Identification and Characterization of Prokaryotic Dipeptidyl-peptidase 5 from Porphyromonas gingivalis

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Background: Dipeptidyl-peptidases (DPPs) are key factors for amino acid metabolism and bacterial growth of asaccharolytic Porphyromonas gingivalis.

Results: DPP5, which is specific for Ala and hydrophobic residues, is expressed in the periplasmic space of P. gingivalis.

Conclusion: DPP5 was discovered in prokaryotes for the first time.

Significance: The discovery of DPP5 expands understanding of amino acid and energy metabolism in prokaryotes.

Porphyromonas gingivalis, a Gram-negative asaccharolytic anaerobe, is a major causative organism of chronic periodontitis. Because the bacterium utilizes amino acids as energy and carbon sources and incorporates them mainly as dipeptides, a wide variety of dipeptide production processes mediated by dipeptidyl-peptidases (DPPs) should be beneficial for the organism. In the present study, we identified the fourth P. gingivalis enzyme, DPP5. In a dpp4-7-11-disrupted P. gingivalis ATCC 33277, a DPP7-like activity still remained. PGN_0756 possessed an activity indistinguishable from that of the mutant, and was identified as a bacterial orthologue of fungal DPP5, because of its substrate specificity and 28.5% amino acid sequence identity with an Aspergillus fumigatus entity. P. gingivalis DPP5 was composed of 684 amino acids with a molecular mass of 77,453, and existed as a dimer while migrating at 66 kDa on SDS-PAGE. It preferred Ala and hydrophobic residues, had no activity toward Pro at the P1 position, and no preference for hydrophobic P2 residues, showed an optimal pH of 6.7 in the presence of NaCl, demonstrated \( K_m \) and \( k_{cat}/K_m \) values for Lys-Ala-MCA of 688 \( \mu M \) and 11.02 \( \mu M^{-1} s^{-1} \) respectively, and was localized in the periplasm. DPP5 elaborately complemented DPP7 in liberation of dipeptides with hydrophobic P1 residues. Examinations of DPP- and gingipain gene-disrupted mutants indicated that DPP4, DPP5, DPP7, and DPP11 together with Arg- and Lys-gingipains cooperatively liberate most dipeptides from nutrient oligopeptides. This is the first study to report that DPP5 is expressed not only in eukaryotes, but also widely distributed in bacteria and archaea.

Porphyromonas gingivalis, a Gram-negative rod, is a bacterium associated with severe forms of periodontal disease, which leads to loss of teeth in adults (1, 2). Over 47% of American adults suffer from periodontitis, the most common infectious disease in humans (3). In addition to dental diseases, recent studies have also reported that P. gingivalis is related with systemic diseases, such as cardiovascular diseases (4), decreased kidney function (5), and rheumatoid arthritis (6). The bacterium does not ferment carbohydrates, but grows under strict anaerobic conditions using proteinaceous substrates as carbon and energy sources. Although anaerobic energy production in P. gingivalis is poorly understood, biochemical examinations (7, 8), whole genome information (9, 10), and transcriptome analysis (11) have led to drafts of the configurations of the entire metabolism and transport pathways.

Glu and Asp, the most consumed amino acids in P. gingivalis (7, 8, 12), are located in the central pathway of amino acid metabolism (9, 13). They are mutually converted in the cytoplasm by \( \delta \)-1-pyrroline-5-carboxylate dehydrogenase (PGN_1401) and ornithine aminotransferase (PGN_1403), respectively. Asp undergoes deamination by aspartate ammonia-lyase (PGN_0377) and is converted to fumarate, which is thought to be a major terminal electron acceptor of the respiratory chain in the organism. In addition to energy production, amino acid metabolism is important for understanding bacterial virulence, because the end products of short-chain fatty acids, e.g. butyrate, exhibit cytotoxicity against periodontal epithelial cells (14). In fact, the concentration of butyrate is increased in subgingival fluid in periodontal patients (15), and these compounds are proposed to be involved in the initiation and progression of these diseases (16). Hence, amino acid metabolism is an important issue for both anaerobic asaccharolytic metabolism and the pathogenicity of P. gingivalis.
To incorporate amino acids, *P. gingivalis* possesses potent proteolytic activities toward extracellular proteinaceous nutrients. First, proteins are degraded to oligopeptides by the cysteine endopeptidases Arg-gingipain (Rgp) and Lys-gingipain (Kgp) (17–20). Subsequently, oligopeptides are cleaved into dipeptides from the N terminus by dipeptidyl-peptidases (DPPs). To date, 3 DPPs, i.e., DPP4, DPP7, and DPP11, have been identified, of which DPP4 is highly specific for Pro and less potently for Ala at the penultimate position from the N terminus (P1) (MEROPS classification: S9.013) (21), whereas DPP7 prefers aliphatic and aromatic residues (22). We recently reported that DPP7 also releases dipeptides with a non-hydrophobic P1 residue, if the N-terminal (P2) residue is hydrophobic (23). The third and novel enzyme DPP11 (S46.002) shares a 38.7% amino acid sequence identity with DPP7 (S46.001), and is specific for Asp and Glu (24). When Pro is located at the third position from the N terminus, DPPs do not cleave the Xaa-Pro bond, although prolyl tripeptidase A (PTP-A) cleaves the Pro-Xaa bond (25). These liberated di- and tripeptides are efficiently incorporated into bacterial cells (8), possibly through oligopeptide transporters (13). The importance of DPPs and PTP-A for bacterial metabolism was confirmed in a study that showed that the growth of *P. gingivalis* with simultaneously deleted dpp4, dpp7, and ptp-A was retarded (26). Growth retardation has also been observed in a dpp11-disrupted strain (24).

The present study was performed to fully clarify the DPP members in *P. gingivalis*. Examination of dipeptide production repertoires among wild-type, gingipain-null, and dpp4, dpp7, and dpp11 triple gene-disrupted strains suggested the existence of additional DPP activities responsible for cleavage of Met-Leu-, Lys-Ala-, and Gly-Phe-methylcoumaryl-7-amide (MCA). Examination of putative peptidases revealed that PGN_0756 (EC = 3.4.14.-, S09.012), which was found to be specific for Ala and hydrophobic amino acids at the P1 position. To our knowledge, this is the first report of DPP5 distribution in a prokaryotic organism.

### EXPERIMENTAL PROCEDURES

**Materials**—Restriction enzymes and DNA-modifying enzymes were purchased from Takara Bio (Ipswich, MA), and KOD-Plus-Neo DNA polymerase from Toyobo (Osaka, Japan). Oligonucleotide primers were from Fasmac (Atsugi, Japan). Commercially unavailable dipeptidyl-MCAs (Leu-Arg-, Val-Arg-, Leu-Lys-, Ala-Arg-, Ala-Asn-, Leu-Gln-, Gly-Arg-, Lys-Lys-, Thr-Asp-, Val-Asp-, Ile-Asp-, and His-Asp-MCA) were prepared from the appropriate pentapeptide-MCA through digestion with thermolysin from *Bacillus thermoproteolyticus rokko*, bovine trypsin (Sigma), or staphylococcal glutamyl endopeptidase GluV8, as previously described (24). Benzyloxy carbonyl (Z)-Phe-Arg-, Arg-Arg-, t-butoxy carbonyl (boc)-Val-Leu-Lys-MCA, and MCA peptides used to produce dipeptidyl MCA were obtained from the Peptide Institute (Osaka, Japan); Met-Leu-, Arg-Arg-, Gly-Phe-, Ser-Tyr-, Thr-Ser-, and Gly-Gly-MCA were from Bachem (Dubendorf, Switzerland). Leu-Asp-, Leu-Glu-, and Z-Leu-Leu-Gln-MCA were synthesized by Thermo Fisher Scientific (Ulm, Germany) and TORAY (Tokyo, Japan); and Lys-Phe-, Lys-Leu-, and Lys-Val-MCA by Scrum (Tokyo, Japan). Alkaline phosphatase-conjugated anti-rabbit Ig and streptavidin were purchased from Promega. Isoleucine thiazolidide hemifumarate (P32/98) was from Focus Biomolecules (Plymouth Meeting, PA), sitagliptin from Sigma, and vildagliptin from LKT Laboratories (St. Paul, MN). SYPRO Ruby protein gel stain was from Invitrogen. Formyl cellulofine was purchased from Seikagaku Corporation (Tokyo, Japan).

**Bacterial Strains and Growth Conditions**—*P. gingivalis* ATCC 33277 was purchased from American Type Culture Collection (Manassas, VA) and other strains used are shown in Table 1. Bacterial cells were grown in anaerobic bacteria culture media (Eiken Chemical, Tokyo, Japan) in the presence of menadione (1 μg/mL, Sigma) with or without appropriate antibiotics (0.7 μg/mL of tetracycline, 10 μg/mL each of ampicillin, erythromycin, and chloramphenicol) at 35 °C under an anaerobic condition (90% N₂, 5% H₂, 5% CO₂).

### Table 1

| Strain | Genotype | Source or Ref. |
|--------|----------|---------------|
| ATCC   | Wild-type|               |
| KDP136 | kgp-2::cat rgaA2::[ermF ermAM] rgaB2::tetQ | 28 |
| NDP200 | dpp4::cepA | This study |
| NDP300 | dpp5::[ermF ermAM] | This study |
| NDP400 | dpp3::tetQ | This study |
| NDP320 | dpp5::[ermF ermAM]dpp7::tetQ | This study |
| NDP210 | dpp4::cepA dpp7::tetQ | This study |
| NDP211 | dpp4::cepA dpp5::[ermF ermAM]dpp7::tetQ | This study |
| NDP212 | dpp4::cepA dpp3::[ermF ermAM]dpp7::tetQ | This study |
| NDP11  | dpp4::cepA dpp7::tetQ dpp11::[ermF ermAM] | This study |

**Plasmids and Expression of DPPs in E. coli**—DNA fragments encoding the full-length form of putative DPP3 (MER034609/PGN_1645: Val^2–Glu^389), putative prolyl oligopeptidase (MER034615/PGN_0756: Asp^2–Lys^691), and putative Ala-DPP (MER034613/PGN_1694: Lys^2–Leu^847) were obtained by PCR using genomic DNA. After digestion with BglII for DPP3, BamHI for prolyl oligopeptidase, and NcoI for Ala-DPP, the DNA fragment was inserted into the BamHI (DPP3 and prolyl oligopeptidase) or NcoI (Ala-DPP) site of pQE60. Expression plasmids for *P. gingivalis* DPP4 (MER004211/PGN_1469: Asp^23–Leu^223), DPP7 (MER014366/PGN_1479: Met^1–Ile^212), and DPP11 (MER034626/PGN_0607: Lys^2–Pro^720) were also used (23, 24). DPPs were expressed in *E. coli* as described (24). Briefly, overnight cultures of *E. coli* were added to 2 volumes of
Discovery of Prokaryotic Dipeptidyl-peptidase 5

| Name                     | Sequence (5'-3')                          |
|--------------------------|------------------------------------------|
| 5pgDPP5V29gII            | AACAGTACTGTTAATTTACGACAGGAGAC           |
| 3pgDPP9E906gII           | GAAAGTACCTGTTTCTTGTCTGAG                |
| 5pgDPP5N2BamHI           | GACATCACTGTTAAGCTTCTGAG                 |
| 3pgDPP5K684BamHI         | CAGCCATACCTGTTAAGCTTCTGAG               |
| 5pgDPP9Alk2NeoI          | CAGCCTAGCTGTTAAGCTTCTGAG                |
| 5pgDPPAlaL841NeoI        | ATAGCTGAGCTGTTAAGCTTCTGAG               |
| DPP4-5R-1                | TTCTGCTGCTGTTAAGCTTCTGAG               |
| DPP4-5R-cep-comp         | TTCTGCTGCTGTTAAGCTTCTGAG               |
| DPP4-5R-cep              | TTCTGCTGCTGTTAAGCTTCTGAG               |
| DPP4-3R-1-cep            | TTCTGCTGCTGTTAAGCTTCTGAG               |
| 3-cep                    | TTCTGCTGCTGTTAAGCTTCTGAG               |
| 3-comp                   | TTCTGCTGCTGTTAAGCTTCTGAG               |
| DPP4-5R-2                | GAGATCATGTACCTGTTAAGCTTCTGAG           |
| DPP4-3R-comp2            | GCTTTTGTGCTGTTAAGCTTCTGAG              |
| DPP5F1p                  | CCAAAACAGCTGTTAAGCTTCTGAG              |
| DPP5P1u-erm              | CCAAAACAGCTGTTAAGCTTCTGAG              |
| DPP5Dwn                  | CCAAAACAGCTGTTAAGCTTCTGAG              |
| 5erm-DPP5P1u             | CCAAAACAGCTGTTAAGCTTCTGAG              |
| 3erm-DPP5Dwn             | CCAAAACAGCTGTTAAGCTTCTGAG              |
| DPP11U1p-2              | CCAAAACAGCTGTTAAGCTTCTGAG             |
| DPP11U1p-catR           | CCAAAACAGCTGTTAAGCTTCTGAG             |
| DPP11U1p-Dwn-catR        | CCAAAACAGCTGTTAAGCTTCTGAG             |
| 3catR-DPP11U1p           | CCAAAACAGCTGTTAAGCTTCTGAG             |
| 3catR-DPP11Dwn          | CCAAAACAGCTGTTAAGCTTCTGAG             |

Luria-Bertani broth containing 75 μg/ml of ampicillin and 0.2 mM isopropyl β-D-thiogalactopyranoside, then further incubated at 30 °C for 4 h. Bacterial cells were lysed with lysis/wash buffer (20 mM Tris-HCl, pH 8.0, 0.1 M NaCl containing 10 mM imidazole) supplemented with 0.4 mg/ml of lysozyme and 10 μg/ml of leupeptin. Recombinant proteins were purified using a Talon metal affinity resin (Takara Bio, Tokyo, Japan) column according to the manufacturer’s protocol. Bound proteins were eluted with 0.1 M imidazole (pH 8.0) containing 10% (v/v) glycerol and stored at −80 °C until use.

Construction of P. gingivalis Strains with Disrupted dpp Genes—To construct a dpp4 (PGN_1469) deletion mutant, 5′- and 3′-untranslated regions (UTR) of dpp4 were PCR amplified from the chromosomal DNA of P. gingivalis ATCC 33277 with sets of primers (DPP4-5R-1 and DPP4-5R-cep, and DPP4-cep-3R-1-comp, respectively) (Table 2). A set of primers (DPP4-5R-1 and DPP4-5R-cep-comp, DPP3-cep-3R-1, 3R-comp) and pCR4-TOPO as a template. Nested PCR was performed with a mixture of these fragments and primers (5DPP5Up, 3CatR-DPP5Dwn). A 3.5-kb DNA fragment composed of these fragments was amplified with a mixture of three DNA fragments and a set of primers (5DPP11Up-2, 3DPP11Dwn-2), then introduced into NDP211, resulting in NDP212 (dpp4::cepA dpp5::[ermF ermAM]).

Hydrolyzing Activity toward Dipeptidyl MCA—Recombinant peptides (1–50 ng) were incubated with 20 μM dipeptidyl-MCA in 200 μl of reaction solution composed of 50 mM sodium phosphate (pH 7.0), 5 mM EDTA, and 0.1 M NaCl at 37 °C for 30 min. In several experiments, pH was varied using 50 mM sodium phosphate buffer and the NaCl concentrations were varied from 0 to 1.6 M. Fluorescence intensity was measured with excitation at 380 nm and emission at 460 nm with a Fluorescence Photometer F-4000 (Hitachi, Tokyo, Japan). To determine the enzymatic parameters, recombinant proteins (5 ng) were incubated with various concentrations of dipeptidyl-MCA (0.5–160 μM) in the presence of 0.1 M NaCl at pH 6.7. Data were analyzed using a nonlinear regression curve fitted to the Michaelis-Menten equation using the GraphPad Prism software program (San Diego, CA). Averages and S.E. were calculated from 4 independent measurements.

Subcellular Fractionations—All procedures were carried out at 4 °C or on ice according to a previously reported method (27), with slight modifications. Briefly, a 20-ml culture of P. gingivalis KDP136, a gingipain-null mutant (28), in the log-phase was centrifuged at 6,000 × g for 15 min. The supernatant was filtered with a 0.45-μm membrane filter, and used for measurement of enzymatic activity as the extracellular fraction. A part of the supernatant was precipitated with 10% trichloroacetic acid on ice for 30 min and collected by centrifugation at 12,500 × g for 30 min, then washed with 30 ml of ice-cold acetone and used for SDS-PAGE and immunoblotting. Bacterial cells were washed with 40 ml of PBS, resuspended in 20 ml of PBS, and an aliquot was used as whole cells. Bacterial cells collected by centrifugation were resuspended in 4 ml of 0.25 M sucrose in 5 mM Tris-HCl (pH 7.5), and left for 10 min on ice. Cells were precipitated at 12,500 × g for 15 min, resuspended in 4 ml of 5 mM Tris-HCl, and mixed gently for 10 min to disrupt the outer membrane. The supernatant was obtained by centrifugation at 6,000 × g for 15 min and collected as the periplasmic...
fraction containing the outer membranes. The resultant precipitate was washed with 40 ml of PBS, then resuspended in 4 ml of PBS and used as spheroplasts. Spheroplast suspensions were sonicated by pulse for 10 times for 10 s each with a 2-s interval in an ice-water bath. After removal of unbroken spheroplasts and debris by centrifugation at 6,000 × g for 20 min, the supernatant (cytosolic fraction) was obtained by ultracentrifugation at 150,000 × g for 1 h, then the precipitate was suspended in 20 ml of PBS, centrifuged at 150,000 × g for 1 h, and then resuspended in 0.1 ml of PBS (inner membrane fraction).

Separately, a total membrane fraction was prepared according to a previously reported method (29), with slight modifications. Briefly, after washing with PBS, bacterial cells from a 200-ml culture were suspended in 20 ml of 10 mM HEPES (pH 7.5) containing 0.15 mM NaCl, 0.2 mM phenylmethylsulfonyl fluoride, and 0.1 mg/ml of leupeptin, then disrupted in a French press at 100 m pascal in the presence of 10 g/ml of DNase through 2 passes. After centrifugation at 100,000 × g for 1 h, the supernatant, the precipitate was suspended in 3 ml of HEPES buffer, and then a 1.5-ml aliquot (total membrane fraction) was separated by sucrose density gradient (26–68%, w/v) centrifugation at 100,000 × g for 48 h in a Hitachi RPS40T. Fractions (0.5 ml) were collected from the top.

Homology Modeling—Sequences for PGN_0756 were submitted to the EsyPred3D web server (30) and SWISS-MODEL. From the homology models constructed, one in pdb format was returned by the EsyPred3D. The program performs all steps of homology modeling including comparisons of amino acid sequences (PSI-BLAST of NCBI NR data bank then scored against the PDB), multiple sequence and pairwise alignments (combination of ClustalW, Dialign2, Match-Box, Multalin, and PRPP), and model building (MODELLER).

Miscellaneous—A polyclonal antibody against P. gingivalis PGN_0756 (DPP5), both DPP5- and anti-DPP5-antibodies cellulofine resins were prepared as previously described (24). Native DPP5 were purified from the periplasmic and cytosolic fractions using anti-DPP5 antibody-cellulofine resin. Protein samples were separated on SDS-PAGE at a polyacrylamide concentration of 10%, and either stained with Coomassie Brilliant Blue or SYPRO Ruby, or electrically transferred to a polyvinylidene fluoride membrane and then subjected to immunoblotting analysis or N-terminal sequencing (24).

Antiserum against the 35-kDa hemin-binding protein (HPB35) localized in the outer membrane (31) was a kind gift from Dr. M. Shoji. The presence of the 13-kDa biotin-containing protein (BCP) localized in the inner membrane was visualized by blotting using alkaline phosphatase-conjugated streptavidin, according to a method reported in a previous study (32). Protein concentrations were determined using the bicinchoninic acid method (Pierce) with BSA as the standard.

RESULTS

Dipeptidyl Peptidase 5

Dipeptide Production in P. gingivalis Wild Type and KDP136—First, we determined cell-associated DPP and endopeptidase activities in P. gingivalis wild-type and gingipain-null mutant KDP136 cells (28). DPP activities in culture supernatant samples from both strains were negligible, as previously reported (24). In addition to activities toward boc-Phe-Ser-Arg-MCA by Rgp and boc-Val-Leu-Lys-/boc-Glu-Lys-Lys-MCA by Kgp, dipeptidyl substrates, such as Arg-Arg-MCA and Leu-Lys-, and Lys-Lys-MCA, were hydrolyzed in the wild-type cells, indicating the presence of Arg- and Lys-specific DPP activities (Fig. 1A). Both endopeptidase and DPP activities toward Arg and Lys were abolished in KDP136 cells, suggesting that Arg and Lys-DPP activities were mediated by Rgp and Kgp, respectively. Activities toward Gly-Pro-MCA by DPP4, Met-Leu-MCA by DPP7, and Leu-Asp-MCA by DPP11 were demonstrated to some extent in the wild-type, and interestingly, these activities were elevated in KDP136 cells, probably to compensate for the loss of gingipain activities. This phenomenon is in accord with a previous report that noted that DPP4 and PTP-A activities were elevated in KDP136 cells (28).

Detailed measurements of DPP activities in KDP136 cells showed that the hydrolyzing activity toward Met-Leu-MCA was abolished in KDP136 cells, suggesting that Arg and Lys-DPP activities were mediated by Rgp and Kgp, respectively. Activities toward Gly-Pro-MCA by DPP4, Met-Leu-MCA by DPP7, and Leu-Asp-MCA by DPP11 were demonstrated to some extent in the wild-type, and interestingly, these activities were elevated in KDP136 cells, probably to compensate for the loss of gingipain activities. This phenomenon is in accord with a previous report that noted that DPP4 and PTP-A activities were elevated in KDP136 cells (28).
was predominant, followed by that for Gly-Pro-, Lys-Ala-, Gly-Phe-, Leu-Arg-, Leu-Glu-, Ser-Phe-, Leu-Asp, Leu-Glu-, and Val-Asp-MCA (Fig. 1B). This profile could be explained by actions of the 3 defined DPPs: i.e. Gly-Pro-MCA by DPP4; Lys-Ala-MCA by both DPP7 and DPP4; Met-Leu-, Gly-Phe-, Leu-Arg-, and Ser-Tyr-MCA by DPP7; and Leu-Asp-, Leu-Glu-, and Val-Asp-MCA by DPP11, whereas Leu-Gln-MCA may be cleaved by DPP7 because of its hydrophobic P2 preference (33). Several substrates such as Thr-Ser- and Gly-Gly-MCA were scarcely released by the bacterium, as suspected based on the substrate preferences of the 3 DPPs and a previous report that noted that no peptidase was found to be responsible for Thr, Ser, and Gly in P. gingivalis (34). Thr-Asp- and His-Asp-MCA were also scarcely hydrolyzed, because DPP11 prefers a hydrophobic P2 residue (24).

To address whether the 3 DPPs and gingipains are full members or an unidentified peptidase is still involved in dipeptide production, we produced mutant strain NDP511, in which dpp3, dpp7, and dpp11 were simultaneously disrupted. Although NDP511 convincingly lost activities for Gly-Pro-MCA (Fig. 4). These results certainly indicated that PGN_0756 is enzymatically distinct from prolyl oligopeptidase mediated by DPP4, and for Leu-Asp- and Leu-Glu-MCA mediated by DPP11, we unexpectedly found significant hydrolyzing activities toward Met-Leu-, Lys-Ala-, Gly-Phe-, and Ser-Tyr-MCA, which had been implicated to be mediated by DPP7 (Fig. 1C). This result indicated the existence of an unidentified DPP possessing a DPP7-like substrate specificity.

Dipeptidyl Peptidase Activity of PGN_0756—We searched for candidates among the 2,090 protein-coding sequences of P. gingivalis ATCC 33277 (10), based on sequence similarity to the defined DPPs generally composed of 650–750 amino acid residues. Consequently, 3 genes for putative DPP3 (PGN_1645), putative prolyl oligopeptidase (PGN_0756), and putative Ala-DPP (PGN_1694) emerged. We expressed these in E. coli and studied the properties of the recombinant proteins. Apparent molecular weights were in accord with their calculated molecular masses (Fig. 2A). An attempt to detect the peptidase activity of recombinant P. gingivalis DPP, labeled as an unassigned peptidase in the S9C family (MER034613) in the MEROPS database (35), was not successful using currently available substrates, thus we did not analyze it further. The 100-kDa putative DPP3 (M₉ = 102,897 in Val²-Glu⁹⁰⁶ with vector tag Mᵣ = 1416.5) exhibited activity with a preference for Arg at the P1 position and the highest activity for Arg-Arg-MCA (Fig. 2B), in accord with the properties of eukaryotic (36) and Bacteroides thetaiotaomicron DPP3 (37). Therefore, PGN_1645 truly represents P. gingivalis DPP3. However, the observation that KDP136 did not hydrolyze Arg-Arg-MCA strongly suggested that DPP3 did not participate in extracellular dipeptide production in P. gingivalis and that it may be localized in the cytoplasm, as reported for B. thetaiotaomicron DPP3 (37).

In contrast to these unpromising results, the recombinant form of PGN_0756/MER034615 (Mᵣ = 77,453 at Met¹–Lys⁶⁸⁴) migrating as a 66-kDa band on SDS-PAGE demonstrated high dipeptidyl peptidase activity toward Lys-Ala-, Gly-Phe-, Met-Leu-, and Ser-Tyr-MCA, and it slowly hydrolyzed Val-Tyr-MCA (Fig. 2C). Interestingly, this repertoire substantially coincided with that of NDP511 (compare Figs. 1C and 2C).

Although the PGN_0756 gene has been annotated as prolyl oligopeptidase, because of sequence identity to the S9-family Pro-specific peptidases, it never hydrolyzed Gly-Pro-MCA (Fig. 2C) and we also found that it shares a 28.5% amino acid sequence identity with another S9 family member, Aspergillus fumigatus DPP5 (38). This homology value is even higher than that of Pro-specific P. gingivalis DPP4 (14.2%) and PTP-A (14.8%) (Fig. 3). Furthermore, in accordance with no preference for Pro, inhibitor analysis using P32/98 (39), sitagliptin (40), and vildagliptin (41), potent inhibitors of human DPP4, demonstrated that neither had an effect on Lys-Ala-MCA hydrolysis by PGN_0756, whereas these compounds completely abrogated the activity of P. gingivalis DPP4 toward Lys-Ala- and Gly-Pro-MCA (Fig. 4). These results certainly indicated that PGN_0756 is enzymatically distinct from prolyl oligopeptidase and DPP4, but it represents an orthologue of A. fumigatus DPP5. Thus, we designated PGN_0756 as DPP5, the fourth P. gingivalis DPP involved in extracellular production of dipeptides. Because DPP5 has been reported to be distributed only in
Discovery of Prokaryotic Dipeptidyl-peptidase 5

The amino acid sequences of *P. gingivalis* DPPs (PgDPP5, pgnP:PGN_0756), *A. fumigatus* DPP5 (AdDPP5, DPP5 ASLc), *P. gingivalis* DPP4 (PgDPP4, pgnP:PGN_1469), and *P. gingivalis* PTP-A (PgPTPA, pgnP:PGN_1149) were aligned by COBALT. Hyphens represent gaps introduced for maximal matching. Common and conserved amino acids are marked by asterisks and dots, respectively. Underlines represent the N-terminal amino acid sequences determined with recombinant DPP5, and the yellow box is that of native DPP5. The signal peptide in *A. fumigatus* DPP5 is written in bold italics. Three amino acids essential for serine proteases are boxed in red. The amino acid sequence chosen for three-dimensional homology modeling is that of native DPP5. The signal peptide and the three-dimensional homology modeled is that of native DPP5.

FIGURE 3. Comparison of amino acid sequences related to S9 family peptidases. The amino acid sequences of *P. gingivalis* DPPs (PgDPP5, pgnP:PGN_0756), *A. fumigatus* DPP5 (AdDPP5, DPP5 ASLc), *P. gingivalis* DPP4 (PgDPP4, pgnP:PGN_1469), and *P. gingivalis* PTP-A (PgPTPA, pgnP:PGN_1149) were aligned by COBALT. Hyphens represent gaps introduced for maximal matching. Common and conserved amino acids are marked by asterisks and dots, respectively. Underlines represent the N-terminal amino acid sequences determined with recombinant DPP5, and the yellow box is that of native DPP5. The signal peptide in *A. fumigatus* DPP5 is written in bold italics. Three amino acids essential for serine proteases are boxed in red. The amino acid sequence chosen for three-dimensional homology modeling is that of native DPP5. The signal peptide and the three-dimensional homology modeled is that of native DPP5.
Discovery of Prokaryotic Dipeptidyl-peptidase 5

that NDP211 lost hydrolyzing abilities with Lys-Ala- and Ser-Tyr-MCA, indicating an additive contribution of DPP4 for their degradation. Taken together, P. gingivalis DPP5 truly participated in hydrolysis of the 4 substrates.

We also found that Met-Leu-MCA hydrolyzing activity was markedly (9.8-fold) increased in the ∆dpp4-5-7-11 quadruple-disrupted strain (NDP212) as compared with triple-disrupted NDP211 and was even higher than that of the wild-type. Similarly, Ser-Tyr-MCA hydrolysis was increased by 6.3-fold in NDP212 over that in NDP211. The overshoot of activity for larly, Ser-Tyr-MCA hydrolysis was increased by 6.3-fold in NDP211 and was even higher than that of the wild-type. Similarly, Ser-Tyr-MCA hydrolysis was increased by 6.3-fold in NDP212 over that in NDP211. The overshoot of activity for

that NDP211 lost hydrolyzing abilities with Lys-Ala- and Ser-Tyr-MCA, indicating an additive contribution of DPP4 for their degradation. Taken together, P. gingivalis DPP5 truly participated in hydrolysis of the 4 substrates.

We also found that Met-Leu-MCA hydrolyzing activity was markedly (9.8-fold) increased in the ∆dpp4-5-7-11 quadruple-disrupted strain (NDP212) as compared with triple-disrupted NDP211 and was even higher than that of the wild-type. Similarly, Ser-Tyr-MCA hydrolysis was increased by 6.3-fold in NDP212 over that in NDP211. The overshoot of activity for Met-Leu-MCA strongly suggested expression induction of an unknown peptidase(s) to compensate for the four-DPP defects. We also observed an increase in activities for succiny1-Leu-Leu-Val-Tyr- and Z-Leu-Leu-Leu-MCA in NDP212 (data not shown), suggesting that the entity mediating the hydrolysis of Met-Leu-MCA was not DPP, but likely an endopeptidase with preference for hydrophobic residues.

N-terminal Sequence Analysis—The N terminus of the recombinant 66-kDa molecule expressed from Asn2 to Lys64 was Lys4 (Fig. 3), indicating cleavage at the Lys3–Lys4 bond in E. coli, and thus, a non-requirement of N-terminal processing to exert the DPP activity. Moreover, a minor species with the N terminus of Ala27 was also present. On the other hand, the N terminus of native 66-kDa DPP5 purified by anti-DPP5 antibody-cellulose was Thr25 (Fig. 3), indicating the cleavage at the Gly24–Thr25 bond.

Size Exclusion Gel Chromatography—Recombinant DPP5 was subjected to size exclusion gel chromatography by use of Sephacryl S200HR. DPP5 was eluted as a single peak at 140 kDa, indicating its dimeric conformation under non-denaturing conditions (data not shown).

NaCl and pH Dependence—DPP5 activity for Lys-Ala-MCA showed a bell-shaped dependence on NaCl concentration, with the maximal activity at 0.1 M (Fig. 6A). We also examined the effects of NaCl on DPP4 and DPP7 activities, because both DPPs could hydrolyze Lys-Ala-MCA. DPP4 hydrolyzed Lys-Ala-MCA at the maximal rate in the absence of NaCl and the activity was gradually decreased at higher concentrations. DPP7 showed very low, but constant activity at a wide range of NaCl concentrations.

The salt dependence of DPP5 for Met-Leu-MCA (Fig. 6B), as well as Gly-Phe- and Ser-Tyr-MCA (data not shown) was similar to that of Lys-Ala-MCA, although changes in the activity became moderate and the optimal NaCl concentration was shifted from 0.1 to 0.4 M. Although DPP7 most efficiently hydrolyzed Met-Leu-MCA among the dipeptidyl MCAs tested (23), the activity was nearly constant and substantially lower than that of DPP5 at all NaCl concentrations. DPP4 and DPP11 do not hydrolyze Met-Leu-MCA (23). It should be noted that the activities in P. gingivalis for Lys-Ala- and Met-Leu-MCA exhibited nearly the same NaCl dependence as those of DPP5, strongly suggesting that these activities are mostly mediated by DPP5 in the bacterial cells.

The optimal pH for DPP5 activity toward Lys-Ala-MCA was 6.7 (Fig. 7), which is identical to that of A. fumigatus DPP5 (38). The pH optimum of DPP7 was slightly shifted to basic pH around 7 and that of DPP4 was further to pH 7.5 in consistent to previous studies (21, 22).

Substrate Specificity of DPP5 Compensates That of DPP7—Enzymatic parameters of DPP5 were determined by fitting a nonlinear regression curve to the Michaelis-Menten equation at pH 6.7 in the presence of 0.1 M NaCl (Table 3). DPP5 possessed the smallest Km for Lys-Ala- and Met-Leu-MCA (146.7 μM), followed by Lys-Phe-MCA (151.5 μM), and possessed the largest kcat/Km value for Gly-Phe-MCA (13.01 μM−1 s−1), followed by Lys-Ala-MCA (11.02 μM−1 s−1). As previously reported (33), the substrates with hydrophobic P1 residue could also be hydrolyzed by DPP7 and the order of Thr-Ser hydrolysis activity by DPP7 was Met-Leu-, Ser-Tyr-, Gly-Phe-, and Lys-Ala-MCA, with kcat/Km values of 10.62, 3.92, 1.17, and 0.32 μM−1 s−1, respectively. Therefore, when comparing the activities between DPP5 and DPP7, the superiority of P1 position Ala should be reinforced in DPP5. To further address P1 position specificity, we synthesized 3 additional MCA substrates with Lys fixed as the P2 position residue and altered P1 position residues, i.e. Ala, Phe, Leu, and Val. DPP5 had the largest kcat/Km value for Lys-Ala-MCA, followed by Lys-Phe- and Lys-Leu-MCA, and possessed the smallest value for Lys-Val-MCA (Table 3).
The values obtained for $k_{cat}/K_m$ strongly suggested that Ala and hydrophobic amino acids are suitable at the P1 position for the activity of DPP5. This substrate specificity was roughly in accord with that of _A. fumigatus_ DPP5, although it has been reported to further hydrolyze His-Ser-2-(4-methoxy)naphthylamide (38). Therefore, hydrolyzing activity especially toward His-Ser was a major obstacle for comprehensive understanding of DPP5 substrate specificity. To obtain general information, activities toward 21 dipeptidyl-MCA substrates were plotted against hydrophobic indexes (43) of P1 and P2 position residues (Fig. 8, A and B). Consequently, a strong positive correlation was observed between the log of activities and hydrophobic indexes of the P1 position residues. In contrast, no correlation was recognized with hydrophobic indexes of the P2 position residues. Taken together, we concluded that DPP5 prefers hydrophobic residues and Ala at the P1 position, and has no hydrophobic preference at the P2 position.

When the top 10 cleavable substrates for either DPP5 or DPP7 were aligned in respect to the ratios of DPP5 to DPP7 activities, Gly-Gly-, Lys-Ala-, Gly-Phe-, and Ser-Tyr-MCA were preferentially hydrolyzed by DPP5 (Fig. 2C) and those of DPP7 reported in our previous study (23), i.e. hydrophobic and Ala preference at the P1 position and no preference at P2 of DPP5, and hydrophobic preference at the P1 and P2 positions of DPP7.

**Subcellular Localization of DPP5 and Growth of Four DPP Gene-disrupted P. gingivalis**—Subcellular localization of DPP5 was investigated with KDP136, to avoid the effect of massive proteolytic degradation by gingipains. Western blotting demonstrated that DPP5 and HBP35 were predominantly localized in the periplasm/outer membrane fraction (Fig. 9). The highest
DPP5 activity toward Lys-Ala-MCA was shown in the periplasm/outer membrane fraction, although a part of that activity was also observed in both the cytosol and inner membrane fractions. Noticeably, Lys-Ala-MCA hydrolyzing activity was not observed in the extracellular fraction. Periplasmic localization of DPP5 was ascertained by sucrose density gradient centrifugation of unwashed total membrane fractions. The activity monitored by hydrolyzes of Lys-Ala- and Met-Leu-MCA, and the 66-kDa DPP5 immunoblot were detected in the upper fractions (Fig. 9, C and D, fractions 5–8). In contrast, HPB35 was observed predominantly in the outer membrane (fractions 14–16) and BCP was in the inner membrane fractions (fractions 9–11). These results clearly indicated predominant periplasmic localization of DPP5 in P. gingivalis.

The growth of NDP212, which lost 4 identified DPP genes, was significantly more retarded than the wild-type when the organisms were cultured in anaerobic bacteria culture medium (Fig. 10). However, growth retardation in KDP136
was more remarkable, suggesting that oligopeptidase production by gingipains is more critical under the present culture condition.

### Modeling of Three-dimensional Structure of DPP5

Three-dimensional structures of DPP5 were predicted by submission of the full amino acid sequences to the fully automated protein structure homology-modeling server ESyPred3D (30). As shown in Fig. 11, a predicted structure was obtained by use of *Stenotrophomas maltophilia* DPP4 (Protein Data Bank code 2ecf) (44) as a template, which shares a 21.0% amino acid identity with *P. gingivalis* DPP5. Similar to that structure, this model contained a C-terminal peptidase domain with an α/β hydrolase-fold, and the catalytic triad (Ser542, Asp627, His659) exists as a large cavity formed between an N-terminal propeller domain-like region and the C-terminal peptidase domain.

### DISCUSSION

In the present study, we identified and characterized *P. gingivalis* DPP5 encoded by the PGN_0756 gene, which has been annotated as prolyl oligopeptidase. This is the first report of DPP5 being demonstrated in prokaryotes, and it is the fourth DPP known to be expressed in the bacterium. *P. gingivalis* DPP5 is composed of 684 amino acids, exists as a dimeric form and is predominantly localized in the periplasm. The N terminus of native DPP5 was Thr25, indicating Met1–Gly24 as a signal peptide.
DPP5 is classified in the S9 serine peptidase family (EC = 3.4.14.-), which liberates N-terminal dipeptide Xaa–Yaa from a polypeptide, where Xaa is Ala and hydrophobic residues except Pro, with $k_{\text{cat}}/K_m$ values of 1–13 $\mu\text{M}^{-1}\text{s}^{-1}$, and no preference for Xaa, showing the highest activity in the presence of 0.1–0.4 mM NaCl with an optimum pH of 6.5–7.0. This pH optimum is identical to A. fumigatus DPP5 (38). The substrate specificity of DPP5 partially overlaps and also compensates for that of DPP7, thus expanding the dipeptide production repertoire in P. gingivalis.

The amino acid identity between P. gingivalis and A. fumigatus DPP5 is 28%, although they exhibited similar enzymatic characteristics. Until recently, 26 proteins had been nominated as DPP5 in fungi, including Trichphyton rubrum, Ajellomyces capsulata, and Schizosaccharomyces pombe. Based on the discovery of P. gingivalis DPP5, a homology search by KEGG BLASTP produced a long list of P. gingivalis DPP5-like molecules, in which the 250 proteins were obtained at the amino acid identity higher than 25% (supplemental Table S1). Those are distributed in eubacteria including the phyla Bacteroidetes, Proteobacteria, Firmicutes, Actinobacteria, Chloroflexi, Acidobacteria, Chlorocasptera, Chloroflexi, archaeon (Methanosphaerula palustris), and eukaryotes including Fungi, Plantae, and Animalia. Currently, they are annotated as either prolyl oligopeptidase, Ala-DPP, unclassified DPP, or a hypothetical protein, etc., except for the cases of fungal DPP5. At least, we confirmed that an S9 peptidase from Porphyromonas endodontalis possessed the substrate specificity identical to that of P. gingivalis DPP5.

DPP5, prolyl oligopeptidase, as well as DPP4 belong to the S9 peptidase family, which is divided into four subclasses based on the sequence characteristics around the essential Ser$^{422}$. GGSXGGGL, where X is typically Asn or Ala (S9A), GWSYGGY (9SB), GGSYGG (S9C), and GHGSHGYAFMT (S9D) (35). On this criterion, fungal and P. gingivalis DPP5 are categorized in the S9C. Similarly, 98% of 250 homologues of P. gingivalis DPP5 possess the sequence G(G/A)S(Y/F/W)GG. Thus, if substitution from Gly to Ala at the second position and from Tyr to Phe or Trp at the fourth position are admitted, most of the homologues are members of S9C. In other words,
the S9C subfamily may be equivalent to DPP5. Relevant distance among 34 representatives is observed in a phylogenetic tree (Fig. 12), which indicates that DPP5 is ubiquitously distributed within bacteria, eukaryotic species, as well as archaea and plays a role in amino acid metabolism. In addition, bacterial DPP5 especially in pathogenic bacteria may function as a virulence factor, the same as in fungi (38, 45). It should be prudently noted that molecules from Animalia (Pantherops hodgsonii) and those from Plantae (Ricinus communis and Zea may) will be evaluated in further studies.

In contrast to fungal DPP5 (38) and the P. gingivalis predominant endopeptidase gingipain (17–20, 46), P. gingivalis DPP5 is neither secreted nor included in vesicles, but rather predominantly localized in the periplasm, which seems suitable for its function to produce nutrient dipeptides. In the periplasm, diffusion of products is limited and they could be readily incorporated into the cells via transporters localized in the inner membrane. P. gingivalis DPP5 does not possess a C-terminal conserved sequence for anchoring with the outer membrane. In addition, subcellular translocation of DPP5 was independent of the type IX secretion system (PorS5) that mediates transportation of outer membrane-associated proteins including gingipains (46) and HBP35 (31), because the porT mutant did not show lateralization of the localization and activity of DPP5.5

Although the dpp5 gene PGN_0756 has been annotated as a prolyl oligopeptidase, it did not exhibit Pro specificity, but preferred Ala and hydrophobic amino acids. It has been reported that the S1 pocket of DPP4 is hydrophobic and small, which accounts for the Pro and Ala restrictions at this position (47). Hence, it could be speculated that the S1 pocket shares some similarity between DPP5 and DPP4, whereas that of DPP5 is further specialized to exclude Pro. This speculation was supported by the finding that the DPP4-specific inhibitors had no effect on the activity of DPP5. Furthermore, in contrast to their P1 preference, the similarity between DPP5 and DPP4 could be noted in the S2 pocket, i.e. both had no preference at the P2 residue, indicating similarity of the fine structure of the S2 pocket. Structurally, it has been reported that the Glu-Glu motif localized in the N-terminal propeller domain is conserved in P. gingivalis DPP4 (Glu195–Glu196, Fig. 3) and is essential for recognition of the N-terminal amine in a substrate peptide (48). However, as shown in the multiple sequence alignment, this motif is not present in P. gingivalis DPP5. Instead, Asp144–Glu145 of DPP5 might function as that motif. To understand the molecular mechanism of the substrate preference of DPP5, structural analysis using crystallography is required and a study by our group is in progress.

In conclusion, DPP5 was identified and biochemically characterized for the first time in the prokaryotic species P. gingivalis. In the bacterium, four DPPs, i.e. DPP4, DPP5, DPP7, and DPP11, are the major members together with gingipains responsible for dipeptide production. Such a combination of peptidases with altered substrate specificities certainly expands the dipeptide production repertoire, providing benefit for P. gingivalis to obtain proteinaceous energy sources from the environment.

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