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Characterization of two outer membrane protein antigens of *Porphyromonas gingivalis* that are protective in a murine lesion model

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Porphyromonas gingivalis is a key periodontal pathogen that has been implicated in the aetiology of chronic adult periodontitis. The aim of this study was to characterize two potential vaccine candidates (PG32 and PG33) identified from a previous genomic sequence analysis. Gene knockout studies suggested that these proteins play an important role in bacterial growth and are transcriptionally linked. Analysis of 14 laboratory and clinical isolates of *P. gingivalis* found that in all strains, both genes were present with a high level of conservation and that the two proteins were also expressed *in vitro*. Truncated recombinant PG32 and PG33 proteins were produced in *Escherichia coli* in an attempt to increase the solubility of the proteins while retaining their native conformation. While most of the truncated proteins remained insoluble, two truncated proteins showed good solubility and high levels of protection in the *P. gingivalis* murine lesion model and may be considered as potential vaccine candidates for further testing in models of human periodontal disease.

Key words: outer membrane protein antigen-like; *Porphyromonas gingivalis*; protective; recombinant

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The bacterium *Porphyromonas gingivalis* is a gram-negative anaerobe that has been strongly implicated as an etiologic agent in human chronic periodontitis (14). This disease affects the supporting structures of the teeth and is a major cause of tooth loss in adults. The current treatment of periodontitis is non-specific and is centered on the removal of subgingival dental plaque by mechanical debridement often involving surgical procedures. This on-going therapy is costly, painful, and has a variable prognosis. Alternatives such as systemic antibiotic treatment is largely ineffective when used in isolation but may be beneficial when used as an adjunctive therapy to surgical procedures. Local antibiotic treatment can be effective as a stand-alone short-term treatment, but does not prevent reestablishment of the pathogen (9, 11). The elucidation of the specific bacterial etiology of chronic periodontitis suggests that a vaccine may be an important adjunctive therapy to prevent site recolonization or virulence of the pathogen (15). In order to develop a successful vaccine, conserved bacterial targets preferably on or near the cell surface need to be identified. Previously, we have reported the genomic analysis of *P. gingivalis* to identify potential vaccine candidate antigens with these desired characteristics (17). Following the bioinformatic analysis of the genome, 120 proteins were expressed in *Escherichia coli* and assessed where possible for their protective efficacy in a murine lesion model. Two novel proteins, termed PG32 and PG33, demonstrated significant levels of protection. The genes for these proteins are adjacent on the genome, have predicted molecular masses of 41 kDa and 40 kDa, respectively, exhibit 45% sequence identity and both contain an *E. coli* outer membrane protein antigen (OmpA) motif near the C-terminus (18). It has further been shown that these two proteins are predominant in preparations of outer membranes obtained following the sarcosyl fractionation of

P. gingivalis W50 cells (23). It is likely that these two proteins form heterodimers in the outer membrane of *P. gingivalis* and function as porins involved in the uptake of essential nutrients, based on their structural similarity to OmpA and OprF and their thermodynamic behavior (23).

In this paper we further characterize these two proteins by investigating their conservation in various strains of *P. gingivalis* and the effects of inactivation of one or both of the genes on growth of *P. gingivalis in vitro*. Truncations of PG32 and PG33 recombinant proteins were also prepared in an approach to produce soluble proteins in *E. coli* that could be easily purified and used in a vaccine.

Material and methods

Bacterial strains

The *E. coli* host ER1793 (New England Biolabs, Beverly, MA) was used as the host strain for making plasmid constructs for protein expression and gene knockout experiments. The bacterial strain *E. coli* BL21 (DE3) (Novagen, Madison, WI) was used for the expression of C-terminal $(His)_6$-tagged proteins. Molecular biology techniques were performed as previously described (17).

A restriction-deficient and transformation competent strain of *P. gingivalis* YH522 was used for the gene knockout experiments. This strain was kindly provided by Professor Toshio Umemoto, Department of Oral Microbiology, Kanagawa Dental College, Yokosuka, Japan. The W50 (ATCC53978) and 33277 (ATCC33277) strains of *P. gingivalis* were obtained from the American Type Culture Collection. A panel of 12 *P. gingivalis* strains representing different serotypes (7, 19) and ribotypes (1–3) consisting of strains 7BTORR, A7A1-28, AFR5B1, 84-3, 381, 74-1, 3-3, 3A1, 15-9, 13-1, 10B, 11A was kindly provided by Prof. Roy Page, Department of Periodontics, School of Dentistry, University of Washington, Seattle. All clinical samples in this panel were obtained from subjects by paper point sampling of the subgingival plaque found in periodontal pockets at least 6 mm deep in subjects that had at least four pockets 6 mm deep, gingival bleeding on probing, no previous periodontal treatment, no antibiotic use in the past 6 months and good systemic health. Pure cultures were derived from each of these isolates and ribotyped as previously reported (3). The isolates were obtained from periodontitis patients residing in the United States, Sudan, Romania and Norway (1–3).

Accession numbers

The GenBank accession numbers of genes referred to in this article are AF175714 (PG32) and AF175715 (PG33). In the complete genome of *P. gingivalis* released by The Institute for Genome Research (TIGR) (6) these genes are referenced as PG0695 (PG32) and PG0694 (PG33). The gene PG28 used as a control in gene knockout experiments is a putative thiol protease and is referenced in the TIGR complete genome as PG1427.

Construction of gene knockout cassettes

To inactivate the genes of *P. gingivalis*, plasmid DNA constructs were made containing an erythromycin resistance gene flanked by *P. gingivalis* gene fragments. The erythromycin resistance gene of *Bacteroides fragilis* was subcloned from plasmid pVA2193 (kindly provided by Prof. Toshio Umemoto, Department of Oral Microbiology, Kanagawa Dental College, Yokosuka, Japan) by PCR amplification and inserted into the *Xba*I/*Kpn*I sites of plasmid pMOD (Invitrogen). The restriction enzyme sites *Pst*I, *Xba*I, *Kpn*I and *Eco*RI in the pMOD plasmid were used to subclone fragments of the *P. gingivalis* genes flanking the erythromycin resistance gene. The primers used for PCR amplification and cloning are listed in Table 1. The resultant recombinant plasmids were then transformed into *P. gingivalis* strain YH522.

Transformation of *P. gingivalis*

DNA was transformed into *P. gingivalis* strain YH522 by electrophoresis as pre-

Table 1. PCR primers used in this study

Sequence	Gene	orientation	use
CGCAGAATTCCAGGAGAATACTGTACCGGCAACG	PG32	F	Expression (A, B, D)
TTTTGAATTCCCTTTCTTTGCAACTCGT	PG32	F	Expression (C, G, H)
TTTTGAATTCTTCATCGGTAGCGAATGG	PG32	F	Expression (E, I)
TTTTGAATTCGCTCACTCCAATCTCAAT	PG32	F	Expression (F, J)
GATCGAATTCGCTACAGCAGGTCTTAATTTCC	PG32	F	Expression (K, L)
GATCGAATTCACTAAGACAGAAAATATACTGA	PG32	F	Expression (M)
CTATGCGGCCGCCTTGGAGCGAACGATTACAACAC	PG32	R	Expression (A, K)
TTTTGCGGCCGCACGATTCCAAGCTTTCTT	PG32	R	Expression (M)
AAAAGCGGCCGCCTCGTTAGTTTCTTTTAC	PG32	R	Expression (F, H, I)
TTTTGCGGCCGCCAATTGATCTTTGTCCAC	PG32	R	Expression (E, G, J, L)
TTTTGCGGCCGCCATCCCCTGGAATCCATT	PG32	R	Expression (B, C)
AAAAGCGGCCGCTTTGTGTTGGTAGCCAAC	PG32	R	Expression (D)
TTTTTTCTGCAGGCTCAACATTGGTTGGAGCAA	PG32	F	Knockout (32, 32/33)
TTTTTCTAGATACAAACTGAGCTACGTCAT	PG32	R	Knockout (32/33)
TTTTGGTACCAATCTAAGTCGCGCATACAA	PG32	F	Knockout (32)
TTTTGAATTCCTTGGAGCGAACGATTACAA	PG32	R	Knockout (32)
TTTTTCTAGAGAGATTGGAGTGAGCTGCTT	PG32	R	Knockout (32)
TGCAGAATTCCAAGAAGCTACTACACAGAACAAA	PG33	F	Expression (A, B, D)
TTTTGAATTCCCTTATTTCGGTACTCGT	PG33	F	Expression (C, G, H)
TTTTGAATTCCATAGCGAAAACGCCAA	PG33	F	Expression (E, I)
TTTTGAATTCTTTGCCGGAAAGATGAAC	PG33	F	Expression (F, J)
GATCGAATTCGCTACAGCAGGTCTAACGTTCAA	PG33	F	Expression (K, M)
GATCCGAATTCGAATAGTGCAAAGATTGAT	PG33	F	Expression (L)
GATCGAATTCAAGACCAACAACGCACCGATCA	PG33	F	Expression (N)
CTATGCGGCCGCTTCCGCTGCAGTCATTACTACAA	PG33	R	Expression (A, K, L)
TTTTGCGGCCGCACGATTCCAAGCGTTCTC	PG33	R	Expression (N)
AAAAGCGGCCGCTGCGTTGTTGGTCTTCGC	PG33	R	Expression (F, H, I)
TTTTGCGGCCGCGATACGGAAGTAAACCAC	PG33	R	Expression (E, G, J, M)
TTTTGCGGCCGCCATTACAGGGAAGTCTGC	PG33	R	Expression (B, C)
AAAAGCGGCCGCGAATTTATAACCAAATCC	PG33	R	Expression (D)
TTTTGAATTCTTATTCCGCTGCAGTCATTACTA	PG33	R	Knockout (33, 32/33)
TTTTGGTACCTCGTCTAAGTATCGTTCCTA	PG33	F	Knockout (32/33)
TTTTGGTACCAGACTTCAATATTGAAGGACA	PG33	F	Knockout (33)
TTTTTCTAGAACGACTCTTGATAGGCGGAA	PG33	R	Knockout (33)
TTTTTTCTGCAGAGCACTTGCGGGTCTCGCAT	PG33	F	Knockout (33)
TTTGGTACCGAACATGTTCCCCGATAGCTTCCGCT-ATTG	Ery^R	R	Knockout vector
TTTTCTAGAGAACATGGTTCGGATCCCCGAA-GCTGTCAGT	Ery^R	F	Knockout vector

The sequences of the oligonucleotides used as PCR primers to amplify and clone DNA fragments for expression and gene knockout studies. The sequence of the oligonucleotides, the gene target of the primer, the orientation of the primer as forward (F) or reverse (R), and how the primer was used. The letters in brackets refer to constructs in Fig. 5 (Expression) and Fig. 2 (Knockout).

viously described (24) with modifications. Briefly, *P. gingivalis* was resuscitated from storage at −70°C in 10% glycerol by plating onto Brain Heart Infusion (BHI) agar plates containing 37 g/l Brain Heart Infusion (Oxoid, Basingstoke, UK), 12 g/l Bacto Agar (Oxoid), 5% horse blood and 2 mg/l Vitamin K. Plates were incubated anaerobically at 37°C for 5 days. A single colony was inoculated into 10 ml of BHI broth containing 37 g/l Brain Heart Infusion (Oxoid), 12 g/l Bacto Agar (Oxoid), 5% Horse Blood and 2 mg/l Vitamin K. After incubation anaerobically for 24 h at 37°C, the broth was inoculated into 250 ml of BHI broth and incubated anaerobically for 18 h at 37°C. The cells were washed twice in an equal volume of cold distilled H_2O and resuspended in 1 ml of 1 mM $MgCl_2$ containing 10% glycerol. DNA samples of 5 μl (10 μg) of DNA were added to 80 μl of cell suspension and electrophoresed in a 0.1-cm cuvette at 200 Ω, 1.8 kV and 25 μF. The cells were immediately diluted with 1 ml of BHI broth and plated onto BHI agar containing 0.1 mg/l clindamycin (Sigma-Aldrich, St. Louis, MO). Plates were incubated for 5–7 days anaerobically at 37°C to develop colonies.

Construction of pET24a (+) expression vectors containing *P. gingivalis* recombinant proteins and truncations of PG32 and PG33

PG32 and PG33 along with a number of truncations of these recombinant proteins were constructed by PCR amplification from genomic DNA of *P. gingivalis* W50 using the oligonucleotide primers listed in Table 1. The *Eco*RI and *Not*I sites on the 5'- and 3' ends of each of the amplified gene segments were used for insertion into the same sites of pET24a (+) (Novagen) in frame with an N-terminal T7 tag and a C-terminal hexahistidine $(His)_6$ tag. All plasmid constructs were confirmed by restriction fragment analysis and sequencing using an ABI prism BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA).

Expression of recombinant proteins in *E. coli*

Single colony transformants were grown overnight at 37°C and rotated at 180 r.p.m. in 10 ml Luria Broth with 50 μg/ml kanamycin. A 1-ml aliquot of each culture was inoculated into 15 ml of Terrific Broth containing potassium phosphates and 50 μg/ml kanamycin. Expression was induced by adding IPTG to 0.1 mM when the cultures reached an OD_{600} of 1.8–2.0. After incubation at 30°C or 37°C for 1–4 h, the cultures were harvested by centrifugation at 4000 r.p.m. for 10 min, and the bacterial pellets were stored at −20°C. For purification purposes, soluble proteins were extracted from 500-ml cultures.

Recombinant protein solubility studies

Bacterial pellets were thawed on ice and resuspended in 1.5 ml TE (pH 8.0) and sonicated (Virosonic Digital 475 ultrasonic cell disruptor, The Virtis Company, NY) in an ice bath for two 10-s pulses. Lysates were then centrifuged at 13,000 r.p.m. for 15 min at 4°C to separate soluble and insoluble protein fractions. The insoluble pellet was resuspended in TE (pH 8.0). The protein samples were analyzed in SDS–PAGE under reducing conditions. The percentage of soluble and insoluble fractions of recombinant proteins was estimated by densitometric analysis (Molecular Dynamics, Amersham Biosciences, Sunnyvale, CA). For murine lesion model studies, 500-ml *E. coli* cultures were pelleted and resuspended in 40-ml binding buffer (5 mM imidazole, 500 nM NaCl, 20 mM Tris-HCl (pH 7.9)). Cells were sonicated for six 10-s pulses and centrifuged as described above. The supernatant containing the soluble recombinant protein was collected for purification. For insoluble recombinant proteins, pellets were resuspended in binding buffer containing 6 M urea and incubated on ice for 1 h while stirring to solubilize proteins. The remaining insoluble protein was removed by centrifugation at 18,000 r.p.m. for 20 min and the process of protein solubilization and centrifugation repeated. The supernatant was filtered through a 0.45-μm membrane prior to nickel nitrilotriacetic acid chromatography.

Recombinant protein purification by nickel nitrilotriacetic acid (Ni-NTA) chromatography

Ni-NTA metal affinity chromatography was used to purify the recombinant proteins via the H_6 tag. Protein was batch bound to an equilibrated Ni-NTA resin (Qiagen, Hilden, Germany), which was loaded into a column, and unbound proteins were eluted under gravity. The column was washed with 10 volumes of binding buffer and 6 volumes of wash buffer (60 nM imidazole, 500 mM NaCl, 20 mM Tris-HCl (pH 7.9)). The bound protein was eluted in buffer containing 1 M imidazole, 500 mM NaCl, 20 mM Tris-HCl (pH 7.9). The wash and elution buffers contained 6 M urea for purification of insoluble recombinant proteins. This denaturant was removed by dialysis from 6 M to 4 M to 2 M to 0.5 M to 0 M in 50 mM Tris-HCl (pH 7.4), 0.5 M NaCl, 8% glycerol. For soluble recombinant proteins a final dialysis was performed to remove the imidazole from the eluted material. In an attempt to improve protein folding, for some purifications detergents such as 1% NOG (n-octylglucoside; Sigma-Aldrich) were added to all steps of the solubilization and purification. The final protein recovery in the dialyzed sample was measured using a Coomassie Protein Assay Reagent Kit (Pierce, Rockford, IL).

Polyacrylamide gel electrophoresis and Western blotting

Samples were mixed with an equal volume of 2 times sample reducing buffer (Invitrogen, Carlsbad, CA), boiled for 10 min at 100°C and electrophoresed on 4–20% SDS-polyacrylamide gels in Tris-glycine buffer (Invitrogen). Proteins were electroblotted onto nitrocellulose filters for 1 h at 100 V. Membranes were blocked with 5% skim milk-PBS before incubation with one of the following: mouse monoclonal antibody to hexahistidine (Roche Molecular Systems Inc, Pleasanton, CA) diluted to 1:5000; rabbit polyclonal antiserum to *P. gingivalis* sarcosyl insoluble proteins diluted to 1:5000; rat polyclonal antiserum to whole *P. gingivalis* cells (protected, post-challenge serum) diluted to 1:1000; mouse polyclonal antiserum to recombinant PG32 protein diluted to 1:1000; or mouse monoclonal antibody to recombinant PG33 protein diluted to 1:500. The immunoreactive bands were detected with a goat anti-rabbit-HRP conjugate (KPL, Gaithersburg, MD) or a goat anti-mouse-HRP conjugate (KPL) and developed with TMB membrane peroxidase substrate (KPL).

Murine lesion model

Recombinant *P. gingivalis* proteins PG32 (1-391; full length), PG23K (244–391), PG32L (224–306), PG32M (281–384), PG33 (1-380; full length) and PG33K (213–380) were assessed for their ability to protect groups of BALB/c mice ($n = 10$, 6–8 weeks old; ARC, Perth, Australia) from *P. gingivalis* challenge by immunizing mice intramuscularly with two doses of 10–20 μg of recombinant protein in Freund's incomplete adjuvant (Sigma-

Aldrich), 3 weeks apart. Control mice were given two doses of 20 μg of *E. coli* lysate (Promega) or 7.5×10^9 formalin-killed *P. gingivalis* strain 33277 in Freund's incomplete adjuvant to act as the negative and positive control, respectively. Mice were challenged 1–2 weeks later with approximately 2.5×10^9 live *P. gingivalis* strain 33277 cells subcutaneously on the ventral surface of the abdomen at a midline point (13). Mice were examined daily for lesions and the number and sizes of the lesions were recorded by measuring the length and width and converting this into an area measurement in mm^2. Maximum lesion size was analyzed statistically using a one-way ANOVA with Dunnett's post test with Graphpad Instat software (San Diego, CA).

Results

Sequence conservation

A series of 14 *P. gingivalis* strains representing distinct geographic, serologic and genetically diverse isolates were examined for their amino acid sequence conservation of PG32 and PG33. PCR amplification and sequencing of PG32 and PG33 from these strains demonstrated that these proteins were highly conserved within the species (Fig. 1) with all 14 strains having the gene and expressing full length PG32 and PG33 proteins. By sequence analysis, 97 and 94% of amino acids in PG32 and PG33, respectively, were completely conserved among all 14 strains tested. In addition, all 14 strains when grown in liquid culture also expressed PG32 and PG33 proteins as determined by Western blotting against whole *P. gingivalis* lysates using mouse polyclonal and monoclonal antisera raised to recombinant PG32K and PG33K (data not shown).

Gene knockouts

Studies to determine whether PG32 and PG33 proteins were essential for *P. gingivalis* growth and survival were carried out by insertionally inactivating the genes encoding these proteins. The strategy used for this inactivation is depicted in Fig. 2. Erythromycin resistance was used as the selective marker for homologous recombination between the knockout cassette and chromosomal DNA. Single gene knockout cassettes (PG32KO, PG33KO) were constructed by inserting the erythromycin resistance gene into the center of the target gene (Fig. 2). As PG32 and PG33 are separated by only 46 bp on the genome, a single knockout cassette was also created (PG32/33KO) to knock out both genes using a construct which contained 60% of the 5' end of PG32 and 60% of the 3' end of PG33 (Fig. 2). The design of these cassettes ensured there was sufficient DNA flanking the erythromycin gene for efficient recombination. It is believed that these two proteins are transcriptionally linked, as an mRNA transcript containing both sequences has been isolated (E. Reynolds, unpublished results). As a control to ensure that erythromycin resistance did not affect PG32 and PG33 expression, a knockout cassette was made to an unrelated gene PG28, which encodes a putative thiol protease (17) and is approximately 700 kb from the genes encoding PG32 and PG33.

Following transformation of *P. gingivalis* YH522, multiple colonies were selected from each knockout group for further analysis. The presence of a 2.2 kb insertion in the PG32, PG33 and PG28 genes was confirmed by PCR and Southern blot hybridization (results not shown). To determine the effect of gene inactivation on protein expression, Immunoblot analysis was performed on cell lysates of the mutant strains (Fig. 3). PG32 and PG33 were expressed in both the parent strain and the strain with PG28 inactivated (Fig. 3, lanes 2 and 3). However, in the mutant strains with either PG32 or PG33 or both genes inactivated, neither of the proteins was expressed (Fig. 3, lanes 4–6). Although the polyclonal antiserum against PG32 was cross reactive with other *P. gingivalis* proteins (Fig. 3, panel a), it was clear that PG32 was not expressed in the knockout mutant.

The most obvious characteristic of the mutant strains was the slow growth rate in culture. When grown on solid media, colony formation was notably slower and this slower growth rate was clearly shown in liquid media. The growth curves of the parent strain and the control YH522-PG28KO strain compared with YH522-PG32KO, YH522-PG33KO or YH522-PG32/33KO are depicted in Fig. 4. This data indicated that the growth rate was impaired by at least two doublings in the logarithmic phase.

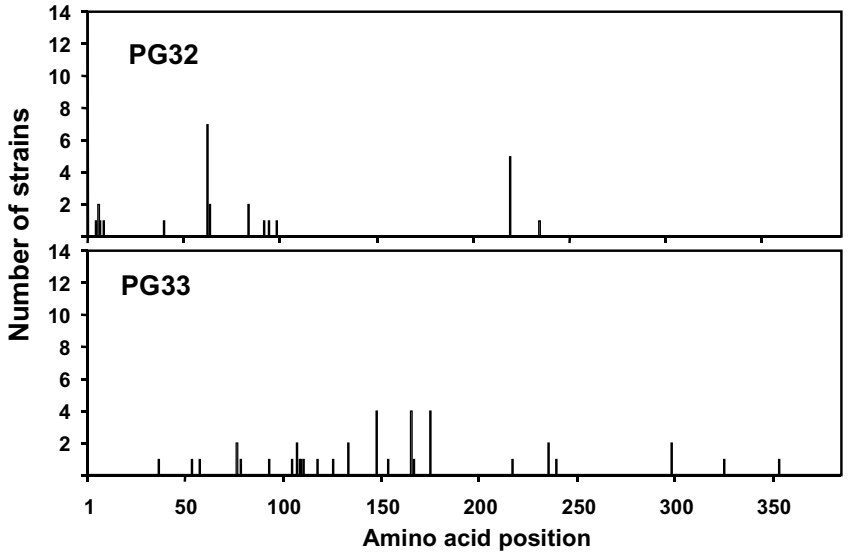

Fig. 1. Schematic representation of the sequence variability of PG32 and PG33 among 14 different strains of *P. gingivalis*. The height of the lines indicates the number of strains having a different amino acid at that position compared with the W83 strain.

Solubility of *E. coli* expressed recombinant PG32and PG33 proteins

Figure 5 depicts schematic diagrams of the truncated expression constructs of PG32 and PG33. PG32K (residues 224–391) and PG33K (residues 213–380) contain the entire OmpA-like C-terminal region from the proline-rich sequence, which has a potential flexible hinge region. PG33L (residues 286–380) is a further truncation of PG33K with the hydrophobic regions, just downstream of the proposed removed hinge region. PG32B (residues 21–223) and PG33B (residues 22–212) contain the entire N-terminal region before the proposed hinge region. All constructs were sequenced and verified to be in frame with the C-terminal H_6 tag. The recombinant plasmids obtained were introduced into the *E. coli* strain BL21 (DE3). PG32 and PG33 fragments were expressed, with varying amounts of protein observed in the whole cell lysates. The relative molecular weight

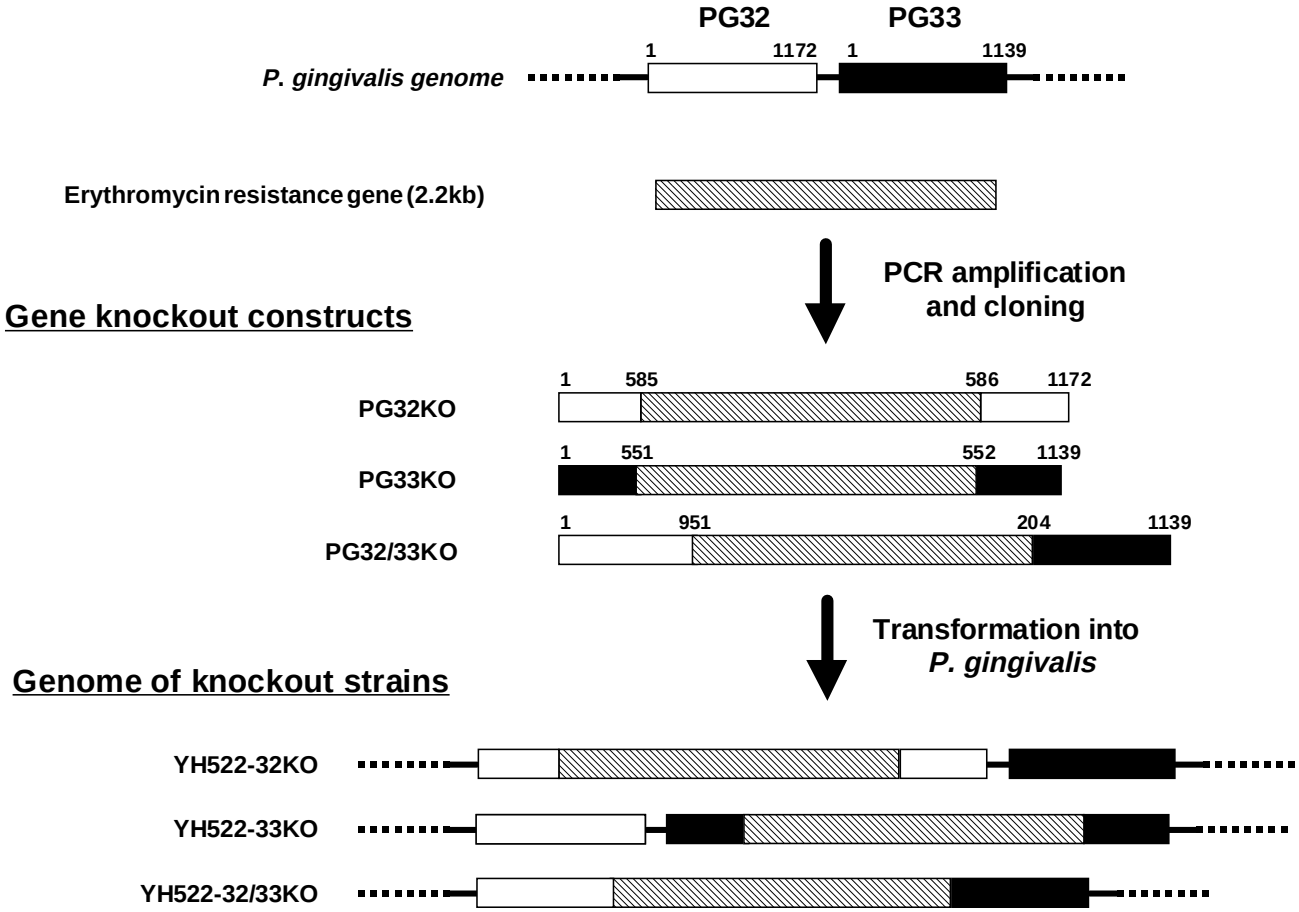

Fig. 2. Diagrammatic representation of the gene knockout process used to inactivate the PG32 and PG33 genes. The original configuration of the *P. gingivalis* genomic DNA is represented at the top of the figure showing these genes as adjacent in the genome, 46 bp apart. PCR amplification and cloning was used to make the gene knockout constructs for insertional gene inactivation. These are designated PG32KO, PG33KO and PG32/33KO. The numbers indicate the nucleotide positions of the genes included in the constructs. At the bottom of the figure is the resultant genomic configuration of the three mutant strains after insertional inactivation. Mutants were made from the *P. gingivalis* YH522 strain, and are designated YH522-32KO, YH522-33KO, YH522-32/33KO.

of each polypeptide coincided with the expected molecular mass from the amino acid sequence. Aliquots of both supernatants and resuspended pellets obtained after mild sonication were analyzed by SDS–PAGE. Densitometric scans of the Coomassie blue-stained SDS–PAGE gels were performed to estimate protein ratios in soluble and insoluble fractions and were expressed as a percentage of the total recombinant protein expressed (Fig. 5). Solubility ranged from zero (e.g. full length PG32, PG33 and a large proportion of the N-terminal truncations) to partial solubility with lower recovery (PG32C 1%, PG32F 2%, PG32J 0.4%, PG32L 9% and PG33L 8%) and partial solubility with higher recovery for the C-terminal fragments (PG32K 18%, PG32M 47%, PG33K 25%, PG33M 15% and PG33N 29%). Attempts to improve protein solubility included varying temperature and time of induction, as well as IPTG concentration, but very limited improvements in solubility were observed. Interestingly, the partially soluble proteins from the PG32K and PG33K truncations which were constructed from the proposed hinge region were both only partially soluble but were reactive with all *P. gingivalis* antisera by Western blot analysis (Fig. 6). These were the only truncations that were as immunoreactive as their full length counterparts with the rat antiserum to *P. gingivalis* whole cells and rabbit antiserum to *P. gingivalis* outer membrane proteins (Fig. 6), suggesting that the target antibody epitopes were located in the C-terminal regions of PG32 and PG33. Purified recombinant $(His)_6$-tagged PG32 and PG33 protein fragments yielded protein concentrations for PG32K and PG33K following final dialysis of 2.75 mg/ml and 491 μg/ml respectively, with 50–60% purity. PG32L and PG32M were purified to 75% homogeneity with protein concentrations of 1.65 and 13.98 mg/ml, respectively.

Murine lesion model

Full length versions of PG32 and PG33 were insoluble in aqueous solutions and were therefore solubilized and purified in 6 M urea, dialyzed to remove part of the urea and formulated with Freund's incomplete adjuvant in the presence of 0.5 and 2 M urea, respectively. Neither of the full length recombinant proteins were protective when tested as immunogens in the murine lesion model (data not shown). As many of the truncated versions of these molecules were also insoluble, only those truncated constructs with solubilities above 5% and good yields were tested in the mouse abscess model. Immunization with PG32K (224-391) and PG33K (213-380) both showed significant protection as immunogens when compared to the control mice immunized with *E. coli* lysate ($P < 0.05$) and challenged with live *P. gingivalis* strain 33277 (Fig. 7). PG32K gave

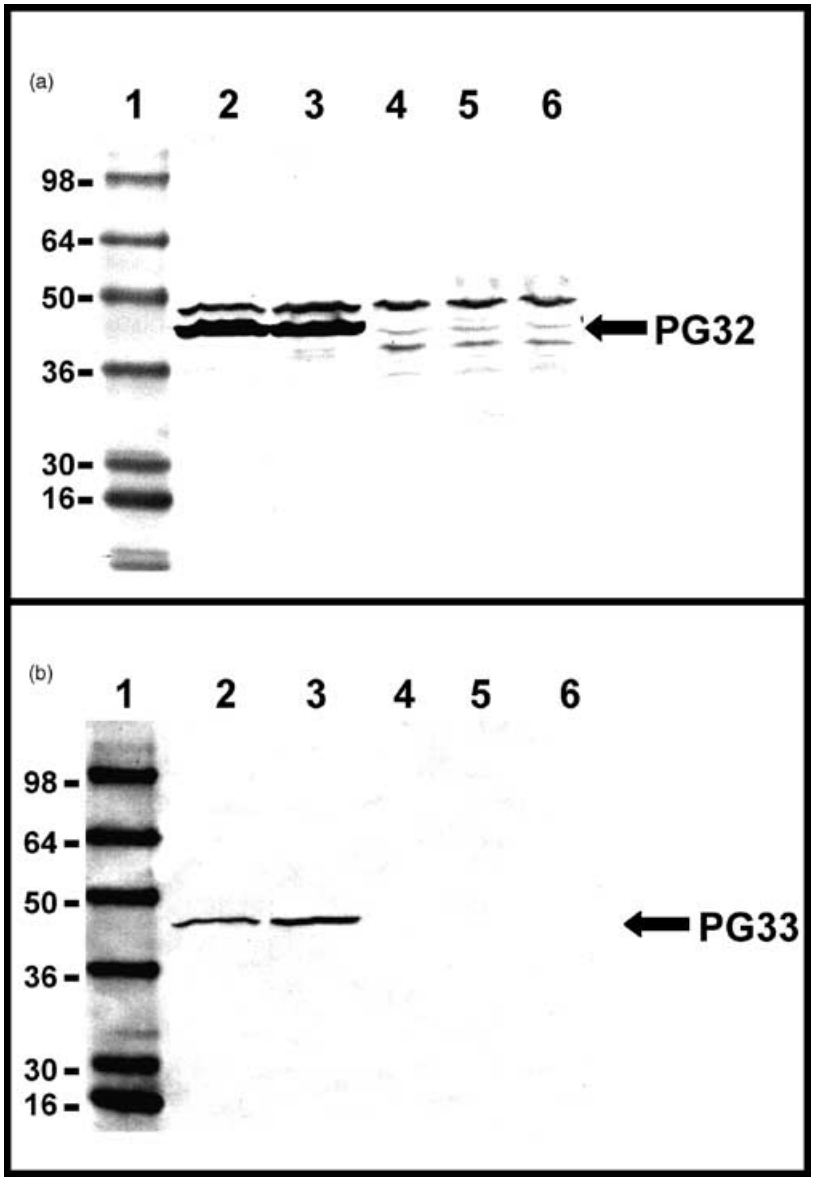

Fig. 3. Immunoblot results of gene knockout strains. Cell lysates were prepared from the parent strain YH522 (lane 2), a strain with an inactivated irrelevant gene PG28 (lane 3), and inactivated genes encoding PG32 (lane 4), PG33 (lane 5), and PG32/33 (lane 6). Immunoblots were probed with either a mouse polyclonal antiserum against recombinant PG32 (Panel a) or a mouse monoclonal against recombinant PG33 (Panel b). The location of PG32 and PG33 is indicated with arrows and the size of molecular weight markers (kDa) in lane 1 are listed.

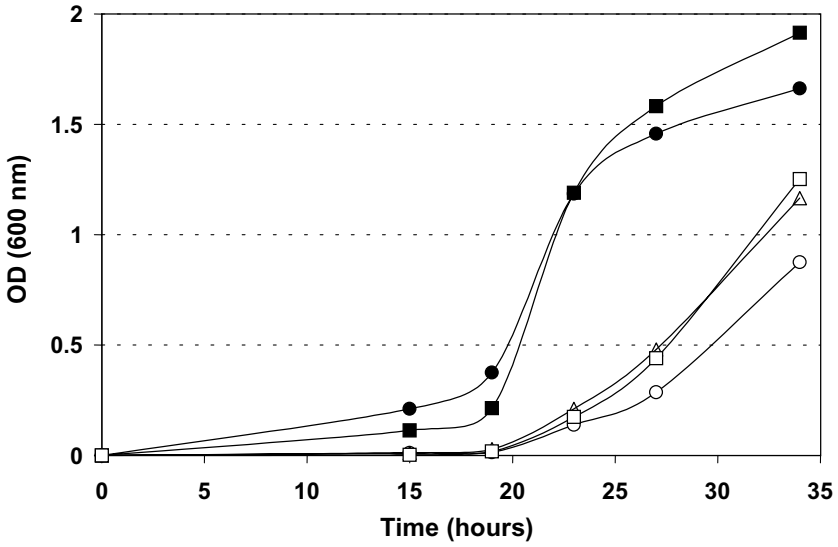

Fig. 4. Growth kinetics of various strains in culture with genes disrupted by insertional inactivation. Strains derived from *P. gingivalis* YH522 have either no insertion (wild type YH522; ●), insertion in a single gene (YH522-PG28KO; ■, or YH522-PG32KO; ○, or YH522-PG33KO; △), or insertion in two genes (YH522-PG32/PG33KO; ○).

approximately 70% reduction in lesion size while PG33K gave a 52% reduction. PG32 K solubilized and purified in the presence of 1% NOG also gave significant protection (78% reduction) as did the mice immunized with formalin-killed whole *P. gingivalis* 33277 cells (82% reduction). The other recombinant fragments tested, PG32L and PG32M, did not confer significant protection when used as immunogens in the mouse lesion model. PG33L, M and N were not tested in the mouse lesion model as a consequence of these results.

Discussion

In this paper we have described the characterization of two proteins from *P. gingivalis* which were identified as potential vaccine antigens from a genomic DNA analysis (17). Subsequently, these proteins, termed PG32 and PG33, have been identified from *P. gingivalis* cell fractions suggesting an outer membrane location and have been proposed to form a heterodimeric porin structure involved in the assimilation of nutrients (23). To confirm the utility of these proteins as potential vaccine antigens we examined the

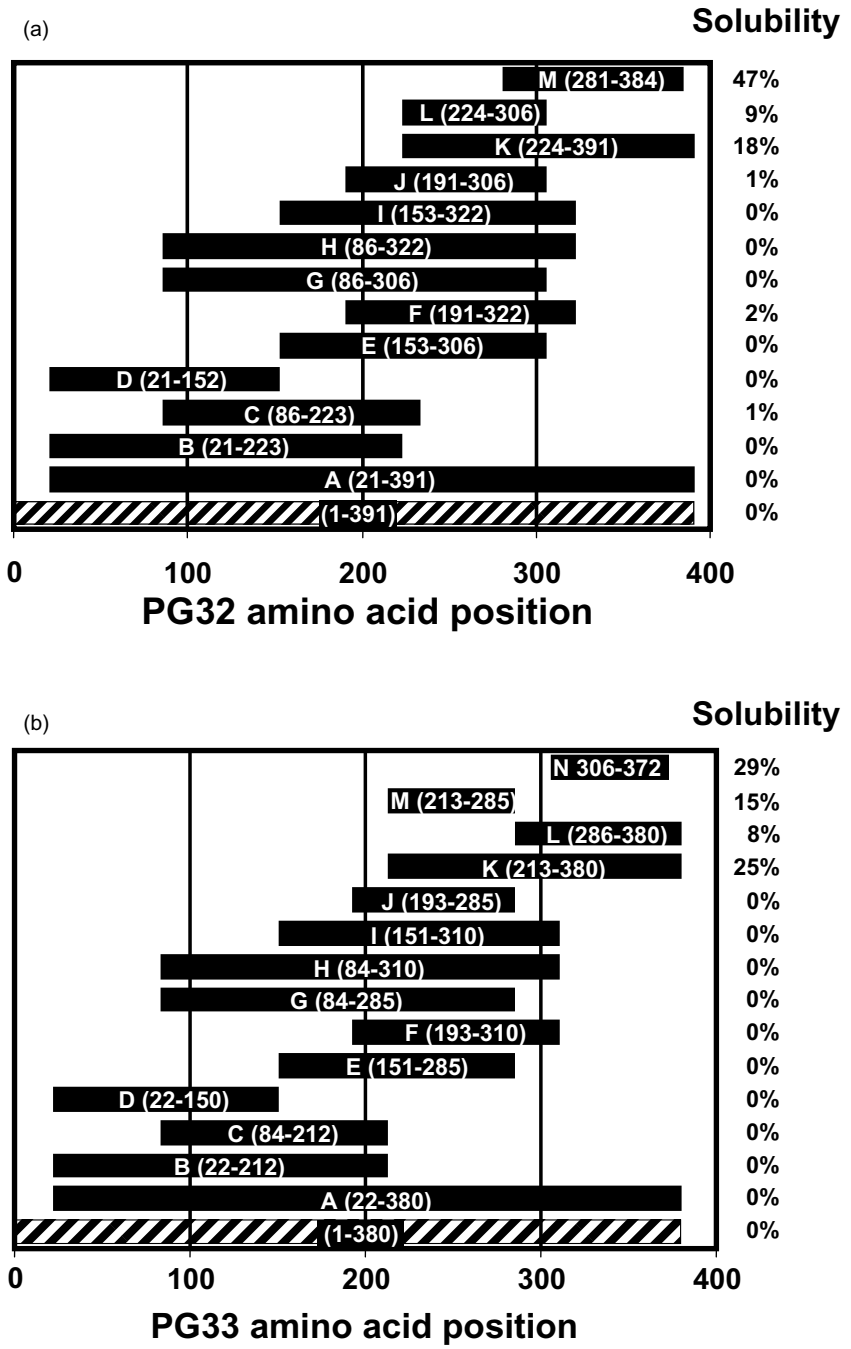

Fig. 5. Diagrammatic representation of the truncated expression constructs of PG32 (a) and PG33 (b). The bars represent the location of each truncated protein in the gene and the number ranges indicate the start and end amino acid positions of the proteins. At the right of the diagrams is the solubility of each protein as a percentage of the total amount of protein expressed. The hatched constructs (1-391 for PG32 and 1-380 for PG33) represent the full length proteins.

sequence conservation in various human isolates and laboratory strains of *P. gingivalis*, investigated the functional role of the proteins on growth *in vitro* and tested recombinantly expressed proteins and truncations for solubility and protection in a mouse lesion model.

The importance of PG32 and PG33 to *P. gingivalis* is supported by the finding that these genes were conserved and the proteins constitutively expressed in 14 geographically and temporally diverse clinical and laboratory strains of *P. gingivalis*. The gene knockout results where PG32, PG33 or both genes were insertionally inactivated, showed that while they were able to survive *in vitro* if one or both genes were inactivated, there was a significant impairment in growth in a laboratory strain of *P. gingivalis* (YH522). The absence of both proteins in single knockout strains was consistent with the proposed co-transcription of these proteins and the requirement that both of these proteins need to be present for the formation of a functional heterodimer (23). Further *in vivo* growth studies will be required to truly determine the absolute requirement of these proteins in this organism, but given their abundance, location and conservation among various strains of *P. gingivalis*, they would appear to be good vaccine candidate targets for antibodies or other immune mediators.

The ideal attributes of a bacterial vaccine antigen to take into human trials are difficult to define. However, some preferred features might include surface localization to enable access by antibodies, the provision of an essential function to the organism so that the gene product cannot be downregulated, sequence conservation amongst different strains to ensure universal efficacy, protection in animal models, and expression of a soluble recombinant protein to facilitate vaccine manufacture.

To some extent, the two proteins described in this study fulfill all of the above criteria. However, full length PG32 and PG33 are hydrophobic transmembrane proteins which, when expressed in *E. coli,* form insoluble aggregates requiring resolubilization by strong detergents, followed by refolding processes, which are unlikely to generate correctly folded recombinant proteins. It is important for the recombinant protein to have the correct conformation so that any antibodies raised against this protein will recognise the native protein on *P. gingivalis* bacterium and target its destruction. To express PG32 and PG33 as predominantly soluble proteins, we generated truncated versions of each gene construct by inserting PCR-amplified gene fragments into the *Eco*RI and *Not*I sites of pET24a (+).

Truncation design was largely based on hydrophobicity data, but certain truncations were based upon proposed 3-D models of OmpA-like proteins such as *Pseudomonas aeruginosa* OprF protein (16).

The production of recombinant protein derivatives of transmembrane proteins such as OmpA-like proteins for vaccine manufacture can be problematic. Their complex hydrophobic β-barrel structure is difficult to mimic in a soluble form. OmpA has been described as a two-domain protein with an N-terminal membrane-spanning β-barrel domain and a C-terminal

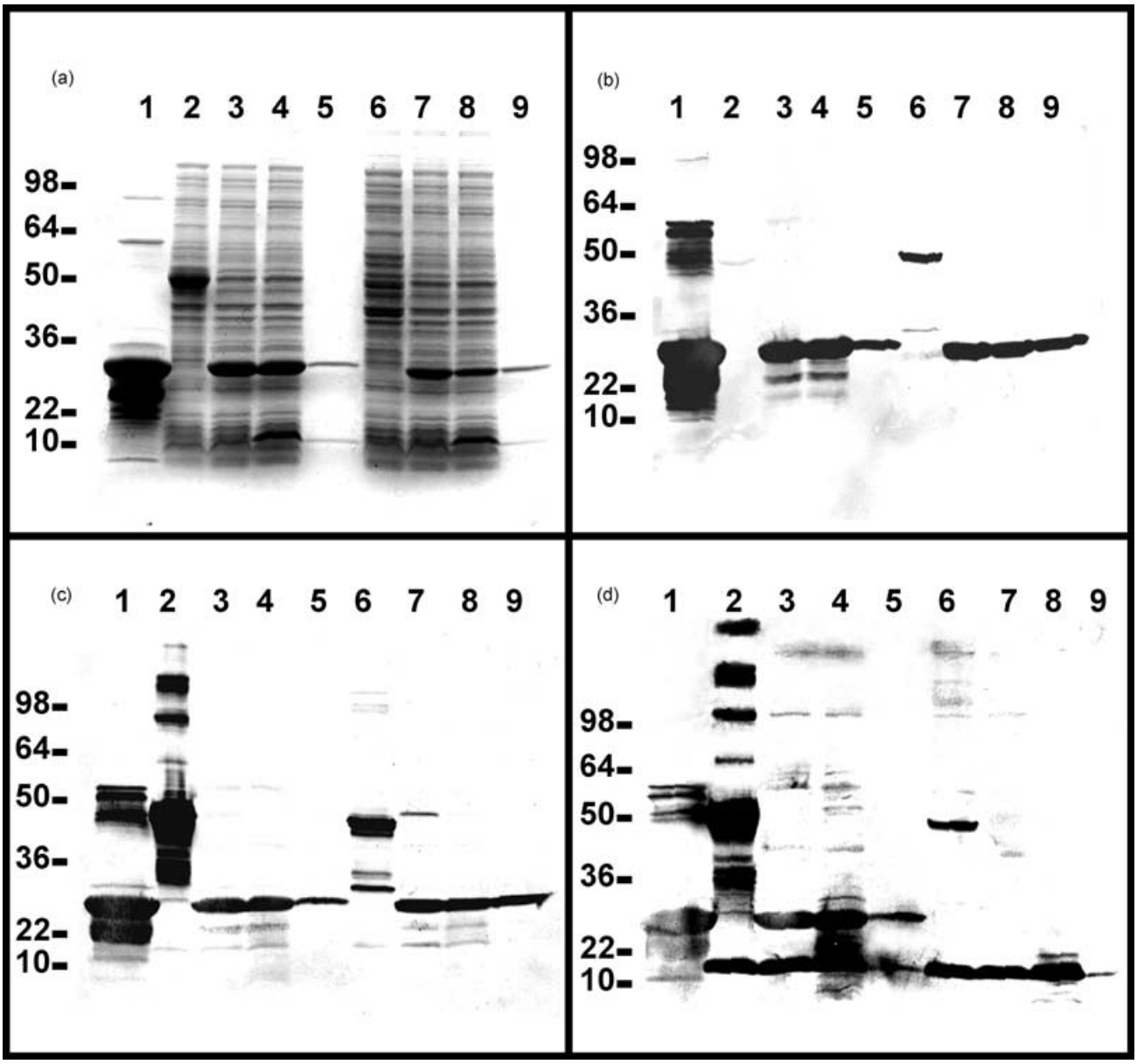

Fig. 6. Analysis of PG32, PG33 and various truncations expressed in *E. coli*. Panel a is a Coomassie-stained SDS–PAGE gel, panel b is an immunoblot reacted with a mouse antiserum to hexahistidine, panel c is an immunoblot reacted with a rabbit antiserum to *P. gingivalis* sarcosyl insoluble proteins and panel d is an immunoblot reacted with a rat antiserum to *P. gingivalis* whole cells. Lanes 1–5 contain PG32 expression products and lanes 6–9, PG33 expression products. Lane 1 contains purified PG32C, which was used as a positive control for the immunoblots. Full length recombinant proteins PG32 and PG33 prepared from whole cell lysate (lanes 2 and 6, respectively), whole cell lysates from the PG32K and PG33K truncations (lanes 3 and 7), the soluble proteins from the PG32K and PG33K truncations (lanes 4 and 8) and the insoluble proteins from the PG32K and PG33K truncations (lanes 5 and 9). The location and size (kDa) of molecular weight markers are indicated.

globular periplasmic domain (22). However, the precise structure and function of OmpA-like proteins is controversial. The C-terminal globular domain from OmpA was in fact represented in these experiments by the recombinants PG32K and PG33K, which were partially soluble, immunoreactive with various antisera to raised whole *P. gingivalis* cells and conferred protection against *P. gingivalis* challenge when used as immunogens in the murine lesion model. The N-terminal β-barrel domain was represented by PG32B and PG33B, which were completely insoluble and were therefore not tested in this model. It is somewhat surprising that a purported periplasmic domain would confer protection when used as an immunogen. However, this C-terminal domain has also been suggested to be transmembrane and surface accessible in an alternative model of the OmpA structure (4, 21). It is likely, therefore, that this alternative structure to the *P. gingivalis* OmpA-like heterodimeric protein and the C-terminal segment is exposed at the cell surface, possibly explaining the protection obtained in this current study. This alternative model is also supported by previous studies on the OmpA-like porin OprF of *P. aeruginosa*. Vaccination with OprF induced protection in animal models of infection (8, 12) and of note was that all the B-cell epitopes were localized in the C-terminal 160 amino acids (20). One of these epitopes was also shown to be surface loca-

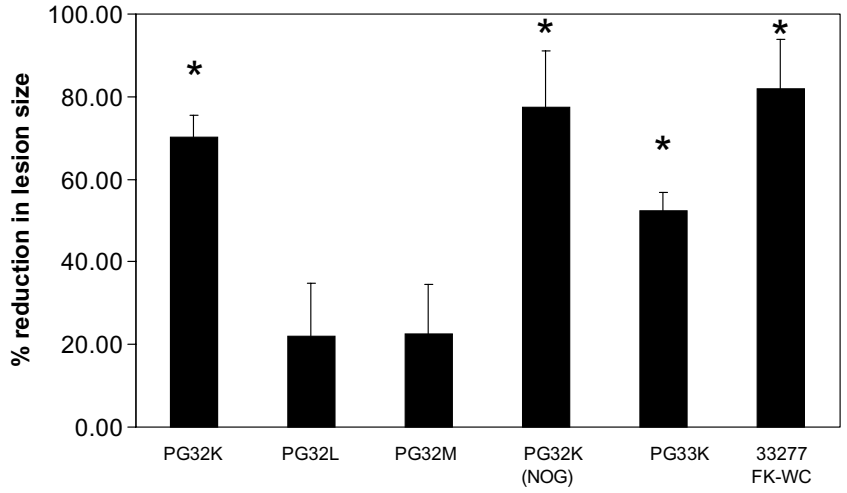

Fig. 7. Protection of mice from lesion formation when challenged with live *P. gingivalis* 33277. Data is derived from a number of individual experiments and the values are mean ±standard error reductions in the maximum lesion size. Significant protection was obtained with the truncated PG32K with or without solubilization and purification in NOG (*n*-octylglucoside) and PG33K recombinant proteins ($*P < 0.05$). Reduction in maximum lesion size is obtained by comparison with mice immunized with *E. coli* lysate. FK-WC = whole *P. gingivalis* strain 33277 cells, which also give very significant protection in this model.

lized on intact *P. aeruginosa* cells (20). Interestingly, the 160 amino acids containing the B-cell epitopes of OprF correspond to the C-termimal 167 amino acids of the *P. gingivalis* constructs used in the PG32K and PG33K truncations. The C-terminal fragment PG32K showed moderate solubility and good protection in the animal model. However, when two smaller subfragments covering this region (PG32M and PG32L) were tested as immunogens in mice they showed no significant protection, even though PG32M had enhanced solubility. This suggests that these two subfragments of the globular domain were too small for correct folding and, as such, protective epitopes were either not exposed or not present in these subfragments. This loss of epitope domains is a significant problem when using truncated versions of proteins as vaccines. In the absence of data to make rational decisions about which portions of a protein to remove, the best protein to use is a full length recombinant protein which contains all potential epitopes. Such a protein is likely to provide optimal protective efficacy. However, we were unable to express a full length, correctly folded, soluble protein for PG32 or PG33 in *E. coli* and further work with other expression systems should be pursued.

Our approach to designing truncated versions of PG32 and PG33 was to use standard bioinformatic analyses such as low hydrophobicity to select more soluble protein segments. Although we achieved some success in this regard, there is significant scope to improve on the results achieved in this study. Further work to enhance the solubility and correct folding of these truncations may involve the use of fusion proteins such as maltose-binding protein, which in some cases has been shown to aid the refolding of recombinant proteins into their correct, active conformation (10).

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