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# Participation of the secreted dipeptidyl and tripeptidyl aminopeptidases in asaccharolytic growth of *Porphyromonas gingivalis*

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*Background and Objective: Porphyromonas gingivalis* secretes gingipains, endopeptidases essential for the asaccharolytic growth of this bacterium. *P. gingivalis* also secretes dipeptidyl aminopeptidases (DPPIV and DPP-7) and a tripeptidyl aminopeptidase (PTP-A), although their role in asaccharolytic growth is unclear. The present study was carried out to elucidate the role of these dipeptidyl/tripeptidyl aminopeptidases on the asaccharolytic growth of *P. gingivalis*.

*Material and Methods:* Knockout mutants for the *DPPIV* (*dpp*), *dpp7* and/or *PTP-A* genes were constructed. Brain–heart infusion medium supplemented with sterile hemin and menadione (BHIHM) was used as a complex medium, and the minimal medium used was GA, in which the sole energy source was a mixture of immunoglobulin G and bovine serum albumin. Growth of *P. gingivalis* was monitored by measuring the optical density of the culture.

*Results:* All knockout mutants for *DPPIV*, *dpp7* and *PTP-A* grew as well as strain W83 in BHIHM. In GA, growth of single-knockout and double-knockout mutants was similar to that of W83, whereas growth of a triple-knockout mutant (83-47A) was reduced. We purified recombinant DPPIV and recombinant PTP-A from recombinant *Escherichia coli* overproducers, and purified DPP-7 from the triple-knockout mutant 83-4A. GA supplemented with the three purified dipeptidyl/tripeptidyl aminopeptidases supported the growth of 83-47A.

*Conclusion:* DPPIV, DPP-7 and PTP-A contribute to the normal growth of *P. gingivalis* by cleaving substrate peptides into short-chain polypeptides that are efficient energy sources for *P. gingivalis*.

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*Porphyromonas gingivalis* is an anaerobic gram-negative bacterium that normally dwells in the gingivodental sulcus of healthy-toothed individuals. As well as being an indigenous bacterium, *P. gingivalis* is an endogenous infectious agent associated with aggressive and chronic periodontitis, which causes inflammation of the ligaments and bones that support the teeth (1–3). *P. gingivalis* has very strict growth requirements. It requires hemin and menadione as growth factors, and utilizes peptides as its sole energy source (4). As external peptides may be scarce in oral environments, *P. gingivalis* probably generates peptide fragments from external proteins through the secretion of various types of proteases. Indeed, *P. gingivalis* secretes various types of proteases, including gingipains, a metallocarboxypeptidase and aminopeptidases. The Arg-gingipains RgpA and RgpB

are endopeptidases that cleave $NH_2$-$(Xaa)_n$-Arg-↓-$(Xaa)_n$, where ↓ represents the cleaved peptide bond and Xaa represents any amino acid residue (5). The Lys-gingipain Kgp is also an endopeptidase but cleaves $NH_2$-$(Xaa)_n$-Lys-↓-$(Xaa)_n$ (5). CPG70 is a metallocarboxypeptidase that cleaves $(Xaa)_n$-↓-Lys/Arg-COOH (6). Gingipains and CPG70 are secreted to the surface of the cells and/or released into the medium (5,6). DPPIV and DPP-7 are dipeptidyl aminopeptidases, whereas PTP-A is a tripeptidyl aminopeptidase. DPPIV and DPP-7 cleave $NH_2$-Xaa-Pro/Ala-↓-Yaa-$(Xaa)_n$ and $NH_2$-Xaa-Zaa-↓-$(Xaa)_n$, respectively (7,8), whereas PTP-A cleaves $NH_2$-Xaa-Xaa-Pro-↓-Yaa-$(Xaa)_n$ (9), where Yaa represents any residue except proline, and Zaa represents any residue except glycine, proline, or a charged residue. DPPIV, DPP-7 and PTP-A are secreted to the surface of the outer membrane and localized there (7–9). Generally, a defined medium that contains protein as a sole energy source can be used to investigate the roles of these proteases on the asaccharolytic growth of *P. gingivalis*. Unfortunately, the complex media for culturing *P. gingivalis* contain proteolytic degradatives and are of no use for this purpose (10). Therefore, a well-designed minimal medium, KGB, was developed (11). KGB contains bovine serum albumin as a sole carbon and nitrogen source and has been used as a minimal medium for *P. gingivalis* (11). We recently found that KGB does not adequately support the growth of wild-type strains of *P. gingivalis* (12), so we developed a novel minimal medium, GA, that supports good growth of three wild-type strains (W50, W83 and ATCC33277) and three clinical isolates of *P. gingivalis* (12). GA contains a mixture of bovine serum immunoglobulin (IgG) and bovine serum albumin as a sole energy source (12). Using GA as a minimal medium, we carefully investigated the growth of gingipain knockout mutants, and definitively demonstrated that gingipains are essential for the growth of *P. gingivalis* (12).

In the present study, we constructed knockout mutants for the peptidase genes of *P. gingivalis*, examined the growth of these knockout mutants in the minimal medium GA and determined the functional roles of the dipeptidyl/tripeptidyl aminopeptidases on the asaccharolytic growth of *P. gingivalis*.

## Material and methods

### Bacterial strains and growth conditions

*Escherichia coli* strains ER2566 (New England Biolabs, Ipswich, MA, USA), BL21 and JM109 are our laboratory stock strains. An *E. coli* strain harboring a derivative of pUC19, pUC119 or pUC118 was grown in Luria–Bertani broth supplemented with ampicillin (75 μg/mL). Erythromycin-resistant *E. coli* was selected on Luria–-Bertani agar supplemented with erythromycin (150 μg/mL) and ampicillin (50 μg/mL). *P. gingivalis* W83 is our laboratory stock. *P. gingivalis* was grown anaerobically (10% $CO_2$, 10% $H_2$ and 80% $N_2$) at 37°C. Brain–heart infusion medium (Becton Dickinson, Franklin lakes, NJ, USA) supplemented with sterile hemin (7.67 μM) and menadione (2.91 μM) (BHIHM medium) or BHIHM agar was used for routine culture of *P. gingivalis*. An *ermF–ermAM* insertion mutant and a $tet^r$ insertion mutant of *P. gingivalis* were selected by supplementation of BHIHM with erythromycin (5 μg/mL) and tetracycline (0.7 μg/mL), respectively. The minimal medium was sterilized GA (10 mM $NaH_2PO_4$, 10 mM KCl and 10 mM $MgCl_2$, pH 7.0) containing sterile hemin (7.67 μM), menadione (2.91 μM), IgG (22.5 mg/mL; catalogue no. G-5009; Sigma-Aldrich, St Louis, MO, USA) and bovine serum albumin (7.5 mg/mL; catalogue no. A-2934; Sigma-Aldrich) (12). To make trypsin-treated GA, GA was supplemented with trypsin (50 μg/mL) and incubated at 37°C for 4 h prior to use. A complementation test for the dipeptidyl/tripeptidyl aminopeptidases was performed using GA medium supplemented with purified dipeptidyl/tripeptidyl aminopeptidases: recombinant DPPIV (0.04 μg/mL), DPP-7 (21 μg/mL) and recombinant PTP-A (1.1 μg/mL).

### Measurement of bacterial growth

*P. gingivalis* cells were grown to stationary phase in BHIHM medium, diluted 40-fold with GA 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, Saitama, Japan) under anaerobic conditions. Data were collected in duplicate, and at least three independent experiments were performed.

### Purification of recombinant DPPIV

Recombinant DPPIV was purified as reported previously (13). Briefly, an overnight culture of BL21(pYKP406) was diluted 20-fold with Luria–-Bertani medium, cultured for 3 h at 30°C, mixed with isopropyl-β-D thiogalactopyranoside at a final concentration of 0.1 mM and cultured for 4 h at 30°C. Cells were harvested, freeze–thawed, sonicated using an Ultrasonic Generator (Nihonseiki, Nagano, Japan) and centrifuged (15,000 *g*, 30 min, 4°C). Recombinant DPPIV in the supernatant fraction was purified using hydroxyapatite chromatography (Macro-Prep Ceramic Type I; Bio-Rad, Hercules, CA, USA), as previously described (13).

### Expression and purification of recombinant PTP-A

A pTD-lac (14) derivative was constructed for expressing the *PTP-A* gene. The 0.7-kbp M13 *ori* region of pTD-lac was amplified by the polymerase chain reaction (PCR) using pTD-lac as a template and the primers 5′-GGGTT*CCGCGG*TAAACGTTAA TAT-3′ (italics, a unique *Sac*II site of pTD-lac) and 5′-C*GAATTC*GTAAT CATGGT*CATATG*TGTTTCCTGTG TG-3′ (bold, corresponding to the first Met in the lac promoter of pTD-lac; italics, a unique *Eco*RI site and an inserted *Nde*I site). This amplified 0.7-kbp DNA fragment was digested with *Sac*II and *Eco*RI, and subcloned into *Sac*II/*Eco*RI-digested pTD-lac, generating the construct pTD-lac–Nde. The *PTP-A* gene was then amplified by PCR using *P. gingivalis* W83 chromosomal DNA as a template and

5′-CC*CATATG*CAGTCTCCTGAAA-CGAGTG-3′ (italics, an inserted *Nde*I site) and 5′-GG*TCTAGA*GGATATT-CATAAGTGATCTGTG-3′ (italics, an inserted *Xba*I site) as primers. This amplified 2.1-kbp DNA fragment was digested with *Nde*I and *Xba*I, and subcloned into *Nde*I/*Xba*I-digested pTD-lac–NdeI, creating pTD-lac–NdeI–PTPA-L2R1, which carries the *PTP-A* gene corresponding to the region from Gln25 to Leu732 (end) of PTP-A. Recombinant PTP-A was purified as follows. An overnight culture of JM109(pTD-lac–NdeI–PTPA-L2R1) was diluted 40-fold with Luria–-Bertani broth supplemented with isopropyl-β-D thiogalactopyranoside (0.1 mM) and glycerol (0.8%), and cultured for 6 h at 37°C. Cells were harvested by centrifugation (10,000 ***g***, 20 min, 4°C), washed twice with 33 mM Tris–HCl (pH 7.5), collected by centrifugation (18,000 *g*, 10 min, 4°C) and frozen at −80°C. Pooled frozen cells were thawed in 10 mM Tris–HCl (pH 7.5), sonicated and centrifuged (12,000 *g*, 20 min, 4°C). An insoluble portion was removed by centrifugation (110,000 *g*, 60 min, 4°C). The resultant supernatant was applied to a DEAE Sephacel column (GE Healthcare Bio-Sciences, Uppsala, Sweden) equilibrated with 10 mM Tris–HCl (pH 7.5)−50 mM NaCl. Bound proteins were eluted with a linear gradient of 50–400 mM NaCl in 10 mM Tris–HCl (pH 7.5). Active fractions were pooled and applied to a hydroxyapatite column equilibrated with 10 mM potassium phosphate (pH 7.5). Retained proteins were eluted with a linear gradient of 200–600 mM potassium phosphate (pH 7.5). Active fractions were pooled, extensively dialyzed against excess 10 mM Tris–HCl (pH 7.5), concentrated using a centrifugal device (MACROSEP 30k OMEGA; Pall Life Sci., Ann Arbor, MI, USA) and stored at −80°C.

### Purification of native DPP-7

*P. gingivalis* W83-4A (*DPPIV*$^{-}$ *PTP-A*$^{-}$) cells were grown to an optical density of 1–1.5 at 620 nm in BHIHM medium, harvested by centrifugation (10,000 *g*, 40 min), washed twice in 50 mM potassium phosphate (pH 7.0) and resuspended in 50 mM potassium phosphate (pH 7.0) with one-tenth the volume of the original BHIHM broth. Triton X-100 was slowly added to the suspension to a final concentration of 0.05%, and cells were gently stirred for 2 h at 4°C. Solubilized proteins were separated from the suspension by centrifugation (10,000 *g*, 40 min, 4°C) and stored at −80°C. The pooled frozen fractions were thawed and applied to a hydroxyapatite column equilibrated with 50 mM potassium phosphate (pH 7.0). The column was extensively washed with 300 mM potassium phosphate (pH 7.0) −0.05% Triton X-100. Retained proteins were eluted with 400 mM potassium phosphate (pH 7.0)−0.05% Triton X-100, pooled and concentrated using a Centricon Plus-70 Ultracel PL-30 centrifugal device (Millipore, Billerica, MA, USA). The concentrated samples were diluted with a 14-fold excess of 0.1% sucrose monolaurate (SM-1200; DOJINDO Lab, Kumamoto, Japan), and passed twice through a DEAE Sephacel column equilibrated with 27 mM potassium phosphate (pH 7.0)−0.1% SM-1200. The flow-through fractions were pooled and applied to an sulphopropyl Sepharose Fast Flow column (GE Healthcare Bio-Sciences) equilibrated with 27 mM potassium phosphate (pH 7.0)−0.1% SM-1200. The column was extensively washed with 20 mM sodium phosphate (pH 7.5)−0.05% SM-1200, followed by washing with 20 mM sodium phosphate (pH 7.5) −0.1 M NaCl. Retained proteins were eluted with 20 mM sodium phosphate (pH 7.5)−0.4 M NaCl. Sodium phosphate and NaCl in the eluate were removed by concentration–dilution (two rounds) using an Ultra-15 Ultracel-50k centrifugal device (Millipore), concentrated in 2 mM sodium phosphate (pH 7.5), diluted with an equal volume of 2 mM sodium phosphate (pH 7.5)−0.05% SM-1200 and stored at −80°C.

### Construction of plasmids for disruption of the *dpp7* and *PTP-A* genes

A plasmid for disruption of the *dpp7* gene was constructed by PCR amplifying the *dpp7* gene from the *P. gingivalis* W83 chromosomal DNA template using the primers 5′-ATA-AACACATATGCAAATGAAATT-AAAAAGTATTC-3′ and 5′-AGA-GATCT*AAGCTT*AGATCAACTTC-AGCTCTTGG-3′ (italics, an inserted *Hin*dIII site). This amplified 2.1-kbp DNA fragment was digested with *Sac*I (in the *dpp7* gene) and *Hin*dIII, and subcloned into *Sac*I/*Hin*dIII-digested pUC119, creating pUC119–SH–*dpp7*, which carries the *dpp7* gene corresponding to the region from Glu44 to Ile712 of DPP-7. The 5.1-kbp *Sma*I/*Bam*HI (both sites are in the cloned *dpp7* gene) fragment of pUC119–SH–dpp7 was ligated with the 2.8-kbp *Sma*I/*Bam*HI fragment containing the *tet*$^{r}$ gene from pYKP028 (15). The resultant plasmid pUC119–SH–dpp7Δ-tet carries the disrupted *dpp7* gene, in which an internal 1.0-kbp region (corresponding to the region from Ser276 to Gln602) was replaced with the *tet*$^{r}$ gene. A plasmid for disruption of the *PTP-A* gene was constructed as follows: pTD-lac–NdeI–PTPA-L2R1 was digested with *Kpn*I (corresponding to Gly484 and Thr485 of PTP-A), and ligated to the 2.1-kbp *Kpn*I–*Kpn*I *ermF–ermAM* from pKS1 (16), creating pTD-lac–NdeI–PTPA-L2R1–erm-R, in which the *ermF–ermAM* genes were inserted into *PTP-A* in the opposite orientation. pUC119–SH–dpp7Δ-tet and pTD-lac–NdeI–PTPA-L2R1–erm-R were linearized and used for disruption of *dpp7* and *PTP-A*, respectively, by electroporation (16).

### Electroporation of *P. gingivalis*

*P. gingivalis* cells were harvested from 2 mL of a saturated growth culture, washed five times in electroporation buffer (10% glycerol, 1 mM $MgCl_2$; filter sterilized) at 4°C, suspended in 0.2 mL of electroporation buffer and mixed with approximately 1 μg of a purified DNA fragment. A mixture of cells and a DNA fragment was placed in an electrode cuvette (0.2 cm gap) and pulsed using a GENE PULSER II (Bio-Rad) at 2500 V. The cell suspension was immediately placed in a test tube (1 mL of BHIHM added)

and cultured for 4 h. *P. gingivalis* cells were harvested, spread onto BHIHM agar supplemented with erythromycin (5 μg/mL) or tetracycline (0.7 μg/mL) and incubated for 7 d. The site-specific insertion of the *ermF-AM* genes or the *tet*$^r$ gene in an erythromycin-resistant clone or a tetracycline-resistant clone was confirmed by Southern blotting and PCR analyses (data not shown).

### Enzymatic assays

DPPIV, DPP-7 and PTP-A activities were determined in 20 mM potassium phosphate buffer (pH 7.5) using Gly-Pro-*p*-nitroanilide (Peptide Institute, Osaka, Japan), H-Ala-Phe-*p*-nitroanilide (Bachem, Bulendorf, Switzerland) and H-Ala-Phe-Pro-*p*-nitroanilide (Bachem), respectively, as a substrate. Kgp activity was determined in 100 mM Tris–HCl buffer (pH 8.0) containing 10 mM $CaCl_2$ and 10 mM L-cysteine using 0.1 mM $N^{\alpha}$-benzyloxycarbonyl-L-lysine-*p*-nitroanilide (Novabiochem, Merck Biosciences, Darmstadt, Germany) as a substrate. Rgp activity was determined in 20 mM sodium phosphate buffer (pH 7.5) containing 5 mM L-cysteine using $N^{\alpha}$-benzoyl-DL-Arg-*p*-nitroanilide (Sigma-Aldrich) as a substrate. All reactions were performed at 30°C, and absorbance was measured at 405 nm.

### Other methods

PCR was performed in a Programmable Thermal Controller PTC-100 (MJ Research, Watertown, MA, USA) using Vent DNA polymerase (New England Biolabs). Protein concentration was determined with a BCA Protein Assay Kit (Pierce, Rockford, IL, USA) using bovine serum albumin as a standard. The N-terminal amino acid sequence of the purified DPP-7 was determined using the Procise 494HT Protein Sequencing System (Applied Biosystems, Foster, CA, USA). Statistically significant differences in the median of the values were evaluated using the Mann–Whitney *U*-test. Differences were considered significant at $p < 0.01$ or $p < 0.05$.

## Results

### Construction and characterization of knockout mutants for *DPPIV*, *dpp7* and *PTP-A*

To determine the role of DPPIV, DPP-7 and PTP-A in the asaccharolytic growth of W83, the *dpp7* and *PTP-A* genes were disrupted. A null deletion mutant of the W83 *DPPIV* (*dpp*) gene, named 4351, was previously constructed by Kumagai *et al.* and used in this study, but was referred to as 83-4 for consistency in nomenclature (13). The *dpp7* gene and the *PTP-A* gene were cloned, disrupted by insertion of the *tet*$^r$ gene and the *ermF–ermAM* cassette, respectively, and introduced into *P. gingivalis* cells by electroporation. Resulting single, double and triple knockout mutants for *DPPIV*, *dpp7* and *PTP-A* were W83-isogenic and referred to as 83-7 (*dpp7*$^-$), 83-A (*PTP-A*$^-$), 83-47 (*DPPIV*$^-$ *dpp7*$^-$), 83-4A (*DPPIV*$^-$ *PTP-A*$^-$), 83-7A (*dpp7*$^-$ *PTP-A*$^-$) and 83-47A (*DPPIV*$^-$ *dpp7*$^-$ *PTP-A*$^-$). These seven knockout mutants specifically lacked activity for their disrupted aminopeptidase genes, indicating that the knockout mutations had been successfully introduced (Table 1). Arg-gingipain and Lys-gingipain activities in all knockout mutants, except for 83-4, 83-47 and 83-47A, were similar to those of W83. The Lys-gingipain activities of 83-4 and 83-47 exhibited an approximate 50% increase, while the Arg-gingipain and Lys-gingipain activities of 83-47A decreased by about 30% and 40%, respectively (Table 1). The single, double and triple knockout mutants grew as well as W83 in BHIHM (Fig. 1A). The doubling times for 83-7 and 83-A were 3.9 and 4.3 h, respectively, and not significantly different from the doubling time for W83 (4.1 h). Interestingly, other knockout mutants showed faster growth in BHIHM than W83. The doubling times for 83-4, 83-47, 83-4A, 83-7A and 83-47A were 3.5, 3.6, 3.7, 3.7 and 3.8 h, significantly faster than for W83 (4.1 h) ($p < 0.05$). Therefore, DPPIV, DPP-7 and PTP-A are

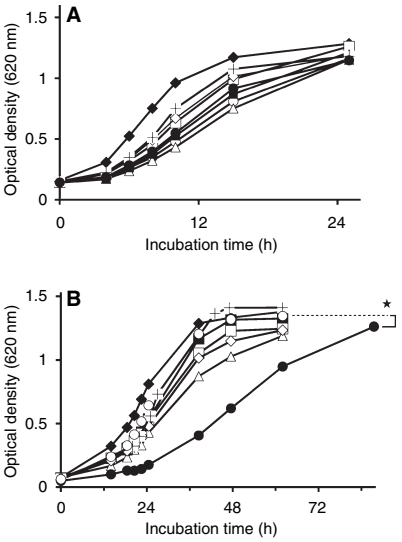


*Fig. 1.* Growth of the dipeptidyl/tripeptidyl aminopeptidase-deficient mutants in complex medium (A) and in minimal medium (B). *Porphyromonas gingivalis* was cultured anaerobically in Brain–heart infusion medium supplemented with sterile hemin and menadione (BHIHM) (A) and minimal medium GA (B). Growth was monitored by measuring the optical density at 620 nm. W83, ○; 83-4, ◆; 83-7, ■; 83-A, △; 83-47, +; 83-4A, □; 83-7A, ◇; 83-47A, ●. ★, $p < 0.01$.

*Table 1.* Relative protease activities of the knockout mutants (%)

| | DPPIV | DPP-7 | PTP-A | Rgp | Kgp |
|---|---|---|---|---|---|
| 83-4 (4351) | — | 92 | 100 | 109 | 159 |
| 83-7 | 69 | — | 90 | 95 | 79 |
| 83-A | 108 | 106 | — | 93 | 81 |
| 83-47 | — | — | 104 | 97 | 140 |
| 83-4A | — | 96 | — | 81 | 74 |
| 83-7A | 79 | — | — | 91 | 94 |
| 83-47A | — | — | — | 67 | 57 |

Data were collected from two experiments. Protease activity in fresh cell extracts is indicated relative to W83 (set at 100%).
'—' indicates undetectable levels of activity.

dispensable for the growth of *P. gingivalis* in BHIHM.

### Growth of knockout mutants in GA minimal medium

The effects of the knockout mutations on the asaccharolytic growth of *P. gingivalis* were determined by investigating the growth properties of the knockout mutants in minimal medium GA, which contains a 3:1 mixture of IgG and bovine serum albumin as a sole carbon and nitrogen source (12). In GA, growth of the single-knockout and double-knockout mutants was comparable to that of W83 (Fig. 1B). The doubling times for 83-4, 83-7, 83-A, 83-47, 83-4A and 83-7A were 6.7, 6.5, 7.0, 6.3, 7.0 and 7.0 h, respectively, not significantly different from the doubling time for W83 (6.7 h). By contrast, a triple knockout mutant, 83-47A, showed significantly slower growth (10.9 h) than W83 (6.7 h) in GA (Fig. 1B) ($p < 0.01$) (Fig. 1B). These results indicated that the combined activities of DPPIV, DPP-7 and PTP-A are required for efficient growth of *P. gingivalis* in minimal medium.

### Complementation of the triple knockout mutant 83-47A by purified dipeptidyl/tripeptidyl aminopeptidases

If slow growth of 83-47A in minimal medium GA was caused by defects in the aminopeptidase activities, growth of 83-47A should be restored by supplementation of GA with purified dipeptidyl/tripeptidyl aminopeptidases. For such a complementation test, we purified recombinant DPPIV and recombinant PTP-A from *E. coli*. Recombinant DPP-7 expressed in *E. coli* lost its activity (data not shown) and therefore native DPP-7 was purified from 83-4A, as described in the Material and Methods. Electrophoresis of the aminopeptidases on a sodium dodecyl sulfate–polyacrylamide gel showed that recombinant DPPIV, DPP-7 and recombinant PTP-A were purified to homogeneity as 76-kDa protein bands (Fig. 2). The expected molecular weight of the

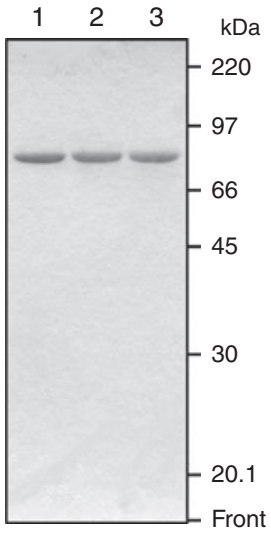


*Fig. 2.* Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of purified peptidases. Two micrograms of recombinant DPPIV (lane 1), DPP-7 (lane 2) and recombinant PTP-A (lane 3) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (12.5% gel) followed by staining with Coomassie Brilliant Blue R-250.

mature forms of DPPIV, DPP-7 and PTP-A were calculated as 80, 78 and 80 kDa, respectively. The N-terminal sequence of a 76-kDa protein band for DPP-7 was Asp-Lys-Gly-Met-Trp-Leu-Leu, identical to the Asp25 to Leu31 region of DPP-7, indicating that the correct protein was purified. Furthermore, each purified sample showed high specific peptidase activity, indicating that purification of recombinant DPPIV, DPP-7 and recombinant PTP-A was successful. The specific activities for recombinant DPPIV, DPP-7 and recombinant PTP-A were 70, 131 and 60 units/mg, respectively, where 1 unit is defined by Δabsorbance at 405 nm/min/μL. As shown in Fig. 3, the growth defect of 83-47A in GA was restored by addition of the combination of recombinant DPPIV, DPP-7 and recombinant PTP-A to the minimal medium GA (GA + 47A). The doubling time for 83-47A in GA + 47A was 7.2 h, not significantly different from the doubling time for W83 in GA (6.7 h). By contrast, trypsin had little effect on the slow growth of 83-47A in minimal medium GA

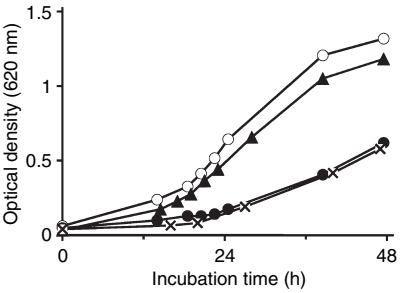


*Fig. 3.* Complementation test for growth of the triple knockout mutant in minimal medium GA. W83 was cultured in GA (○), whereas 83-47A was cultured in GA (●), in GA supplemented with purified peptidases recombinant DPPIV, DPP-7 and PTP-A (▲), or in GA supplemented with trypsin (×).

(Fig. 3). The doubling time for 83-47A was not significantly different in GA vs. trypsin-treated GA (10.9 and 10.0 h, respectively), indicating that decreased gingipain activities (Table 1) did not noticeably affect the asaccharolytic growth of W83-47A.

## Discussion

In this study, we found that DPPIV, DPP-7 and PTP-A activities are required for proper growth of *P. gingivalis* in minimal medium GA. The doubling times for W83 and the triple knockout mutant 83-47A were 6.7 and 10.9 h in GA, respectively, indicating a 40% decrease in growth rate following disruption of the *DPPIV*, *dpp7* and *PTP-A* genes. The optical density of 83-47A in the stationary phase was similar to that of W83 (Fig. 1B), suggesting that DPPIV, DPP-7 and PTP-A are required for efficient growth of *P. gingivalis*. To our knowledge, this is the first report to elucidate the participation of DPPIV, DPP-7 and PTP-A in the asaccharolytic growth of *P. gingivalis*. DPPIV, DPP-7 and PTP-A break their substrate peptides into N-terminal dipeptides/tripeptides and C-terminal truncated peptides. DPPIV and PTP-A produce the N-terminal dipeptides and tripeptides Xaa-Pro and Xaa-Xaa-Pro, respectively. However, neither Xaa-Pro nor Xaa-Xaa-Pro is probably utilized by *P. gingivalis* because it rarely consumes

proline-containing peptides (4). DPP-7 produces many kinds of Xaa-Zaa dipeptides as N-terminal dipeptides, where Zaa represents any residue except for Gly/Pro/Arg/Lys/His/Glu/Asp. A single knockout mutant (83-7) grew well in GA (Fig. 1B) (the doubling time of 83-7 and W83 was 6.5 and 6.7 h, respectively), suggesting that Xaa-Zaa dipeptides are not critical for growth of *P. gingivalis*. Therefore, this evidence suggests that C-terminal truncated peptides, but not N-terminal dipeptides/tripeptides, are the primary contributors to growth of *P. gingivalis*. By contrast, Takahashi *et al*. reported that *P. gingivalis* utilizes Asp–Asp and Glu–Glu preferentially as metabolic substrates for *P. gingivalis*, and proposed that dipeptides are a good energy source for *P. gingivalis* (17). These inconsistent results indicate that the profile of peptides used as an energy source for *P. gingivalis* may not be simple. However, it should be noted that the production of Asp–Asp and Glu–Glu may be rare during the growth of *P. gingivalis* because none of the secreted proteases (RgpA, RgpB, Kgp, DPPIV, DPP-7, PTP-A) cleaved $NH_2$-Asp-Asp-↓-$(Xaa)_n$ or $NH_2$-Glu-Glu-↓-$(Xaa)_n$. Further studies will be required to determine the properties of the peptides required for growth of this bacterium. At present, we propose the following model to explain the contribution of DPPIV, DPP-7 and PTP-A in the growth of *P. gingivalis*: DPPIV, DPP-7 and PTP-A truncate their substrate peptides to separate the C-terminal portions from the N-terminal dipeptides/tripeptides. Among the truncated C-terminal peptides, some are readily taken up by *P. gingivalis* and metabolized, while others may undergo structural changes after truncation, or may be synergistically digested by secreted proteases and then broken into short-chain polypeptides that are metabolized. Therefore, DPPIV, DPP-7 and PTP-A contribute to the growth of *P. gingivalis* in oral environments where external peptides may be scarce. The nutritional purposes of these aminopeptidases easily render them important pathogenic factors of this bacterium. Furthermore, DPPIV, DPP-7 and PTP-A may contribute to the induction of periodontal disease in a different way. DPPIV participates in the degradation of human type I collagen and gelatin in combination with human matrix metalloproteinase 1 (MMP-1) and MMP-2, respectively (18). Working in concert with other proteases, DPP-7 and PTP-A may contribute to the degradation of connective tissue. Interestingly, DPPIV is a virulence factor and its peptidase activity is essential, but not sufficient, and other activity, such as fibronectin-binding activity, is essential for full virulence (15). Similar cryptic activity might localize in DPP-7 or PTP-A.

We have constructed an experimental system to determine the properties of asaccharolytic growth of *P. gingivalis*, and have demonstrated, through our previous work and the present study, that the secreted proteases of *P. gingivalis* are important for the asaccharolytic growth of *P. gingivalis* (12). Novel types of bactericidal agents specific for *P. gingivalis* may be discovered using our experimental system to screen for inhibitors of proteases secreted by this bacterium.

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