Generation of Lys-gingipain protease activity in *Porphyromonas gingivalis* W50 is independent of Arg-gingipain protease activities

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*Porphyromonas gingivalis*, a black-pigmenting anaerobe implicated in the aetiology of periodontal disease, contains two loci, *rgpA* and *rgpB*, encoding the extracellular Arg-X specific proteases (RGPs, Arg-gingipains), and *kgp*, which encodes a Lys-X specific protease (KGP, Lys-gingipain). The *rgpA* and *kgp* genes encode polyproteins comprising pro-peptide and catalytic domain with large N- and C-terminal extensions which require proteolytic processing at several Arg and Lys residues to generate mature enzymes. The product of *rgpB* contains only a pro-peptide and the catalytic domain which requires processing at an Arg residue to generate active enzyme. An *rgpA* *rgpB* double mutant (E8) of *P. gingivalis* was constructed to study the role of RGPs in the processing of KGP. A *kgp* mutant (K1A) was also studied to investigate the role of KGP in the generation of RGPs. E8 was stable in the absence of the antibiotics tetracycline and clindamycin (selection markers for *rgpA* and *rgpB*, respectively) and exhibited the same pigmentation, colony morphology and identical growth rates to the parent W50 strain in the absence of antibiotics, in both complex and chemically defined media. The KGP activity of E8, grown in the absence of tetracycline, in whole cultures and in culture supernatants (up to 6 d) was identical to levels in W50. However, in the presence of tetracycline in the growth medium, the level of KGP was reduced to 50% of levels present in whole cultures of W50. Since tetracycline had no effect on RGP or KGP activity when incorporated into assay buffer, this effect is most likely to be on the synthesis of Kgp polypeptide. K1A was also stable in the absence of antibiotics but was unable to pigment, and remained straw-coloured throughout growth. RGP activity in whole cultures of K1A was identical to levels in W50, but RGP activity in 6 d culture supernatants was reduced to 50% of levels present in W50. Thus, although KGP is not required for generation of RGP activity from RgpA and RgpB polypeptides, its absence affects the release/transport of RGP into culture supernatant.

**Keywords:** *Porphyromonas gingivalis*, extracellular proteases, isogenic mutants, polypeptide maturation/processing

**INTRODUCTION**

The black-pigmenting Gram-negative oral anaerobe

*Porphyromonas gingivalis* is present in the subgingival plaques of patients with progressive periodontitis and is strongly implicated in the aetiology of this disease (Slots *et al*., 1986; Holt *et al*., 1988; Dzink *et al*., 1988). Proteases, haemagglutinins, fimbriae and superoxide dismutase (Nakayama *et al*., 1996; Lamont & Jenkin-
son, 1998; Curtis et al., 1999) are among the many virulence factors produced by this organism. Several physiologically important proteins, including collagens (Birkedal-Hansen et al., 1988; Kato et al., 1992), fibronectin (Smalley et al., 1988; Uitto et al., 1989), plasma protease inhibitors (Carlsson et al., 1984), immunoglobulins (Kilian, 1981; Sundqvist et al., 1985; Sato et al., 1987) and complement factors (Sundqvist et al., 1985; Schenkein & Berry, 1988; Wingrove et al., 1992), have been shown to be degraded by proteases from P. gingivalis and it is plausible that these proteases may play a major role in evading host defence mechanisms in addition to causing destruction of the tissues supporting the teeth. Proteases are also essential for generating free amino acids and peptides, which form the main carbon and nitrogen source for growth of this organism.

Two major classes of extracellular cysteine proteases specific for Arg-X and Lys-X peptide bonds [Arg-gingipains (RGP) and Lys-gingipain (KGP)] have been characterized (Curtis et al., 1999). The RGP s are encoded by two homologous genes, rgpA and rgpB, and KGP by kgp. The rgpA and kgp genes encode long polyproteins which require proteolytic processing at multiple sites to generate the mature enzymes. The initial translation product of rgpA contains 1704 or 1706 amino acids, depending on the strain (Aduse-Opoku et al., 1995; Pavloff et al., 1995) and is composed of a pro-region, an α catalytic domain, a β-adhesin domain and a C-terminal γ domain. Three mature enzyme isoforms are produced from the precursor: HRgpA (αβ heterodimer, ~110 kDa), RgpAcat (α monomer, ~55 kDa) and mt-RgpAcat (a highly post-translationally modified α monomer, ~70–80 kDa) (Rangarajan et al., 1997a). Proteolytic processing of the RgpA translation product is thought to occur at Arg227-Tyr229 and Arg271-Ser270 to release the α catalytic chain and probably at Arg110, Phe126 to generate the β domain of HRgpA. An alternative form of HRgpA has been described by Pike et al. (1994) which is composed of the α catalytic domain in association with three different sets of lower molecular mass polypeptides derived from the RgpA C terminus (~95 kDa). In this case, the RgpA polypeptide is likely to be processed at Arg227-Tyr229, Arg110-Ser119, Arg113, Ala119, Lys1273-Pro1274 and Arg1431-Ala1432.

KGP is generated as a polypeptide of 1732 amino acids (Curtis et al., 1999) or 1723 amino acids which is processed at Arg229, Asp229 and Arg273-Ala278 to generate the catalytic domain and at Arg1153-Ala1156, Lys1380, Pro1391 (Pavloff et al., 1997; Slakeski et al., 1999) and Arg1418-Ala1419 (Pavloff et al., 1997) to produce the C-terminally derived polypeptide analogous to the HRgpA described by Pike et al. (1994). Unlike RgpA and Kgp, the product of rgpB contains only a pro-peptide and catalytic domain which requires processing only at the junction of these two domains, Arg272-Tyr273 (Nakayama, 1997), to release the active monomeric enzyme.

Since generation of mature enzymes from the initial translation products of these three genes involves multiple cleavages at Arg-X and Lys-X peptide bonds, it has been suggested that the maturation pathways of these proteases may be interdependent. Thus the RGP s may be involved in processing the products of all three genes and KGP may have an autoprocessing role (Pavloff et al., 1997; Slakeski et al., 1999) and also be required for the generation of the HRgpA multimer described by Pike et al. (1994). The requirement for rgpA rgpB and kgp-derived products in the production of Arg-X and Lys-X protease activity has been addressed through the construction of single, double and triple mutants of these loci using a variety of antibiotic-resistance cassettes as selection markers. However, the results of these studies have generated contradictory conclusions.

Okamoto et al. (1996) reported that Lys-X protease activity in both cell extracts and supernatants of an RGP-null mutant was reduced to approximately 20–30% of levels in the wild-type parent strain. This was confirmed by Kadowaki et al. (1998), who suggested that abnormal processing produced KGP in the RGP-null mutant with reduced activity or rapid degradation of the processed enzyme. Conversely, in a more recent report, Shi et al. (1999) observed that an rgpA kgp mutant of P. gingivalis (KDP133) produced higher levels of KGP activity compared to the parent strain. Similar discrepancies have been reported with respect to Arg-X activity in kgp mutant strains. An insertion mutant of kgp in strain W83 has been shown to be associated with reduced Arg-X activity (Lewis & Macrina, 1999), whereas loss of function of kgp in strain ATCC 33277 did not affect the Arg-X specific protease activity (Okamoto et al., 1998). The variation in the results obtained could be due to differences in strains used, although all the P. gingivalis strains examined so far have been shown to possess rgpA rgpB and kgp which are remarkably similar (Curtis et al., 1999).

In this report we present the construction and systematic analysis of a P. gingivalis W50 rgpA rgpB double mutant in order to establish the role(s) of RGP(s) in the processing of KGP, and a kgp mutant in order to assess the role of KGP in the processing of the RGP s.

**METHODS**

**Growth of bacteria and maintenance of plasmids.** Bacteria and plasmids used in this paper are listed in Table 1. *P. gingivalis* strains were grown in BHI (Oxoid) or CDM supplemented with bovine serum albumin (Milner et al., 1996) in an atmosphere of N₂/H₂/CO₂ (80:10:10 by vol; Don Whitley anaerobic chamber) or blood agar base medium containing 5% defibrinated horse blood as previously described (Rangarajan et al., 1997b) and 0.5 µg haemin ml⁻¹. For the study of the growth of *P. gingivalis* and mutants, the bacteria were initially cultured on blood agar plates. These were then inoculated into appropriate broths, grown overnight and used as inocula in relevant experiments. *E. coli* XL-1 Blue (Stratagene) and TH2 (Takara) were grown in Luria–Bertani broth. When necessary, Technical agar (Oxoid no. 3) was added to 1.5% (w/v). Plasmid DNA was prepared using the ion-exchange chromatographic columns of Qiagen. DNA was purified from agarose gels using Qiaquick (Qiagen). Ampicillin (selection for pUC-derived plasmids), tetracycline...
Table 1. Bacteria and plasmids used

<table>
<thead>
<tr>
<th>Bacterium/plasmid</th>
<th>Relevant genotype</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><strong>Bacteria</strong></td>
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<tr>
<td>P. gingivalis</td>
<td></td>
<td></td>
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<tr>
<td>W50</td>
<td>Wild-type</td>
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<tr>
<td>W501</td>
<td>rgpA::erm</td>
<td>Rangarajan et al. (1997b)</td>
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<tr>
<td>D7</td>
<td>rgpB::erm</td>
<td>Rangarajan et al. (1997b); Aduse-Opoku et al. (1998)</td>
</tr>
<tr>
<td>E8</td>
<td>rgpA::tetQ rgpB::erm</td>
<td>This paper</td>
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<tr>
<td>K1A</td>
<td>kgp::erm</td>
<td>This paper</td>
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<tr>
<td><strong>E. coli</strong></td>
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<tr>
<td>XL-1 Blue</td>
<td>Cloning strain</td>
<td>Stratagene</td>
</tr>
<tr>
<td>TH2</td>
<td>Cloning by positive selection using pKF rpsL. (Sp’)</td>
<td>Takara</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pKPl</td>
<td>pUC18 derivative containing the 5’ end of rgpA; Amp’</td>
<td>Aduse-Opoku et al. (1995)</td>
</tr>
<tr>
<td>pKF3</td>
<td>Positive selection by inactivation of lethal rpsL gene; Sp’ Chl’</td>
<td>Takara</td>
</tr>
<tr>
<td>pNJRI2</td>
<td>E. coli–Bacteroides shuttle plasmid containing a 2.5 kb SstI fragment of tetQ; kan’ (E. coli)</td>
<td>Maley et al. (1992); plasmid was kindly supplied by Dr A. Salyers</td>
</tr>
<tr>
<td>pKFT2</td>
<td>pKF3 containing tetQ marker from pNJRI2</td>
<td>This paper</td>
</tr>
<tr>
<td>pAG2</td>
<td>pKPl containing tetQ from pKFT2</td>
<td>This paper</td>
</tr>
</tbody>
</table>

(XL-1 Blue), chloramphenicol (pKF3) and spectinomycin (inactivated rpsL of pKF3 in TH2 cells) were used at 100, 20, 15 and 30 µg ml−1, respectively. For P. gingivalis, selection for erm used 5 µg clindamycin ml−1, and for tetQ, 1 µg tetracycline ml−1 was incorporated into the medium.

**SDS-PAGE and Western blotting.** These were performed in 10% polyacrylamide gels as described by Laemmli (1970). Samples were treated with either leupeptin (1 mM) or TLCK (1 mM) for 10 min at room temperature to inhibit RGPs and KGP, respectively, prior to SDS-PAGE. The specificity of antiserum Rh3158 has been described (Aduse-Opoku et al., 1995).

**Enzyme assays.** Enzyme assays for Arg-X and Lys-X protease activities using N-α-benzoyl-DL-arginine-p-nitroanilide (BAPNA) and N-α-acetylsalysine-p-nitroanilide (AcKpNA), respectively, were performed as described by Rangarajan et al. (1997a). One unit of enzyme catalyses an increase in A410 of 1.0 min−1 at 30 °C.

**Generation of rgpA rgpB double mutant of P. gingivalis W50.** DNA manipulation techniques were according to Sambrook et al. (1989). Plasmid pNJRI2 (Maley et al., 1992) was kindly supplied by Dr Abigail Salyers, Microbiology Department, University of Illinois at Urbana-Champaign, USA. Both rgpA and rgpB have previously been cloned in pUC18 and single knockout mutants have been generated by insertion of an Ern cassette within the region encompassing the catalytic domain (Rangarajan et al., 1997b). A 2.8 kbp HpaI–SpI fragment of pKFT2, encoding TetQ resistance, was excised from a preparative agarose gel and cloned into an EcoRV–SpI site of pKPl at the 5’ end of rgpA (Aduse-Opoku et al., 1995). The resulting construct (pAG2) was linearized with restriction with KpnI and electroporated into P. gingivalis D7 (rgpB) to achieve allelic exchange in rgpA via homologous recombination. Mutants were selected following anaerobic growth on blood agar plates containing tetracycline (TetQ selection) and clindamycin (Ern selection).

**Generation of kgp knockout mutant, P. gingivalis K1A.** Plasmid pNS1 contains a BamHI fragment of P. gingivalis W50 chromosomal DNA in pUC18 (nt 1–3516; Slakeski et al., 1999). This was linearized with BstXI, blunted and a 2.1 kbp Kpnl–BamHI Ern cassette (blunted) was cloned into the site (nt 1928) to generate pNSE1 to inactivate the catalytic domain of kgp in vitro. The 5.6 kbp BamHI fragment was then retrieved for electroporation into W50. Twelve random clindamycin-resistant mutants were further screened by PCR and P. gingivalis K1A was chosen for further analysis. PCR was performed using Reddy Load amplification reagent (Advanced Biotechnologies) with primer pairs: PRTKF1, 5’-AGCCGAGAAAAAGGAAAG-3’ and PRTKRI1, 5’-GTAGCATCATTAAAGACCCTAG-3’ with P. gingivalis DNA as the template, using 25 cycles of 94 °C (1 min), 55 °C (1 min) and 72 °C (4 min) (Omnigene, Hybaid). A 680 bp fragment was amplified from the parent strain while all mutants yielded a 2.78 kb fragment indicative of insertions of the 2.1 kb cassette at this locus. Chromosomal DNA was prepared with Puregene DNA isolation reagent (Flowgen).

**Southern hybridization.** Chromosomal DNA was restricted using enzymes from Amersham Pharmacia Biotech, and transferred to Hybond-N+ (Amersham Pharmacia Biotech) using Vacu-Aid (Hybaid). Hybridization was performed in Rapid Hyb buffer (Amersham Pharmacia Biotech) as previously described (Aduse-Opoku et al., 1995).

**RESULTS**

**Construction of mutants**

Eight mutants (rgpA rgpB) were initially selected for analysis. Southern hybridization (not shown), using
**Table 2. Arg-X and Lys-X protease activities in isogenic mutants of *P. gingivalis***

*P. gingivalis* strains were grown in BHI and haemin as described in Methods. Enzyme activities, normalized with respect to OD$_{590}$ of the cultures, are expressed as a percentage of the activity present in *P. gingivalis* W50. Arg-X and Lys-X activities (units ml$^{-1}$ per OD$_{590}$ unit) are 3.9 and 1.2, respectively, for 1-d-old W50 cultures. In culture supernatant, the corresponding Arg-X and Lys-X values are 2.17 and 0.5, respectively, after 6 d.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Phenotype</th>
<th>One-day whole culture</th>
<th>Six-day supernatant</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Arg-X</td>
<td>Lys-X</td>
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<tr>
<td>W50</td>
<td></td>
<td>Black</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>W501</td>
<td><em>rgpA::erm</em></td>
<td>Black</td>
<td>~50</td>
<td>100</td>
</tr>
<tr>
<td>D7</td>
<td><em>rgpB::erm</em></td>
<td>Black</td>
<td>~50</td>
<td>100</td>
</tr>
<tr>
<td>E8</td>
<td><em>rgpA::tetQ</em></td>
<td>Black</td>
<td>0–2</td>
<td>100</td>
</tr>
<tr>
<td>E8 + Tet</td>
<td><em>rgpA::tetQ</em></td>
<td>Black</td>
<td>0</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td><em>rgpB::erm</em></td>
<td>Beige</td>
<td>100</td>
<td>0–2</td>
</tr>
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</table>

* Antibiotics were excluded from the media as the mutants are stable.

*Smal* restricted chromosomal DNA, indicated that all eight mutants possessed the *tetQ* gene which was present once in the expected position on the genome. The hybridization profile also established that gene replacement had occurred via a double crossover event and homologous *rgpB::erm* was not affected by this process. *P. gingivalis* E8 was selected for detailed analysis.

*P. gingivalis* (*kgp*) was generated following *in vitro* inactivation of the catalytic domain of *kgp*. Twelve mutants were analysed by PCR and assayed for Arg-X and Lys-X activities. One mutant, K1A, was selected for detailed analysis.

**Characterization of *P. gingivalis* W50 (*rgpA* *rgpB*)/E8**

The stability of the *tetQ* marker in E8 was tested by subculturing the strain every 24 h over 4 d in BHI in the absence of tetracycline and using 10% of inoculum from the previous culture. Chromosomal DNA was prepared from the first and fourth cultures and was subjected to restriction digestion and Southern hybridization. The Southern hybridization profiles were the same for the first and fourth samples, suggesting that the mutants were stable in the absence of antibiotic selection. Similarly, there were no differences in Arg-X and Lys-X protease activities between the first and fourth cultures. Hence, tetracycline and clindamycin were usually omitted from the growth medium after the initial culture on blood agar-antibiotic plates.

*P. gingivalis* E8 exhibited the same colony morphology and rate of pigmentation as wild-type W50 on blood agar plates. The only obvious phenotypic difference was the delayed ability of E8 to show visible zones of haemolysis but this was not accompanied by any perceptible alteration in growth.

**Arg-X and Lys-protease activities in mutants**

The results of Arg-X and Lys-X protease assays in several *P. gingivalis* strains, grown in BHI with added haemin, are shown in Table 2. Inactivation of either *rgpA* or *rgpB* reduced the Arg-X activity in whole cultures by 50% without affecting the Lys-X enzyme activity (Rangarajan et al., 1997b; Aduse-Opoku et al., 1998). Loss of both *rgpA* and *rgpB* in *P. gingivalis* E8 abolished virtually all Arg-X protease activity without any significant effect on the Lys-X protease activity in both day-old whole cultures and 6 d culture supernatants. Thus, the RGPs are not required for generation of KGP activity. However, growth of the E8 mutant (*rgpA* *rgpB*) in the presence of tetracycline, with or without clindamycin, led to a dramatic reduction in Lys-X activity in whole cultures to 50% of the wild-type values. Tetracycline had no effect on the Lys-X activities of pure KGP or of whole cultures of W50 or E8 when it was incorporated into the assay solution at concentrations comparable to those present in the growth medium. Therefore, tetracycline appears to exert a non-antibiotic effect on the synthesis/activation of KGP.

Inactivation of *kgp* (K1A) abolished Lys-X activity with no perceptible change in Arg-X activity in 1-d-old whole cultures. However, Arg-X activity in 6 d culture supernatants of K1A only reached a maximum of 50% of levels in W50, implying that although KGP is not required for the activation of RGPs, it may have a role in the transport/release of these enzymes into the culture.
supernatant (Table 2). Fig. 1 shows the Arg-X enzyme activity in whole cultures and in culture supernatant of *P. gingivalis* W50 and K1A (kgp) strains grown in BHI and haemin over a period of 6 d. Although the cell mass and total Arg-X activity in whole cultures of the two strains were comparable, the enzyme activity in the culture supernatants of strain kgp was only ~50% of levels present in the culture supernatant of the parent W50 strain. K1A showed greater cell lysis, especially after 72 h.

**Examination of protein profiles by Western blots**

Coomassie brilliant blue staining of SDS-PAGE of total proteins of *P. gingivalis* W50 and isogenic mutants did not appear to be significantly different. Therefore, Western blotting using characterized antiserum was used to detect differences between W50 and mutants. Antiserum Rb3158 recognizes the haemagglutinin (β) domain of HRgpA and immunologically related polypeptides from HAgA, Kgp and Tla (Curtis et al., 1999). A Western blot developed with Rb3158 gave a characteristic ladder of bands in W50 (Fig. 2). The adhesin band of HRgpA at ~55 kDa is present in D7 (rgpB) and K1A (kgp) but absent in W501 (rgpA) and E8 (rgpA rgpB). However, the ~44 kDa adhesin domain of KGP is present in W50, W501 (rgpA), D7 (rgpB) and E8 (rgpA rgpB) but absent in K1A (kgp). In E8 there were no Rb3158 immunoreactive protein bands above 55 kDa, which would be indicative of abnormal Kgp processing/maturation since the initial translation product is 188 kDa. Thus, neither RGPA nor RGPB is required for processing or generation of KGP activity. Similarly, the appearance of the ~55 kDa adhesin band in K1A indicated that there were no major effects on processing of the RgpA polypeptide in the absence of KGP activity.

**Growth of E8 in chemically defined medium**

Growth of the mutant strains was also examined in a chemically defined medium (CDM; Milner et al., 1996) containing bovine serum albumin as the sole source of protein in order to determine whether RGPs and KGPs are required for degradation of large macromolecules for nutrition. Whereas the rates of growth of all three strains (W50, E8 and K1A) were indistinguishable from each other in BHI, significant differences were seen during growth in the defined medium (Fig. 3). W50 and E8 grew at a much lower rate in this medium than in BHI although the final cell mass reached levels seen in BHI.

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**Fig. 1.** Comparison of the growth and Arg-X specific enzyme activity in whole cultures and culture supernatants of *P. gingivalis* W50 and K1A (kgp) grown in BHI and haemin. Cultures were grown as described in Methods and samples withdrawn at the indicated time points. The optical densities were measured at 540 nm and cultures and culture supernatants were assayed for Arg-X activity using DL-BRpNA as the chromogenic substrate as described in Methods. W50: ●, OD<sub>540</sub>; ◯, Arg-X activity in whole culture; □, Arg-X activity in supernatant. K1A: ▲, OD<sub>540</sub>; △, Arg-X activity in whole culture; ■, Arg-X activity in supernatant.

**Fig. 2.** Western blot analysis of proteins of *P. gingivalis* W50 and isogenic mutants. Proteins from whole cells of *P. gingivalis* W50, W501 (rgpA), D7 (rgpB), E8 (rgpA rgpB) and K1A (kgp) grown in BHI and haemin were separated on 10% SDS-PAGE, blotted onto nitrocellulose membranes and probed with antiserum Rb3158 (RgpA-β-region specific). The strains are denoted above the lanes. The molecular masses of marker proteins are indicated alongside the gel. Arrowheads indicate the positions of HRgpA (β region) and Kgp (adhesin domain).

**Fig. 3.** Comparison of the growth of *P. gingivalis* W50, E8 (rgpA, rgpB) and K1A (kgp) in BHI or CDM. The media, both with added haemin (5 μg ml<sup>-1</sup>), were inoculated with W50, E8 (rgpA rgpB) and K1A (kgp) strains and incubated in an anaerobic cabinet. Culture was withdrawn at different time points and the optical density was measured at 540 nm. BHI: ●, W50; ■, E8; ▲, K1A. CDM: ○, W50; □, E8; △, K1A. No samples were withdrawn between 20 h and 36 h and this is represented by dotted lines.
after 36 h. However, K1A showed a reduced growth rate in CDM and the OD$_{540}$ after 36 h was approximately 50% of levels seen with W50 and E8, suggesting that KGP is nutritionally important in this medium.

The results shown in Table 2 suggest that tetracycline influences the amount of KGP activity in \textit{P. gingivalis} (rgpA rgpB), so the effect of this antibiotic on the growth of W50 and E8 in the chemically defined medium was also investigated. The growth of \textit{P. gingivalis} W50 and E8 in CDM with added haemin, and E8 in CDM with haemin and tetracycline, after 24 h and 48 h is shown in Fig. 4. Even after 48 h, E8 in CDM with haemin and tetracycline showed poor growth and low levels of KGP activity. The poor growth in this medium could be a consequence of the reduced levels of KGP (due to the effect of tetracycline) and hence the inability to hydrolyse BSA to provide nutrients. In another experiment, \textit{P. gingivalis} W50 was grown in BHI and haemin in the presence of tetracycline (0–2 $\mu$g ml$^{-1}$) and growth (OD$_{540}$) and Arg-X and Lys-X activities in whole cultures were measured after 48 h to study the effect of subinhibitory concentrations of tetracycline. At 0-008 $\mu$g tetracycline ml$^{-1}$, neither growth nor the level of the two enzyme activities was affected. However, at concentrations of tetracycline between 0.04 and 0.2 $\mu$g ml$^{-1}$, the growth of \textit{P. gingivalis} W50 was severely restricted, as were the levels of the two enzymes. However, the ratios of Arg-X activity to Lys-X activity remained the same over the entire range of tetracycline concentration.

**DISCUSSION**

The ability of the RGP's and KGP's of \textit{P. gingivalis} to inactivate/deregulate a wide range of host proteins critical to the defence and integrity of the periodontal tissues \textit{in vitro} suggests that these enzymes may be important factors in disease associated with this organism. However, the benefit to the bacterium of the production of these activities has not been conclusively demonstrated, although recent work has indicated some potential roles for KGP.

Data in the current report confirmed that inactivation of \textit{kgp} leads to loss of the black-pigmenting, haemolytic phenotype on blood agar, suggesting that KGP is involved in the acquisition/storage of haemin. Support for this hypothesis has emerged from the work of Lewis \textit{et al.} (1999), which demonstrates that this enzyme is an efficient haemoglobinase and hence may serve to release protein-bound haemin into the extracellular environment. Furthermore, Nakayama \textit{et al.} (1998) have shown that a haemin-binding peptide is intragenically encoded by \textit{kgp} and this may function as part of a haemin storage mechanism at the cell surface. However, data in this report demonstrate that KGP is not required for growth in complex medium with added haemin. In addition to a role in haemin acquisition, KGP also appears to be important for growth of \textit{P. gingivalis} on large proteins since \textit{kgp} mutants grow very poorly in a defined medium containing albumin as the sole source of carbon and nitrogen (Fig. 3) and this effect can be reversed by pretreatment of the medium with trypsin (Shi \textit{et al.}, 1999). Hence there is reasonable evidence that KGP has an important nutritional role.

In contrast, characterization of a \textit{P. gingivalis} \textit{rgpA rgpB} double mutant has failed to demonstrate a similar role in nutrition for the RGP's either in this study or in an earlier report (Shi \textit{et al.}, 1999). Clearly the possibility that the RGP's play an important nutritional role \textit{in vivo} through degradation of specific host proteins or release of important protein-bound micronutrients cannot be excluded. However, it is equally plausible that an important function of these enzymes \textit{in vivo} is an immunosubversive role through the proteolytic inactivation of neutrophil opsonins and other agents of the host's defensive armoury.

Whilst the extracellular functions of the RGP's remain uncertain there are significant data to support a role in cell-associated 'house-keeping' duties. Kadokaki \textit{et al.} (1998) described the importance of RGP's in the processing of fimbrillin, a major component of fimbriae, which remained in an unassembled precursor form in an \textit{rgpA rgpB} double mutant. In addition, the proteolytic maturation of a 75 kDa outer-membrane protein was also shown to be dependent on functional \textit{rgpA/rgpB}. It has also been suggested that the RGP's are required for appropriate processing and activation of KGP (Lewis & Macrina, 1999). The data in the present report conclusively demonstrate that processing and activation of KGP is not dependent on products of \textit{rgpA} and \textit{rgpB} and also that processing and activation of the RGP's does not require \textit{kgp} function. Western blotting experiments did not demonstrate a discernible difference in the size of fragments derived from RgpA and Kgp polypeptides in the K1A and E8 mutants, respectively, versus the parent strain. It is possible that there are minor differences in
the processing sites of the polyproteins in the mutants that do not result in significant alterations to the apparent molecular mass of the resultant polypeptides on SDS-PAGE. However, if this is the case, altered processing does not influence the total activity of the mature enzymes as was suggested by Kadokawa et al. (1998), who reported that KGP was abnormally processed in an rgpA rgpB double mutant of P. gingivalis ATCC 33277 at Leu225-Phe226 instead of Arg226-Asp228 and that this resulted in a significant reduction in total KGP activity. 

Whilst RGP activity in day-old whole cultures of P. gingivalis (kgp) was identical to those of the parent W50, enzyme activity in 6 d culture supernatants was reduced to 50% of wild-type levels. Hence, although KGP activity is not required for the processing and activation of the RGPs, it may be involved in the export of this activity into culture supernatant.

A plausible explanation for the differences between the present study and previous examination of the P. gingivalis (rgpA rgpB) mutant phenotype concerns a non-antibiotic effect of tetracycline on KGP activity in the RGP-null background. Stability testing of the E8 mutant confirmed that it was not necessary to maintain antibiotic selection pressure during growth of this mutant to maintain the rgpA and rgpB insertions. This enabled a comparison of the effects of the antibiotics on enzyme activity, which demonstrated that tetracycline in the growth medium causes a significant decrease in KGP activity. Since tetracycline has no effect on preformed KGP (or on RGP) activities when incorporated into the assay solution at levels present in the growth medium, the reduction of KGP activity in the rgpA rgpB mutant probably represents either a direct effect on synthesis or an indirect effect on activity. This non-antibiotic effect of tetracycline may explain some of the discrepancies in the earlier literature on the inter-relationships between RGP and KGP activity. Hence, the absence of an effect on KGP activity in the parent strain at subantibiotic concentrations suggests it is unlikely to be clinically relevant.

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REFERENCES


Okamoto, K., Kadowaki, T., Nakayama, K. & Yamamoto, K.
biochemically distinct enzymes.


