Original Research article

Identification of a new subtype of dipeptidyl peptidase 11 and a third group of the S46-family members specifically present in the genus *Bacteroides*

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ABSTRACT

Peptidase family S46 consists of two types of dipeptidyl-peptidases (DPPs), DPP7 and DPP11, which liberate dipeptides from the N-termini of polypeptides along with the penultimate hydrophobic and acidic residues, respectively. Their specificities are primarily defined by a single amino acid residue, Gly^{673} in DPP7 and Arg^{673} in DPP11 (numbering for *Porphyromonas gingivalis* DPP11). Bacterial species in the phyla *Proteobacteria* and *Bacteroidetes* generally possess one gene for each, while *Bacteroides* species exceptionally possess three genes, one gene as DPP7 and two genes as DPP11, annotated based on the full-length similarities. In the present study, we aimed to characterize the above-mentioned *Bacteroides* S46 DPPs. A recombinant protein of the putative DPP11 gene BF9343_2924 from *Bacteroides fragilis* harboring Gly^{673} exhibited DPP7 activity by hydrolyzing Leu-Leu-4-methylcoumaryl-7-amide (MCA). Another gene, BF9343_2925, as well as the *Bacteroides vulgatus* gene (BVU_2252) with Arg^{673} was confirmed to encode DPP11. These results demonstrated that classification of S46 peptidase is enforceable by the S1 essential residues. *Bacteroides* DPP11 showed a decreased level of activity towards the substrates, especially with P1-position Glu. Findings of 3D structural modeling indicated three potential amino acid substitutions responsible for the reduction, one of which, Asn650Thr substitution, actually recovered the hydrolyzing activity of Leu-Glu-MCA. On the other hand, the gene currently annotated as DPP7 carrying Gly^{673} from *B. fragilis* (BF9343_0130) and *Bacteroides ovatus* (Bovatus_03382) did not hydrolyze any of the examined substrates. The existence of a phylogenic branch of these putative *Bacteroides* DPP7 genes classified by the C-terminal conserved region (Ser^{571}-Leu^{700}) strongly suggests that *Bacteroides* species expresses a DPP with an unknown property. In conclusion, the genus *Bacteroides* exceptionally expresses three S46-family members; authentic DPP7, a new subtype of DPP11 with substantially reduced specificity for Glu, and a third group of S46 family members.

Highlights

- Classification by the S1 residue Arg^{673}/Gly^{673} is reliable for S46 peptidase.
- Three S46 DPPs are exceptionally present in the genus *Bacteroides*.
- One of *Bacteroides* DPP with Gly^{673} represented authentic DPP7.
- Another DPP with Gly^{673} is a third group of S46 members with an unknown property.
- The *Bacteroides* DPP with Arg^{673} is a new type of DPP11.

Keywords: *Bacteroides*, DPP7, DPP11, *Porphyromonas gingivalis*, S46 peptidase

Introduction

Dipeptidyl-peptidase (DPP) 7 and DPP11 were first discovered in *Porphyromonas gingivalis* [1, 2] and *Porphyromonas endodontalis* [2], Gram-negative asaccharolytic anaerobes implicated as causative agents of chronic [3, 4] and acute [5, 6] periodontal diseases, respectively. These periodontal pathogens solely metabolize amino acids as carbon and energy sources, hence, their bulk degradation process of extracellular peptides is important for microorganism survival. In addition, another characteristic feature of *Porphyromonas* species is incorporation of amino acids mainly as dipeptides, not as single amino acids. These features seem to convey an advantage for *Porphyromonas* species to form subgingival periodontopathic microbial symbioses with other bacterial species, such as saccharolytic *Aggregatibacter actinomycetemcomitans* and *Prevotella intermedia*, and also *Fusobacterium* and *Prevotella* species that incorporate single amino acids.

In *P. gingivalis*, extracellular nutritional proteins are initially digested to oligopeptides by two potent types of cysteine endopeptidases, Arg-gingipain [7] and Lys-gingipain (C25.002) [8], then further degraded into di- and tri-peptides by exopeptidases, *i.e.*, DPPs [1, 2, 9-11] and prolyl tripeptidyl-peptidase A (PTP-A) [12, 13], respectively, together with the dipeptide-releasing activity of Arg- and Lys-gingipains [11]. To date, five DPPs have been identified in *P. gingivalis*, *i.e.*, DPP4, which mainly releases an Xaa-Pro (Xaa, any amino acid) dipeptide and also Xaa-Ala to a lesser extent [9, 10, 14, 15], DPP5, with a preference for Ala and aliphatic residues at the penultimate position from the N-terminus [11], DPP7 [1], and DPP11 [2]. Although the gene encoding DPP3 (generally referred to as DPPIII) specific for Arg (M49.003) is also present, this DPP does not seem to be involved in extracellular protein metabolism, because DPP3 appears to be located in the cytoplasm [11]. Additionally, acylpeptidyl oligopeptidase (AOP) produces di- and tri-peptides preferentially from N-terminally acylated peptides [16]. Therefore, in consideration of the fact that

most extracellular polypeptides derived from human serum are N-terminally acylated, proteinaceous nutrients in gingival crevicular fluid should be effectively degraded into di- and tri-peptides, and then incorporated into *P. gingivalis* by co-operative actions of these peptidases [17]. DPP4, DPP5, PTP-A, and AOP belong to the peptidase family S9, and DPP7 and DPP11 are classified into the S46 peptidase.

Currently, the S46 family is composed of three members, DPP7 (S46.001), DPP11 (S46.002), characterized in *P. gingivalis* (PgDPP7, PgDPP11, respectively), and DAPBII from *Pseudoxanthomonas mexicana* (S46.003) [18, 19]. Since the biochemical property and substrate preference of DAPBII are nearly identical to those of PgDPP7, the S46 family should be composed solely of two members, *i.e.*, DPP7 preferentially liberates dipeptides with both P1 and P2 hydrophobic residues, and DPP11 is specific for P1 Asp and Glu. Interestingly, we previously found that each of their P1 substrate specificities is primarily defined by a single amino acid residue, Gly^{673} in DPP7 and Arg^{673} in DPP11 (numbering in PgDPP11) [20]. In *Shewanella* DPP11, Arg^{673} is substituted by Ser, then Lys^{680} plays the role of S1. Recently, the 3D structure of DPP11 was reported [21, 22], and analysis of the complex of di-, tri-, and tetra-peptides suggested that an increase in conformational entropy of DPP11 is the primary driving force for substrate recognition [22].

Orthologs of DPP7 and DPP11 are widely distributed in the phyla *Proteobacteria* and *Bacteroidetes*, and these bacteria generally possess one each of the DPP7 and DPP11 genes. However, it was unexpectedly observed that the genus *Bacteroides* contain three members of the S46 family, one is DPP7 while the other two are DPP11 genes, annotated based on full-length similarity. In contrast, our previous proposal regarding classification by Gly^{673} and Arg^{673} indicates the existence of two DPP7 and one DPP11 gene in *Bacteroides* species. Accordingly, biochemical and enzymatic analyses are indispensable to determine these entities.

In the present study, recombinant forms of putative *Bacteroides* DPP7 and DPP11 genes were expressed exogenously, and their substrate specificities were determined for classification. Our results clearly show the validity of essential Gly^{673} and Arg^{673} residues, and the inappropriateness of full-length similarities for classification of the family of S46 DPPs. Furthermore, we found that *Bacteroides* DPP11 specifically exhibits decreased activity compared to authentic DPP11, especially for substrates with P1-position Glu. Also, 3D modeling and substitution analysis demonstrated that Asn^{650} is partially responsible for this phenomenon. Additionally, the present findings indicate the existence of the third S46 member, though its real role remains unknown.

2. Materials and methods

2.1. Materials

pQE60 (Qiagen Inc., Chatsworth, CA) and pTrcHis2-TOPO (Invitrogen, Carlsbad, CA) were used as expression vectors. Restriction and DNA-modifying enzymes were purchased from Takara Bio (Tokyo, Japan) and New England Biolabs (Ipswich, MA), respectively. Quick Taq HS DyeMix and KOD-Plus-Neo DNA polymerase came from Toyobo (Tokyo, Japan). Met-Leu- and Leu-Asp-MCA were obtained from the Peptide Institute (Osaka, Japan). Leu-Glu-, Leu-Leu-, Phe-Leu-, and His-Leu-MCA were synthesized by Thermo Fisher Scientific (Ulm, Germany) and Scrum (Tokyo, Japan). Oligonucleotide primers were synthesized by FASMAC (Atsugi, Japan). Low-molecular-weight marker was obtained from GE Healthcare (Little Chalfont, UK). Genomic DNA from *B. fragilis* strain NCTC 9343 (JCM 11019^T), *Bacteroides vulgatus* JCM 5826^T, *Bacteroides thetaiotaomicron* JCM 5827^T, *Bacteroides ovatus* RDB 09006, and *Bacteroides dorei*

JCM 13471 were provided by the RIKEN BRC through the National Bio-Resource Project of the MEXT, Japan. Bacterial strains *B. fragilis* YCH10, *B. thetaiotaomicron* KYU12, and *Bacteroides uniformis* KYU12 were generous gifts from Dr. Mariko Naito of Nagasaki University.

2.2. Culture Conditions

P. gingivalis strain ATCC 33277, *B. fragilis* YCH10, *B. thetaiotaomicron* KYU12, *B. uniformis* KYU12, and *B. dorei* were grown anaerobically (80% N_2, 10% CO_2, 10% H_2) in Anaerobic Bacterial Culture Medium broth (Eiken Chemical, Tokyo, Japan), supplemented with 0.5 μg/ml of menadione. Following centrifugation, bacterial cells were suspended in phosphate-buffered saline (PBS) at pH 7.4, then centrifuged at 6000 x *g* for 15 min at 4 ˚C. Cell pellets were re-suspended in PBS to adjust absorbance at 600 nm to 5.0, then used for DPP assays.

2.3. Construction of Expression Plasmids

The DNA fragment of BVU_2252, deduced as *B. vulgatus* S46 peptidase (Asp^{20}-Glu^{728}) (UniProtKB, YP_001299533.1/MEROPS code MER034614), was amplified by PCR using a set of primers (Table 1), with genomic DNA used as a template, then the PCR product was ligated with pTrcHis2-TOPO according to the manufacturer's protocol. DNA fragments of the *B. ovatus* genes, Bovatus_00118 (Asn^{2}-Glu^{719}) (MEROPS code, MER109242) and Bovatus_03382 (Lys^{2}-Arg^{713}) (MER09141), were amplified by PCR using sets of primers. The former and latter fragments were digested by *Bam*HI and *Bgl*II, respectively, then inserted into the *Bam*HI site of pQE60. DNA fragments of the *B. fragilis* genes, BF9343_0130 (Asn^{2}-Gly^{725}, MEROPS code MER039992), and BF9343_2925 (Met^{2}-Glu^{721}, MER039991), were amplified by PCR using sets of primers. The PCR

fragments were digested by *Bam*HI, then inserted into the *Bam*HI site of pQE60. Expression plasmids of *P. gingivalis* DPP7 and DPP11, *B. fragilis* DPP7 (BF9343_2924), *P. endodontalis*, *B. vulgatus*, *Capnocytophaga gingivalis*, *Flavobacterium psychrophilum*, and *Shewanella putrefaciens* DPP11 have been reported [2, 11, 20, 23].

2.4. Amino Acid Numbering of DPP7 and DPP11

The amino acid numbers of all recombinant proteins were counted as those of PgDPP11 to avoid confusion. Accordingly, since Gly^{666} of PgDPP7, Gly^{678} of BF9343_0130, Gly^{674} of BF9343_2924, Arg^{674} of BF9343_2925, and Arg^{670} of *P. endodontalis* DPP11 (PeDPP11) are equivalent to Arg^{673} of PgDPP11, they were numbered as 673. In addition, Asn^{651}, Gly^{671}, and Leu^{679} of BfDPP11 are equivalent to PgDPP11 Thr^{650} Asn^{670}, and Val^{678}, respectively, therefore their numbers were unified to 650, 670, and 678, respectively.

2.5. In vitro *Mutagenesis*

PCR-based *in vitro* mutagenesis was carried out to substitute Gly^{673} to Arg in the expression plasmids of *B. fragilis* BF9343_0130 and BF9343_2924 using primer sets (Table 1), as previously reported [2, 20]. Substitutions of Asn650Thr, Gly670Asn, and Leu678Val in BfDPP11 (BF9343_2925) were performed, and Thr650Asn and Asn670Gly in the plasmid for PgDPP11 were inversely produced using sets of primers. Additionally, the double mutations of Asn650Thr and Leu678Val were introduced into the plasmid for BfDPP11.

2.6. Expression and Purification of Recombinant Proteins

Escherichia coli XL-1 Blue cells carrying respective expression plasmids were cultured in Luria-Bertani broth supplemented with 75 μg/ml of ampicillin at 37 ˚C. Recombinant proteins were induced with 0.2 mM isopropyl-β-thiogalactopyranoside at 30 ˚C for 4 h, then purified from cell lysates using Talon affinity chromatography, as previously reported [2]. Purified samples were stored at -80 ˚C until use.

2.7. Measurement of DPP Activity

DPP7 activity was measured with Phe-Leu- and Leu-Leu-MCA, and DPP11 activity was determined using Leu-Asp- and Leu-Glu-MCA, as previously reported [11]. Briefly, the reaction was started by addition of recombinant proteins (4 − 200 ng) or bacterial cell suspensions (4 μl of A_{600} = 5) in a reaction mixture (200 μl) composed of 50 mM sodium phosphate (pH 7.0), 5 mM EDTA, and 20 μM dipeptidyl MCA. After 30 min at 37 ˚C, fluorescence intensity was measured with excitation at 380 nm and emission at 460 nm. Activity is expressed as pmol min^{-1} μg^{-1} of protein.

To determine enzymatic parameters, recombinant proteins were incubated with various concentrations of dipeptidyl MCA. Data were analyzed using a nonlinear regression curve fitted to the Michaelis-Menten equation with the GraphPad Prism software program (San Diego, CA). Values were calculated from three independent measurements and are shown as average ± S.D.

2.8. 3D Modeling of BfDPP11

In order to obtain insight regarding the 3-dimensional structure of BfDPP11 and particularly

its active site, we employed homology modeling. The model was generated via the server Phyre2 (Protein Homology/Analogy Recognition Engine) [24] using the one-to-one threading tool in expert mode. Similar to PeDPP11, BfDPP11 is expected to undergo a conformational change upon peptide binding, resulting in complete formation of the active site. Therefore, PeDPP11 in complex with a peptide was chosen as a template for the modeling routine. The coordinates of PeDPP11 in complex with the dipeptide Arg-Asp (PDB code: 5JWG) [22] were subjected to structural alignment and further model building.

2.9. Construction of Phylogenic Trees

In the MEROPS database (release 9.6), 264 members of the S46 family were chosen for construction of phylogenic trees, with ClustalX software [25] used to align the sequences. The conserved amino acid sequence at the C-terminal region (Pro^{571}-Leu^{700} of PgDPP11) was used for generating the phylogenic trees. Phylogenic analysis with the neighbor-joining (NJ) algorithm [26] was conducted using MEGA version 5 [27].

2.10. Miscellaneous

Recombinant proteins (0.4 μg) were separated by PAGE in the presence of 0.1% (w/v) of SDS with a polyacrylamide concentration of 10% (w/v). Separated proteins were stained with Coomassie Brilliant Blue. Protein concentrations were determined by the Coomassie Brilliant Blue dye method (Bio-Rad) using bovine albumin as a standard.

3. Results

3.1. Characterization of Three S46-Family Members in the Genus Bacteroides

To date, commercially available synthetic substrates, Ala-Phe-*p*-nitroanilide [1], and Lys-Ala- and Met-Leu-MCA [28], had been used for measuring DPP7 activity. However, since we established that DPP7 prefers hydrophobic residues at the P2 as well as P1 position [23], we initially synthesized Leu-Leu- and Phe-Leu-MCA with hydrophobic residues at both positions to test the potentials as DPP7 substrates. The k_{cat}/K_m values of PgDPP7 were 37.7 μM^{-1} sec^{-1} for Leu-Leu-MCA and 15.3 μM^{-1} sec^{-1} for Phe-Leu-MCA, which were much higher than those for Met-Leu-, His-Ala- (Table 2), and Lys-Ala-MCA (data not shown). Thus, Leu-Leu- and Phe-Leu-MCA were used in the present study for measurement of DPP7 activity.

Species of the genus *Bacteroides* frequently contain three genes of the S46 family (Supplemental Table S1), and their annotation based on the full-length similarity to PgDPP7 and PgDPP11 was confusing. For example, two genes (BF9343_2924, BF9343_2925) in *B. fragilis* are annotated as DPP11 (Table 3); BF9343_2925 with Arg^{673} seemed to be DPP11, while BF9343_2924 carrying Gly^{673} could be DPP7 according to the classification of DPP7 with the S1 Gly^{673} residue. Similarly, the *B. ovatus* putative DPP11 gene (Bovatus_00118) with Gly^{673} was considered to be DPP7. To determine the enzymatic characteristics, three S46-family members of *B. fragilis* and two from *B. ovatus* were expressed in *E. coli*, and purified to homogeneity (Fig. 1). One of the putative DPP11 genes, BF9343_2925 (MER039991) (Arg^{673}), expressed a protein truly hydrolyzing Leu-Asp-MCA efficiently and Leu-Glu-MCA weakly, confirming its DPP11 entity (BfDPP11). Meanwhile, another DPP11 gene, BF9343_2924 (MER039993) with Gly^{673}, expressed a protein specifically hydrolyzing Leu-Leu-MCA and not Leu-Asp/Glu-MCA, exhibiting its entity as DPP7

(BfDPP7). These findings demonstrated that the presence of Gly^{673} is a definitive feature of DPP7, while the annotation based on the full-length amino acid sequence similarity misled the identification.

In addition, the enzymatic property of the putative DPP7 gene BF9343_0130 (MER039992) (Gly^{673}) was examined. BF9343_0130, which shows approximately 43% amino acid similarity to PgDPP7, was successfully expressed and purified (Fig. 1). However, the purified protein hydrolyzed neither Leu-Leu- nor Leu-Asp/Glu-MCA, and did not hydrolyze any other synthetic substrates for DPPs, such as Gly-Pro-, Ser-Tyr-, Gly-Phe-, Met-Leu-, Lys-Leu-, and Lys-Val-MCA (data not shown). Since we previously demonstrated that PgDPP7 bearing the mutation Gly673Arg partially acquired a DPP11-like property [20], the Gly673Arg substitution was introduced into BF9343_0130 as well as BF9343_2924/BfDPP7. As a result, BF9343_2924/BfDPP7 Gly673Arg slowly hydrolyzed Leu-Glu-MCA, showing a change into the DPP11 type. However, BF9343_0130 Gly673Arg was still not able to hydrolyze any substrates. These results further suggested a distant relationship of BF9343_0130 (Gly^{673}) with BF9343_2924/BfDPP7 (Gly^{673}) as well as BF9343_2925/BfDPP11 (Arg^{673}).

As another DPP7 gene in *Bacteroides* species carrying Gly^{673}, we examined Bovatus_00118, annotated as DPP11, and Bovatus_03382, annotated as DPP7, in *B. ovatus*. As shown in Fig. 1, Bovatus_00118 (Gly^{673}) was not correctly annotated, but was truly DPP7 (BvDPP7) and selectively hydrolyzed Leu-Leu-MCA. In addition, similar to *B. fragilis* BF9343_0130 (Gly^{673}), putative *B. ovatus* DPP7 (Bovatus_03382, Gly^{673}) did not hydrolyze any synthetic MCA substrates. The sequence similarity between Bovatus_03382 and BF9343_0130 was close to 77%, and that between BF9343_2924/BfDPP7 and Bovatus_00118/BvDPP7 was around 84%, while the similarities between all other combinations of two proteins within the species were lower (Table 3). Therefore, BF9343_0130 from *B. fragilis* and Bovatus_03382 from *B. ovatus* are not DPP7, and considered

classifiable as novel DPP. Taken together, our findings revealed that gene annotation based on full-length amino acid similarities did not match biochemical identification of the S46 peptidases DPP7 and DPP11. In addition, the *Bacteroides* S46 DPP genes carrying Gly^{673}, which are currently classified as DPP7, should be considered as a third group of S46 family members.

3.2. Hydrolyzing Properties of DPP11 from Genus Bacteroides

We previously reported the presence of two types of DPP11 in respect to substrate specificity; DPP11 with Arg^{673} from *P. gingivalis*, *P. endodontalis*, *C. gingivalis*, and *F. psychrophilum*, with higher k_{cat}/K_m values for Leu-Asp-MCA, and DPP11 with Ser^{673}/Lys^{680} from *S. putrefaciens* with a higher k_{cat}/K_m value for Leu-Glu-MCA [20]. In order to comprehensively understand the Asp/Glu preference in DPP11, we reexamined the enzymatic characteristics of DPP11s from *P. gingivalis*, *P. endodontalis*, *C. gingivalis*, *F. psychrophilum*, *S. putrefaciens B. fragilis*, and *B. vulgatus* (Fig. 2). Purified recombinant DPP11s were found to migrate at 70- to 85-kDa positions on SDS-PAGE, reflecting their calculated molecular masses. All DPP11 molecules except for *S. putrefaciens* DPP11 showed hydrolyzing activities more preferential for Leu-Asp- to Leu-Glu-MCA, though *B. vulgatus* DPP11 (BvDPP11) displayed extremely low activities. The ratios of activity toward Leu-Glu-MCA per those for Leu-Asp-MCA of *P. gingivalis*, *P. endodontalis*, and *C. gingivalis* DPP11 were 0.47, 0.34, and 0.34, respectively, while the ratios of BfDPP11 and BvDPP11 were substantially lower (0.014 and 0.018, respectively). The k_{cat}/K_m value of BfDPP11 for Leu-Asp-MCA was 40.9 ± 4.4 (μM^{-1} sec^{-1}) and for Leu-Glu-MCA was 0.87 ± 0.04 (μM^{-1} sec^{-1}), indicating that BfDPP11 cleaves Leu-Asp-MCA at a level 47-fold more efficiently as compared to Leu-Glu-MCA. Hence, even though the substantially low k_{cat}/K_m value of BfDPP11 for Leu-Glu-MCA was caused by both a decrease in k_{cat} and an increase in K_m, it was evident that the increase in K_m had a larger impact on

that change (Table 2).

Next, we investigated DPP11 activity in *Bacteroides* cells. In *P. gingivalis,* Leu-Asp- and Leu-Glu-MCA were shown to be primarily hydrolyzed by DPP11. If the same is also true for *Bacteroides* species, the inefficient Leu-Glu-MCA hydrolysis could be reproduced in these cells. Hence, we compared cellular activities toward the two substrates, and found that the ratio of hydrolysis of Leu-Glu-MCA per hydrolysis of Leu-Asp-MCA in *P. gingivalis* cells (0.496) was comparable to that of recombinant PgDPP11 (0.468) (Fig. 3). On the other hand, the ratio with *B. fragilis* cells was 0.17, which was larger than that of BfDPP11 (0.014), though substantially smaller than the ratio of PgDPP11 and *P. gingivalis* cells. Accordingly, it was confirmed that the ratio determined with bacterial cells principally reflected that of DPP11, though the differential extent was rather neutralized, possibly by the presence of minor Asp- and Glu-hydrolyzing activities in the cells. Similarly, the cellular ratios of *B. thetaiotaomicron*, *B. uniformis*, and *B. dorei* ranged at 0.09-0.23, which indicated that *Bacteroides* DPP11 commonly possesses very low activities for Leu-Glu-MCA.

3.3. BfDPP11 3D Structural Modeling and Mechanism of its Inefficient Hydrolysis

We performed 3D modeling of BfDPP11 using the structure of PeDPP11 (PDB code: 5JXK) [22], which indicated that BfDPP11 also has a bilobal architecture with an entirely helical domain capping the catalytic domain bearing a typical chymotrypsin double β-barrel fold. PeDPP11 3D structures in complexes with peptides (5JY0, 5JWG, 5JWI, 4XZY) and PgDPP11 showed that the S1 subsite is formed by Arg^{337}, His^{649}, Thr^{650}, Gly^{652}, Asn^{670}, Arg^{673}, Gly^{677}, Gly^{680}, Asp^{681}, and Ser^{691} (PgDPP11 numbering) [21, 22]. Among the ten residues, Gly^{652}, Arg^{673}, Gly^{677}, Gly^{680}, and Asp^{681} are conserved, while the others, *i.e.*, $Arg^{337,}$ His^{649}, Thr^{650}, Asn^{670}, and Ser^{691} are substituted

by Asn, Asp, Asn, Gly, and Ala, respectively, in BfDPP11 (Fig. 4). Hence, we subsequently analyzed our model of BfDPP11 in order to structurally explain the inefficient cleavage of Leu-Glu-MCA.

The role of Arg^{337} of PgDPP11 has been extensively examined [21] and its substitution to Asn, Ala, and Gly was shown to lead to a significant increase in enzymatic activity. Thus, the Arg337Asn substitution of BfDPP11 could not explain the activity reduction of BfDPP11. Furthermore, His^{649} and Ser^{691} appear to be rather distant from the P1-position of a dipeptide, thus the influence of the two substitutions seems to be limited. In the rest two residues, Thr^{650} in PgDPP11 stabilizes the glutamic acid of incoming peptides via a hydrogen bond (Fig. 5). By superimposing BfDPP11 to PeDPP11 in a complex with Arg-Glu, we verified that the substitution Thr^{650} of PgDPP11 to Asn in BfDPP11 results in steric clashes according to the side chain orientation. In this manner, binding of incoming peptides with glutamic acid in the P1 position would be unfavorable in BfDPP11. Asn^{670} in PgDPP11 also stabilizes the Glu of incoming peptides via a hydrogen bond. However, Asn^{670} is replaced by Gly in BfDPP11, preventing the stabilization of the substrate Glu, resulting in decrease of the activity verified for Leu-Glu-MCA. On the other hand, Val^{678} in PgDPP11 is replaced by Leu in BfDPP11. Although Val^{678} does not directly form an S1 subsite, we speculate that the larger side chain of Leu of BfDPP11 influences the conformation of Arg^{673}, which would explain the decrease in activity for Leu-Glu-MCA.

To verify our hypothesis, we expressed PgDPP11 and BfDPP11 with these substitutions (Fig. 6). The activity of BfDPP11 Asn650Thr displayed a 3.1-fold increase in Leu-Asp-MCA, with a further increase (8.1-fold) observed for the activity of Leu-Glu-MCA. As a result, the Glu/Asp ratio of the activity was increased by 2.6-fold in BfDPP11 Asn650Thr. In contrast, BfDPP11 Gly670Asn revealed little change. The third mutant, BfDPP11 Leu678Val, lost the activity for the two substrates, while the double mutation (Asn650Thr/Leu678Val) showed an intermediate amount of

activity between those of the respective mutants. We also performed two reverse mutations with PgDPP11, Thr650Asn, and Asn670Gly. As a result, PgDPP11 Thr650Asn completely lost the activities for Leu-Asp-MCA and Leu-Glu-MCA, and PgDPP11 Asn670Gly revealed extremely low activities for those (0.53% and 0.18%, respectively). Therefore, all mutations except for Gly670Asn in BfDPP11 had effects on the activities of BfDPP11 or PgDPP11. In particular, it should be emphasized that mutual exchanges between Thr^{650} of PgDPP11 and Asn^{650} of BfDPP11 partially mimicked the change between PgDPP11 and BfDPP11.

When the amino acid substitutions at positions 650, 670, and 678 were reexamined in the sequences, only Thr^{650} and Asn^{650} were found to be strictly distributed in non-*Bacteroides* and *Bacteroides* DPP11, respectively (Fig. 4). In contrast, residues at positions 670 and 678 had variations even among listed DPP11s, findings compatible with our biochemical data (Fig. 6). Nevertheless, it should be noted that the Leu-Glu-MCA/Leu-Asp-MCA ratio of PgDPP11 (0.468) was still much greater than that of BfDPP11 Asn650Thr (0.038), suggesting that other factors may be involved in this phenomenon.

4. Discussion

To date, the enzymatic properties of both DPP7 and DPP11 have been characterized in only three bacterial species, *i.e.*, *P. gingivalis* [1, 2], *P. endodontalis* [2, 28], and *C. gingivalis* [20], while either one of DPP7 or DPP11 has been characterized in *B. fragilis*, *B. vulgatus*, *F. psychrophilum*, *S. putrefaciens* [20], *P. mexicana* [18], and *Capnocytophaga canimorsus* [29]. At present, though their homologues are widely distributed among eubacteria, most S46-family members are tentatively classified by their sequence similarity to authentic S46 members of *P. gingivalis*, and their enzymatic characteristics remain to be elucidated.

In the present study, we confirmed the validity of the classification criteria for the S1 amino acid residues Gly^{673}/Arg^{673} for identification of DPP7 and DPP11, and found that classification of the S46 peptidase based on full-length similarity misleads the annotation. For example, putative *Bacteroides* DPP11 genes (BF9343_0130, Bovatus_00118), which exhibit a higher similarity to PgDPP11 than PgDPP7 but carry Gly^{673}, encode DPP7. Similarly, Hack *et al*. [29] recently reported that the gene Ccan_08540 from *C. canimorsus* encoding a DPP with Gly^{673} convincingly exhibited DPP7 activity, though the gene was tentatively annotated as DPP11.

We also demonstrated that *Bacteroides* DPP11 exhibited decreased hydrolyzing activity, especially toward the substrate with P1-position Glu. Accordingly, there are three types of DPP11 based on substrate specificity, with the first being Arg^{673} possessing greater preference for the P1-position Asp of a substrate (Asp>Glu type) represented by *P. gingivalis*, the second *Shewanella* type DPP11 possessing Ser^{673} and Lys^{684}, and exhibiting a higher Glu preference (Glu>Asp type), and the third *Bacteroides* type carrying Arg^{673}, which is much less potent for Glu (Asp>>Glu). The present and previous 3D structural modeling and substitution analyses revealed that alterations in the P1-position preference among the three types appeared to be mediated by a few amino acid substitutions. Hence, essential amino acid residues can be used for simple discrimination of the 3 DPP11 types; Thr^{650} and Arg^{673} for the Asp>Glu type, Asn^{650} and Arg^{673} for the Asp>>Glu type, and Thr^{650}, Ser^{673}, and Lys^{684} for the Glu>Asp type.

The present findings also indicate the existence of the third group in S46 peptidase, which has been annotated simply as DPP7 in *Bacteroides* species. Although these putative DPP7 genes, such as BF9343_0130 and Bovatus_03382, showed more than 40% sequence similarity to PgDPP7 and carrying Gly^{673}, they showed no hydrolytic activity for the dipeptidyl MCA currently available in our laboratory. *In vitro* mutagenesis analysis further indicated that BF9343_0130 and Bovatus_03382 are substantially distant from DPP7 as well as DPP11. The possibility of a

pseudogene cannot be excluded in this entity, however, general distribution of the third S46 gene in the genus *Bacteroides* strongly suggests its physiological significance. Among previously reported DPPs, DPPX/DPP10, possessing structural similarity to DPP4, has no peptidase activity due to a point mutation in its active site [30]. In this analogy, the third group of S46 members might have no peptidase activity. A further study should be needed for characterization of this group.

Taken together, we propose that there are three members of the S46 family, DPP7, DPP11, and an uncharacterized group. We previously reported that the phylogenic tree of the S46 family, with construction based on the C-terminal conserved region (Ser^{571}-Leu^{700}) rather than the tree based on the full-length form, matches the distribution of DPP7 and DPP11 [20]. Since the present analysis demonstrated the presence of *Bacteroides*-type DPP11 and the third S46 group members, we reexamined the phylogenic tree. As shown in Fig. 7, S46 peptidases have two branches of DPP7, one that includes *Porphyromonas* and *Bacteroides* species, and the other with *Shewanella* and *Xanthomonas* species. DPP7 and DPP11 of the genus *Bacteroides* form branching links independent of other DPP7 and DPP11 groups, respectively, and the third group is arranged at the opposite end from *Bacteroides* DPP7. As a result, classification of most parts of S46 peptidases was ascertained in the present study, except for Group 1, which is composed of an unassigned S46 peptidase possessing Gly^{673} or Ser^{673}. The members of this branch seem to have sequence variations, and compose a mixture of DPP7 and DPP11.

In *P. gingivalis,* the two S46-family genes are localized at separate loci as PGN_0607 (DPP11) and PGN_1479 (DPP7). In contrast, two of the three S46 family genes of *Bacteroides* species are adjacently located, *i.e.*, BF9343_2924/DPP7 and BF9343_2925/DPP11 in *B. fragilis* are one-base overlapped at the stop codon TAA of the former and start codon, ATG, of the latter (overlap underlined). Moreover, the genes of Bovatus_00117/BfDPP11 and Bovatus_00118/BfDPP7 in *B. ovatus* are directly connected without a gap. Hence, these two genes form an operon. These

observations suggest that the gene duplication possibly occurred in an S46-family ancestor gene, then became to the DPP7 and DPP11 genes. In contrast, the third S46-family gene is separately localized from the other two S46-family genes on the genome, and amino acid identity between BfDPP7 and BfDPP11 (42.8%) was found to be higher than that between BF9343_0130 (unidentified group) and BfDPP7 (37.5%), as well as that between BF9343_0130 (unidentified group) and BfDPP11 (35.7%) (Table 3).

Finally, it is important to emphasize that newly synthesized Phe-Leu- and Leu-Leu-MCA are more potent DPP7 substrates compared to Lys-Ala- and Met-Leu-MCA, which were previously used. These substrates should be valuable for examining the DPP7 activities of oral and intestinal bacterial cells. However, we would like to note that k_{cat}/K_m of PgDPP7 for Leu-Leu-MCA was still at a one to 34 ratio of that of *P. gingivalis* DPP4 for Gly-Pro-MCA [15] and one to 14.5 of that of PgDPP11 for Leu-Asp-MCA. We suppose that this may be due to the necessity in DPP7 for exertion of the broad specificity. In other words, in contrast to DPP4 and DPP11, which are highly specific for Pro and Asp/Glu, respectively, DPP7 is able to accept P1-position hydrophobic residues with various residual groups. This redundancy of DPP7 may be achieved by a sacrifice in k_{cat}/K_m value.

Acknowledgments

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Author contributions

Author contributions for this study were as follows: conceptualization (T.K.N.), methodology (T.K.N., Y.O-N., A.G.B., T.O.), investigation (H.N., T.F., A.G.B., Y.O-N., T.K.N. T.O.), writing of original draft (T.K.N.), review and editing (Y.O-N., T.K.N., A.G.B.), and funding acquisition (T.K.N., Y.O-N., N.H.).

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Table 1

Primers and plasmids used in this study. Restriction and mutated sites are shown by italics and underlining, respectively.

Name	Sequence	Plasmid (substituted nucleotides)
5BF9343_0130N2Bam	TTTATA*GGATCC*AATAAAATGAAAGTGATTA	pTrcHis2BF0130
3BF9343_0130G725Bam	GTGATG*GGATCC*CCCCTCTATTTTCAGTTCT	
5BF9343_2925M2Bam	ATATA*GGATCC*ATGAAAAGAAACTTATTATC	pQE60BF2925
3BF9343_2925E721Bam	TCTGT*GGATCC*TTCTTCTACAATATCCAGCT	
5BVU_2252D20	GACGAGGGGATGTGGATGCTGACTGA	pTrcHis2BV2252
3BVU_2252E728	TTCCACAATGGTCATTTCATCTACCA	
5BF9343_0130G673R	CGAAACTGGGAAGCCATGAGCAG	pTrcHis2BF0130Gly673Arg (GGA>CGA)
3BF9343_0130D672	ATCGAACGCCAATCCCAGCAATT	
5BF9343_2924G673R	CGTAACTGGGAATCACTGAGCGG	pTrcHis2BF2924Gly673Arg (GGT>CGT)
3BF9343_2924D672	ATCGAATGCACAACCGATCAATT	
5 PgDPP11T650N	AATACCGGCGGCAACTCAGGCAGTCCGGTC	pQPgDPP11Thr650Asn (ACA>AAT)
3 PgDPP11H649	ATGTGTGGTGGCACAAAAGGCGACAGG	
5PgDPP11N670G	GGATTCGATCGTAACTGGGAGGGAGTCGG	pQPgDPP11Asn670Gly (AAC>GGA)
3PgDPP11L669	GAGACCGATCAGTTCGCCGTTGGCATTCA	
5BfDPP11N650T	ACAACAGGAGGAAATTCCGGAAGCC	pQBF2925Asn650Thr (AAT>ACA)
3BfDPP11D649	GTCTGTATTGACAATGAAGCAAACAGGCA	
5BfDPP11G670N	AACTTCGATCGTAATTATGAAGGCCTGAC	pQBF2925Gly670Asn (GGA>AAC)
3BfDPP11T669	GTCCCGATCAATTGCCCTTTTCCATTGA	
5BfDPP11L678V	GTCACAGGAGACATTGCTTTCCGG	pQBF2925Leu678Val (CTG>GTC)
3BfDPP11G677	GCCTTCATCATTACGATCGAATCCGGTC	

Table 2

Enzymatic parameters of *P. gingivalis* and *B. fragilis* DPPs.

Dipeptidyl peptidase	MCA peptide	k_{cat} (sec^{-1})	K_m (μM)	k_{cat}/K_m ($\mu M^{-1}\ sec^{-1}$)	Reference
P. gingivalis					
DPP4	Gly-Pro-	121,752 ± 2,974	94.9 ± 2.1	1,283.3 ± 22.5	[15]
DPP5	Lys-Ala-	1,948 ± 165	185 ± 21	10.5	[11]
DPP7	Met-Leu-	394 ± 79	39.6 ± 16.0	10.6 ± 2.5	[23]
	Leu-Leu-	753.9 ± 1.9	20.0 ± 0.6	37.7 ± 1.1	this study
	Phe-Leu-	2,044 ± 254	134.2 ± 18.9	15.3 ± 0.6	this study
	His-Leu-	1,137 ± 203	648 ± 136	1.73 ± 0.05	this study
DPP11	Leu-Asp-	10,707 ± 140	19.5 ± 0.4	547.4 ± 6.3	[17]
	Leu-Glu-	13,587 ± 577	80.9 ± 5.7	167.7 ± 5.3	this study
B. fragilis					
DPP11	Leu-Asp-	7,379 ± 994	183.3 ± 41.1	40.9 ± 4.4	this study
	Leu-Glu-	25,865 ± 2,473 $x10^3$	29,710 ± 1,629 $x10^3$	0.87 ± 0.04	this study

Table 3

Essential amino acids, sequence identity, and identification of *Bacteroides* S46 DPP members.

					Similarity (%)							
Species	Gene	MEROPS ID	Annotation[a]	Xaa[673]	2924 (BfDPP7)	2925 (BfDPP11)	00118 (BoDPP7)	03382 (Bo-third)[b]	PgDPP7	PgDPP11	Identification	Reference
B. fragilis	0130	039992	DPP7	Gly	37.5	35.7	37.1	76.9	43.0	34.2	third group[b]	this study
	2924	039993	DPP11	Gly	100	45.8	84.3	38.2	39.5	44.7	DPP7	[20]
	2925	039991	DPP11	Arg		100	44.9	35.3	37.2	45.1	DPP11	this study
B. ovatus	00118	0109242	DPP11	Gly			100	37.6	37.5	44.5	DPP7	this study
	03382	0109141	DPP7	Gly				100	40.5	34.9	third group[b]	this study
P. gingivalis	1479	0014366	DPP7	Gly					100	36.7	DPP7	[1]
	0607	0034628	DPP11	Arg						100	DPP11	[2]

[a]Based on comparison of full-length amino acid sequence (MEROPS). Genomic sequence data shown are from *B. fragilis* ATCC 25285 (BF9343), *B. ovatus* ATCC 8483 (Bovatus), and *P. gingivalis* ATCC 33277 (PGN). [b]See text in detail.

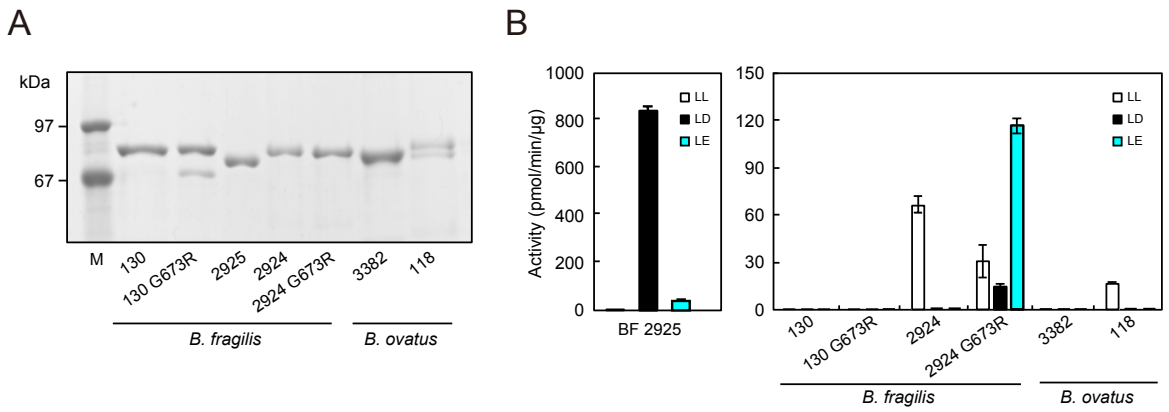

Fig. 1. Activities of *B. fragilis* and *B. ovatus* DPPs in S46 family.

(**A**) Putative S46 peptidase genes from *B. fragilis* (130, BF9343_0130; 2925, BF9343_2925; 2924, BF2925_2924), *B. ovatus* (3382, Bovatus_03382; 118, Bovatus_03382), and Gly673Arg mutants of BF9343_0130 and BF2925_2924 were expressed. Purified DPPs and their mutants (0.4 μg) were separated on SDS-PAGE. M, low-molecular-weight marker. (**B**) DPP activities were measured with Leu-Leu-, Leu-Asp-, and Leu-Glu-MCA. Values are shown as the mean ± S.D. (n = 3).

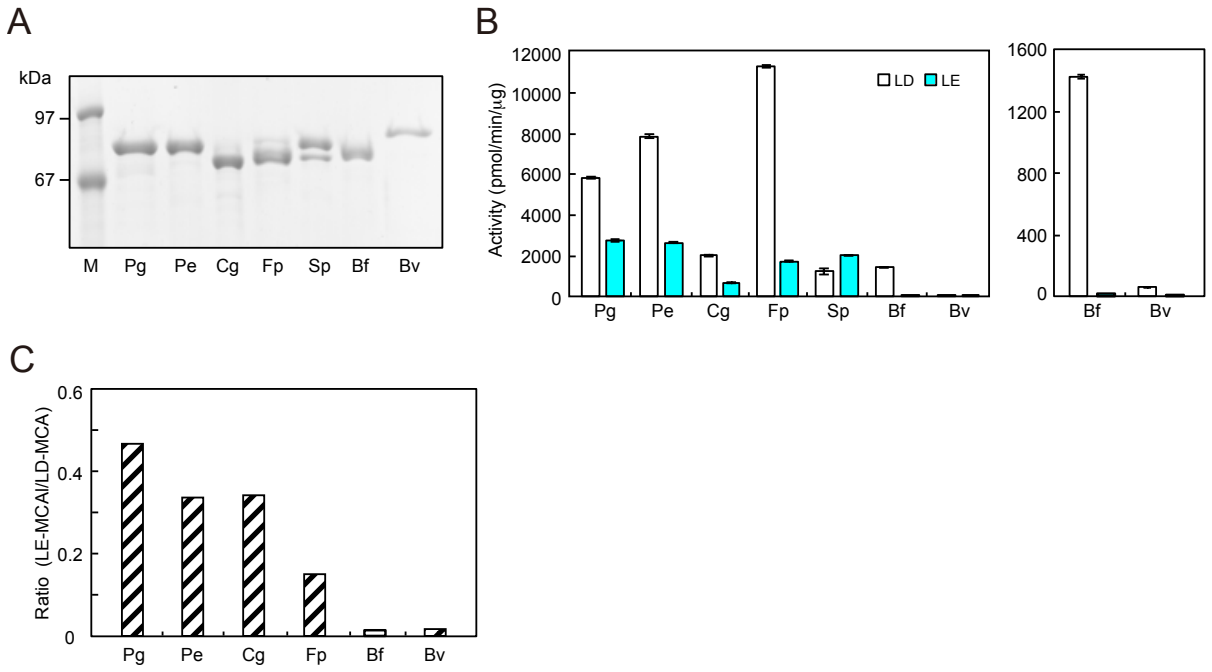

Fig. 2. Expression and characterization of three types of DPP11s.

(**A**) Recombinant forms of DPP11 (0.4 μg) from *P. gingivalis* (Pg), *P. endodontalis* (Pe), *C. gingivalis* (Cg), and *F. psychrophilum* (Fp), which carry Arg^{673}, that from *S. putrefaciens* (Sp) with Ser^{673} and Lys^{680}, and putative DPP11 from *B. fragilis* (Bf) (BF9343_2925) and *B. vulgatus* (Bv) (BVU_2252) with Arg^{673} were separated on SDS-PAGE. M, low-molecular-weight marker. (**B**) Activities of recombinant DPP11s were measured with Leu-Asp- and Leu-Glu-MCA. Values are shown as the mean ± S.D. (n = 3). Data for *B. fragilis* (Bf) and *B. vulgatus* (Bv) DPP11 are expanded in the right panel. (**C**) Ratio of activity for Leu-Glu-MCA to that for Leu-Asp-MCA is plotted, except for *S. putrefaciens* DPP11.

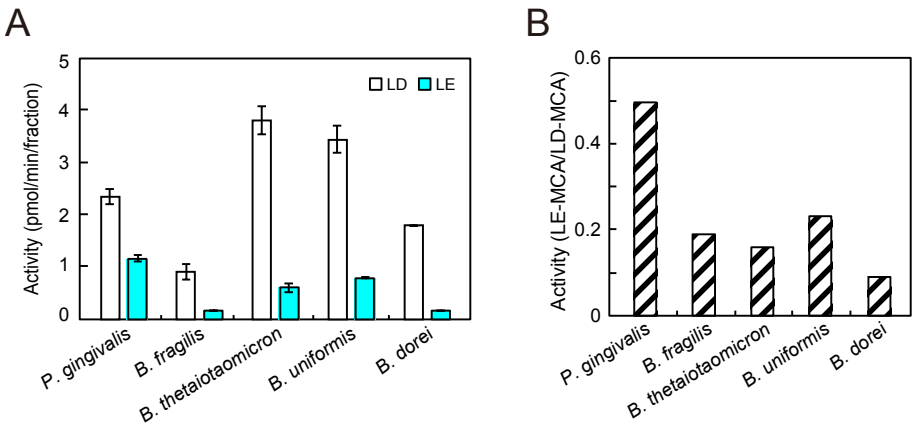

Fig. 3. DPP11 activity in *Bacteroides* cells.

(**A**) Hydrolyses for Leu-Asp- and Leu-Glu-MCA were determined using bacterial cell suspensions (4 μl, A_{600} = 5). Values are shown as the mean ± S.D. (n = 3). (**B**) Ratio of activity for Leu-Glu-MCA to that for Leu-Asp-MCA is plotted. *P. gingivalis* ATCC 33277, *B. fragilis* YCH10, *B. thetaiotaomicron* KYU12, *B. uniformis* ATCC 8492, and *B. dorei* JCM 13471 cells were used.

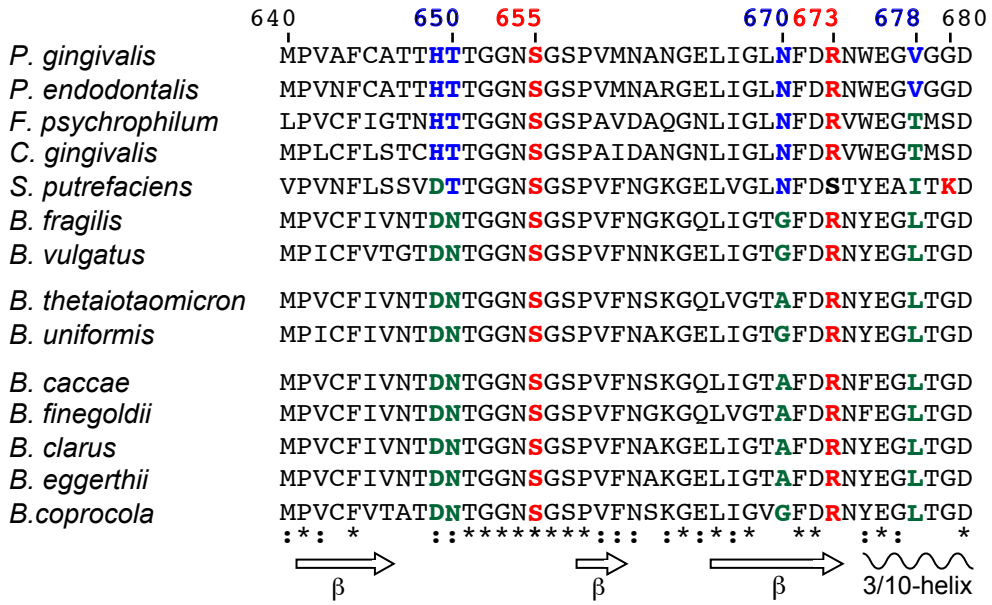

Fig. 4. Comparison of amino acid sequences around essential serine residue in DPP11s.

The amino acid sequences at Met^{640}-Asp^{681} of 7 DPP11s expressed in this study are shown at the top, 2 *Bacteroides* DPP11 are shown at the middle, of which cell activities were measured, and 5 other *Bacteroides* DPP11s are shown at the bottom, and were compared. Identical amino acid residues are indicated by asterisks and those with a conserved property are indicated by colons under the sequences. The β-strand and 3/10-helix regions are indicated at the bottom. Ser^{655} composing the charge relay system, and Arg^{673} and Lys^{680}, essential for the P1 Asp/Glu specificity of DPP11, are shown in red. His^{649}, Thr^{650}, Asn^{670}, and Val^{678} (blue) of PgDPP11 are substituted by other amino acids (green) in *Bacteroides*, and other bacterial DPP11s. Among them, Thr^{650}, Asn^{670}, and Val^{678} are potentially responsible for the alteration in the activity in *Bacteroides* DPP11 (see text).

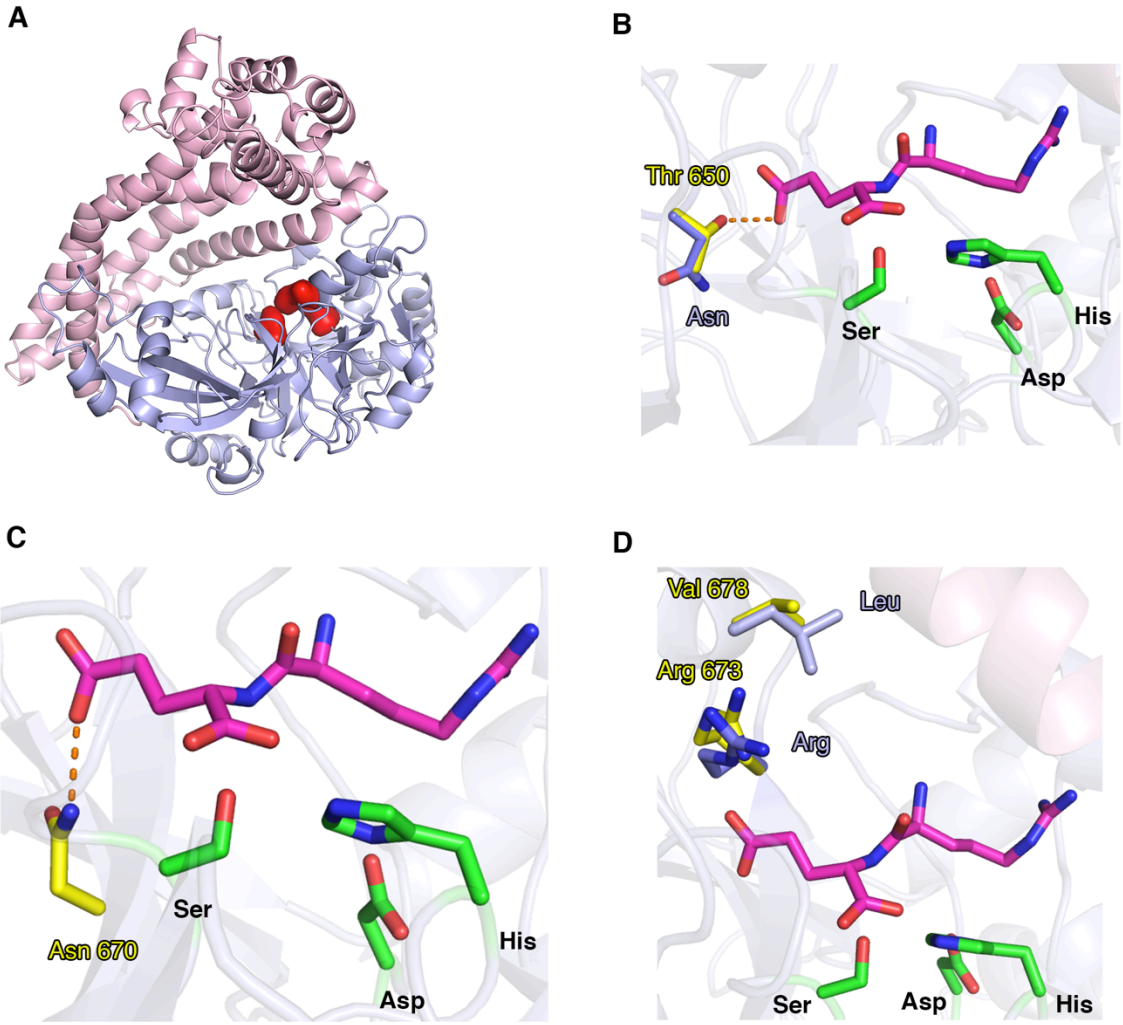

Fig. 5. 3D-homology model of BfDPP11.

(**A**) The catalytic fold in the overall structure of BfDPP11 is represented in blue and the helical fold in pink. The location of the catalytic triad is shown in red. (**B-D**) BfDPP11 is superimposed to PeDPP11 in the complex with Arg-Glu (magenta). The catalytic triad (His^{85}, Asp^{227}, Ser^{655}) is shown as green sticks. Nitrogen and oxygen are represented by blue and red, respectively. (**B**) Thr^{650}, (**C**) Asn^{670}, and (**D**) Val^{678} are substituted by Asn, Gly, and Leu (yellow), respectively, in BfDPP11. All amino acid numbers are indicated as those of PgDPP11.

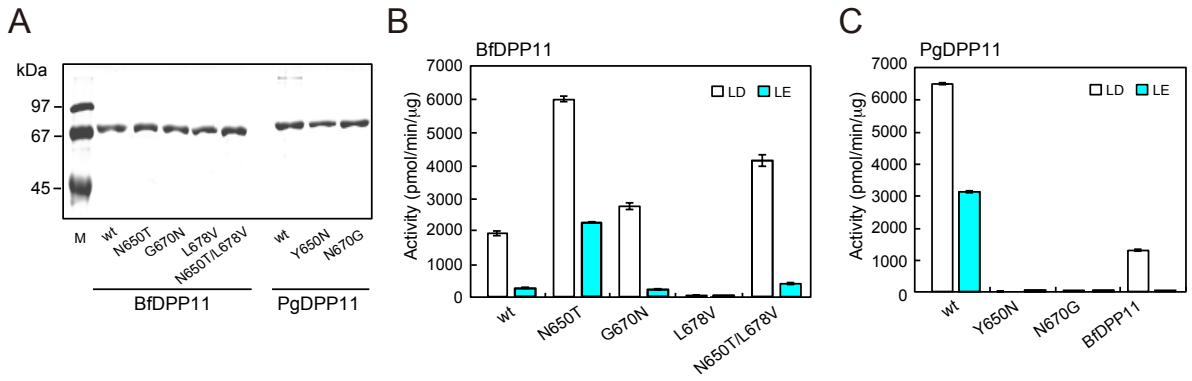

Fig. 6. Effect of in *vitro* mutagenesis of PgDPP11 and BfDPP11 at positions 650, 670, and 678. (**A**) Purified proteins (0.4 μg) of BfDPP11 and PgDPP11, and their mutants were separated on SDS-PAGE. M, low-molecular-weight marker. (**B**) Activities of BfDPP11 and its mutants, and (**C**) those of PgDPP11 and its mutants were determined with Leu-Asp- and Leu-Glu-MCA. Values are shown as the mean ± S.D. (n = 3).

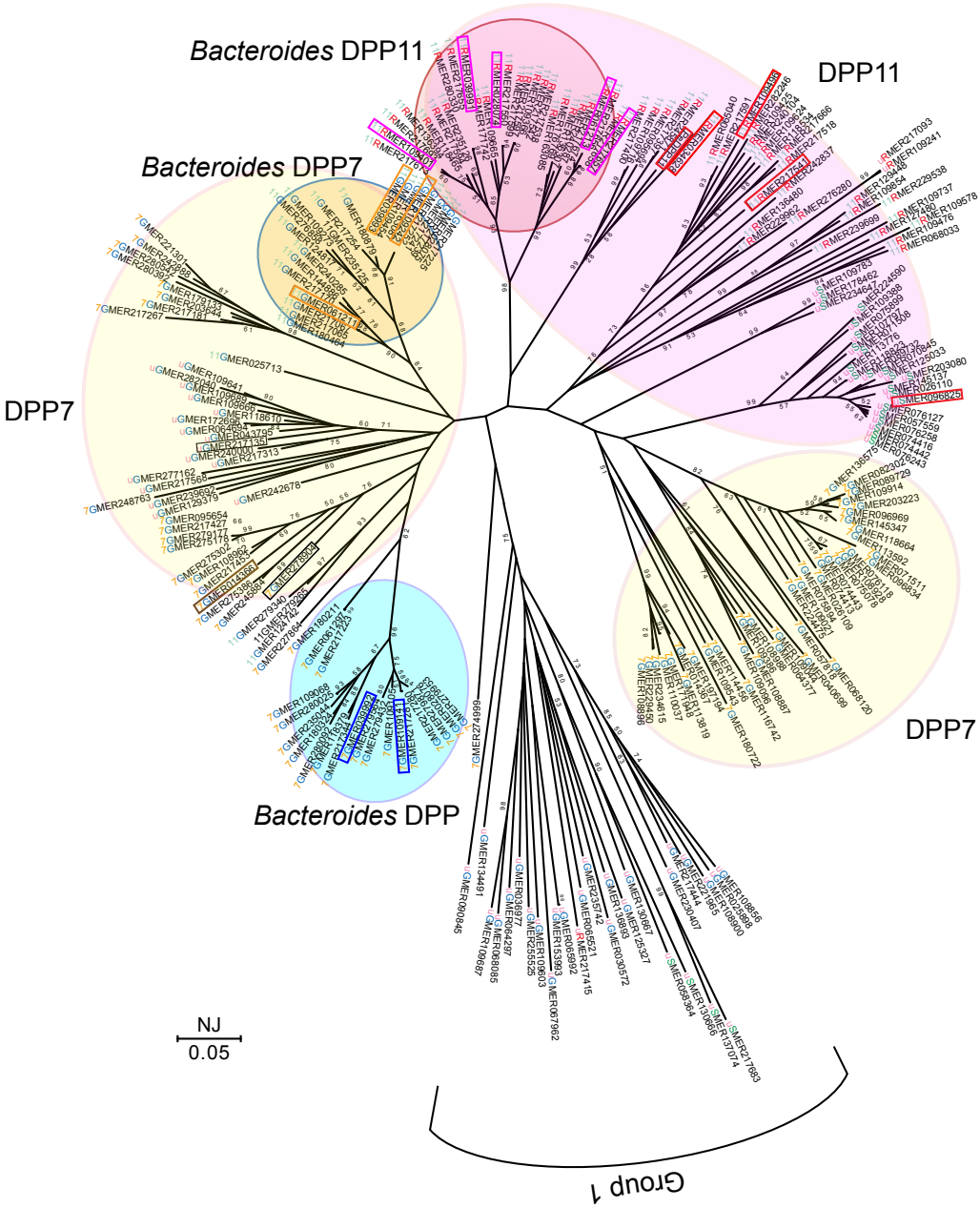

Fig. 7. Phylogenic tree of S46 family based on sequences of C-terminal conserved region.

A phylogenic tree was constructed using the NJ method [25] with ClustalX software [26] based on the C-terminal conserved region (Ser^{571}-Leu^{700} of PgDPP11). Basic information for DPPs is included in the names, as follows: first 7 (DPP7), 11 (DPP11), or u (unassigned) in the MEROPS database (Release 9.6), followed by G (Gly^{673}), R (Arg^{673}), or S (Ser^{673}), then finally the MEROPS codes. For example, PgDPP11 with Arg^{673} is written as 11RMER034628. DPPs

expressed in the present study are boxed in brown (PgDPP7), orange (*Bacteroides* DPP7), red (DPP11), pink (*Bacteroides*-type DPP11), and blue (third group). *P. endodontalis* (7GMER27890) and *C. gingivalis* (uGMER218135) DPP7, boxed in black, were previously characterized [20]. DPP7 (light yellow), DPP11 (light pink), *Bacteroides* DPP as the third group of S46 members (blue), *Bacteroides* DPP7 (cream), and *Bacteroides*-type DPP11 (red) clusters are indicated by circles.