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ORIGINAL ARTICLE

Cloning and Sequencing of Alginate Lyase Genes from Deep-Sea Strains of *Vibrio* and *Agarivorans* and Characterization of a New *Vibrio* Enzyme

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Abstract Four alginate lyase genes were cloned and sequenced from the genomic DNAs of deep-sea bacteria, namely members of *Vibrio* and *Agarivorans*. Three of them were from *Vibrio* sp. JAM-A9m, which encoded alginate lyases, A9mT, A9mC, and A9mL. A9mT was composed of 286 amino acids and 57% homologous to AlxM of *Photobacterium* sp. A9mC (221 amino acids) and A9mL (522 amino acids) had the highest degree of similarity to two individual alginate lyases of *Vibrio splendidus* with 74% and 84% identity, respectively. The other gene for alginate lyase, A1mU, was shotgun cloned from *Agarivorans* sp. JAM-A1m. A1mU (286 amino acids) showed the highest homology to AlyVOA of *Vibrio* sp. with 76% identity. All alginate lyases belong to polysaccharide lyase family 7, although, they do not show significant similarity to one another with 14% to 58% identity. Among the above lyases, the recombinant A9mT was purified to homogeneity and characterized. The molecular mass of A9mT was around 28 kDa. The enzyme was remarkably salt activated and showed the highest thermal stability in the presence of NaCl. A9mT favorably degraded mannuronate polymer in alginate. We discussed substrate specificities of family 7 alginate lyases based on their conserved amino acid sequences.

Keywords Alginate lyase · PL family 7 · Deep-sea · *Vibrio* · *Agarivorans*

Introduction

Alginate, which is biosynthesized as a cell wall component and intracellular material of brown seaweeds, is composed of 1,4 linked α-L-guluronic acid (G) and β-D-mannuronic acid (M). These residues are arranged in block structures that are composed of homopolymeric G or M blocks, alternating G and M blocks, and heteropolymeric GM blocks (Gacesa 1988). Alginate lyases degrade alginate by a β-elimination mechanism (Gacesa 1992). They have been isolated from various sources, including marine algae, marine mollusks, fungi, bacteria, bacteriophages, and viruses (Wong et al. 2000). The enzyme is a useful bioagent for preparation of protoplasts from brown alga (Boyen et al. 1990; Butler et al. 1990). Furthermore, reaction products of alginate lyases increase the germination efficiency and shoot elongation of some plants and also repress the proliferation and/or differentiation of HeLa cells and *Chlamydomonas* (Yonemoto et al. 1993). Recently, we found a new high-alkaline alginate lyase from a deep-sea bacterium, *Agarivorans* sp. (Kobayashi et al. 2009) and also a deep subseafloor bacterium, *Shewanella* sp., as a potent enzyme producer (Kobayashi et al. 2008).

Alginate lyases are organized into seven polysaccharide lyase (PL) families, namely, PL families 5, 6, 7, 14, 15, 17, and 18 (http://www.cazy.org/fam/acc_PL. html). They are classified into two types according to substrate specificity: one is a G block-specific lyase (polyguluronate lyase; EC4.2.2.11), and the other is an M block-specific one (polymannuronate lyase; EC4.2.2.3). This classification has been widely accepted, but some enzymes preferably

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degrade G and GM blocks or M and GM blocks (Shimokawa et al. 1997a, b; Yamasaki et al. 2004; Kobayashi et al. 2009). Among the known alginate lyases belonging to PL family 7, which substrate lyase will prefer to degrade can be predicted by its amino acid sequence. Indeed, Osawa et al. (2005) demonstrated that the substrate specificities can be classified depending on three conserved regions in PL family 7 enzymes. We are interested in the fact that the same organism produces several alginate lyases, all of which are thought to efficiently degrade alginates (Wong et al. 2000; Sugimura et al. 2000; Miyake et al. 2004). During the screening of alkaline alginate lyase-producing bacteria from deep-sea sediments, we selected two candidates and cloned several genes for alginate lyases belonging to the PL 7 family. In this report, we describe cloning and sequencing four alginate lyase genes and purification and some properties of one of the alginate lyases that shows a relatively low similarity to known enzymes. We also discuss their predicted substrate specificities.

Materials and Methods

Bacterial Strains, Plasmids, and Propagation Alkaline alginate lyase-producing bacteria, strains A1m and A9m, were isolated from deep-sea sediments off Cape Nomamisaki at the southwestern tip of Kyushu Island (representative sampling site: 31º18.522N, 129º59.372E), Japan, at a depth of 254 m in July 2005. The isolation procedure was described previously (Kobayashi et al. 2009). The isolates were propagated aerobically in marine broth 2216 (Difco) plus 0.2% sodium alginate at 30ºC for 24 h. *Escherichia coli* DH5α and TOP10 (Invitrogen) were grown in Luria–Bertani (LB) agar or broth supplemented with 100 μg/ml ampicillin at 30ºC for 24 h. The plasmids pUC18 and pCR2.1 (Invitrogen) were used as the cloning vectors.

Determination of 16S rRNA Gene Sequence of Isolate The 16S rRNA gene sequence of strain A9m was determined by colony PCR as previously described for strain A1m (Kobayashi et al. 2009). Briefly, the gene was amplified using the universal primers 27f and 1492r and a colony of strain A9m as the template in a DNA thermal cycler (Gene Amp PCR system 9700, ABI PRISM) with an LA Taq DNA polymerase. Nucleotide sequencing was done with a DNA sequencer (Mega BACE 1000, GE Healthcare) using a DYEnamic ET Terminal Sequencing kit (GE Healthcare). The nucleotide sequence of the 16S rRNA gene for strain A9m has been submitted to GenBank/EMBL/DDBJ databases under the accession number AB472064.

Cloning and Sequencing Alginate Lyase Genes Shotgun cloning of alginate lyase genes of *Agarivorans* sp. JAM-A1m was done essentially as described previously (Kobayashi et al. 2009). Two genes for alginate lyases A1m and A1mU were cloned and sequenced. In the case of *Vibrio* sp. A9m, alginate lyase genes were cloned by PCR. Primers 1 (5′-CGNTCNGARCTNCGNGAG/AATG-3′) and 2 (5′-YTGRTTRTANACNCCNGCYTT-3′) were designed from two highly conserved amino acid sequences, R-X-E-L-R and Y-F-K-A-G-X-Y-X-Q, in A1m and several PL family 7 enzymes (X, variable amino acid). PCR was done using a combination of designed primers and genomic DNA of strain A9m as the template. PCR conditions were 2 min at 96ºC, followed by 30 cycles of 30 s at 96ºC, 30 s at 55ºC, and 90 s at 72ºC. The three amplified fragments were ligated into pCR2.1 using a TA Cloning kit (Invitrogen), and then *E. coli* TOP10 cells were transformed with the resultant plasmids. After blue/white colony selection of transformants, several plasmids were purified using a High Pure Plasmid Isolation kit (Roche), and each insertion was sequenced using a DYEnamic ET Terminal Sequencing kit and a DNA sequencer. The entire genes were cloned by inverse PCR using appropriate primers and *Aat*II, *Eco*RI, or *Sma*I digested genomic DNA of strain A9m as the template. The amplified fragments were sequenced, and consequently, the sequences of three individual alginate lyase genes were sequenced. The entire genes were amplified by PCR with additional *Eco*RI sites and ligated into pUC18. Each resultant plasmid was transformed into *E. coli* DH5α. To confirm the enzyme activity, transformants were grown at 30ºC for 18 h on LB agar containing 0.1% sodium alginate plus 100 μg/ml ampicillin, then a soft agar solution (0.2% alginate, 0.2 M NaCl, 50 mM glycine-NaOH buffer, and 0.8% agar, pH 9) with or without lysozyme (5 mg/ml) was poured over the agar plates. The agars were incubated at 30ºC for 18 h, and then 0.1% cetylpyridinium chloride solution was poured onto the soft agars.

Nucleotide sequence data of A1mU, A9mT, A9mC, and A9mL genes have been submitted to the GenBank, EMBL, and DDBJ databases under the accession numbers AB472333, AB473598, AB473599, and AB473600.

Phylogenetic Analyses The similarity of 16S rRNA gene of strain A9m was analyzed using BLAST databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Nucleotide sequences of the 16S rRNA genes were automatically aligned, and alignment positions with gaps were ignored. To construct the phylogenetic tree, nucleotide substitution rates (*knuc* values) were determined, and a distance matrix tree was constructed by bootstrapping neighbor-joining method (Saitou and Nei 1987) using the CLUSTAL_X program (Thompson et al. 1997). The topology of the phylogenetic tree was evaluated by bootstrap analysis with 1,000 replications. The phylogenetic tree of alginate lyases belonging to PL family 7 was constructed in the same way.

Expression of A9mT Gene and Purification of Recombinant Enzyme *E. coli* DH5α harboring pUC-A9mT was propagated aerobically at 30ºC for 18 h in a 500 ml shaking flask containing 100 ml of LB broth plus 100 μg/ml ampicillin. The cultures were centrifuged (8,000×g for 20 min at 4ºC), and ammonium sulfate was gently added to the supernatant (2.4 l) with stirring to 75% saturation on an ice bath. The precipitates were removed by centrifugation (10,000×g for 30 min at 4ºC), and ammonium sulfate was further added to the centrifugal supernatant to 85% saturation. After standing at 4ºC overnight, the precipitates were collected by centrifugation and dissolved in a small amount of 10 mM Tris-HCl buffer (pH 8.0). The solution was dialyzed against the same buffer for 18 h, and the retentate (62.5 ml) was applied to a DEAE-Toyopearl column (2.5×20 cm; Tosoh) equilibrated with 10 mM Tris-HCl buffer (pH 8.0). The adsorbed proteins were eluted with a 600-ml linear gradient of 0.08 to 0.23 M NaCl in the equilibrating buffer. Alginate lyase activity was eluted around 0.13 M NaCl. The active fractions were concentrated and desalted by ultrafiltration using YM3 membrane (Millipore).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) SDS-PAGE was performed by the method of Laemmli (1970) using a 12.5% acrylamide slab gel (Bio-Rad). The protein was stained with Coomassie Brilliant Blue R-250. Precision Plus protein standards (Bio-Rad) were used as the molecular mass markers.

Enzyme Assay and Protein Concentration Alginate lyase activity was measured in 0.25 ml of a reaction mixture composed of 0.2% (*w*/*v*) sodium alginate, 100 mM Tris-HCl buffer (pH 7.5), 200 mM NaCl, and a suitably diluted enzyme solution. After incubation at 30ºC for 15 min, the reaction was terminated by adding 1 ml of 5 mM HCl, and then, the absorbance of the solution was measured at 235 nm. One unit of enzyme activity was defined as the amount of protein that increased absorbance by 0.1 per min at 235 nm. Mannuronic acid (M), guluronic acid (G), and GM blocks were also used as the substrates, which were basically prepared according to the method of Haug et al. (1966, 1967). The protein concentration was quantified by a DC protein assay kit (Bio-Rad) using bovine serum albumin as the standard. All experiments of enzymatic properties were done at least duplicate, and their mean values were shown.

Results

Analysis of 16S rRNA Gene Sequence of Strain JAM-A9m The 16S rRNA gene of strain JAM-A9m was amplified and sequenced. A 1,497-bp fragment of the gene was compared with those of other strains. Its best match was the 16S rRNA gene of *Vibrio supersteus* strain G3-15 (AY155584) with 98.5% identity (1,462 of 1,484 bases matched). The next match was observed on the 16S rRNA genes of *Vibrio comitatus* strain NHM14 (DQ922919), NHG111 (DQ922918), and NHG13 (DQ922917) with 99.2% identity (1,419 of 1,430 bases matched). The 16S rRNA gene sequences of *Mucus bacterium* strains 19 (AY654742), 45 (AY654784), 54 (AY654792), and 98 (AY654827) also revealed a high degree of similarity (>98%) to the 16S rRNA gene of strain JAM-9m; however, these strains are not validated and should be classified into the genus *Vibrio*, as judged by the phylogenetic affiliation (Fig. 1). These results indicate that strain JAM-A9m is closely related to the genus *Vibrio*. As described previously, strain JAM-A1m belongs to the genus *Agarivorans* (Kobayashi et al. 2009).

Nucleotide and Deduced Amino Acid Sequences for Alginate Lyases Three alginate lyase genes were cloned by PCR and sequenced. The gene for A9mT consisted of 861 bp starting from an ATG codon and ending at a TAA codon. The G+C content of the open reading flame (ORF) was 42.3%. The ORF encoded 286 amino acids including a putative signal sequence of 23 amino acids. The mature enzyme had a calculated molecular mass of 29,643 Da and a p*I* value of 7.15. A9mT was most homologous to an alginate lyase precursor (P39049) of *Photobacterium* sp. with 57.8%

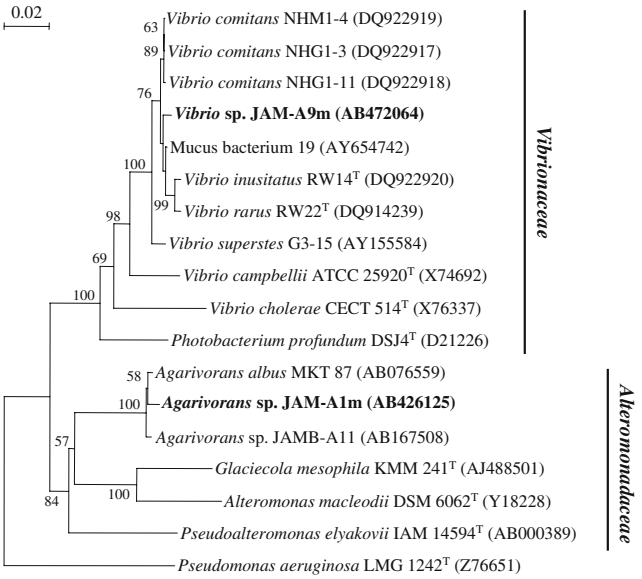

Fig. 1 Phylogenetic tree of alginate lyase-producing isolates and related bacteria based on 16S rRNA gene sequences. The percentage of 1,000 bootstrap resamplings that support branching points above 50% confidence is indicated. GenBank accession numbers of 16S rRNA gene sequences are shown in *parentheses*. *Bar* indicates 0.02 nucleotide substitutions per site

identity, followed by an alginate lyase (AlyVOA; DQ235160) of *Vibrio* sp. O2 and a putative alginate lyase (ZP_00990009) of *V. splendidus* 12B01 with 57.6% and 42.6% identity, respectively.

The gene for A9mL started from an ATG codon and terminated at a TGA codon in the 1,569 bp ORF corresponding to a putative 26-amino-acid signal peptide and a 496-amino-acid mature enzyme. The G+C content of the ORF was 45.8%. A9mL had a molecular mass of 54,426 Da and a p*I* value of 4.94. A9mL showed the highest level of similarity to a putative alginate lyase (ZP_00990010) of *V. splendidus* 12B01 with 83.9% identity. The next similarity was observed in a putative alginate lyase (Q21HU1) of *Saccharophagus degradans* 2–40 with 46.5% identity. The crude recombinant A9mL (rA9mL) showed the highest lyase activity at pH 8 to 9 in Tris-HCl buffer. The relative activities at pH 9.5 and 10 in glycine-NaOH buffer were approximately 50% and 20%, respectively, of the maximal activity.

A9mC was encoded by an ORF of 666 bp that started from an ATG codon and terminated at a TAA codon. The G+C content of ORF was 43.7%. A9mC consisted of 221 amino acids without any signal peptide-like sequence. Indeed, rA9mC was intracellularly expressed in *E. coli* DH5α. The molecular mass and a p*I* value of A9mC were calculated to be 25,009 Da and 4.66, respectively. A9mC showed the highest degree of similarity to a putative alginate lyase (ZP_00990009) of *V. splendidus* 12B01 with 77.2%, followed by alginate lyases of *Vibrio* sp. QY101 (AY221030) and *V. haliticoli* IAM 14596T (AF114037) with 48.0% and 42.5% identity, respectively.

A shotgun-cloned DNA fragment encoding A1mU of *Agarivorans* sp. JAM-A1m contained one ORF of 861 bp. It started from an ATG codon and terminated at a TAG codon. The G+C content of ORF was 40.8%. The ORF encoded 286 amino acids including a putative signal sequence of 20 amino acids. Thus, the mature enzyme had a calculated molecular mass of 30,218 Da and a p*I* value of 6.36. A1mU exhibited higher levels of similarity to AlyVOA (DQ235160) of *Vibrio* sp. O2 and a (poly ManA) alginate lyase (P39049) of *Photobacterium* sp. ATCC43367 with 76.1% and 75.8% identity, respectively. The properties of each enzyme are summarized in Table 1. The similarity among the five enzymes including A1m (Kobayashi et al. 2009) was relatively low with between 14.2% and 58.1% identity (Supplementary Table). Based on the low similarity among the five lyases in this study, their phylogenetic positions are separated as shown in Fig. 2.

Biochemical Characterization of rA9mT rA9mT was purified to homogeneity as judged by SDS-PAGE from a culture of *E. coli* DH5α harboring pUC-A9mT. Ammonium sulfate precipitation was very effective for enzyme purification, and the enzyme was simply and completely purified by only one anion-exchanged column chromatography. A typical purification was summarized in Table 2. The purification increased the specific activity 311 times with 11.6% recovery of the initial activity. The molecular mass of the enzyme was around 28 kDa by SDS-PAGE (Fig. 3) and 31 kDa by Bio-Gel-A0.5 m gel filtration, indicating that the enzyme is a monomeric protein. rA9mT preferably degraded the M block to the G block in alginate. The relative activities for alginate, M, G, and GM blocks were 100%, 75%, 21%, and 15%, respectively, under the standard assay conditions at pH 7.5. rA9mT was remarkably activated in the presence of NaCl. When 0.4 M NaCl was added to the reaction mixture, the activity was increased maximally to 24 times the original activity. The activity gradually decreased at higher NaCl concentrations, but was still activated 20 times at 0.8 M and 8 times at 1.6 M NaCl. We noticed that the enzyme was also activated by rare salts, such as LiCl, RbCl, and CsCl, as shown in Table 3. All salts tested at 0.2 M increased the original activity 18 to 25 times.

Effects of pH on rA9mT The maximum activity of rA9mT was observed between pH 7.6 and pH 9 in 100 mM Tris-HCl buffer, MOPS buffer, and glycine-NaOH buffer in the presence of 0.2 M NaCl. In glycine-NaOH buffer with 0.2 M NaCl at pH 10, the relative activity was more than

Table 1 Properties of alginate lyases of *Vibrio* and *Agarivorans*

Enzyme	Number of amino acid (Number of signal amino acids)	Molecular mass (Da)	Calculated p*I*	The highest identity (%)	
A9mT	286 (23)	29,643	7.15	57.8	Alginate lyase precursor of *Photobacterium* sp. (P39049)
A9mL	522 (26)	54,426	4.94	83.9	Putative alginate lyase of *Vibrio splendidus* 12B01 (ZP_0099009)
A9mC	221 (0)	25,009	4.66	77.2	Putative alginate lyase of *Vibrio splendidus* 12B01 (ZP_0090010)
A1mU	286 (20)	30,218	6.36	76.1	AlyVOA of *Vibrio* sp.O2 (DQ 235160)
A1m[a]	309 (20)	32,295	5.66	53.7	AlyA of *Klebsiellae pneumoniae* sub sp. *aerogenes* (QO 59478)

[a] Kobayashi et al. 2009

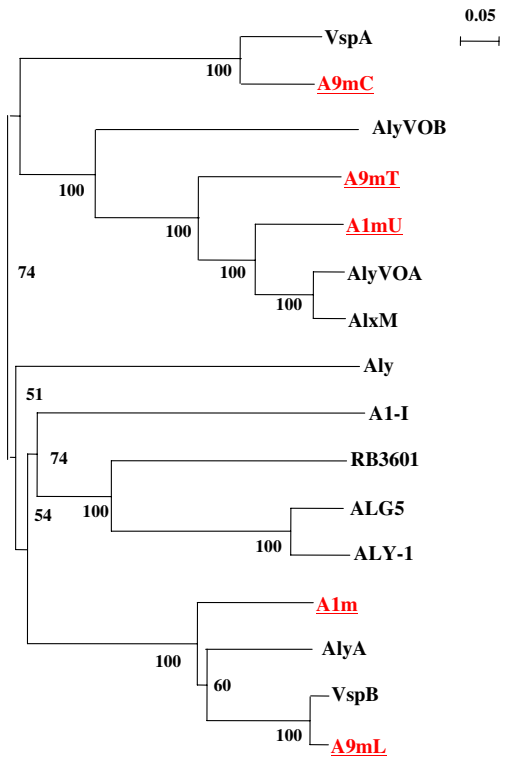

Fig. 2 Phylogenetic tree of alginate lyases belonging to PL family 7. Sources of sequences *aligned*: ALY-1 (AB030481) from *Corynebacterium* sp. strain ALY-1; A1-I (BAB03312) from *Sphingomonas* sp. A1; AlyA (L19657) from *Klebsiella pneumoniae* subsp. *aerogenes*; Aly (AF082561) from *Pseudoalteromonas elyakovii* IAM 14594; AlxM (P39049) from *Photobacterium* sp. ATCC43367; AlyVOA (DQ235160) and AlyVOB (DQ235161) from *Vibrio* sp. O2; A1m (AB426616) from *Agarivorans* sp. JAM-A1m; VspA (ZP_00990009) and VspB (ZP_00990010) of *Vib. splendidus* 12B01; ALG5 (ABS59291) of *Streptomyces* sp. ALG5; and RB3601 (BX294139) of *Rhodopirellula baltica* SH1. *Bar* represents *knuc* unit

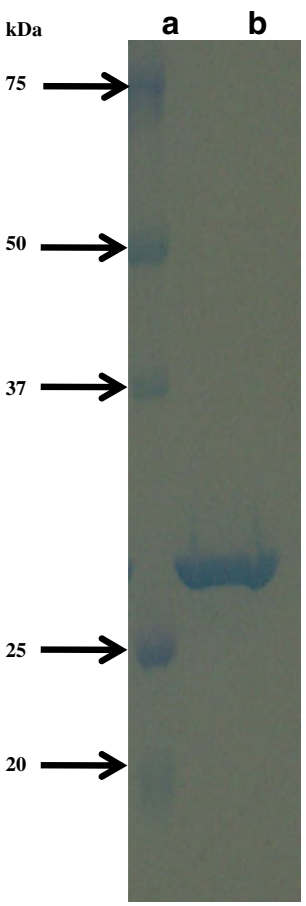

Fig. 3 SDS-PAGE of purified rA9m. *Lane A* protein mass markers, *lane B* purified enzyme (6.3 μg). The proteins were electrophoresed on 12.5% acrylamide gel and stained with Coomassie Brilliant Blue R-250

80% of the maximal activity (Fig. 4). This is the reason why *Vibrio* sp. JAM-A9m was selected as a potential alkaline alginate lyase producer. The enzyme was stable between pH 7 and 10 in various 20 mM buffers without NaCl after incubation at 30ºC for 15 min. In the presence of 0.2 M NaCl, however, the residual activities from pH 6 to 10 were decreased 40–75% of those observed in the absence of NaCl (Fig. 5). This phenomena was very puzzling, but the residual activity was completely recovered when the enzyme solution was diluted 10 times with distilled water after preincubation in 20 mM Tris-HCl buffer (pH 7) plus 0.2 M NaCl at 30ºC for 15 min. This indicates that preincubation of the enzyme in the presence of 0.2 M NaCl without a substrate caused a reversible inactivation. The results suggest that the enzyme was able to bind substrate at high ionic strength, with consequent stabilization due to substrate induced conformational change.

Effects of Temperature of rA9mT The optimal temperature for the reaction of rA9mT was around 30ºC in the presence of 0.2 M NaCl at pH 7.5. The enzyme worked well at lower temperatures; the relative activities at 10ºC and 2ºC were around 45% and 30% of the maximal activity at 30ºC, respectively. rA9mT was rapidly inactivated above 30ºC when the enzyme was incubated at 5ºC to 50ºC for 15 min in 100 mM Tris-HCl buffer (pH 7.5) with or without 0.2 M

Table 2 Summary of purification of rA9mT

Step	Volume (ml)	Total protein (mg)	Total activity (units)	SA (units/mg)	Yield (%)	Fold
Culture broth	2,400	17,910	81,100	4.53	100	1
Ammonium sulfate precipitation	62.5	358.8	56,070	156.3	69.1	34.5
DEAE-Toyopearl (concentrate)	10.5	6.7	9,387	1,401	11.6	309.3

Table 3 Effects of salts on rA9mT activity

Salts	Concentration (mM)	Relative activity (%)
None	–	100
NaCl	200	2,190
KCl	200	2,490
LiCl	200	1,880
RbCl	200	2,500
CsCl	200	2,500
NH4Cl	200	2,270
NaBr	200	2,180
NaF	200	2,170
$(NH_4)_2SO_4$	200	2,350
CH_3COONa	200	2,100
CH_3COONH_4	200	2,090

rA9mT (0.32 μg) was incubated with 200 mM salts in 100 mM Tris-HCl buffer (pH 7.5) containing 0.2% alginate at 30°C for 15 min

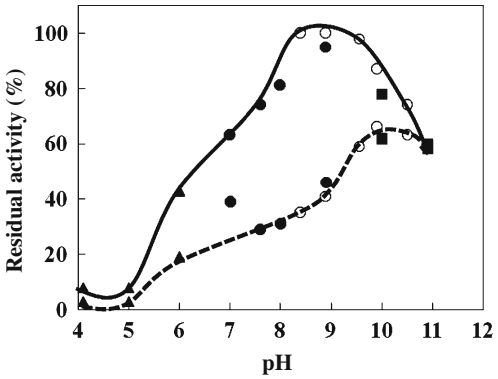

Fig. 5 Effect of pH on rA9m stability. Purified rA1m (0.8 μg) was preincubated at 30ºC for 15 min in various 20 mM buffers with (*dotted line*) or without (*straight line*) 0.2 M NaCl. After preincubation, the residual activity was measured at 30ºC for 15 min in 100 mM Tris-HCl buffer (pH 7.5) plus 0.2 M NaCl. The buffers used were acetate (pH 4–6, *filled triangles*), Tris-HCl (pH 7–8.9, *filled circles*), glycine-NaOH (pH 8.4–10.5, *open circles*), and carbonate (pH 10–10.9, *filled squares*). The highest residual activity is taken as 100%

NaCl, and the enzyme was rapidly inactivated when incubated at above 40ºC in the absence of NaCl. On the other hand, in the presence of NaCl, the response of the residual activity to the temperature was unexpected as shown in Fig 6. The enzyme showed a higher stability, and more than 30% of the original activity remained even after incubation at 80ºC for 15 min.

Effects of Metal Ions and Surfactants on A9mT Activity

rA9mT activity was measured in the presence of various metal ions (1 mM each). Ca^{2+}, Mn^{2+}, and Mg^{2+} did not influence enzyme activity, whereas, Ni^{2+}, Co^{2+}, Sr^{2+}, Zn^{2+}, and Cu^{2+} ions inhibited the enzyme by 14% to 35% (Table 3). rA9mT was not affected by EDTA and EGTA (5 mM each) at all. After the enzyme was preincubated in 100 mM Tris-HCl buffer (pH 7.5) with 100 mM surfactants at 30ºC for 15 min, the residual activity was measured in the standard assay conditions. An anionic surfactant, namely SDS, strongly inhibited the enzyme activity by 90%, while the

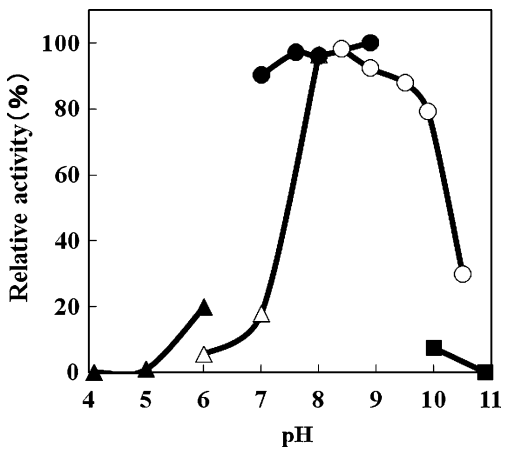

Fig. 4 Effect of pH on rA9m activity. The activity was measured at 30ºC for 15 min in a total volume of 0.25 ml containing 0.4 μg of purified rA9mT in the presence of 0.2 M NaCl. The following 100 mM buffers were used: *open triangles* indicate acetate (pH 4–6); *filled triangles*, MOPS (pH 6–7.9); *filled circles*, Tris-HCl (pH 7.1–8.9); *open triangles*, glycine-NaOH (pH 8–10.8); and *filled squares*, carbonate buffer (pH 10–11). The highest activity, obtained at pH 9 in Tris-HCl buffer, is taken as 100%

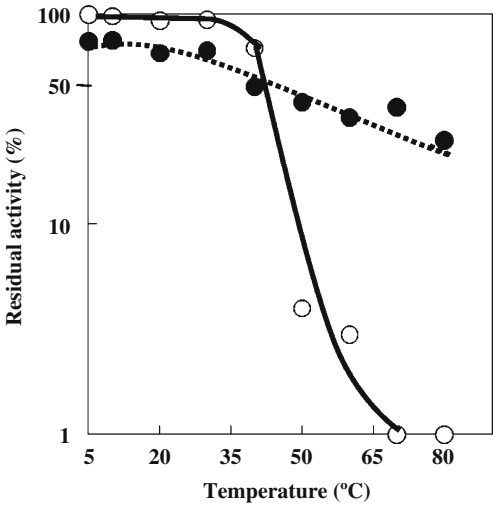

Fig. 6 Effect of temperature on rA9m stability. Purified rA9mT (0.4 μg) was treated with the indicated temperature for 15 min in 20 mM Tris-HCl buffer (pH 7.5) with (*dotted line*) or without (*straight line*) 0.2 M NaCl. After treatment, the solution was put in an ice bath; then, the residual activity was measured under the standard assay conditions described in text. The residual activity (with or without NaCl) after treatment with 4ºC is taken as 100%

Table 4 Effects of metal ions and chelators on rA9mT activity

Additives	Concentration (mM)	Relative activity (%)
None	–	100
$CaCl_2$	1	104
$MnCl_2$	1	100
$MgCl_2$	1	103
$CoCl_2$	1	86
$SrCl_2$	1	86
$NiCl_2$	1	65
$ZnCl_2$	1	81
$CuCl_2$	1	84
$SnCl_2$	1	96
EDTA	5	101
EGTA	5	103

rA9mT (0.4 μg) was incubated with the indicated concentration of metal ions and chelators in 50 mM Tris-HCl buffer (pH 7.5) containing 0.2% alginate plus 0.2 M NaCl at 30°C for 15 min

enzyme was moderately activated by nonionic surfactants, Tween20 by 139% and Nonidet P40 by 134%.

Discussion

We cloned and sequenced four alginate lyase genes from deep-sea bacteria, *Agarivorans* and *Vibrio*. Among them, rA9mT was purified to homogeneity and characterized. rA9mT was a salt-activated enzyme, like several alginate lyases reported to date (Horikoshi and Akiba 1982; Lange et al. 1989; Brown and Preston 1991; Kitamikado et al. 1992; Wong et al. 2000; Xiao et al. 2006). A guluronate lyase from *Vibrio harveyi* AL-128 is the greatest salt activation, being increased 24 times at 1 M NaCl (Kitamikado et al. 1992), whereas rA9mT is the greatest one (24 times activation at 0.4 M NaCl) among M block-degrading lyases reported previously. A9mT and A1m work well in marine environment Furthermore, the thermal stability of rA9mT was protected by NaCl and the enzyme possesses the highest thermal stability among alginate lyases reported to date (Wong et al. 2000). rA9mT was not activated by Ca^{2+} or Mg^{2+} ions unlike other M block-specific alginate lyases from marine bacterium (Romeo and Preston 1986), *Vibrio alginolyticus* (Kitamikado et al. 1992) and *Pseudomonas* sp. (Xiao et al. 2006), whose activities are strongly enhanced by both metal ions. rA9mT was unaffected by EDTA and EGTA. Several M block-degrading enzymes are slightly activated or unaffected with chelators (Shimokawa et al. 1997a, b; Ertesvåg et al. 1998; Miyake et al. 2004; Xiao et al. 2006), although many enzymes that prefer G blocks are inactivated by EDTA (Kitamikado et al. 1992; Nibu et al. 1995; Wong et al. 2000; Kobayashi et al. 2009). It is thought that the original strain extracellularly produces A9mT in the presence of alginate because of the optimal pH values and salt-activated property of the enzyme. Thus, the characteristics of A9mT are appropriate for activity in marine environments, like A1m (Kobayashi et al. 2009).

As shown in Fig. 6, PL family 7 alginate lyases including the four enzymes in this study contain three highly conserved amino acid sequences, (R/E)(S/T/N)EL, Q(I/V)H, and YFKAG(V/I)YNQ. According to the crystal structures and

Enzyme	I			Region II			III			Substrate specificity
ALY-1	··· 72	RSELREM	78 ···	112	HLVGAQIH	119 ···	189	YFKAGAYTQ	197 ···	G
A1-1	···479	RSELREM	485 ···	517	KTIVMQIH	524 ···	610	YFKAGNYLQ	618 ···	G
AlyA	··· 93	RSELREM	99 ···	157	RVIIGQIH	164 ···	274	YFKAGVYNQ	282 ···	G,GM
Aly	···217	RHEYKVK	223 ···	250	KTIISQHH	257 ···	365	YFKFGNYLQ	373 ···	GM
A1-II'	···146	RSELREM	153 ···	184	KIIVAQIH	191 ···	278	YFKAGNYLQ	286 ···	GM
PA1167	··· 59	RSELRET	65 ···	97	RMIIGQIH	104 ···	193	YFKAGLYLQ	201 ···	GM
AlxM	··· 97	RTDLPDH	103 ···	139	EITFLQVH	146 ···	257	YFKAGVYNQ	265 ···	M
AlyVOA	··· 97	RNELRVQ	103 ···	139	EITFLQVH	146 ···	257	YFKAGVYNQ	265 ···	M
AlyVOB	··· 83	RSELRQV	89 ···	119	EITFLQVH	126 ···	220	YFKAGVYNQ	228 ···	M
A1m	··· 94	RSELREM	100 ···	159	RVIIGQIH	166 ···	276	YFKAGVYNQ	284 ···	G, GM
A1mU	··· 46	ESELQIS	52 ···	139	EITFLQVH	146 ···	257	YFKAGVYNQ	265 ···	M*
A9mC	··· 20	RSELREL	26 ···	74	KVVLGQIH	81 ···	191	YFKAGIYPD	199 ···	G*
A9mT	··· 99	RNELRVH	105 ···	141	EITYLQVH	148 ···	258	YFKAGVYNQ	266 ···	M**
A9mL	···316	RTELREM	322 ···	380	RFIIGQIH	387 ···	487	YFKAGVYNQ	495 ···	G*

*Predicted substrate specificity; **this study

Fig. 7 Conserved amino acid sequences among PL family 7 alginate lyases. The amino acid residues that are highly conserved among PL family 7 enzymes are represented by a *single letter*. The amino acid numbers in each sequence are counted from the initial methionine of each enzyme. Substrate specificities are indicated as G block-specific or block-preferable enzyme (*G*), M block-specific or block-preferable enzyme (*M*), and GM block-specific or block-preferable enzyme (*GM*). Sources of sequences aligned: ALY-1, A1-I, AlyA, Aly, AlxM, AlyVOA, and AlyVOB are the same as in the legend for Fig. 2

the sequence analyses of several PL family 7 lyases (Yamasaki et al. 2004, 2005; Osawa et al. 2005), the conserved regions I, II, and III form the cavity composed of a jelly roll β-sandwich structure, and the cavity is assumed to bind to a suitable substrate. Thus, conserved amino acid residues are thought to play an important role in catalytic activity or folding of the structure. Osawa et al. (2005) suggested that Y^{195}, H^{119}, Q^{117}, and R^{72} may be involved in the catalytic site (ALY-1 numbering in Fig. 7). Therefore, amino acid residues in catalytic or substrate binding sites would be highly conserved depending on the substrate specificity. Actually, known G block- and M block-degrading lyases contain the amino acid sequences, QIH and QVH, in the conserved region II, respectively. Furthermore, G block-degrading enzymes retain more conserved amino acid residues in region I, RSELREM, than M block-degrading enzymes. Indeed, A1m preferably degrade G and GM blocks (Kobayashi et al. 2009), which shows the amino acid sequence, QIH, in the region II. Besides, A9mT favorably degrades M blocks, possessing QVH in the region II. From this perspective, the substrate specificities of the enzymes in this study were classified. As predicted in Fig. 7, A1mU of *Agarivorans* sp. JAM-A1m preferably degrades M blocks, A9mC and A9mL of *Vibrio* sp. JAM-A9m preferably degrade G blocks. Both organisms produce several alginate lyases showing the different substrate specificity, thus, they can effectively degrade alginate in nature. It is interested that all alginate lyases of both bacteria belong to the same family, although, they show a relatively low similarity one another.

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