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Research paper

# 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.



## 1. 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,

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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 tripeptides 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 xg 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