J Periodont Res 2007; 42: 438–442

JOURNAL OF PERIODONTAL RESEARCH
doi:10.1111/j.1600-0765.2006.00966.x

Effect of γ-immunoglobulin on the asaccharolytic growth of *Porphyromonas gingivalis*

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Oda H, Saiki K, Numabe Y, Konishi K. Effect of γ-immunoglobulin on the asaccharolytic growth of Porphyromonas gingivalis. *J Periodont Res 2007; 42: 438–442. © 2007 The Authors. Journal compilation © 2007 Blackwell Munksgaard*

Background and Objectives: A minimal medium is indispensable for examining the growth properties of the asaccharolytic bacterium, *Porphyromonas gingivalis*. The purpose of the present study was to improve the widely used KGB medium to support good growth of *P. gingivalis*.

Material and Methods: Growth of *P. gingivalis* (W50, W83, and ATCC33277) in a minimal medium was monitored by measuring the optical density of the culture during incubation.

Results: W50, W83, and ATCC33277 grew poorly with bovine serum albumin as the sole carbon and nitrogen source, and α-ketoglutarate had little or no effect on this poor growth. In contrast, $FeCl_3$ improved the growth of W83 and ATCC33277; however, the use of a high concentration of $FeCl_3$ elicited black pigmentation of the cells. Bovine γ-immunoglobulin greatly recovered the growth defect. None of α-ketoglutarate, citrate, or trace metal ions, when used to supplement KGB medium, was required for growth. We determined the optimal conditions for growth, and developed a new simple minimal medium for *P. gingivalis* (GA medium). Growth of ATCC33277 in GA medium was dependent on gingipains; Arg-gingipains and Lys-gingipain contributed comparably to proliferation of the bacterium.

Conclusion: These data indicate that GA medium is currently the most reliable minimal medium for examining the growth properties of *P. gingivalis*.

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Key words: gingipain; minimal medium; *Porphyromonas gingivalis*

Accepted for publication October 10, 2006

Porphyromonas gingivalis, a gram-negative anaerobe, is a rare member of normal human oral commensals. However, *P. gingivalis* has been strongly implicated as a major cause of aggressive and chronic periodontitis, involving infection and inflammation of the ligaments and bones that support the teeth (1–3). *P. gingivalis* is asaccharolytic and is dependent on an external peptide source for growth. As peptides exist poorly in the oral environment, the bacterium is thought to create peptide pools by digesting exogenous protein sources. Indeed, *P. gingivalis* secretes various proteases that are probably capable of digesting external proteins into peptides. Among these proteases, trypsin-activity-like proteases, arginine-specific Arg-gingipains (RgpA and RgpB) and lysine-specific Lys-gingipain (Kgp) have been studied in most detail and are believed to play an important role in proliferation of the bacterium (4). Unfortunately, complex media routinely used for the culture of *P. gingivalis* are not suitable for investigating its asaccharolytic growth (5). Several types of minimal medium have been developed (6–8); however, these have a complex composition, contain protein hydrolysates, and/or poorly support the growth of *P. gingivalis* (9). In 1996, Milner *et al.* developed a new minimal medium that contains bovine serum albumin as the sole carbon and nitrogen source (KGB medium) (9). KGB medium is reported to support well the growth of 15 *P. gingivalis* strains, including W50 and ATCC33277 (9). The asaccharolytic property of

P. gingivalis has been characterized using KGB medium (5,10–12).

In this study, we investigated, in detail, the growth of *P. gingivalis* with bovine serum proteins as the sole source of carbon and nitrogen. Surprisingly, we found that the growth of *P. gingivalis* in KGB medium was too poor for growth studies to be carried out. Therefore, we developed a new minimal medium that was able to support good growth of *P. gingivalis*, and characterized the growth properties of the bacterium in this medium.

Material and methods

Bacterial strains and growth conditions

P. gingivalis W50, W83, and ATCC33277 are our laboratory stocks, whereas gingipain mutants (*kgp* mutant KDP129, *rgpA rgpB* mutant KDP133, *kgp rgpA rgpB* mutant KDP136) and their parent wild-type strain, ATCC33277, were provided by Dr K. Nakayama (Nagasaki University, Japan). *P. gingivalis* was routinely grown anaerobically (10% CO_2, 10% H_2, and 80% N_2), in brain heart infusion medium (Difco, Detroit, MI, USA) supplemented with sterile hemin (7.67 μM) and menadione (2.91 μM) (BHIHM medium), or on BHIHM agar. A saturated culture (i.e. having an optical density, at 620 nm, of 1–1.5) of *P. gingivalis* in BHIHM medium was diluted 40-fold with a minimal medium and incubated anaerobically at 37°C. Cell growth was monitored by measuring the optical density, at 620 nm, using a Mini Photo 518R (Taitec Co., Tokyo, Japan) under anaerobic conditions. Data were collected from duplicate cultures, and at least four independent experiments were performed for each culture condition.

Minimal media

The bovine serum albumin used in this study was electrophoretically 98% pure, contained low endotoxin, and was essentially γ-globulin free (catalogue no. A-2934; Sigma-Aldrich Co., St Louis, MO, USA). The γ-immunoglobulin used in this study was electrophoretically 99% pure and is available from Sigma-Aldrich Co. (catalogue No. G-5009). A stock solution for bovine serum albumin or γ-immunoglobulin was prepared as follows. Bovine serum albumin or γ-immunoglobulin was dissolved in deionized water and then sterilized using a 0.45-μm membrane filter Minisart-plus (Vivascience, Göttingen, Germany). The protein concentration of the filtered bovine serum albumin or γ-immunoglobulin solution was then determined with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA) using bovine serum albumin as the standard. KGB medium was prepared as described by Milner *et al.* (9); the modified Evans salt base (10 mM NaH_2PO_4, 10 mM KCl, 2 mM citrate, 1.25 mM $MgCl_2$, 20 μM $CaCl_2$, 0.1 μM Na_2MoO_4, 25 μM $ZnCl_2$, 50 μM $MnCl_2$, 5 μM $CuCl_2$, 10 μM $CoCl_2$, 5 μM H_3BO_3, and 20 mM α-ketoglutarate), pH 7, was filter sterilized and supplemented with sterile hemin (7.67 μM), menadione (2.91 μM), and bovine serum albumin (30 mg/mL). FB medium was supplemented with $FeCl_3$ in place of α-ketoglutarate used in KGB medium. KG medium is KGB medium minus bovine serum albumin. GA medium was prepared as follows: basal buffer (10 mM NaH_2PO_4, 10 mM KCl, and 10 mM $MgCl_2$), pH 7, was filter sterilized and supplemented with sterile hemin (7.67 μM), menadione (2.91 μM), γ-immunoglobulin (22.5 mg/mL), and bovine serum albumin (7.5 mg/mL). To make trypsin-treated minimal medium, a minimum medium was supplemented with trypsin (50 μg/mL) and incubated at 37°C for 4 h prior to use.

Results

Growth of *P. gingivalis* in KGB medium

Milner *et al.* reported that W50 grew well in KGB medium (9). We examined the growth of *P. gingivalis* W50, W83, and ATCC33277 in KGB medium. As shown in Fig. 1A–C, our KGB medium poorly supported the growth of

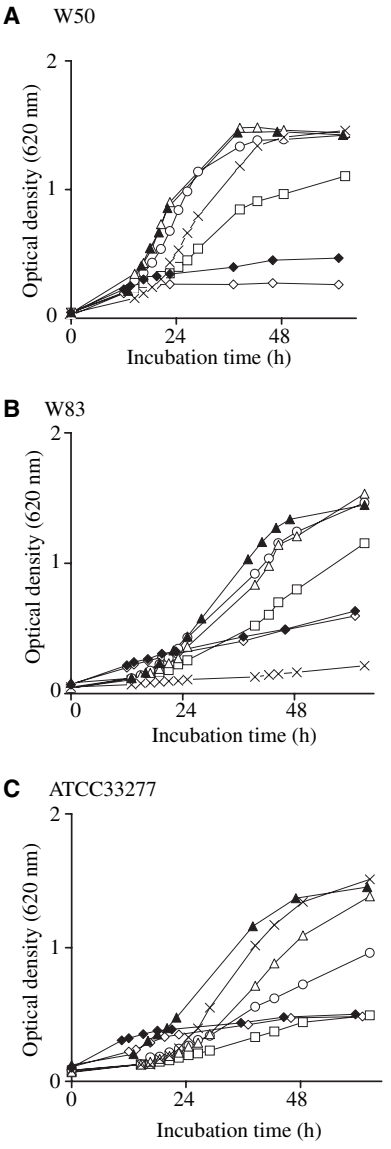

Fig. 1. Growth of *Porphyromonas gingivalis* on bovine serum proteins as the sole carbon/nitrogen source. W50 (A), W83 (B), or ATCC33277 (C) was cultured anaerobically in a minimal medium, and growth was monitored by measuring the optical density at 620 nm. The different types of minimal medium were as follows: KG medium supplemented with bovine serum albumin (KGB medium; ◇), γ-immunoglobulin (×), or a mixture of γ-immunoglobulin and bovine serum albumin (1 : 3, □; 1 : 1, ○; 3 : 1, △); and GA medium (▲); trypsin-predigested-KGB medium (◆).

P. gingivalis. The observed growth defect was independent of the lot of bovine serum albumin (three different lots were tested; data not shown). W83, W50, and ATCC33277 also grew

poorly in a trypsin-treated KGB medium (Fig. 1A-C). These results suggest that the peptide pools derived from bovine serum albumin in KGB medium are not suitable for the growth of *P. gingivalis*.

Effect of $FeCl_3$ on the growth of *P. gingivalis*

Milner *et al*. reported that W50 also grew well in FB medium (100 μM $FeCl_3$) although a black pigment accumulated heavily on the surface of the growing cells (9). This black pigment was proposed to be an FeS compound produced by a reaction with $FeCl_3$ and volatile sulfur compounds, such as H_2S (9). We tested the effect of $FeCl_3$ on the growth of *P. gingivalis*. In our FB medium (100 μM $FeCl_3$), W83 grew slowly, whereas W50 and ATCC33277 grew poorly (Fig. 2A). In these cultures, black pigmentation of the cells was not observed (Fig. 2B, a–c). These results indicate that 100 μM $FeCl_3$ is not sufficient for the growth of W50 (and ATCC33277) with bovine serum albumin as the sole carbon and nitrogen source. We found that $FeCl_3$ affected the growth of W83 and ATCC33277 in a concentration-dependent manner (0.3 mM, Fig. 2A; and 0.5 and 1 mM, data not shown) and that the black pigmentation was caused by supplementation of $FeCl_3$ (1 mM) (Fig. 2B, b,c). In contrast, the growth of W50 was still slower in the presence of $FeCl_3$ (0.3 mM, Fig. 2A; and 0.5 and 1 mM, data not shown), and no black pigmentation was observed (Fig. 2B, a), indicating that $FeCl_3$ had a only minor effect on the growth and pigmentation of W50.

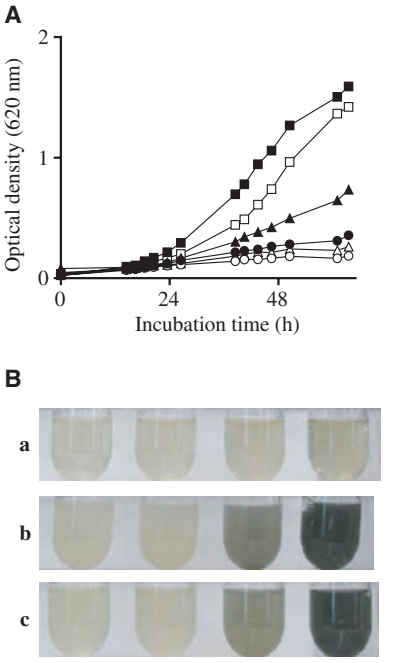

Fig. 2. Effect of $FeCl_3$ on the growth of *Porphyromonas gingivalis*. (A) Growth of W50 (○, ●), W83 (□, ■), and ATCC33277 (△, ▲) in FB medium supplemented with 100 μM $FeCl_3$ (○, □, △) or 300 μM $FeCl_3$ (●, ■, ▲). (B) Black pigmentation of cells in the 72-h culture of W50 (a), W83 (b), or ATCC33277 (c) in FB medium supplemented with 100, 300, 500, or 1000 μM $FeCl_3$ (from the left to the right test tube).

Effect of γ-immunoglobulin on the growth of *P. gingivalis*

We tested the effect of bovine γ-immunoglobulin on the growth of *P. gingivalis* in KGB medium. γ-Immunoglobulin and/or bovine serum albumin were added to KG medium to achieve a total protein concentration of 30 mg/mL. As shown in Fig. 1, addition of γ-immunoglobulin to the medium greatly recovered the growth of *P. gingivalis*. ATCC33277 showed concentration-dependent growth on γ-immunoglobulin, and its growth was fastest on γ-immunoglobulin alone (30 mg/mL) but comparable to a 3 : 1 mixture of γ-immunoglobulin (22.5 mg/mL) and bovine serum albumin (7.5 mg/mL) as the sole carbon/nitrogen source (Fig. 1C). The growth of W50 was fastest in KG medium supplemented with a 3 : 1 mixture of γ-immunoglobulin and bovine serum albumin (Fig. 1A). The growth of W83 was fastest on either a 1 : 1 mixture of γ-immunoglobulin (15 mg/mL) and bovine serum albumin (15 mg/mL), or a 3 : 1 mixture of γ-immunoglobulin and bovine serum albumin (Fig. 1B). KG medium, supplemented with γ-immunoglobulin alone, supported the growth of W50 comparably, but the growth of W83 poorly. Therefore, in this study, a 3 : 1 mixture of γ-immunoglobulin (22.5 mg/mL) and bovine serum albumin (7.5 mg/mL) was used as the sole carbon and nitrogen source for the growth experiments of *P. gingivalis*.

Development of a new simple minimal medium, GA

KGB medium contains α-ketoglutarate, a proposed essential factor, although our KGB medium poorly supported the growth of *P. gingivalis*. KGB medium also contains citrate and trace metal ions ($CaCl_2$, Na_2MoO_4, $ZnCl_2$, $MnCl_2$, $CuCl_2$, $CoCl_2$, and H_3BO_3), the functional roles of which have yet to be determined regarding the growth of *P. gingivalis*. We examined the effects of these supplements on the growth of *P. gingivalis* in our minimal medium containing a 3 : 1 mixture of γ-immunoglobulin and bovine serum albumin as the sole carbon and nitrogen source. In KG medium supplemented with a 3 : 1 mixture of γ-immunoglobulin and bovine serum albumin, but with no α-ketoglutarate, citrate, or trace metal ions, W50, W83, and ATCC33277 showed growth comparable to that in KG medium supplemented with a 3 : 1 mixture of γ-immunoglobulin and bovine serum albumin (data not shown). As we found that the growth of *P. gingivalis* ATCC33277 was slightly concentration-dependent on $MgCl_2$ (data not shown), the concentration of $MgCl_2$ was increased from 1.25 to 10 mM. The resultant minimal medium, designated GA medium (a minimal medium containing bovine γ-globulin and bovine serum albumin), supported the fastest growth of W50, W83, and ATCC33277 (Fig. 1A–C). Growth of W50, ATCC33277, and W83 in GA medium was independent of the lot of γ-immunoglobulin (four different lots were tested). The growth was supported in over 10 repeated subcultures (data not shown).

Growth property of gingipain mutants in GA medium

P. gingivalis secretes Arg-gingipains (RgpA and RgpB) and Lys-gingipain (Kgp). These proteases are thought to play an important role in the proliferation of *P. gingivalis* (4), and there-

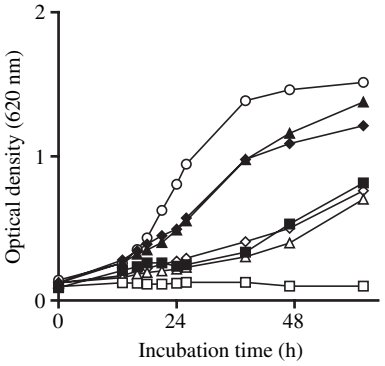

Fig. 3. Growth of gingipain mutants in GA medium. Growth of ATCC33277 (○), KDP129 (◇, ◆), KDP133 (△, ▲), and KDP136 (□, ■) in GA medium (○, ◇, △, □) or trypsin-predigested GA medium (◆, ▲, ■).

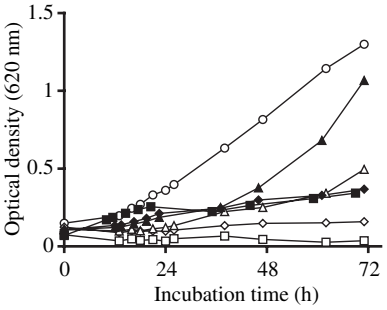

Fig. 4. Growth of gingipain mutants in FB medium containing 300 μM $FeCl_3$. Growth of ATCC33277 (○), KDP129 (◇, ◆), KDP133 (△, ▲), and KDP136 (□, ■) in FB medium (○, ◇, △, □) or trypsin-predigested FB medium (◆, ▲, ■).

fore we examined the growth of gingipain-deficient *P. gingivalis* mutants in GA medium. As shown in Fig. 3, KDP136 (*kgp rgpA rgpB*) showed no apparent growth in GA medium, indicating that gingipains are essential for the assacharolytic growth of ATCC33277. Interestingly, KDP129 (*kgp*) and KDP133 (*rgpA rgpB*) showed poor growth in GA medium. The doubling time for ATCC33277, KDP129, and KDP133 was about 8, 24, and 28 h, respectively. The poor growth of KDP129 and KDP133, and the growth defect of KDP136, was recovered by incubation in trypsin-treated GA medium (Fig. 3). These results indicated that both Rgp and Kgp function in the degradation of a mixture of γ-immunoglobulin and bovine serum albumin to create peptide pools to support the growth of *P. gingivalis*.

Discussion

Bovine serum albumin contains a high concentration of sulfur-containing amino acids (6.18 mol% of cysteine and 0.71 mol% of methionine), and the metabolism of bovine serum albumin probably results in the production of harmful volatile sulfur compounds, such as H_2S (9). Therefore, evasion or suppression of harmful volatile sulfur compounds is probably an important growth property that enables *P. gingivalis* to grow with bovine serum albumin as the sole carbon and nitrogen source. In this study, three types of minimal medium were analyzed. First, KGB medium contains α-ketoglutarate, which has been proposed as essential for the growth of *P. gingivalis* with bovine serum albumin as the sole carbon and nitrogen source (9). However, we found that α-ketoglutarate (20 mM) had little or no effect on the growth of *P. gingivalis* on bovine serum albumin. An increased concentration of α-ketoglutarate (50 and 100 mM) in KGB medium showed no apparent effect on the growth defect of W50, W83, and ATCC33277 (data not shown). Furthermore, KGB medium poorly supported the growth of clinical isolates of *P. gingivalis* (three strains of our laboratory stock were investigated; data not shown). Therefore, KGB medium was considered not to be a useful minimal medium for *P. gingivalis*. The results reported by Milner *et al.* (9), and those of the present study, were totally discrepant. The discrepancy might be caused by the different purification grade of bovine serum albumin used for the medium. (Note that the bovine serum albumin used in this study was electrophoretically 98% pure.) Second, FB medium contains another proposed essential factor – $FeCl_3$ – for the growth of *P. gingivalis* on bovine serum albumin (9). FB medium supplemented with $FeCl_3$ (1 mM) supported well the growth of W83 and ATCC33277; however, a black pigment, which strongly perturbed optical density measurements, was accumulated on the surface of the cells (Fig. 2B, b,c). Therefore, $FeCl_3$ is probably effective in evasion of the harmful volatile sulfur compounds by converting them to an insoluble black pigment (9). We found that FB medium supplemented with $FeCl_3$ (300 μM) supports the slow growth of W83 and ATCC33277 (Figs 2A and 4) without causing apparent black pigmentation of the cells (Fig. 2B, b,c). However, FB medium poorly supported the growth of W50. Third, we developed a new minimal medium – GA – which contains a 3 : 1 mixture of γ-immunoglobulin and bovine serum albumin. GA medium has a simple composition but supports good growth of the three laboratory strains (Fig. 1) and the three clinical isolates (data not shown) of *P. gingivalis* without causing any black pigmentation. The addition of γ-immunoglobulin into the protein (bovine serum albumin) pool may result in a reduction of the relative amount of cysteine and methionine in peptide pools and, consequently, suppress production of the harmful volatile sulfur compounds. Therefore, GA medium is probably a suitable minimal medium for using to examine the property of the asaccharolytic growth of *P. gingivalis*.

Strain-to-strain differences were observed in the utilization of γ-immunoglobulin and bovine serum albumin. W50 and ATCC33277 grew well on γ-immunoglobulin as the sole carbon and nitrogen source, whereas W83 grew poorly in the same medium (Fig. 1). This suggests that the expression level of gingipains affects the utilization of γ-immunoglobulin. By contrast, W83 grew faster than ATCC33277 and W50 in FB medium containing 100 μM $FeCl_3$ (Fig. 2A). As $FeCl_3$ probably helps *P. gingivalis* to evade the harmful volatile sulfur compounds, this finding suggests that W83 is more resistant to harmful volatile sulfur compounds, such as H_2S. Indeed, W83 (but not W50 and ATCC33277) showed a slight, but successive, increase of growth in KGB medium (Fig. 1). We also tested the effect of human γ-globulin (electro-

phoretically 99% pure, catalogue no. G4386; Sigma-Aldrich) on the growth of *P. gingivalis*. W83 showed comparable growth in GA medium supplemented with human γ-globulin instead of bovine γ-globulin (hGA medium), whereas W50, ATCC33277, and the three clinical isolates showed somewhat retarded growth in hGA medium (the doubling time for them was increased by 1.3–2; data not shown). As *P. gingivalis* is a human oral commensal, human γ-globulin may be able to bind to the surface components (e.g. fimbriae such as FimA) of the intact cells of *P. gingivalis*. This binding activity probably affects the growth of *P. gingivalis* in a strain-dependent manner (e.g. W83 is known not to express FimA).

Functional roles of gingipains on the growth of *P. gingivalis* have previously been investigated. Shi *et al.* reported that ATCC33277 deficient in *rgpA rgpB kgp* (KDP128 and KDP136) showed no growth in KGB medium (5). However, they also reported that ATCC33277 deficient in *rgpA* and *rgpB* (KDP112 and KDP133), and ATCC33277 deficient in *Kgp* (KDP129), showed growth comparable to that of ATCC33277 in KGB medium (5). In contrast, Grenier *et al.* reported that KDP112 showed poor growth, whereas KDP129 showed comparable growth, in KGB medium supplemented with human serum albumin (10). Curtis *et al.* reported that W50 deficient in *kgp* (K1A) showed an apparent reduction of growth in KGB medium (4). These results are probably inconsistent with each other. In this study, we clearly showed that the growth of KDP129 and KDP133 is impaired in GA medium (Fig. 3). Furthermore, we examined the growth of gingipain mutants in FB medium (300 μM $FeCl_3$), which contains bovine serum albumin as the sole carbon and nitrogen source. As shown in Fig. 4, KDP136 (*kgp rgpA rgpB*) showed no growth, and KDP133 (*rgpA rgpB*) and KDP129 (*kgp*) showed a severe reduction in growth. This growth property is similar to that observed GA medium. Therefore, these results definitively support the strict requirement of Arg-gingipains and Lys-gingipain for asaccharolytic growth of *P. gingivalis* ATCC33277 with bovine serum protein(s) as the sole carbon and nitrogen source.

In conclusion, we have successfully developed a new minimal medium – GA – and definitively determined the functional roles of Arg-gingipain and Lys-gingipain on the growth of *P. gingivalis*. Furthermore, we have also shown that KGB medium is not a suitable minimal medium for *P. gingivalis*. Our results have provided a better understanding of the growth properties of *P. gingivalis*.

Acknowledgements

We thank Dr Koji Nakayama (Nagasaki University) for kindly providing KDP129, KDP133, KDP136, and their parent wild-type strain, ATCC33277.

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