polysaccharide biosynthesis protein in *Vibrio cholerae* (PG0111), and a putative polysaccharide transport protein or flippase in *B. thetaiotaomicron* (PG0117). Chen et al. (5) originally suggested that in *P. gingivalis* this region represents the K-antigen locus of the organism (5), and our recent study of mutations in this region supports this hypothesis (2). Deletions were made from PG0116 to PG0120 (*P. gingivalis* GPA) and from PG0109 to PG0119 (*P. gingivalis* GPC), and a mutation was made in PG0117 (*P. gingivalis* GPB) by insertion of an *erm* cassette (13). Two further mutants were constructed by insertion of *erm* cassettes in the second region of homology (14). TIGR identifiers were as follows: *porR* (PG1138), a regulator of pigmentation in *P. gingivalis*, and *porS* (PG1137), a region downstream from *porR* (24, 34). In addition, a seven-gene deletion was made of the entire region (PG1135 to PG1141), which includes *porR* and *porS* (*P. gingivalis* BEG). The involvement of the *porR* region in polysaccharide synthesis or regulation in *P. gingivalis* 33277 has previously been demonstrated by Shoji et al. (34), who produced a mutation in this region which led to a deficiency in phenol-extractable polysaccharide compared to the parent strain. We have now characterized this polysaccharide as an anionic branched mannan (APS) (26). Finally, a mutant was constructed by insertion of an *erm* cassette within *wbpB* (PG2119), which has homology to an LPS biosynthetic gene in *Pseudomonas aeruginosa* (4). Gingipain activities of all these mutants are summarized in Table 2.

All strains and mutants were stored in 10% skim milk (Oxoid) on sterile glass beads at −70°C. They were resuscitated from frozen stocks directly onto blood agar base supplemented with 5% horse blood (Oxoid) and maintained by weekly subculture. Liquid cultures were prepared by inoculation of bacteria from plates into brain heart infusion medium (BHI) (Oxoid) supplemented with 5 mg/liter hemin and 1 mg/liter menadione (Sigma) and maintained by a 1:10 subculture after 24 h. All cultures were grown anaerobically at 37°C in a Don Whitley cabinet, Mark II, in an atmosphere of 80% N_2, 10% H_2, and 10% CO_2.

Serum collection. Serum (normal human serum [NHS]) for the bacterial killing assay was extracted from the blood of four normal donors. After venipuncture, the blood was allowed to clot and the serum was separated by centrifugation. Serum was dispensed in aliquots and frozen at −70°C within 2 h of blood collection. Serum immunoglobulin G (IgG) antibody to *P. gingivalis* whole cells was detectable by enzyme-linked immunosorbent assay in all samples, and levels were comparable. For the killing assays, an aliquot was defrosted immediately before addition to the bacterial suspensions and any surplus thawed serum was discarded. Heat-inactivated control serum was prepared by incubating aliquots of the serum at 56°C for 30 min. Serum for antibody serology was extracted from the blood of a group of 16 patients with moderately advanced chronic periodontal disease described previously (3).

Treatment of bacterial samples with serum. Overnight cultures of bacteria grown in BHI were subcultured 1:10 and grown to early exponential phase (5 h). Cells were harvested, washed once in complement fixation diluent (CFD) (tablet form; Oxoid), and adjusted to an optical density at 595 nm (OD_{595}) of 0.5 (approximately 10^7 CFU ml^{-1}). Conditions for measurement of bacterial killing were established with *P. melaninogenica*, ATCC 25845, which has been shown to be sensitive to complement (37). Aliquots of 0.8 ml bacteria in triplicate were incubated anaerobically with 0.2 ml serum for 0 and 60 min and with heat-inactivated serum for 60 min. Sample aliquots were removed at the fixed time points, serially diluted, and plated onto blood agar. Serum, heat inactivated by heating to 56°C for 30 min, was used as a serum control. Plates were incubated for 6 days before colonies were counted and percentage of surviving bacteria calculated. Means and standard deviations of the replicates were calculated. Comparisons between *P. gingivalis* W50 and mutants were made using a two-

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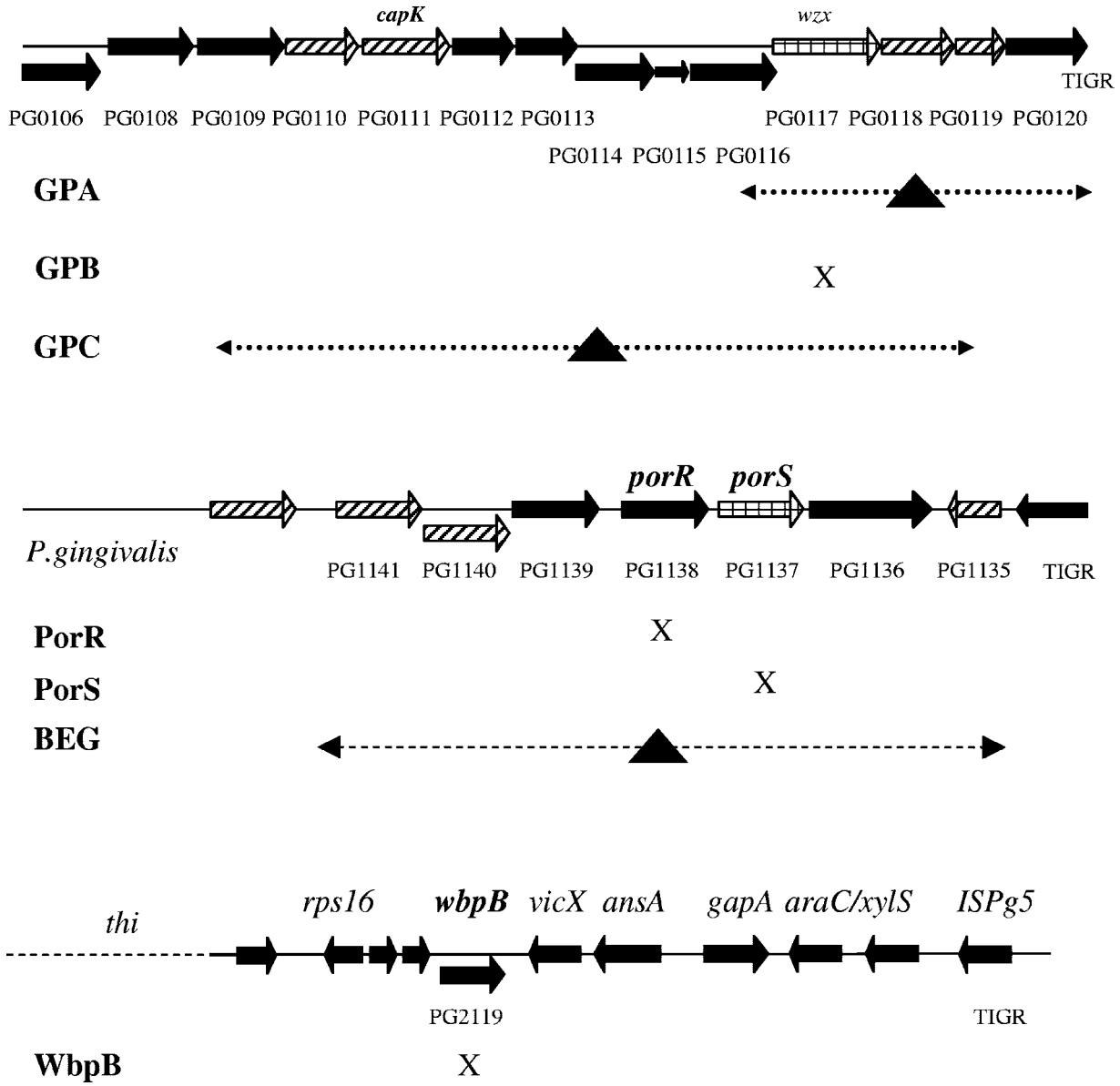

FIG. 1. Construction of *P. gingivalis* strains with mutations in polysaccharide synthesis genes. *Porphyromonas gingivalis* polysaccharide loci showing single gene knockouts (X) and deletions (▲). ▨, encodes glycosyl transferase; ⊞, encodes translocase/flippase; ■, other open reading frames. Details of other genes within these loci are described elsewhere (2, 14).

tailed Student's *t* test. Differences of *P* values of <0.005 were considered significant.

Inhibition of protease activity. The inhibitors TLCK (Sigma) and tyrosine-alanine-lysine-arginine chloromethyl ketone (YAKR-CK) (a generous gift from E. Shaw, Friedrich Meischer Institute, Basel, Switzerland) were used at final concentrations of 1 mM and 40 μM, respectively. The inhibitors were added to the bacterial suspensions and incubated for 10 min at room temperature. The suspensions were centrifuged, washed in CFD, and resuspended in 0.8 ml CFD. Serum was then added, and the procedure continued as described above.

Protease activity assay. Arginine-specific and lysine-specific protease activities of whole cultures and culture supernatants were determined by assaying samples, diluted 1/10 in Tris-cysteine buffer (pH 7.5) (Tris, 50 mM; $CaCl_2$, 7.5 mM; cysteine-HCl, 1 mM), using the synthetic chromogenic substrates *N*-benzoyl-L-arginine-*p*-nitro-aniline (L-BAPNA) and *N*-α-acetyl-L-lysine-*p*-nitroanilide at a concentration of 0.4 mM (each). The rate of substrate hydrolysis was followed spectrophotometrically at a wavelength of 405 nm using a Uvikon 930 spectrophotometer.

Western blotting. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed as described by Laemmli (20) using 10% acrylamide/bis-acrylamide (37.5:1). Bacteria in BHI were harvested after 48 h culture by centrifugation of 1.5 ml aliquots at 10,000 × g and resuspended at ten times concentration in SDS (0.2%) containing leupeptin (0.1 mg/ml). After incubation for 10 min at room temperature, the suspensions were diluted 1/10 in reducing sample buffer, incubated at 100°C for 5 min and loaded at 5 to 10μl/lane for SDS-polyacrylamide gel electrophoresis.. Transfer onto a polyvinylidene difluoride membrane was carried out by Western blotting for 2 h at 400 mA in carbonate-bicarbonate transfer buffer, pH 9.9. Blots were blocked by incubation for 1 h with 5% bovine albumin (Pentex) in phosphate-buffered saline (PBS) before being incubated overnight with monoclonal antibody (MAb) 1B5 in 1% bovine albumin or with serum from patients with adult periodontitis diluted 1/500 in 1% bovine albumin. Production of MAb1B5 has been described previously (8). The antibody was raised to *P. gingivalis* W50 $RgpA_{cat}$ and was found to recognize covalently linked carbohydrate additions to the catalytic chain of the enzyme. The blots were washed five times in 0.05% Tween 20 in PBS and

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TABLE 2. Relative gingipain activities of whole cultures of *P. gingivalis* and mutants grown for 48 h[a]

P. gingivalis strain[b]	Relative gingipain activity		Reference(s)
	Arg-X	Lys-X	
W50	100	100	1
E8	0–2	100	1
K1A	100	0–2	1
PorR*	20–30	25–30	14, 34
PorS	70–80	~60	Unpublished
BEG*	~50	~70	Unpublished
WbpB*	35	50	Unpublished
GPA	100	100	2
GPB	100	100	2
GPC	100	100	2
BE 1*	20–25	20	6

[a] Activity for *P. gingivalis* W50 wild type = 100%. For W50, Arg-X and Lys-X activities correspond to 2.6 and 0.8 U, respectively, where units of enzyme activity are measured as an increase in absorbance at 405 nm/min/OD_{540}.

[b] *, these cells do not produce APS.

incubated for 2 h in rabbit antimouse IgG or with rabbit antihuman IgG conjugated with horseradish peroxidase (Dako). After being washed as before, the blots were developed in diaminobenzidine (0.05%) in PBS with hydrogen peroxide (0.0002%).

C3d and C5b-9 deposition on bacterial cell surface. Bacterial suspensions, pretreated with serum, were centrifuged in Eppendorf tubes, and the pellets were washed three times and resuspended in CFD. Fifty microliters of the suspension in CFD was incubated with 50 μl monoclonal anti-C5b-9, an antiserum to the neoepitope in activated C9 which is not exposed in native C9 (Dako, Glostrup, Denmark), or rabbit anti-C3d (Dako) in 1% bovine albumin–CFD for 90 min. Samples were then washed three times with CFD–0.05% Tween before incubation with horseradish peroxidase-conjugated antimouse IgG (Southern Biotech) or antirabbit immunoglobulins (Dako) for 90 min. This was followed by a second wash step (three times in CFD–0.05% Tween) and incubation with the substrate, hydrogen peroxide and *o*-phenylenediamine. Color development was measured at 405 nm.

Electron microscopy. Bacteria were pelleted at 10,000 × *g* in 1.5-ml microcentrifuge tubes for 5 min in a refrigerated centrifuge before fixation in 0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4 for 2 h at 4°C. Pellets were carefully removed from the tubes, cut into approximately 0.5-mm cubes, and postfixed in 1% osmium tetroxide in distilled water for 1 h. They were then briefly rinsed with distilled water, dehydrated through ascending grades of ethyl alcohol, infiltrated with increasing strengths of embedding resin (TAAB) in propylene oxide over a period of 48 h, and then finally embedded and polymerized in pure embedding resin at 60°C for a further period of 48 h. Thin sections were cut using a Leica Ultracut E ultramicrotome, collected onto 400-mesh copper grids, and stained for 20 min with a solution of saturated aqueous uranyl acetate followed by staining with Reynold's lead citrate for 5 min. Sections were examined and micrographs taken using a Jeol 1200 EX11 transmission electron microscope operating at 80 kV.

RESULTS

Aliquots of a *P. melaninogenica* cell suspension with an OD_{595} of 0.5 treated with heat-inactivated NHS (diluted in series from 10^{-1} to 10^{-7}) plated onto blood agar plates contained approximately 10^7 CFU per ml. CFU were reduced to less than 5% of this number after incubation for 1 h with 20% normal serum. Deposition of C3d from serum onto the cell surfaces of wild-type *P. gingivalis* W50 and mutants was compared in the same assay using peroxidase-labeled anti-C3d antiserum, measuring color development in phenylenediamine with the substrate at 405 nm and subtracting the color development in control samples which had been incubated with heat-inactivated sera. After incubation with 20% normal serum, the surface deposition of C3d on *P. melaninogenica* gave an absorbance value of 0.648 (±0.014) and on *P. gingivalis* W50 a value of 0.055 (±0.02). Deposition of C5b-9 was compared using peroxidase-labeled anti-C5b-9 and measuring color development in the substrate as before. Surface deposition of C5b-9 on *P. melaninogenica* gave an absorbance value of 1.65 (±0.133), and that on *P. gingivalis* W50 gave an absorbance value of 0.177 (±0.013).

Strains of *P. gingivalis* were found to give somewhat better growth in heat-inactivated NHS than *P. melaninogenica*, and numbers of CFU after 60 min ranged from 10^7 to 10^9 per ml. There was greater than 90% survival of all 11 of the laboratory strains tested in the presence of NHS (results not shown). However, the beige mutant of W50 (W50 BE1) was extremely sensitive to normal serum. Levels of survival of W50 BE1 varied between assays from 15% to a level where no surviving bacteria were detected after 60 min of incubation in serum. Since the growth characteristics of *P. gingivalis* W50 were more closely mirrored by W50 BE1 than by *P. melaninogenica*, the former was subsequently used as a control for serum killing efficiency. W50 BE1 samples in triplicate, treated in the same manner as the test samples, were included in every assay. After the assay conditions were established, results were expressed as a ratio of bacterial survival by dividing every replicate from incubation in normal serum by every replicate from incubation in heat-inactivated serum.

Complement deposition onto the surface of *P. gingivalis* W50 BE1, although measurable, gave very poor replicates, perhaps because the cells were disrupted by the lytic process.

Exponential cultures of W50 BE1 at optical densities similar to that of the parent strain, W50, contained smaller amounts of Arg-X protease activity, measured with the chromogenic substrate L-BAPNA (23%) (6). We therefore investigated whether pretreatment of the parent strain, *P. gingivalis* W50, with protease inhibitors affected complement deposition and serum killing. Incubation of the organisms with TLCK (1 mM) and YAKR-CK (0.04 mM) was found to give total inhibition of Arg-X and Lys-X protease activity, and greatly increased levels of C3d and C5b-9 were shown to be present on the cell surface after treatment with these inhibitors (Fig. 2A). However, pretreatment with protease inhibitors had no effect on the resistance of *P. gingivalis* to killing (Fig. 2B). The protease inhibitors at these concentrations did not appear to interfere with the proper functioning of the complement cascade, since treatment with TLCK and YAKR did not affect the killing of *P. melaninogenica* and *P. gingivalis* W50 BE1 (not shown).

Incubation of the Arg-gingipain A- and B-deficient mutant (*rgpA rgpB*) of *P. gingivalis* W50 with human serum led to an increased level of C3d on the bacterial cell surface. Under similar conditions, the Lys-gingipain mutant (*kgp*) of *P. gingivalis* W50 showed an increased level of deposition of C5b-9 (Fig. 3A). However, the two mutants showed levels of survival after incubation with serum equal to that of the parent strain (Fig. 3B). Therefore, while Arg- and Lys-gingipain activity was able to reduce the level of complement deposition on *P. gingivalis*, the cells retained their resistance to serum killing when these enzyme activities were either inhibited or genetically inactivated. Serum resistance in this organism is therefore achieved via a mechanism independent of the Arg- and Lys-gingipains.

Hence, in the next experiments we examined the influence of

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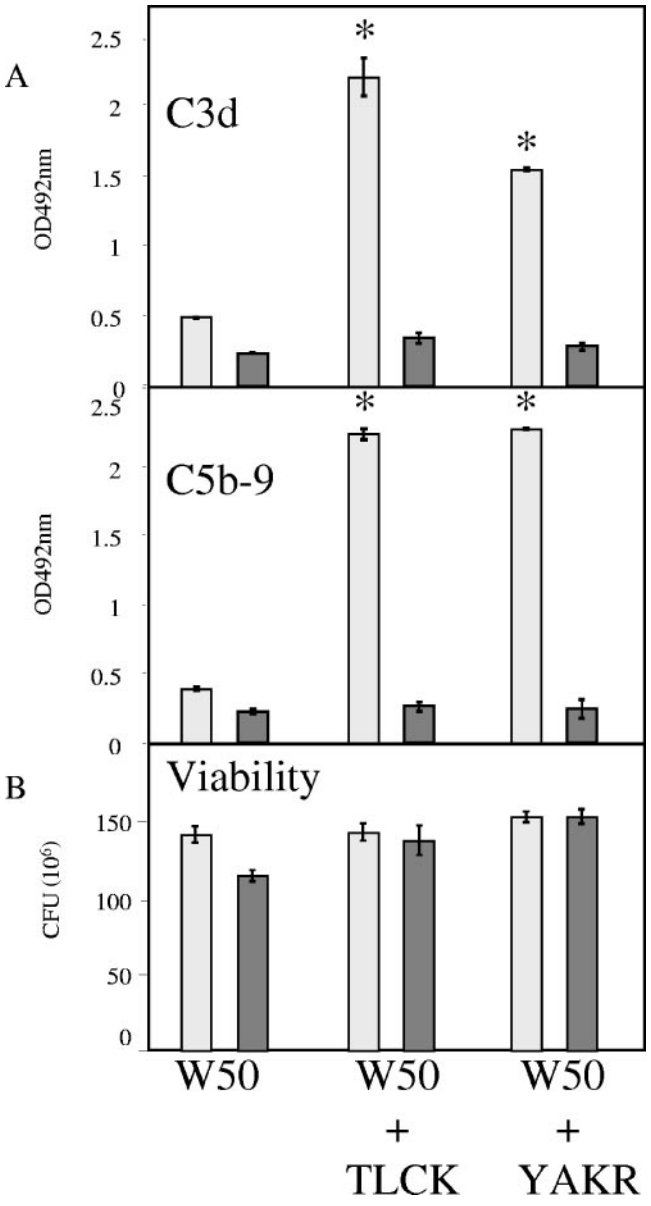

FIG. 2. A. Effect of protease inhibitors on deposition of C3d and C5b-9 on *P. gingivalis* W50 after 60 min of incubation with 20% serum. Deposition of complement factors was demonstrated by enzyme-labeled antibodies and measurement of color development using a chromogenic substrate at 405 nm. B. Effect of protease inhibitors on the serum sensitivity of *P. gingivalis* W50. Viability of bacteria preincubated with serum for 60 min was measured in CFU from serial dilutions on blood agar plates. Numbers of bacteria following incubation with NHS are shown by columns with lighter shading; numbers for incubation with heat-inactivated serum are shown by columns with darker shading. *, results significantly different ($P = <0.005$) from those for *P. gingivalis* W50.

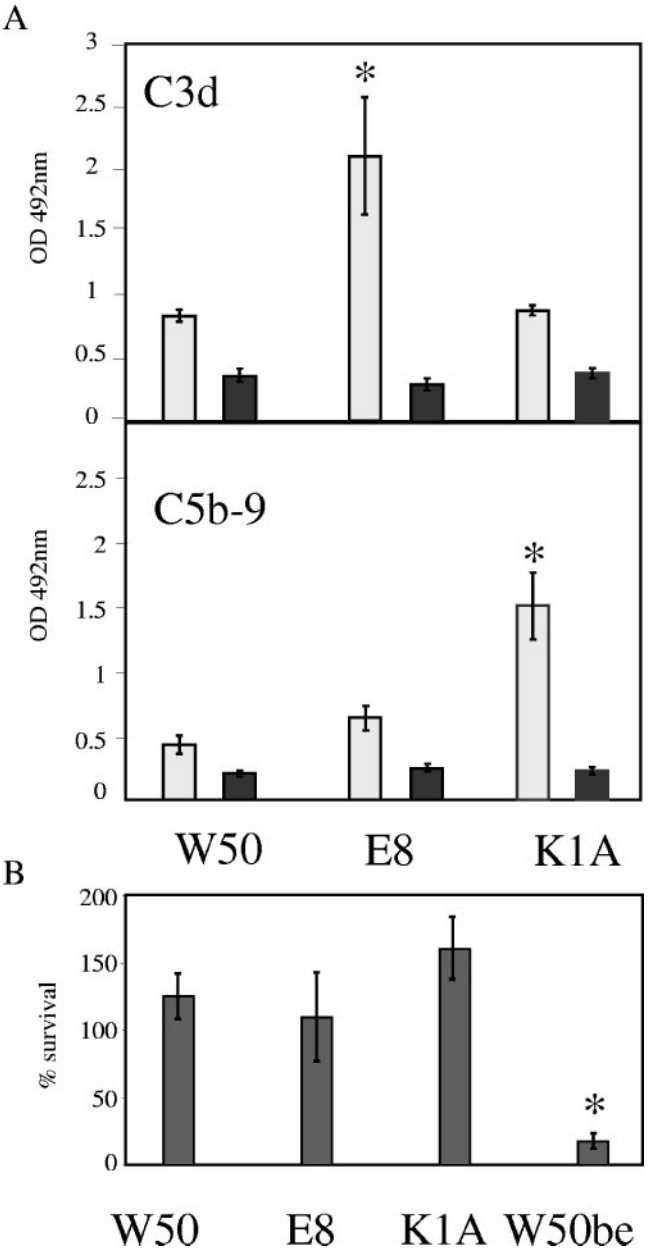

FIG. 3. A. Deposition of C3d and C5b-9 from normal human serum onto *P. gingivalis* W50 and protease mutants after 60 min of incubation. Deposition of complement factors was demonstrated by enzyme-labeled antibodies and measurement of color development using a chromogenic substrate at 405 nm. B. Survival of protease mutants of *P. gingivalis* W50 after 60 min of incubation in serum. Viability of bacteria preincubated with serum was measured as CFU from serial dilutions onto blood agar plates. Results for bacteria incubated with NHS are shown by columns with lighter shading; results for bacteria incubated with heat-inactivated serum are shown by columns with darker shading. *, results significantly different ($P < 0.005$) from those for *P. gingivalis* W50.

the loss of the K-antigen and APS biosynthesis on serum sensitivity. We recently described the identification of the K-antigen locus of *P. gingivalis* W50 at PG0106 to PG0120 and the generation of two deletion mutants (GPA [PG0116 to PG0120] and GPC [PG0109 to PG0119]) which fail to produce a capsule antigen based on double immunodiffusion and capsule staining using India ink and fuchsine stain (2). A partial effect on serum sensitivity was observed with the major deletion mutant GPC. After 60 min of incubation, the number of CFU of this mutant was reduced to 67% of the level with the heat-inactivated serum control. The GPA mutant, in which only the 3′ end of the capsule locus has been deleted, was unaffected with respect

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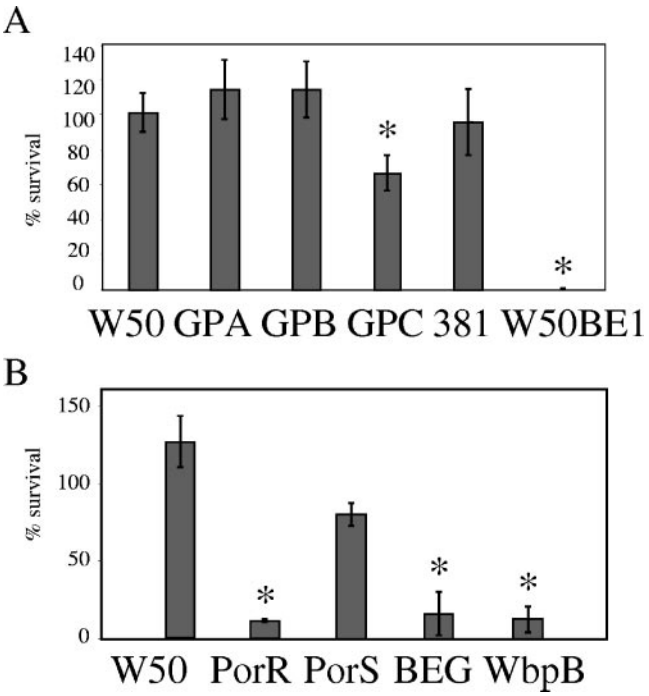

FIG. 4. A. Survival of mutants of *P. gingivalis* W50 (K-antigen locus: PG0106 to PG0120) after 1 h of incubation in serum. B. Survival of mutants of *P. gingivalis* W50 (*porR* locus, PG1135 to PG1141; and *wbpB*, PG2119) after 1 h of incubation in serum. Viability of bacteria preincubated with serum was measured as CFU from serial dilutions on blood agar plates. *, results significantly different ($P < 0.005$) from those for *P. gingivalis* W50.

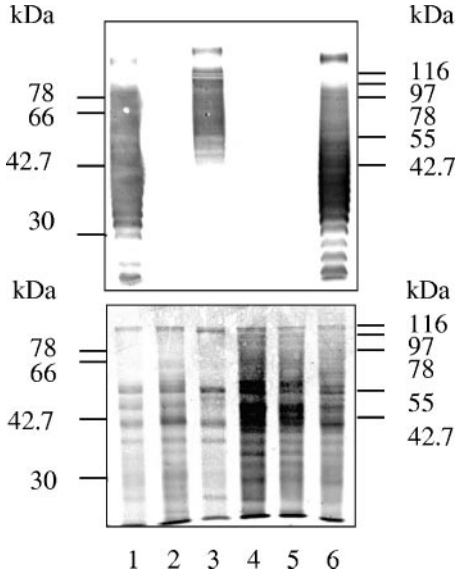

FIG. 5. Western blots of *P. gingivalis* W50 mutants in carbohydrate and Arg-X protease synthesis. Upper blot, incubation with antibody MAb 1B5. Lower blot, staining with Coomassie blue. Lanes: 1, W50; 2, PorR; 3, PorS; 4, BEG; 5, WbpB; 6, E8.

to serum killing, as was the mutant GPB (an insertion mutant of PG0117), which still retained K-antigen expression (Fig. 4A). Hence, even in the absence of K-antigen expression, the organism retained either full or partial protection from serum killing.

We recently described the production of another cell surface carbohydrate polymer by *P. gingivalis*: a branched phosphomannan or APS, which is distinct from the K-serotype and LPS (26). MAb 1B5 reacts with an epitope on APS. The reactivity of MAb 1B5 with *P. gingivalis* W50 and some of the mutants used in the current study is shown in Fig. 5. As we have previously reported, there was no reactivity with a *porR* mutant (34). Similarly, deletion of the *porR*-containing locus (PG1135 to PG1141) in *P. gingivalis* BEG leads to a loss of reactivity with MAb 1B5. *porS* retained reactivity over a more limited range of molecular weight than the W50 wild type. K- serotype mutants retained reactivity with MAb 1B5, as did the protease mutants (results for the *rgpA rgpB* mutant are shown). However, MAb 1B5 showed no reactivity with W50 BE1 or with the *wbpB* mutant, implying that this glycosyltransferase is required for the production of APS.

Transmission electron microscopy (Fig. 6) showed that cells of W50 have an organized surface layer which extends to approximately 10 nm from the cell surface. This surface layer is distinct from the K-antigen capsule, which is a looser network extending much further from the outer membrane and is visualized using India ink exclusion (2). Hence, the electron-microscopic appearance of the K-antigen mutant (GPC) was identical to that of the parent strain, and a surface layer was also evident in the natural K-antigeo strain 381 although it appeared to be rather less dense than that for W50. The double Arg-gingipain mutant (E8) and the Lys-gingipain mutant (K1A; results not shown) showed no irregularities of the surface layer or outer membrane and were of similar appearance to W50. Both PorR and BEG lacked the organized surface layer, and it was partially disrupted in WbpB. PorS retained the surface layer, although it appeared somewhat less uniform than that of the parent strain (results not shown).

When *P. gingivalis* isogenic mutants PorR (PG1138), WbpB (PG2119), and BEG (PG1135 to PG1141) were incubated in normal human serum, they showed survival rates of less than 10%. PorS had 75% survival (Fig. 4B), a statistically significant loss of resistance ($P = 0.02$) but also significantly more resistance than that demonstrated by PorR, WbpB, BEG, and W50 BE1. These mutants retained the K serotype (K1) of the parent strain, *P. gingivalis* W50, as shown by Western blotting and double immunodiffusion (results not shown). Hence, sensitivity to human serum correlates with loss of expression of APS.

In order to determine whether APS biosynthesis is liable to be a feature of the in vivo growth of *P. gingivalis* and hence relevant to serum resistance in periodontal disease, we examined the serum IgG antibody response to *P. gingivalis* W50 and the *porR* mutant. In all cases, adult periodontitis patients showed more serum IgG antibody reactivity over a wider molecular weight range with *P. gingivalis* W50 than with the PorR mutant (Fig. 7).

DISCUSSION

Since the work of Sundqvist and Johansson (37) showed that *Bacteroides asaccharolyticus*, later reclassified as *P. gingivalis*, is resistant to killing by complement in serum, several groups have investigated the cause of this resistance. Sundqvist et al.

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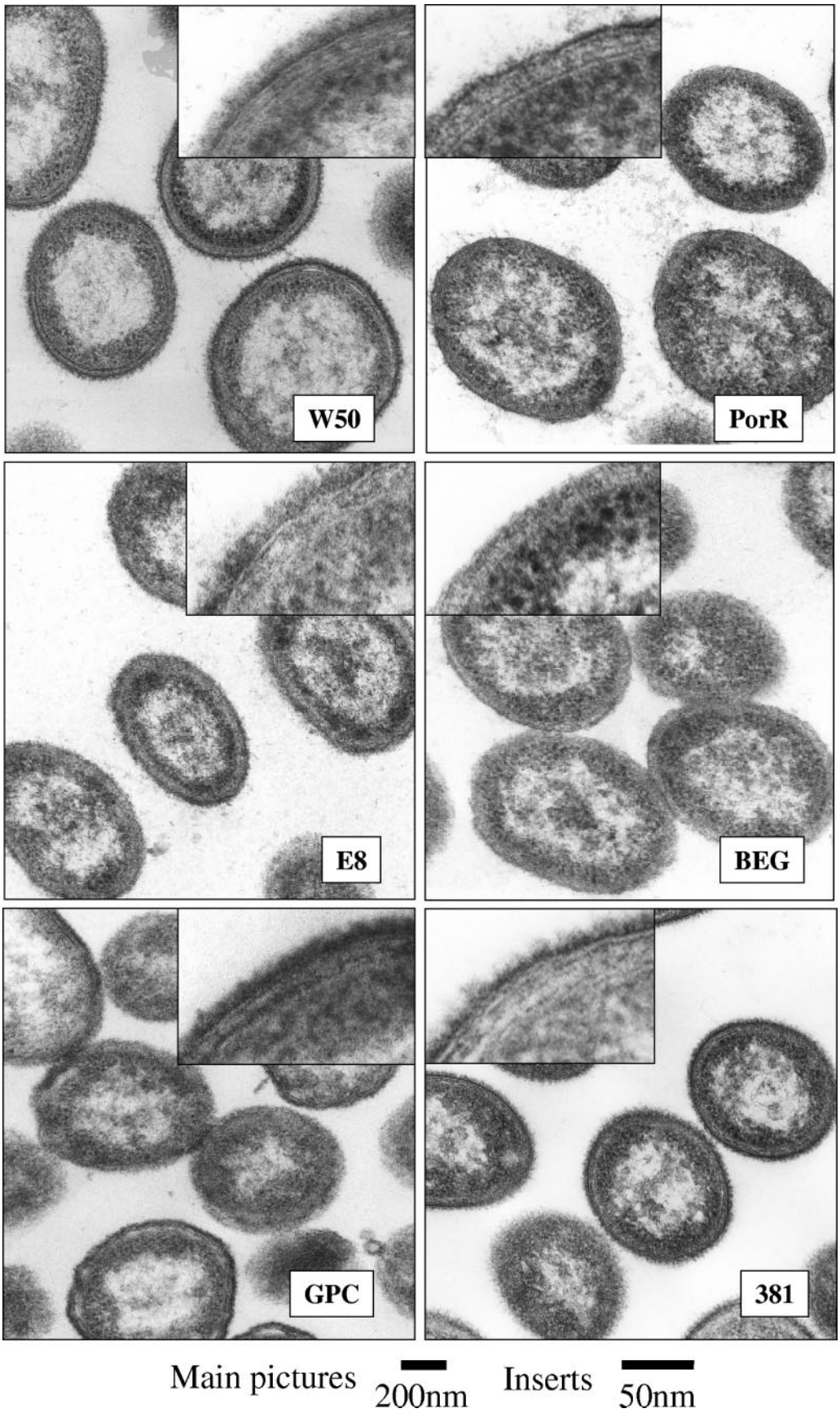

FIG. 6. Transmission electron microscopy of *P. gingivalis* strains and mutants. Note significantly diminished surface layer in both the *porR* mutant (PG1138) and BEG (PG1135 to PG1141) compared to the parent, W50.

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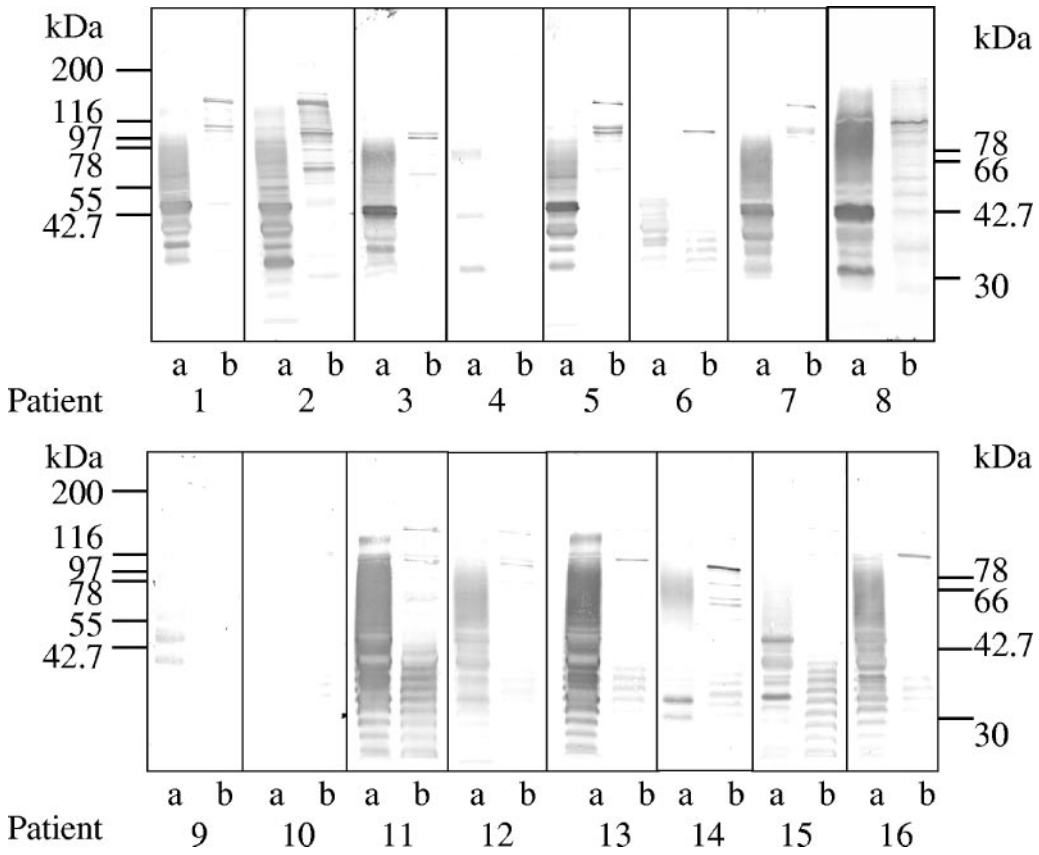

FIG. 7. Western blots of adult periodontitis patients' serum IgG versus *P. gingivalis* W50 and the *porR* mutant. Whole-cell lysates were probed using serum at 1:500 dilution and antibody detected using a rabbit antihuman IgG. Lanes: a, *P. gingivalis* W50; b, PorR.

(38) showed that complement components are susceptible to degradation by protease(s) produced by *P. gingivalis*. Schenkein (33) demonstrated consumption and inactivation of purified complement components C3, C4, and factor B. He further described a failure to accumulate bound C3 on *P. gingivalis* when cultures were incubated with ^{125}I-C3 in normal human serum. Treatment with the protease inhibitor TLCK led to an increased deposition.

However, successful deposition of functional C3 onto the cell surface was implicated in studies of opsonophagocytosis of *P. gingivalis* strain A7436 by human polymorphonuclear leukocytes (10). In the present study, we therefore investigated deposition of complement components on the surface of the organism by using antibody to C3d, the least labile component of C3. We also investigated whether the complement cascade was able to proceed to cell surface assembly of the terminal complement complex C5b-9 by using an antibody which detects a neoantigen on complexed C9. In both cases, we found that the levels of C3d and C5b-9 on the surfaces of *P. gingivalis* were at least equivalent to those on bacteria susceptible to killing. We cannot rule out the possibility that susceptible bacteria were disrupted such that levels on the surface were underestimated, but since levels of C5b-9 present in serum were also higher after incubation with most strains of *P. gingivalis* tested than with susceptible organisms (results not shown), this seems unlikely.

The resistance of *P. gingivalis* to complement deposition reported by Schenkein was not decreased following treatment with leupeptin, antipain, soybean trypsin inhibitor, or phenylmethylsulfonyl fluoride (32). This would suggest that resistance to C3 deposition does not depend on cysteine or serine proteases. However, the beige mutant of strain W50, which produces low levels of protease as measured by the chromogenic substrate L-BAPNA, shows greater susceptibility to killing by serum than the native strain. We have previously used chloromethyl ketone inhibitors to label the Arg-X proteases and obtained complete loss of enzyme activity (7). We therefore decided to investigate the effect on complement deposition and killing of the organism by inactivation of these enzymes with YAKR-CK and TLCK.

Levels of C5b-9 on the surface of the cell and in serum were substantially increased after treatment with YAKR-CK and TLCK, but this did not result in increased killing of *P. gingivalis* strains. Levels of surface C5b-9 and susceptibility to killing of *P. melaninogenica* were unchanged, suggesting that there was no gross disruption of the complement pathway by the inhibitors, although disruption of serum complement inhibitory processes cannot be excluded.

The Arg- and Lys-gingipain protease mutants maintained resistance to killing by complement, confirming that complement resistance does not depend on protease activity alone. These results differ from those reported by Grenier et al. (17), who showed killing of both Arg - and Lys-gingipain mutants of strain ATCC 33277. The strain-to-strain variation of cell sur-

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face glycans of *P. gingivalis* has yet to be investigated fully and may contribute to this discrepancy.

In the present study, mutants with mutations in genetic loci involved in APS synthesis were much less resistant to serum killing than the parent strain. Electron microscopy of the mutant strains confirmed that they lack an organized dense surface. Loss of resistance is also associated with a more fragile cell phenotype, evidenced by a rapid decrease in culture optical density in late stationary phase of mutants nonreactive with MAb 1B5 (26). However, since levels of protease activity in these mutants were also reduced, some additional involvement of protease activity cannot be ruled out.

Several mechanisms of polysaccharide-mediated resistance to serum killing have been found in other bacteria, including some where there is direct involvement of cell surface polysaccharide, whether capsule or LPS. In a study of *Burkholderia pseudomallei*, the addition of purified capsule to serum-sensitive strain SLR5 increased survival by 1,000-fold (31). The authors demonstrated that a capsule mutant of strain 1026B showed more deposition of C3b than the wild type and concluded that the increased serum resistance was mediated by the capsule.

In *Neisseria gonorrhoeae*, sialylation of LPS by host CMP-*N*-acetylneuraminic acid catalyzed by bacterial sialyltransferase renders gonococci resistant to absorption of complement component C3, to killing by immune serum and complement, to killing by phagocytes, and to entry into epithelial cell lines (15). In strains of *Neisseria meningitidis*, sialic acid incorporation into LPS is endogenous, and this organism also possesses a strain-specific polysaccharide capsule which prevents insertion of the complement membrane attack complex. Similarly endogenous sialylation of lipopolysaccharide in *Haemophilus influenzae* has been shown to contribute to serum resistance when wild-type strains were compared with nonsialylated mutants (19). Ram et al. (30) have suggested that the complement control protein factor H acts as a lectin for sialic acid and other polyanions, such as heparin and most sulfated glycosaminoglycans. Factor H acts as a cofactor for factor I-mediated cleavage of C3b, so the enhanced interaction of factor H with the sialic acid of the cell surface could promote the local inactivation of the complement cascade.

Complement resistance brought about by binding of soluble host-derived complement control proteins factor H and C4 binding protein onto cell surfaces has been confirmed for several strains, including *N. gonorrhoeae* (28, 29) and *Streptococcus pyogenes* (27). *Escherichia coli* can acquire resistance by binding of host-derived glycophosphoinositol-anchored protectin (CD59), which inhibits C5b-9 neoantigen expression. There is no evidence to date that any similar binding of control proteins occurs in *P. gingivalis*, but this possibility has not been fully investigated. This study shows that complement resistance in *P. gingivalis* is associated with the presence on the cell surface of an anionic branched mannan. It has been suggested by this group that the charge is conferred by phosphorylation (26), but whether the anionic nature of the mannan is the determining factor of the complement resistance is yet to be established.

We conclude from this study that *P. gingivalis* protease activity prevents the deposition of C3d and C5b-9 on the bacterial cell surface. However, increased complement deposition on *P. gingivalis* W50 in the presence of protease inhibitors or on *P. gingivalis* protease mutants does not lead to bacterial killing, suggesting another mechanism of resistance. *P. gingivalis* mutants defective in biosynthesis of a cell surface polysaccharide are serum sensitive, suggesting that the main mechanism of complement resistance in this organism is mediated via a polysaccharide surface layer which is independent of the K capsular antigens described by Laine et al. (21). The demonstration that this polysaccharide is a major antigen in periodontal disease suggests that this mechanism of serum resistance may play a significant role in vivo.

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