Unexpected Diversity of pepA Genes Encoding Leucine Aminopeptidases in Sediments from a Freshwater Lake

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(Received July 30, 2015—Accepted December 25, 2015—Published online March 3, 2016)

We herein designed novel PCR primers for universal detection of the pepA gene, which encodes the representative leucine aminopeptidase gene, and investigated the genetic characteristics and diversity of pepA genes in sediments of hypereutrophic Lake Kasumigaura, Japan. Most of the amino acid sequences deduced from the obtained clones (369 out of 370) were related to PepA-like protein sequences in the M17 family of proteins. The developed primers broadly detected pepA-like clones associated with diverse bacterial phyla—Alpha-, Beta-, Gamma-, and Deltaproteobacteria, Acidobacteria, Actinobacteria, Aquificae, Chlamydiae, Chloroflexi, Cyanobacteria, Firmicutes, Nitrospirae, Planctomycetes, and Spirochetes as well as the archaeal phylum Thaumarcheota, indicating that prokaryotes in aquatic environments possessing leucine aminopeptidase are more diverse than previously reported. Moreover, prokaryotes related to the obtained pepA-like clones appeared to be r- and K-strategists, which was in contrast to our previous findings showing that the neutral metalloprotease gene clones obtained were related to the r-strategist genus Bacillus. Our results suggest that an unprecedented diversity of prokaryotes with a combination of different proteases participate in sedimentary proteolysis.

Key words: leucine aminopeptidase gene, sediments, hypereutrophic freshwater lake, genetic diversity, prokaryotic community

The bacterial enzymatic hydrolysis of proteins (proteolysis) has a prominent influence on nitrogen cycling in lake sediments. The hydrolysis of particulate proteins, which are representative components of the particulate organic matter in sediments (15), is often the rate-limiting step in this protein degradation (4, 5), and the subsequent deamination of amino acids by microbes may strongly contribute to nitrogen regeneration in sediments (11). However, our knowledge of the distribution and diversity of bacterial proteases in sediments is limited.

Extracellular proteases are generally secreted from intact living cells and play a significant role in the proteolysis of particulate proteins outside cells. In natural environments, intracellular (cytosolic) proteases, which are released by cell death or lysis, may also be responsible for this proteolysis. For example, Nannipieri et al. (29) reported that some enzymes liberated by cell death and lysis, particularly those associated with humic substances and minerals, maintained their activity for an unexpectedly long time. Alkaline metalloprotease, neutral metalloprotease (Npr), and serine protease, which are secreted from living cells (10), are representative extracellular proteases derived from bacteria (13). Phylogenetic analyses of the genes encoding these proteases have been conducted in some environments (26–28, 33), and npr-related genes were identified in the sediments of a hypereutrophic lake (38). Since the occurrence of these genes is related to the dominance of the genus Bacillus and high interstitial ammonium concentrations in sediments, proteolysis by sedimentary bacteria has been suggested to play an important role in nitrogen regeneration (38).

Leucine aminopeptidases belong to the M1 and M17 protease families (22). One of the genes encoding the M17 family of leucine aminopeptidases, pepA, has been detected in bacterial isolates including Escherichia coli (35) and Rickettsia prowasekii (40). Although leucine aminopeptidases are generally regarded as intracellular enzymes (17), a recent study identified a pepA gene that encodes a secretory leucine aminopeptidase (16). Thus, the subcellular location of these leucine peptidases remains to be clarified.

Leucine aminopeptidases appear to be significant proteolytic agents in aquatic environments. This enzymatic activity has been detected in lake water (5, 9, 14), groundwater (37), river water (12, 37), intertidal mudflat sediments (23), inlet sediments (30), and lake sediments (6). Furthermore, a previous study reported that all 44 bacterial strains isolated from marine environments exhibited positive leucine aminopeptidase activity, but with marked differences in activity levels among strains (20). However, information on the diversity of bacteria possessing leucine aminopeptidases or the occurrence of functional genes encoding these enzymes in natural aquatic environments is limited.

The aims of the present study were 1) to develop a pepA-specific universal PCR primer set for the detection of leucine aminopeptidase-harboring bacteria; 2) to evaluate the applicability of the designed primers; and 3) to investigate the genetic characteristics and diversity of pepA genes in the sediments of a hypereutrophic lake.

Materials and Methods

Bacterial strains and culture conditions

As representative organisms for evaluating the applicability of the newly designed primer pair, we used pure cultures of E. coli

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JM109 and Pseudomonas stutzeri IFO3773 because other strains of both species are known to possess pepA genes. E. coli JM109 and P. stutzeri IFO3773 were cultured in Luria-Bertani medium and medium containing (L–1) 10 g polypeptide, 2 g yeast extract, and 1 g MgSO₄·7H₂O (pH 7.0), respectively, at 37°C.

**Design of primers**

PCR primers were designed from the alignment of the amino acid sequences encoded by the leucine aminopeptidase gene in 25 bacterial species (Fig. 1). In order to design the primers, we applied the consensus-degenerate hybrid globonucleotide primers (CODEHOP) strategy (http://blocks.fhcrc.org/codehop.html) (32). The parameters for designing the primers were an annealing temperature ≤60°C and primer degeneracies ≤128.

**Collection of sediment core samples and DNA extraction from pure cultures and sediment samples**

The environmental samples used in the present study were freshwater lake sediments from Lake Kasumigaura, Japan. An acrylic tube (30 cm in length, internal diameter=4 cm) was used as a gravity corer in order to collect sediments at the center of Lake Kasumigaura (36°01'57"N, 140°24'25"E). Eight cores were collected at each sampling event. The sediment cores were transported to the laboratory. In the present study, we used sections at a depth of 4–6 cm in the sediment cores collected bimonthly between February and December 2007. Bacterial DNA was extracted from pure cultures and sediment samples using a FastDNA Pro Soil Kit (Q-Biogene, Carlsbad, CA, USA) according to the manufacturer’s protocol.

**PCR conditions**

In order to detect the pepA gene, PCR was performed in a volume of 10 μL containing 1×PCR buffer (with MgCl₂), 0.2 mM of each PCR primer degeneracies ≤128.

**Real-time quantitative PCR (qPCR) assay**

The copy numbers of pepA genes were quantified in sediment samples. Standard samples (554 bp) were constructed from the transformed vector including the pepA gene, PCR was performed in a volume of 10 μL containing 1×PCR buffer with (MgCl₂), 0.2 mM of each dNTP, 1 μM of each primer, 0.05 U TaKaRa Ex Taq (TaKaRa Bio, Otsu, Japan), and the DNA sample. The touchdown PCR program was as follows: initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 94°C for 1 min, annealing for 1 min, and extension at 72°C for 1 min. The annealing temperature decreased from 65°C to 60°C at 0.5°C cycle–1 for the first 10 cycles and was kept constant at 60°C for the last 25 cycles. The PCR reaction was performed using the thermal cycler TaKaRa Thermal Cycler Dice Gradient or TaKaRa Thermal Cycler Dice Touch (TaKaRa Bio). In order to determine whether this primer pair had the ability to amplify the pepA genes, the genomic DNAs of E. coli JM109 and P. stutzeri IFO3773 were used as positive controls.

**Clone library construction, sequencing, and phylogenetic analysis**

Clone libraries were constructed using the February and August samples from a depth of 4–6 cm. The amplified pepA genes were cloned into the pMD20-T vector with the Mighty-TA Cloning Kit (TaKaRa Bio) according to the manufacturer’s instructions. The constructed vectors were transformed into E. coli JM109 competent cells (TaKaRa Bio). The transformed E. coli JM109 was cultured on a Luria-Bertani plate containing ampicillin (100 μg mL⁻¹), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (80 μg mL⁻¹), and isopropyl-β-D-thiogalactopyranoside (100 μM) at 37°C overnight and distinguished by blue–white selection. The white colonies were checked for the presence of an insert fragment of the correct size by direct PCR using the vector primers M13 primer M4 and M13 primer RV. More than 180 E. coli JM109 colonies with a PCR fragment of the correct size were randomly selected for each environmental sample and used in further sequencing analyses. Positive fragments were sequenced using BigDye Terminator Kit v. 3.1 (Applied Biosystems, Carlsbad, CA, USA) and the vector primers described above, and sequences were determined on an Applied Biosystems 3730 DNA Analyzer. Distance matrices were calculated based on the DNADIST program in PHYLOGENY Inference Package (3.695 (http://evolution.genetics.washington.edu/phylip.html) and were used to group the obtained sequences into operational taxonomic units (OTUs) with a distance cut-off of 0.3 using the software Mothur (34). Rarefaction curves were calculated using R version 2.15.2 statistical software (R Development Core Team; http://www.r-project.org/). Evolutionary distance dendrograms were constructed by the neighbor-joining method with the MEGA 6 software package (36). Confidence in the dendrogram topology was evaluated using a bootstrap analysis with 1,000 resamplings.

**Alignment of PepA partial amino acid sequences and consensus amino acid sequences used to design primers for pepA.**

![Alignment of PepA partial amino acid sequences and consensus amino acid sequences used to design primers for pepA.](image-url)
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Peptide aminopeptidase from Escherichia coli JM109, which were amplified using M13 primer M4 and M13 primer RV. The standard samples generated by PCR were purified using the PureLink Quick PCR Purification Kit (Life Technologies, Grand Island, NY, USA) and the single band was visually confirmed by electrophoresis through a 2% agarose gel containing 0.5 mg L⁻¹ ethidium bromide. The concentrations and copy numbers of the standard DNA samples were measured and calculated using the Quant-it dsDNA Broad-Range Assay Kit and a Qubit fluorometer (Invitrogen, San Diego, CA, USA) according to the manufacturer’s specifications. qPCR was performed using TaKaRa Thermal Cycler Dice Real-Time System Single and MightyAmp for Real Time (SYBR Plus) (TaKaRa Bio) according to the manufacturer’s protocol. All analyses were conducted in triplicate with an appropriate dilution of each extracted DNA sample. The qPCR thermal program was as follows: initial denaturation at 98°C for 2 min, followed by 45 cycles of 98°C for 10 s, 64°C for 15 s, and 68°C for 1 min. The specificity of the qPCR products was checked by 1.5% agarose gel electrophoresis. In order to quantify pepA genes in the sediment samples, environmental DNA samples were diluted to reduce the influence of inhibitors on PCR amplification.

Nucleotide sequence accession numbers

The partial pepA gene sequences retrieved have been deposited in the DDBJ/EMBL/GenBank databases under the following accession numbers: LC048882 for E. coli JM109, LC048883 for P. stutzeri IFO3773, and LC048512–LC048881 for environmental clones.

Results

Primer design and amplification of pepA genes from pure cultures

In PepA sequences of approximately 500 amino acids, the CODEHOP program identified only two conserved regions as possible PCR primers: “EVLNTDAEG” and “HLDIAGTA” (Fig. 1). Based on the sequences in these regions, two CODEHOPs were designed: pepAf-codehop (5'-CGAGGTTGCTGAAACCGGAYGCNGARGG-3') and pepAr-codehop (5'-GCGGTGCCGGAYGCNGARGG-3'). The designed primer set successfully amplified the genomic DNA of E. coli JM109 and P. stutzeri IFO3773 at the expected length of approximately 370 bp without any extra bands (Fig. 2). Sequencing showed that the translated amino acid sequence from the amplicon of E. coli JM109 was closely related to the amino acid sequence of a protein from the cytosol aminopeptidase family from E. coli 4-203-08_S3_C3 (accession no. KEK90327) with a similarity value of 100%. The translated amino acid sequence of the pepA amplicon derived from P. stutzeri IFO3773 also showed a high similarity (100%) to multifunctional aminopeptidase A from P. stutzeri ATCC 14405 (accession no. WP003284686).

Diversity and sequence analysis of pepA genes from freshwater lake sediments

The designed primer pair successfully amplified genes with the predicted size (approximately 370 bp) from the sediments of Lake KasumiGaura without any extra bands (Fig. 2). Of these PCR amplicons, the February and August samples were cloned, sequenced, and then phylogenetically analyzed; 182 clones from February and 188 clones from August were analyzed. The 75 OTUs from February and 69 OTUs from August were grouped into a clone library with 118 OTUs at a similarity cut-off value of 70%. Multiple diversity indices on the pepA gene clone libraries are presented in Table 1. Shannon-Weaver (H') and the reciprocal of Simpson diversity indices (1/D) of the retrieved pepA clones were both slightly higher in February than in August. This difference was also supported by rarefaction curves of the pepA clone libraries (Fig. S1).

We performed an alignment of the residues surrounding the catalytic amino acid residue (Fig. S2). All of the retrieved sequences possessed the catalytic amino acid residue of leucine aminopeptidases: arginine (“R”). Moreover, the amino acid residues in the retrieved PepA sequences next to the catalytic amino acid residue, which may influence leucine aminopeptidase activity (3), were of diverse types: histidine (“H”), isoleucine (“I”), leucine (“L”), methionine (“M”), glutamine (“Q”), and valine (“V”).

An evolutionary distance dendrogram was generated incorporating reference sequences from the NCBI database (http://www.ncbi.nlm.nih.gov/) based on partial amino acid sequences after removing the primer sequences from the retrieved clones (approximately 109 amino acids) (Fig. 3). All of the sequences retrieved by PCR showed similarity to the amino acid sequences of aminopeptidases belonging to the M17 family of proteins in the NCBI database and were apparently different from other zinc-dependent proteases in the M28 and M20 families of proteins. Moreover, all sequences were phylogenetically close to PepA-like proteins, except for one clone. The PepA clones retrieved were close to those of various bacterial phyla, including the Alpha-, Beta-, Gamma-, and Deltaproteobacteria, Acidobacteria, Actinobacteria, Aquificae, Chlamydiae, Chloroflexi, Cyanobacteria, Firmicutes, Nitrospirae, Planctomycetes, and Spirochetes as well as the archael phylum Thaumarchaeota (Table S1). The major genotypes of pepA from February were similar to those from August (Table S1). The genera and families affiliated with the obtained PepA sequences showed a large range of 16S rRNA gene copy numbers per cell (Table S1).

Table 1. Diversity indices for pepA clones obtained from sediments of Lake Kasumigaura, Japan.

| Month | No. of clones | Shannon-Weaver (H') | Simpson (1/D) | Coverage |
|-------|---------------|---------------------|---------------|----------|
| February | 182 | 75 | 3.86 | 37.2 | 0.736 |
| August | 188 | 69 | 3.74 | 29.8 | 0.739 |
We used the Basic Local Alignment Search Tool (BLAST) to find matching pepA sequences in the GenBank database. Table 2 presents phylogenetic information for the representative 10 pepA OTUs from sediments of Lake Kasumigaura, Japan.

Table 2. Phylogenetic information for 10 representative pepA OTUs from sediments of Lake Kasumigaura, Japan

| OTU | Cluster | No. of clones | Feb | Aug | Closest BLAST match | Accession No. | Amino acid identity (%) |
|-----|---------|--------------|-----|-----|---------------------|---------------|------------------------|
| OTU 1 | III | 26 | 27 | hypothetical protein Candidatus Magnetobacterium sp. MYR-1 | WP040334930 | 65–73 |
| OTU 2 | I | 12 | 13 | aminopeptidase A Candidatus Competibacter denitrificans Run_A_D11 | CDJ00939 | 75–83 |
| OTU 3 | III | 8 | 5 | leucyl aminopeptidase Chloroflexus aurantiacus J-10-1 | YP001639614 | 66–74 |
| | | 2 | 7 | cytosol aminopeptidase Chloroflexus sp. MS-G | WP031685701 | 67–74 |
| | | 1 | 1 | cytosol aminopeptidase Chloroflexus aggregans | WP012612696 | 65–67 |
| | | 1 | 1 | cytosol aminopeptidase Roseiflexus sp. RS-1 | WP011955470 | 68 |
| OTU 4 | I | 7 | 13 | hypothetical protein OT06_52005 Candidatus Thiomargarita nelsonii | KHD04775 | 74–78 |
| OTU 5 | III | 11 | 7 | leucyl aminopeptidase Beggiatia sp. PS | EDN67650 | 78 |
| | | 1 | 1 | cytosol aminopeptidase Syntrophus aciditrophicus SB | WP041584835 | 59–61 |
| OTU 6 | I | 5 | 15 | cytosol aminopeptidase Methylocaldum szegediense | WP026610414 | 81–88 |
| OTU 7 | I | 7 | 8 | cytosol aminopeptidase Methylococcus capsulatus | WP026610414 | 81–84 |
| OTU 8 | I | 9 | | leucyl aminopeptidase Pseudomonas sp. GM16 | WP007909047 | 98–100 |
| OTU 9 | II | 3 | 4 | aminopeptidase A Caldimena aerophila | WP014434738 | 55–57 |
| | | 1 | 1 | cytosol aminopeptidase Roseiflexus sp. RS-1 | WP011955470 | 54 |
| OTU 10 | IV | 2 | 1 | cytosol aminopeptidase Persephonella sp. IF05-L8 | WP029520707 | 51 |
| | | 2 | 1 | leucyl aminopeptidase Sanguibacter keddieii | WP012865512 | 52 |
| | | 1 | 1 | cytosol aminopeptidase Sulphurospirillum sp. YO3AO1 | WP012458779 | 50 |
| | | 1 | 1 | hypothetical protein Dehalococcoidia bacterium SCGC AB-539-J10 | WP029478441 | 53 |

Phylogenetic analysis of PepA from sediments of a hypereutrophic lake

We used the Basic Local Alignment Search Tool (BLAST) to find matching pepA sequences in the GenBank database. Table 2 presents phylogenetic information for the representative 10 pepA OTUs. Detailed phylogenetic trees were also generated (Fig. S3). Most of the PepA sequences retrieved in this study were assigned to four clusters: cluster I (35.9% of sequences; 133 out of 370), cluster II (5.4%; 20 out of 370), cluster III (42.4%; 157 out of 370), and cluster IV (15.9%; 59 out of 370) (Fig. 3). Gammaproteobacterial methanotroph-related pepA clones were dominant in cluster I (11.6%; 43 out of 370), particularly OTU 6, related to the cytosol aminopeptidase of Methylocaldum szegediense, and OTU 7, multifunctional aminopeptidase A of Methylococcus capsulatus (Fig. S3a). These sequences had 81–88% amino acid similarities to the cytosol aminopeptidase of M. szegediense (Fig. S3b). Cluster II contained the fewest sequences of the four clusters (Fig. S3b). In cluster III, the most abundant sequences (16.2%; 60 out of 370) were associated with the clones related to the hypothetical protein of “Ca. Contendobacter odensis” (65–73% similarities) (Fig. S3c and Table 2). The clones evolutionally close to
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Chloroflexi-related aminopeptidase (65–82% similarities) were also abundant (9.7%; 36 out of 370) (Fig. S3c). The most abundant clones in cluster IV (5.4%; 20 out of 370) were those related to class Dehalococcoidia (66–83% similarities) (Fig. S3d).

At the amino acid level, the sequences retrieved from the sediments had similarities to NCBI database entries of 49–96% in February and 49–100% in August (Fig. 4). The similarity range of 65–70% had the most amino acid sequences as deduced from pepA clones in both February and August. There was also a large number of PepA sequences with low similarities (<65%) to the database sequences. These results indicate that most of the sedimentary PepA sequences were unknown and different from the PepA sequences derived from pure cultures currently in the databases.

Quantification of pepA genes in sediments of a hypertrophic lake

qPCR assays were used to quantify pepA gene copy numbers in the sediments (at a depth of 4–6 cm) of Lake Kasumigaura in February and August 2007. The standard curve constructed from the pepA gene of P. stutzeri IFO3773 was strongly linear ($r^2=0.99$), ranging from $1.18\times10^2$ to $1.18\times10^8$ copies per reaction. qPCR amplification efficiency was 85.3%. The copy numbers of pepA genes were $1.24\times10^8 (±3.07\times10^7)$ mL$^{-1}$ sediment in February and $7.20\times10^7 (±2.17\times10^7)$ mL$^{-1}$ sediment in August, indicating the absence of a significant difference between the sampling periods in pepA gene abundance at a depth of 4–6 cm in Lake Kasumigaura sediments.

Discussion

In the present study, we attempted to develop a pepA-specific universal primer pair. We demonstrated the applicability of the designed primers for environmental samples by investigating the diversity and phylogeny of this gene in sediments from a freshwater lake.

Of the two conserved regions of PepA sequences proposed for primers by the CODEHOP program (Fig. 1), the first region (EVLNTDAEG), which was used to design the forward primer, is a very important amino acid sequence for the functioning of PepA. The sixth (aspartic acid; “D”) and eighth amino acid (glutamic acid; “E”) are zinc-binding residues (2). Although the second region (HLDIAG) was shown to be highly conserved in PepA-like protein sequences in previous studies (3, 17), the function of this amino acid motif is not yet known. In the amplicons generated using the designed primers, the catalytic residue arginine (R) was also found in all the clones retrieved (Fig. S2).

The designed primers permitted the specific detection of the expected amplicons without any extra bands, both from pure cultures and sedimentary DNA (Fig. 2). The results of a phylogenetic analysis indicated that most of the retrieved sequences were related to PepA-like protein sequences in the M17 protein family (Fig. 3). Therefore, the designed primers may be specifically useful for detecting pepA genes. The designed primers have the ability to broadly detect putative pepA genes from diverse phyla including Acidobacteria, Aquificae, Chlamydiae, Cyanobacteria, Nitrospirae, Planctomycetes, and Spirochetes, which were not used to design CODEHOPs (Table S1). A pepA clone related to an uncultured marine Thaumarchaeota was also obtained (Fig. S3d). These results suggest that pepA genes are widely distributed in diverse taxonomic groups of prokaryotes in sediments. Unidentified or hardly cultivable microorganisms (e.g., Candidatus species) may also be significant contributors to leucine aminopeptidase production in sediments because most of the amino acid sequences deduced from the pepA clones retrieved had low identity to those of PepA sequences from pure cultures in the GenBank database (Fig. 4). Thus, the primers that we developed may be a powerful culture-independent tool for examining the community structure of leucine aminopeptidase–producing bacteria and investigating the expression of the pepA gene.

A phylogenetic analysis of the pepA genes found in the present study showed an abundance of those derived from various bacterial groups having specific functions such as methane oxidation (M. szegediense) (25) and glycogen accumulation (“Ca. Competibacter denitrificans”) (24) (Fig. S3a). Moreover, we also found a high abundance of PepA close to the class Dehalococcoidia, which are anaerobic bacteria (18) (Fig. S3d). These results indicate that aerobic and anaerobic microbes may both be relevant to PepA production and that diverse functional groups possess proteolytic functions in sediments. Leucine aminopeptidase activity has been associated with heterotrophic bacteria in aquatic environments on the basis of the strong correlation detected between bacterial secondary production and leucine aminopeptidase activity (5, 39). In the present study, we found pepA clones similar to Sideroxydans lithotrophicus and Gallionella sp., which are chemolithoautotrophic bacteria (1, 19) (Fig. S3a). Thus, our results suggest a markedly higher diversity of prokaryotes possessing leucine aminopeptidase in aquatic environments than previously suspected. The copy numbers of pepA genes obtained in the present study appear to be similar to those of functional genes responsible for inorganic nitrogen metabolism such as nirS, nirK, and amoA, which range from approximately $10^8$ to $10^9$ copies g$^{-1}$ of dry sediment (21); however, the quantification of functional genes with CODEHOPs may be somewhat inaccurate because of differences in the priming efficiencies of primers to the respective target sequences (7). This implies that pepA-mediated organic nitrogen metabolism plays a significant role in nitrogen cycles.

We previously reported that the temporally synchronous occurrence of 16S rRNA and npr-related genes from the
genus *Bacillus* in the same sediment samples used in the present study positively correlated with increased interstitial ammonium concentrations (38). This finding suggests that proteolysis by Npr from the genus *Bacillus* is one of the contributors to ammonium production. However, in that study, the relative abundance of the genus *Bacillus* markedly increased in August, whereas ammonium concentrations increased gradually between April and December (38). Thus, the detection of *pepA* genes in this study may provide an important insight into understanding this apparent contradiction; that is, proteases other than Npr (e.g., PepA) may also act to increase ammonium concentrations.

Fierer et al. (8) previously reported that *r*-strategists are copiotrophs with high 16S rRNA copy numbers per cell (>5) and large nutritional requirements, whereas *K*-strategists are oligotrophs with low 16S rRNA copy numbers per cell (<2) that prefer low nutrient conditions. Based on this characterization, *r*- and *K*-strategists both appear to possess PepA in Lake Kasumigaura because the candidate contributors of PepA in this study have a wide range of 16S rRNA gene copy numbers per cell (Table S1). In contrast, most of the Npr sequences obtained were phylogenetically related to Npr of the genus *Bacillus*, which is a representative *r*-strategist that increased in August in Lake Kasumigaura (38). The sediment in August had higher nutrient concentrations (dissolved organic carbon, ammonium, dissolved total nitrogen, and orthophosphates) than those in February (38). These results suggest that proteolysis by leucine aminopeptidase is a universal function in aquatic sediments, regardless of season and nutrient conditions. Therefore, PepA may play a particularly important role in decomposing particulate proteins during the early stage of relatively low inorganic nitrogen conditions, and appears to act as a trigger for subsequent proteolysis by Npr. These results indicate that proteolysis by sedimentary bacteria is a ubiquitous process, but one that arises from a complex combination of different types of protease-producing microbial communities depending on environmental conditions.

**Conclusion**

We herein developed a novel universal PCR primer set for the detection of leucine aminopeptidase genes (*pepA*) and investigated the genetic characteristics and diversity of *pepA* genes in sediments of a hypereutrophic lake. Our results show that the designed primers have the ability to broadly detect putative *pepA* genes derived from diverse phyla. The diversity of prokaryotes possessing *pepA* in the sediments was markedly higher than previously identified, and *pepA* genes were detected across all seasons and nutrient conditions in the sediments. Our results indicate that proteolysis by sedimentary bacterial communities is a ubiquitous process, but arises from a complex combination of different types of protease-producing microbial communities.

**Acknowledgements**

This study was funded by the Environmental Research and Technology Development Fund of the Ministry of the Environment, Japan (5-1304). We thank the GEMS/Water Trend Monitoring Project at Lake Kasumigaura for financially supporting sampling. We are grateful to Dr. Ayato Kohzu, Dr. Kazuhiro Komatsu, and Dr. Noriko Tomioka for collecting sediment samples. We also thank the associate editor and anonymous reviewers for their constructive comments and suggestions, which helped to improve the quality of this manuscript.

**References**

1. Beckwith, C.R., M.J. Edwards, M. Lawes, L. Shi, J.N. Butt, D.J. Richardson, and T.A. Clarke. 2015. Characterization of MtoD from *Sideroxydans lithoautotrophicus*: a cytochrome c electron shuttle used in lithoautotrophic growth. Front. Microbiol. 6:332.

2. Burley, S.K., P.R. David, R.M. Sweet, A. Taylor, and W.N. Lipscomb. 1992. Structure determination and refinement of bovine lens leucine aminopeptidase and its complex with bestatin. J. Mol. Biol. 224:113–140.

3. Chi, M.C., H.B. Huang, J.S. Liu, W.C. Wang, W.C. Liang, and L.L. Lin. 2006. Residues threonine 346 and leucine 352 are critical for the proper function of *Bacillus kaustophilus* leucine aminopeptidase. FEMS Microbiol. Lett. 260:156–161.

4. Chröst, R.J. 1991. Environmental control of the synthesis and activity of aquatic microbial ectoenzymes, p. 29–59. In R.J. Chröst (ed.), Microbial Enzymes in Aquatic Environments. Springer Verlag, New York.

5. Chröst, R.J. 1992. Significance of bacterial ectoenzymes in aquatic environments. Hydrobiol. 243:244-61–70.

6. Das, B., R. Nordin, and A. Mazumder. 2008. Aminopeptidase activity in lake sediments. Verh. Internat. Verein. Limnol. 30:117–121.

7. De Long, S.K., K.A. Kinney, and M.J. Kirisits. 2010. qPCR assays to quantify genes and gene expression associated with microbial phospho- reduction. J. Microbiol. Met. 83:270–274.

8. Fierer, N., M.A. Bradford, and R.B. Jackson. 2007. Toward an ecological classification of soil bacteria. Ecol. 88:1354–1364.

9. Halemejko, G.Z., and R.J. Chröst. 1986. Enzymatic hydrolysis of proteinaceous particulate and dissolved material in an eutrophic lake. Arch. Hydrobiol. 107:1–21.

10. Häse, C.C, and R.A. Finkelstein. 1993. Bacterial extracellular zinc-containing metalloproteases. Microbiol. Rev. 57:823–837.

11. Herbert, R.A. 1999. Nitrogen cycling in coastal marine ecosystems. FEMS Microbiol. Rev. 23:563–590.

12. Hosen, J.D., O.T. McDonough, C.M. Febria, and M.A. Palmer. 2014. Dissolved organic matter quality and bioavailability changes across an urbanization gradient in headwater streams. Environ. Sci. Technol. 48:7817–7824.

13. Kalisz, H.M. 1988. Microbial proteinases. Adv. Biochem. Eng. Biotechnol. 36:1–65.

14. Kiersztyn, B., W. Siuda, and R.J. Chröst. 2012. Persistence of bacterial proteolytic enzymes in lake ecosystems. FEMS Microbiol. Ecol. 80:124–134.

15. Li, H., and E.C. Minor. 2015. Biogeochemical characteristics of settling particulate organic matter in Lake Superior: A seasonal comparison. Org. Geochem. 85:76–88.

16. Liew, S.M., S.T. Tay, and S.D. Puthucheary. 2013. Enzymatic and molecular characterization of leucine aminopeptidase of *Burkholderia pseudomallei*. BMC Microbiol. 13:110.

17. Lin, L.L., W.H. Hsu, C.P. Wu, M.C. Chi, W.M. Chou, and H.Y. Hu. 2004. A thermostable leucine aminopeptidase from *Bacillus kaustophilus* CCRC 11223. Extremophiles 8:79–87.

18. Löffler, F.E., J. Yan, K.M. Ritalahti, L. Adrian, E.A. Edwards, K.T. Konstantinidis, J.A. Müller, H. Flenn, S.H. Zinder, and A.M. Spormann. 2013. *Dehalococcoides mccartyi* gen. nov., sp. nov., obligately organohalide-respiring anaerobic bacteria relevant to halogen cycling and bioremediation, belong to a novel bacterial class, *Dehalococcoides* classis nov., order *Dehalococcoidales* ord. nov. and family *Dehalococcoidaceae* fam. nov., within the phylum Chloroflexi. Int. J. Syst. Evol. Microbiol. 63:625–635.

19. Lütters-Czeczkalla, S. 1990. Lithoautotrophic growth of the iron bacterium *Gallionella ferruginea* with thiosulfate or sulfide as energy source. Arch. Microbiol. 154:417–421.

20. Martinez, J., D.C. Smith, G.F. Steward, and F. Azam. 1996. Variability in extracellular enzyme activities of pelagic marine bacteria and its significance for substrate processing in the sea. Aquat. Microb. Ecol. 10:223–230.
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21. Martins, G., A. Terada, D.C. Ribeiro, A.M. Corral, A.G. Brito, B.F. Smet, and R. Nogueira. 2011. Structure and activity of lacustrine sediment bacteria involved in nutrient and iron cycles. FEMS Microbiol. Ecol. 77:666–679.

22. Matsui, M., J.H. Fowler, and L.L. Walling. 2006. Leucine aminopeptidases: diversity in structure and function. Biol. Chem. 387:1535–1544.

23. Mayer, L.M. 1989. Extracellular proteolytic enzyme activity in sediments of an intertidal mudflat. Limnol. Oceanogr. 34:973–981.

24. McIlroy, S.J., M. Albertsen, E.K. Andresen, A.M. Saunders, R. Kristiansen, M. Stockholm-Bjerregaard, K.L. Nielsen, and P.H. Nielsen. 2014. ‘Candidatus Competibacter’-lineage genomes retrieved from metagenomes reveal functional metabolic diversity. ISME J. 8:613–624.

25. Medvedkova, K.A., V.N. Khmelena, N.E. Suzina, and Y.A. Trotsenko. 2009. Antioxidant systems of moderately thermophilic methanotrophs Methylocaldum szegediense and Methylococcus capsulatus. Microbiol. 78:670–677.

26. Mrkonjic Fuka, M., M. Engel, A. Gattinger, U. Bausenwein, M. Sommer, J.C. Munch, and M. Schloter. 2008. Factors influencing variability of proteolytic genes and activities in arable soils. Soil Biol. Biochem. 40:1646–1653.

27. Mrkonjic Fuka, M., M. Engel, F. Haesler, G. Welzl, J.C. Munch, and M. Schloter. 2008. Diversity of proteolytic community encoding for subtilisin in an arable field: spatial and temporal variability. Biol. Fertil. Soils 45:185–191.

28. Mrkonjic Fuka, M., M. Engel, A. Hagn, J.C. Munch, M. Sommer, and M. Schloter. 2009. Changes of diversity pattern of proteolytic bacteria over time and space in an agricultural soil. Microb. Ecol. 57:391–401.

29. Nannipieri, P., P. Sequi, and P. Fusi. 1996. Humus and enzyme activity, p. 293–328. In A. Piccolo (ed.), Humic Substances in Terrestrial Ecosystems. Marcel Dekker, New York.

30. Patel, A.B., K. Fukami, and T. Nishijima. 2001. Extracellular proteolytic activity in the surface sediment of a eutrophic inlet. Microb. Environ. 16:25–35.

31. Power, S.D., R.M. Adams, and J.A. Wells. 1986. Secretion and auto-proteolytic maturation of subtilisin. Proc. Natl. Acad. Sci. U.S.A. 83:3096–3100.

32. Rose, T.M., J.G. Henikoff, and S. Henikoff. 2003. CODEHOP (COnsensus-DEgenerate Hybrid Oligonucleotide Primer) PCR primer design. Nucleic Acids Res. 31:3763–3766.

33. Sakurai, M., K. Suzuki, M. Onodera, T. Shinano, and M. Osaki. 2007. Analysis of bacterial communities in soil by PCR-DGGE targeting protease genes. Soil Biol. Biochem. 39:2777–2784.

34. Schloss, P.D., S.L. Westcott, T. Ryabin, et al. 2009. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75:7577–7591.

35. Stirling, C.J., S.D. Colloms, J.F. Collins, G. Szatmari, and D.J. Sherratt. 1989. xerB, an Escherichia coli gene required for plasmid ColE1 site-specific recombination, is identical to pepA encoding aminopeptidase A, a protein with substantial similarity to bovine lens leucine aminopeptidase. EMBO J. 8:1623–1627.

36. Tamura, K., G. Stecher, D. Peterson, A. Filipski, and S. Kumar. 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30:2725–2729.

37. Tiquia, S.M. 2011. Extracellular hydrolytic enzyme activities of the heterotrophic microbial communities of the Rouge River: an approach to evaluate ecosystem response to urbanization. Microb. Ecol. 62:679–689.

38. Tsuibo, S., S. Yamamura, A. Imai, T. Satou, and K. Iwasaki. 2014. Linking temporal changes in bacterial community structures with the detection and phylogenetic analysis of neutral metalloprotease genes in the sediments of a hypereutrophic lake. Microb. Environ. 29:314–321.

39. Vives Rego, J., G. Billen, A. Fontigny, and M. Somville. 1985. Free and attached proteolytic activity in water environments. Mar. Ecol. Prog. Ser. 21:245–249.

40. Wood, D.O., M.J. Solomon, and R.R. Speed. 1993. Characterization of the Rickettsia prowazekii pepA gene encoding leucine aminopeptidase. J. Bacteriol. 175:159–165.