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ORIGINAL ARTICLE

Cloning and Characterization of a Novel Oligoalginate Lyase from a Newly Isolated Bacterium *Sphingomonas* sp. MJ-3

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Abstract A bacterium possessing alginate-degrading activity was isolated from marine brown seaweed soup liquefied by salted and fermented anchovy. The isolated strain was designated as *Sphingomonas* sp. MJ-3 based on the analyses of 16S ribosomal DNA sequences, 16S-23S internal transcribed spacer region sequences, biochemical characteristics, and cellular fatty acid composition. A novel alginate lyase gene was cloned from genomic DNA library and then expressed in *Escherichia coli*. When the deduced amino acid sequence was compared with the sequences on the databases, interestingly, the cloned gene product was predicted to consist of AlgL (alginate lyase L)-like and heparinase-like protein domain. The MJ-3 alginate lyase gene shared below 27.0% sequence identity with exolytic alginate lyase of *Sphingomonas* sp. A1. The optimal pH and temperature for the recombinant MJ-3 alginate lyase were 6.5 and 50°C, respectively. The final degradation products of alginate oligosaccharides were analyzed by electrospray ionization mass spectrometry and proved to be alginate monosaccharides. Based on the results, the recombinant alginate lyase from *Sphingomonas* sp. MJ-3 is regarded as an oligoalginate lyase that can degrade oligoalginate and alginate into alginate monosaccharides.

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Introduction

Alginate is a linear polysaccharide consisting of guluronate (G) and mannuronate (M) as the monomer constituents. Alginate is produced by brown seaweed and some bacteria. Alginate is a commercially useful polysaccharide that is widely used in food and pharmaceutical industry due to its high viscosity and gelling properties (Draget et al. 2005). Alginate has a property to form gel beads in the presence of certain metal ions and thus can be used as immobilization media for animal cell culture. Oligosaccharides prepared from alginates are known to have various physiological properties such as enhancing cytokine production from macrophage cells and growth of human keratinocytes and endothelial cells (Iwamoto et al. 2003, 2005; Kawada et al. 1999; Yamamoto et al. 2007). Acetylated bacterial alginate is an important therapeutic target for preventing lung infection by *Pseudomonas aeruginosa* in cystic fibrosis patients (Boyd and Chakrabarty 1994; Ramsey and Wozniak 2005). Pathogenic *P. aeruginosa* forms biofilm consisting of acetylated alginates, and this biofilm prevents effective antibiotics treatment.

Alginate lyase is an alginate-degrading enzyme. Alginate lyase depolymerizes alginate by cleaving the glycosidic bonds through a β-elimination reaction (Wong et al. 2000). Various alginate lyases have been cloned and characterized (Cao et al. 2007; Gimmestad et al. 2009; Han et al. 2004; Iwamoto et al. 2001; Kawamoto et al. 2006; Matsubara et al. 2000; Suzuki et al. 2006). The mode of degradation of

alginate by alginate lyase can be classified into endo- and exolytic fashion (Fig. 1). Endotype alginate lyase can be further classified as poly-G, poly-M, and poly-MG specific lyases that can degrade poly-G, poly-M, and poly-MG blocks of alginate, respectively. Endotype alginate lyases give rise to unsaturated di-, tri-, and tetra-saccharides as the degradation products (Zhang et al. 2004). Exotype alginate lyase can degrade both oligomeric alginates and alginate polymer (Hashimoto et al. 2000). It has been reported that the exotype oligoalginate lyase from *Sphingomonas* sp. A1 could recognize both unsaturated and saturated non-reducing saccharides in alginate or oligoalginates, and degrades them into saturated or unsaturated monosaccharides (Miyake et al. 2003). The unsaturated monosaccharide is non-enzymatically converted to α-keto acid, 4-deoxy-L-erythro-5-hexoseulose uronic acid (Fig. 1). The X-ray crystallography for the alginate lyase from *Agrobacterium tumefaciens* strain C58 was conducted to analyze 3D structure of exotype alginate lyase (Ochiai et al. 2010).

Recently, microalgae and macroalgae have attracted much attention as the alternative biomass for the production of biofuels such as biodiesel and bioethanol. In the case of macroalgae, laminaran and mannitol are mainly used as the carbohydrate source for the production of bioethanol. In algal biomass, alginate constitutes more than 20% of total biomass, and it can be used as the alternative carbohydrate source. In order to use alginate as the alternative carbohydrate source for the production of biofuels or biochemicals, the saccharification of alginate is a prerequisite requirement. Alginate lyases can play a key role in the saccharification process as the biocatalyst. Therefore, it is of interest to recruit various types of recombinant alginate lyases that can be produced in large quantity. Recently, we have isolated and cloned the endolytic alginate lyases from *Streptomyces* sp. ALG-5 for the development of recombinant biocatalyst for the production of alginate oligosaccharides (Kim et al. 2009). The recombinant ALG-5 alginate lyase was immobilized onto magnetic nanoparticles for the preparation of alginate oligosaccharides in a repeated batch operation (Shin et al. 2010).

In this study, we isolated a bacterium that could use alginate as the sole carbon source for its growth. The isolated bacterium was taxonomically identified, and its microbiological characteristics were analyzed. In order to utilize alginate as the sole carbon source, the isolated strain was expected to have various types of alginate lyases. We cloned a novel alginate lyase that can degrade alginate to unsaturated monosaccharides from the isolated strain, *Sphingomonas* sp. MJ-3. A novel oligoalginate lyase possessing an AlgL domain and a heparinase-like domain was cloned and characterized on the molecular level. The substrate specificity of recombinant oligoalginate lyase and final degradation products were also analyzed.

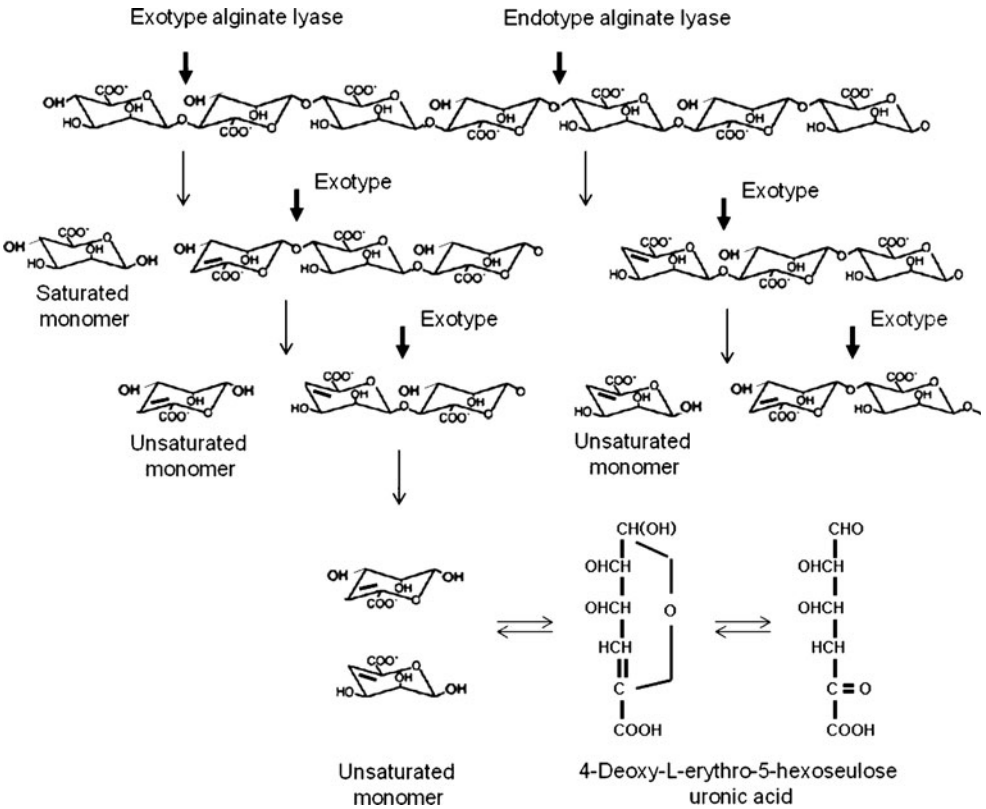

Fig. 1 Schematic diagram of endo- and exolytic cleavage of alginate into monosaccharides and non-enzymatic conversion of the monosaccharide to the corresponding α-keto-deoxy uronic acid (4-deoxy-L-erythro-5-hexoseulose uronic acid)

Materials and Methods

Screening and Cultivation of Alginate-Degrading Bacteria

Alginate-degrading bacteria were isolated from fermented brown seaweed with salted and fermented anchovy. After the sea mustard (*Undaria pinnatifida*) was seasoned with fermented anchovy, the bottle was left outside until the seaweed was liquefied completely. The fermented soup was diluted with deionized water serially and then smeared on M9 agar plate containing 0.8% (*w*/*v*) alginate. The plates were incubated at 20°C for 10 days and stored at 4°C as a master plate. Several colonies with colors grew on the plate. A pale and flat colony with the fastest growth rate was chosen for further study. The isolated bacterium was cultured in M9 media or Luria–Bertani (LB) broth with alginate to analyze the effect of alginate on the cell growth.

Chemotaxonomic and Molecular Characterization of the Isolated Strain MJ-3

The strain MJ-3 was isolated by the standard dilution-plating technique at 25°C on M9 agar plate with 0.8% (*w*/*v*) alginate. The Gram staining was conducted using the Difco Gram staining kit according to the manufacturer's instruction. Enzyme activities and sugar utilization were determined using the API 20 NE kit and API ZYM system (bioMerieux, France). Cellular fatty acid methyl esters were prepared according to the standard protocol of the MIDI Microbial Identification System. Fatty acid analysis was identified by gas–liquid chromatography using the TSBA40 method (Buyer 2002). Sensitivity to antibiotics was tested on a tryptic soy agar plate using discs containing the following antibiotics: norfloxacin (10 μg), vancomycin (30 μg), doxycycline (30 μg), streptomycin (10 μg), chloramphenicol (30 μg), kanamycin (30 μg), ampicillin (30 μg), erythromycin (15 μg), amilkacin (30 μg), cefotaxime (30 μg), cephalothin (30 μg), gentamicin (10 μg), netilmicin (30 μg), tobramycin (10 μg), sulfamethoxazole/trimethoprim (25 μg), rifampin (5 μg), tetracycline (30 μg), and amoxicillin/clavulanic acid (30 μg).

The 16S ribosomal DNA sequence of the isolated strain was determined by the procedure described in the previous work (Kim et al. 2009). The 16S ribosomal DNA (rDNA) was amplified from the genomic DNA by PCR using the bacterial primers 27f (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1518r (5′-AAGGAGGTGATCCANCCRCA-3′). The 16S–23S intergenic transcribed spacer (ITS) DNA was amplified by PCR using the following primers: 16S rDNA 3′-end sequence (5′-GCTGGATCACCTCCTTTCT-3′) and 23S rDNA 5′-end sequence (5′-CTGGTGCCAAG GCATCCA-3′). The PCR product was ligated to T-blunt vector (SolGent, Korea) and sequenced using a BigDye terminator sequencing method. The 16S rRNA gene and 16S–23S ITS region were blasted and aligned with multiple sequence data in GenBank database using BLAST algorithm and ClustalW program. The phylogenetic tree was constructed using neighbor-joining algorithms.

Assay of Alginate Lyase Activity

Enzymatic degradation of alginate was done in 50 mM phosphate buffer containing 0.2% (*w*/*v*) sodium alginate as the substrate at pH 7.0 and 30°C. The alginate lyase activity was determined by measuring an increase in absorbance at 235 nm (Yoon et al. 2000) or using thiobarbituric acid method (Warren 1960). The degradation products were analyzed by thin layer chromatography (TLC) and fast protein liquid chromatography (FPLC). The degraded products on TLC were separated twice with a developing solvent of 1-butanol/acetic acid:water (2:1:1, *v*/*v*/*v*). The degradation products were visualized by dipping into 10% (*v*/*v*) sulfuric acid in ethanol. Since the monomer was not readily detected by TLC, FPLC equipped with Superdex peptide 10/100 GL column (GE Healthcare, USA) was also used with 0.2 M bicarbonate buffer (pH 7.2). The degraded oligomer and monomer with unsaturated non-reducing end were detected by measuring the absorbance at 235 nm with UV detector.

Effects of Alginate on Cell Growth and Alginate Lyase Activity

In order to investigate the effect of alginate on the cell growth, the cell was cultured in a M9 broth or LB broth containing 0–0.8% (*w*/*v*) sodium alginate for 5 days at 30°C and 180 rpm. Fifteen flasks containing each culture media were inoculated with seed culture, and samples were withdrawn periodically to measure the absorbance at 600 nm (for measuring growth rate) and assay the enzyme activity. To assay alginate lyase activity, the cell pellets were sonicated in 50 mM potassium phosphate buffer (pH 7.2) using ultrasonicator Vibra Cell CX400 (Sonics & Materials Inc, USA), and the lysate was centrifuged at 16,000×*g* for 40 min to obtain supernatant.

Construction of Genomic DNA Library of the Isolate MJ-3

Genomic DNA was isolated from the cells using organic chemical solvent extraction technique (Gimmestad et al. 2009). Sheared genomic DNAs with 30–40 kb size were ligated into the fosmid vector, CopyControl™ pCC1FOS™ vector (Epicentre, USA), packaged and plated on EPI300-T1^{R} plating cells by Genotech, Korea. Approximately

10,000 clones on 35 LB agar plates (15 cm id) were picked and arrayed in 96-well microtiter plates. Each microplate with recombinant EPI300-T1^{R} cells was duplicated and cultured in LB broth containing 12.5 μg/ml chloramphenicol. One set of each microplate was made to glycerol stock and then stored at −75°C deep freezer.

Screening of the Fosmid DNA Containing Alginate Lyase Gene

In order to screen a fosmid clone containing the gene of alginate lyase from MJ-3 genomic DNA library, the primers were designed on the basis of homolog sequences from heparinase-like protein domain because oligoalginate lyase from *Sphingomonas* sp. A1 possesses heparinase-like domain in the C-terminal sequence. The following nucleotide sequences were used to design the primers: Smal_2067 (*Stenotrophomonas maltophilia* R551-3), Smlt_2602 (*S. maltophilia* K279a), BresuDraft_1835 (*Brevundimonas subvibrioides* ATCC 15264), Avin_46500 (*Azotobacter vinelandii* DJ), Sde_3284 (*Saccharophagus degradans* 2–40), and Mmar10_0256 (*Maricaulis maris* MCS10). A pair of primers for detection of oligoalginate lyase was 5MJ-3-Hepa790F (5′-GAAGGSCCCTACTACCAGCGCTATGC-3′) and 3MJ-3-Hepa1430R (5′-ACVACCARGGTGTTGTGSGCSAYGGTCTG-3′).

Each colony on 35 fosmid library plates was transferred to a 96-well microplate to store the colonies. After transferring into 96-well plate, the colonies on each agar plate were collected by spreader, respectively. The collected cells were boiled with small amount of water and centrifuged, and then the supernatant was used for PCR to identify the agar plate containing the positive colony and the corresponding 96-well microplate. The cells in every 12 wells of the positive 96-well microplate were mixed and then subjected for next round of PCRs. After identifying the positive lane, the cells in each well of the positive lane were boiled and used for third round of PCRs. Based on this step-by-step procedure consisting of 57 PCRs, we finally identified the positive fosmid clone.

Cloning and Expression of MJ-3 Alginate Lyase

To identify and clone the whole sequence of the putative MJ-3 alginate lyase, the positive fosmid DNAs were sequenced with several primers (Table 1). A partial heparinase-like protein gene without N-terminal sequence, the homology sequence of polymannuronate lyase (5MJ-3-PolyM1170F) and N-terminal sequence of heparinase-like protein (3MJ-3-Hepa300R) were also used to design the primers, 5MJ-3-PolyM1170F and 3MJ-3-Hepa300R, for the amplification of the N-terminal region of MJ-3 alginate lyase. The 3MJ-3-Hepa300R sequence was obtained from the sequences of the target fosmid DNA with 3MJ3-Hepa870R. After the complete nucleotide sequences were determined, the primers with restriction sites were designed for cloning and expressing the MJ-3 alginate lyase gene in the expression vector pET21b(+). The forward primer [5MJ3-OligoMF (*Nde*I)] and the reverse primer [3MJ3-OligoR(*Hin*dIII)] were used to amplify the translational region of MJ-3 alginate lyase without signal peptide sequence and stop codon (Table 1). The genomic DNA of the strain MJ-3 was used as the template for PCR, and the signal peptide cleavage site was predicted using SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/). A PCR was carried out using pfu-X DNA polymerase (SolGent, Korea). The PCR product was ligated to ready-made T-blunt vector (SolGent, Korea) and then transferred to pET21b(+) vector (Novagen, USA).

The recombinant *Escherichia coli* BL21 (DE3) harboring the pET21b/MJ3-alginate lyase plasmid was cultured on an LB medium supplemented with 50 μg/ml ampicillin for 2–3 h up to OD_{600}=0.4–0.6 in a shaking incubator at 250 rpm and 37°C. The cells were cultured for 24 h to express the MJ-3 alginate lyase gene by addition of 1 mM IPTG.

Purification of the Recombinant MJ-3 Alginate Lyase and Analysis of Its Substrate Specificity

The cells were harvested and sonicated in lysis buffer [50 mM potassium phosphate buffer (pH 7.2), 300 mM NaCl, 10 mM imidazole, 10% (*v*/*v*) glycerol, 0.5% (*v*/*v*) Triton X-100, 1 mg/ml lysozyme, 1 mM phenylmethylsulfonyl fluoride]. The cell homogenate containing the expressed $(His)_6$-tagged MJ-3 alginate lyase was loaded on Ni-Sepharose column (Amersham Biosciences, USA) equilibrated with 50 mM phosphate buffer (pH 7.2) and 0.5 M NaCl. The column was washed with the same buffer containing 50 mM imidazole, and the MJ-3 alginate lyase protein was eluted with the same buffer containing 300 mM imidazole. The active fraction was desalted using HiTrap^{TM} desalting column (Amersham Biosciences, USA). The substrate specificity of MJ-3 alginate lyase was determined using alginate, poly-M, poly-G, and poly-MG block substrates, and oligoalginate substrate as described in Haug et al. (1966). MG ratio was calculated from ^{1}H-NMR spectra using the method described in the references (Ertesvåg et al. 1998; Grasdalen et al. 1979).

SDS-PAGE Analysis and Immunoblotting

The proteins were separated on 12% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and transferred onto a nitrocellulose membrane. The blotted membrane was incubated with rabbit polyclonal antibody against

Table 1 Primers used for fosmid screening, determining internal nucleotide sequences of the alginate lyase gene from MJ-3 genomic DNA library, and cloning of the alginate lyase gene from *Sphingomonas* sp. MJ-3

Primer name	Sequence (5′→3′)
For fosmid screening	
5MJ-3-Hepa790F	GAAGG**S**CCCTACTACCAGCGCTATGC
3MJ-3-Hepa1430R	AC**V**ACCA**R**GGTGTTGTG**S**GC**S**A**Y**GGTCTG
3MJ-3-Hepa300R	GTTGAGGCTCTGCCAGAACAGACG
5MJ-3-PolyM1170F	CCSGCAACCTGATCGTCAACCGC
3MJ3-Hepa870R	GAAATAGCCGTCATAGGTCGTCTGG
For determining internal sequences	
5MJ-3-Hepa1330F	CATGCCATCGTGACCGATTACGGCGC
5MJ-3-Hepa2080F	CTCGGCCGATGCCAATGCTGCAC
For cloning of the MJ-3 alginate lyase gene into pET21b(+) vector	
5MJ-3-OligoMF (*Nde*I)	GCATATGCAGACCGCGCCGGGCGATCAG
3MJ-3-OligoR (*Hind*III)	GAAGCTTGTTCCCCTTGCCCGCATCGAAG

Bold letters, S, V, R, and Y, means G+C, G+A+C, A+G, and C+T, respectively. Underlined nucleotides indicate the restriction sites

hexahistidine (H-15, Santa Cruz Biotechnology Inc., USA) and peroxidase-conjugated anti-rabbit IgG (Jackson Immunoresearch, USA), and then visualized with 4-chloronaphthol/3,3′-diaminobenzidine solution (Pierce, USA).

Separation of Alginate-Degraded Products and Identification of Molecular Mass

The lyophilized alginate-degrading products mixture was dissolved in small amount of water and then size-fractionized using BioGel P2 gel filtration chromatography (1.5×360 cm, BioRad, USA). The mixture was eluted with 0.2 M NH_4HCO_3 at 0.3 ml/min flow rate. The fraction was collected as 2 ml/tube. The unsaturated uronate fraction was detected by measuring the absorbance at 235 nm. The alginate degradation fractions were pooled, evaporated, and lyophilized for molecular weight analysis. To analyze alginate degradation pattern of MJ-3 alginate lyase, a partially digested 5% (*w*/*v*) oligoalginate mixture prepared by ALG-5 lyase (poly-G specific lyase; Kim et al. 2009) was also treated using MJ-3 alginate lyase, and then the degradation products were analyzed using TLC and FPLC.

For the determination of the molecular mass of each fraction, electrospray ionization mass spectroscopy (6410 Triple Quadrupole LC-MS, Agilent, USA) was used. Each fraction from gel filtration chromatography was dissolved in methanol/water (1:1, *v*/*v*) and then injected to liquid chromatography–mass spectrometry (LC-MS) with the electrospray source at a 0.5 ml/min flow rate. Mobile phase was 10 mM ammonium acetate/methanol (1:1, *v*/*v*). MS was performed in negative mode with ion spray voltage of 4 kV and source temperature of 350°C.

Chemicals

Sodium alginate (3,500 cps grade) was purchased from Sigma Co. (USA). Homopolymeric block regions, poly-G and poly-M, and poly-G/M or M/G random regions were prepared from sodium alginate using the method of Haug et al. (1966). Dimer, trimer, and oligomer mixture used as the standard oligoalginates were obtained as described previously (Kim et al. 2009).

Results and Discussion

Screening of Alginate-Degrading Bacteria and Identification of the Isolated Strain

In order to isolate a microbial strain possessing a novel alginate-degrading activity, the diluted liquefied sample of seasoned sea mustard with salted and fermented anchovy was spread on M9 agar plate containing 0.8% (*w*/*v*) alginate as the sole carbon source. After incubation at 20°C for 10 days, there were several colonies on the agar plate surface. The strains were tooth-picked, transferred to another M9 agar plate with alginate, and then incubated at 20°C for 5 days. Based on the relative size of colonies on M9 agar plate with alginate, one strain with the fastest growth rate was selected and isolated for further analysis. The isolated strain was assigned as MJ-3.

The strain MJ-3 was a pale yellow and Gram-negative bacterium. Table 2 showed the biochemical characteristics according to the result of a microbial identification kit (VITEK 2 Compact, bioMerieux, France). The biochemical characteristics of the isolate MJ-3 showed 99.7% similarity to *Brevundimonas vesicularis*. The strain MJ-3 had the ability of esculin hydrolysis and can utilize maltose but not sucrose. The optimal temperature was 25–30°C when it was grown in tryptone soy broth media.

The cellular fatty acid composition of the strain MJ-3 was also analyzed as shown in Table 3. The major four fatty acids were $C_{18:1}$ $\omega 7c$ (63.1%), $C_{16:0}$ (24.3%), $C_{14:0}$ 2-OH (4.9%), and 11-methyl-$C_{18:1}$ $\omega 7c$ (2.0%). The known

Table 2 Biochemical characteristics of the strain MJ-3

Gram stain	Negative		
Pigmentation	Pale yellow		
Enzyme activity of		Indole formation	−
Ala-Phe-Pro-arylamidase	+	Acidification	
Glu-gly-arg-arylamidase	−	Glucose	−
L-PyroGlu-arylamidase	−	Assimilation	
Glu-arylaminase pNA	+	Adonitol	−
β-Ala-arylamidase	−	L-Arabitol	−
L-Pro-arylamidase pNA	+	D-Cellobiose	−
Tyr-arylamidase	+	D-Glucose	+
Gly-arylamidase	+	D-Maltose	+
α-glucosidase	+	D-Mannose	−
β-Glucosidase	+	D-Isomaltose	−
β-glucurnoidase	−	D-Sorbitol	−
β-Xylosidase	−	Sucrose	−
Urease	−	D-Tagatose	−
Arginine dihydroase	−	D-Treholose	−
Oxidase	+	Citrate	−
Ellman (thiol group)	+	Malonate	−
Nitrate reduction	−	5-Keto-D-gluconate	−
H_2S production	−	Courmarate	−
		L-Malate	+

members of the alpha-4 subclass of the *Proteobacteria* can be characterized by the presence of α-hydroxymyristic acid ($C_{14:0}$ 2-OH) in their cell membrane (Sly et al. 1999). Since the isolated MJ-3 also possessed α-hydroxymyristic acid as one of the main fatty acids, MJ-3 might be classified into alpha-4 subclass of the *Proteobacteria*. Some *Brevundimonas* sp. and *Caulobacter* sp. have the unusual fatty acid, 11-methyl-*cis*-octadeca-11-enoic acid (11-methyl-$C_{18:1}$ *ω7c*) (Andreev et al. 1986; Yoon et al. 2007). The strain MJ-3 also possessed 11-methyl-$C_{18:1}$ *ω7c* in its fatty acid composition. Compared to the fatty acid profiles to the TSBA40 database of organisms provided with Sherlock software, the fatty acid composition of the strain MJ-3 was closer to *Brevundimonas* sp. (20.1%). The strain MJ-3 was sensitive to various antibiotics described as above except ampicillin (30 μg), cefotaxime (30 μg), and sulfamethoxazole/trimethoprim (25 μg).

Table 3 Cellular fatty acid composition (%) of the strain MJ-3

Fatty acids	Compositions (%)
$C_{14:0}$ 2-OH	4.9
$C_{16:0}$	24.3
$C_{16:0}$ 2-OH	1.1
$C_{17:1}$ *ω6c*	1.0
$C_{18:0}$	1.2
$C_{18:1}$ *ω7c*	63.1
$C_{18:1}$ *ω5c*	1.2
11-methyl-$C_{18:1}$ *ω7c*	2.0
$C_{14:0}$, iso-$C_{15:0}$ 2-OH, $C_{16:1}$ *ω7c*	<1.0

In order to identify the strain MJ-3 more exactly, the 16S rDNA sequence was determined, and the sequence similarity was analyzed using BlastN. The 16S rDNA sequence analysis showed that the strain MJ-3 was taxonomically close to *Caulobacter leidyia* (99.1%), *Caulobacter endosymbiont* (99.0%), *Asticcacaulis excentricus* (98.9%), *Sphingomonas mali* (97.6%), *Sphingomonas pruni* (97.6%), and *Sphingomonas asaccharolytica* (97.2%). Abraham et al. reported that *C. leidyia* was observed to cluster with species of the genus *Sphingomonas* and thus rather belongs to the genus *Sphingomonas* (Abraham et al. 1999). *C. leidyia*, isolated from the hind gut of a millipede, was reported to be unrelated to the other species of *Caulobacter* and belonged to a distinct cluster with *A. excentricus* and *Asticcacaulis biprosthecium* (Sly et al. 1999).

For clear identification of the strain MJ-3, the ITS DNA of 16S–23S rRNA genes was determined, and a phylogenetic tree was constructed. The genes encoding for rRNAs in prokaryotes can be arranged in an operon as 5′-16S-23S-5S-3′, which is separated by two spacer regions known as ITS (Tokajian et al. 2008). The PCR product of the 16S–23S ITS region of MJ-3 was around 900 bp size. Figure 2a showed 16S–23S rDNA ITS structure of MJ-3. The two domains highly conserved (75- and 76-bp size) within ITS were identified as the genes encoding $tRNA^{Ile}$ and $tRNA^{Ala}$. Figure 2b showed the phylogenetic tree on the basis of the nucleotide sequence of tRNA regions in 16S-23S rRNA ITS. The MJ-3 was most closely related to the genus *Sphingomonad* including *Sphingomonas* and *Novosphingobium*. *Sphingomonas wittichii* ITS region (EU334830), which showed the closest relationship with ITS region of MJ-3 has shorter ITS region (482 bp) than MJ-3 (916 bp), but $tRNA^{Ile}$ and $tRNA^{Ala}$ genes were highly conserved. Based on the results on biochemical characteristics and sequences of 16S rDNA and ITS region, we identified the strain MJ-3 as *Sphingomonas* sp. MJ-3. The IST sequence of MJ-3 was submitted to GenBank (JN091778).

Analysis of Modes of Cell Growth and Alginate Lyase Activity in Batch Culture with Alginate as the Sole Carbon Source

Sphingomonas sp. MJ-3 was cultivated on M9 and LB medium containing various concentrations of alginate (Fig. 3). When M9 medium without any kinds of carbon substrate was used, the cells could not grow at all. In the presence of alginate in M9 medium, the cells grew well, and the final amounts of cells were proportionally related

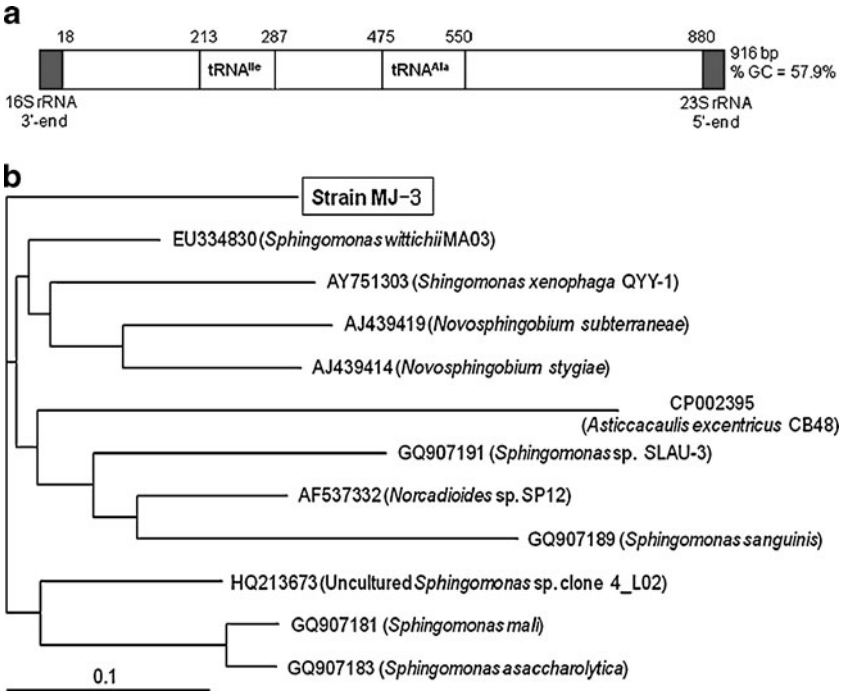

Fig. 2 16S–23S rRNA intergenic transcribed spacer (ITS) region of MJ-3 (**a**) and phylogenetic tree of newly isolated bacterium MJ-3 and other bacteria (**b**). **a** The *number* indicates the order of nucleotide sequence. The sequences of 213–287 and 475–550 were identified as the genes encoding $tRNA^{Ile}$ and $tRNA^{Ala}$. *Black boxes* are 3′-end 16S rRNA and 5′end 23S rRNA sequences. **b** The phylogenetic tree was constructed by using 16S–23S rRNA ITS sequences. The bar labeled 0.1 indicates 1 base change per 10 nucleotides. Each number means the GenBank accession number. Twelve bacteria were chosen by BlastN search

with the amounts of added alginate. This strongly suggested that *Sphingomonas* sp. MJ-3 could use alginate as the sole carbon source very well and has many kinds of alginate-degrading enzymes for its metabolism from alginate. The growth characteristics of *Sphingomonas* sp. MJ-3 in LB medium were also analyzed. Although the cells cultured in LB medium without alginate, the cells grew up well because LB contained enough nutrients such as yeast extract and peptone. Interestingly, the growth rate and extent were higher in the presence of alginate. When LB media containing 0.2, 0.4, 0.6, and 0.8% (*w*/*v*) alginate were used, the cells grew well, and the final growth extent was increased with the increase in alginate concentration up to 0.6% (*w*/*v*) alginate. The growth extent of the cells grown in the LB with 0.6% (*w*/*v*) alginate was almost twofold higher than that grown in the LB without alginate. From these results, we concluded that alginate assimilation was not severely inhibited by the presence of other nutrients such as yeast extract and peptone, and quite different transport and assimilation pathway for alginate might be present in the *Sphingomonas* sp. MJ-3. Further analysis of transport and assimilation metabolic pathway will be interesting for alginate degradation and metabolism in the strain MJ-3. In case of *Sphingomonas* sp. A1, alginate polymer is directly transported into the cell through a channel consisting of a pit on the cell surface, alginate-binding proteins in the periplasm and ATP-binding cassette transporter in the inner membrane. Incorporated alginate is degraded to oligosaccharides including di-, tri-, and tetrasaccharides by three endotype alginate lyases such as A1-I, A1-II, and A1-III. The oligoalginates are degraded to unsaturated monosaccharides by exotype alginate lyase A1-IV (Hashimoto et al. 2005).

Alginate lyase activity was monitored in the batch growth of *Sphingomonas* sp. MJ-3 in M9 and LB media in the presence or absence of alginate. There was negligible alginate lyase activity in the cells incubated in M9 medium without alginate. The alginate lyase activity of cell lysate was detected in the cells grown in the M9 medium with alginate (Fig. 4a). Alginate lyase activity of MJ-3 continuously increased up to stationary phase and then remained constant after the cells entered a stationary growth phase, indicating that alginate lyase activity was directly related with cell growth. The levels of total alginate lyase activity in the same amount of cells were also linearly increased with increase in the alginate concentration. The alginate lyase activities were also analyzed for the cells grown in LB medium containing alginate (Fig. 4b). When the cells were cultured in the LB with alginate, there was about half of alginate lyase activity, compared to the cells grown on M9 medium with alginate. Based on the above results, we could conclude that the newly isolated *Sphingomonas* sp. MJ-3 possessed an alginate-assimilating system consisting of several alginate-degrading enzymes that degrade alginate into monosaccharides for cell growth.

Cloning of Alginate Lyase Gene from *Sphingomonas* sp. MJ-3 and Multiple Sequence Alignment Analysis

Recently, *Sphingomonas* sp. strain A1 was reported to possess an exotype alginate lyase that depolymerizes alginate completely (Hashimoto *et al*. 2000; Miyake et al.

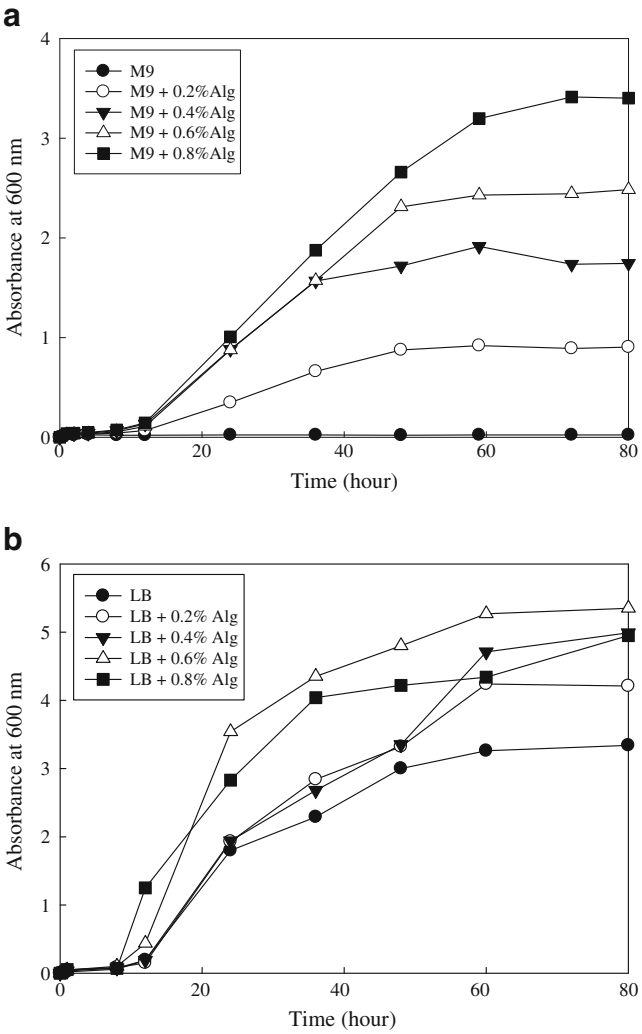

Fig. 3 Batch growth curve of *Sphingomonas* sp. MJ-3 on M9 medium (**a**) and LB medium (**b**) containing various amounts of alginate. The strain MJ-3 was cultured at 180 rpm and 30°C

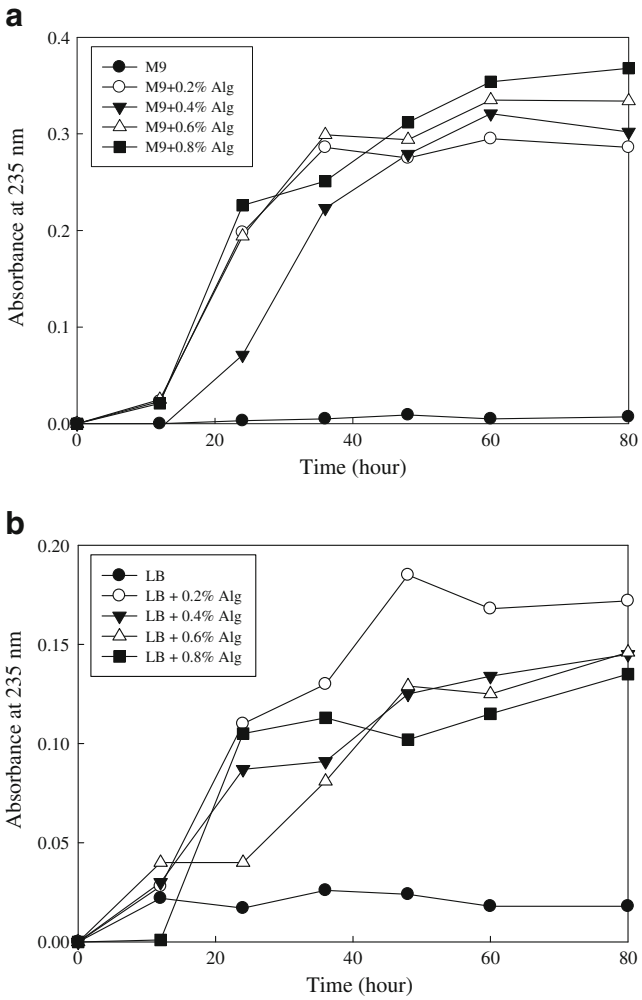

Fig. 4 Alginate lyase activity of *Sphingomonas* sp. MJ-3 cultured on M9 medium (**a**) and LB medium (**b**) in the presence or absence of alginate. The sample was withdrawn from culture flask at indicated time, and then the cell pellet was homogenized by using a sonicator. The alginate lyase activity of the cell lysate was monitored based on the increase in absorbance at 235 nm due to the increase in double bond generated by alginate lyase-catalyzed elimination reaction

2003). Ochiai et al. reported that an alginate lyase from *A. tumefaciens* strain C58 exhibited exolytic activity and its crystal structure had α/α-barrel and anti-parallel β-sheet as a basic scaffold (Ochiai et al. 2006, 2010). When the sequence of exotype oligoalginate lyase of *Sphingomonas* sp. A1 was analyzed, it was shown to possess heparinase II/III family domain in the C-terminal region, while endolytic alginate lyases did not have that domain. Hence, we designed the specific primers for PCR on the basis of the homolog sequences of various heparinase II/III family proteins to screen the fosmid clone containing the alginate lyase that can degrade alginate to unsaturated monosaccharides.

When PCR was carried out for the MJ-3 genomic DNA with 5MJ-3-Hepa790F and 3MJ-3-Hepa1430R (refer to Table 1), we obtained a DNA fragment that possesses 60% homology with heparinase-like family protein, which indicates that MJ-3 most probably harbors oligoalginate lyase. Based on this result, we prepared a MJ-3 fosmid library. All fosmid library colonies were transferred to microplates. The colonies on each agar plate were pooled, boiled with small amount of water, centrifuged, and then the supernatant was used for PCR with 5MJ-3-Hepa790F and 3MJ-3-Hepa1430R to identify the agar plate containing the positive colony and the corresponding 96-well plate. Two positive microplates (14-1 and 19-2) were chosen for further screening because the original master agar plates exhibited positive PCR products with 640-bp size. From the two microplates, the cells in 12 wells of eight lanes (designated as lanes A to H) were pooled and then used for the second round of PCRs to search out the positive colony in a lane. The 14-1A and 19-2H lanes were shown

to have 640 bp PCR products. In order to identify the positive well containing the positive colony, the cells in each well of 14-1A and 19-2H lanes were used for the third round of PCRs. The PCR products of 14-1A-2 and 19-2H-2 wells showed DNA bands with 640-bp size. When the DNAs of 14-1A-2 and 19-2H-2 fosmids were sequenced, only the DNA of 14-1A-2 has 70% and 60% protein sequence identity with alginate lyase of *A. vinelandii* DJ and heparinase II/III family protein of *B. subvibrioides* ATCC 15264, respectively. Conclusively, fosmid 14-1A-2 clone was elucidated to have the partial C-terminal heparinase-like protein gene (from nt 256 to nt 2,196) and other downstream genes.

In order to clone the N-terminal of the MJ-3 alginate lyase from the genomic DNA, two primers of 5MJ-3-PolyM1170F and 3MJ-3-Hepa300R (refer to Table 1) were designed and used for PCR. We obtained 560-bp PCR product that contain the N-terminal of the MJ-3 alginate lyase that has 48% identity to heparinase II/III family protein of *S. maltophilia* R551-3. The 560-bp PCR product also contained C-terminal part of a putative poly-(β-D-mannuronate) lyase. Based on the DNA sequence information of the N- and C-terminal sequences of heparinase-like protein gene, forward and reverse primers with restriction sites could be designed, as shown in Table 1. The complete gene was cloned and is composed of 2,196 nucleotides. The putative MJ-3 alginate lyase protein consists of 731 amino acids (Fig. 5). Signal 3.0 server predicts that MJ-3 alginate lyase protein has a signal peptide sequence (^{1}Met–^{20}Ala). The nucleotide sequence of MJ-3 alginate lyase was submitted to GenBank (accession number JN091777).

Deduced amino acid sequence of MJ-3 alginate lyase was analyzed by multiple sequence alignment with other alginate lyases or heparinase family protein (Fig. 6). The amino acid sequence of MJ-3 alginate lyase showed the highest homology to the deduced sequence of alginate lyase

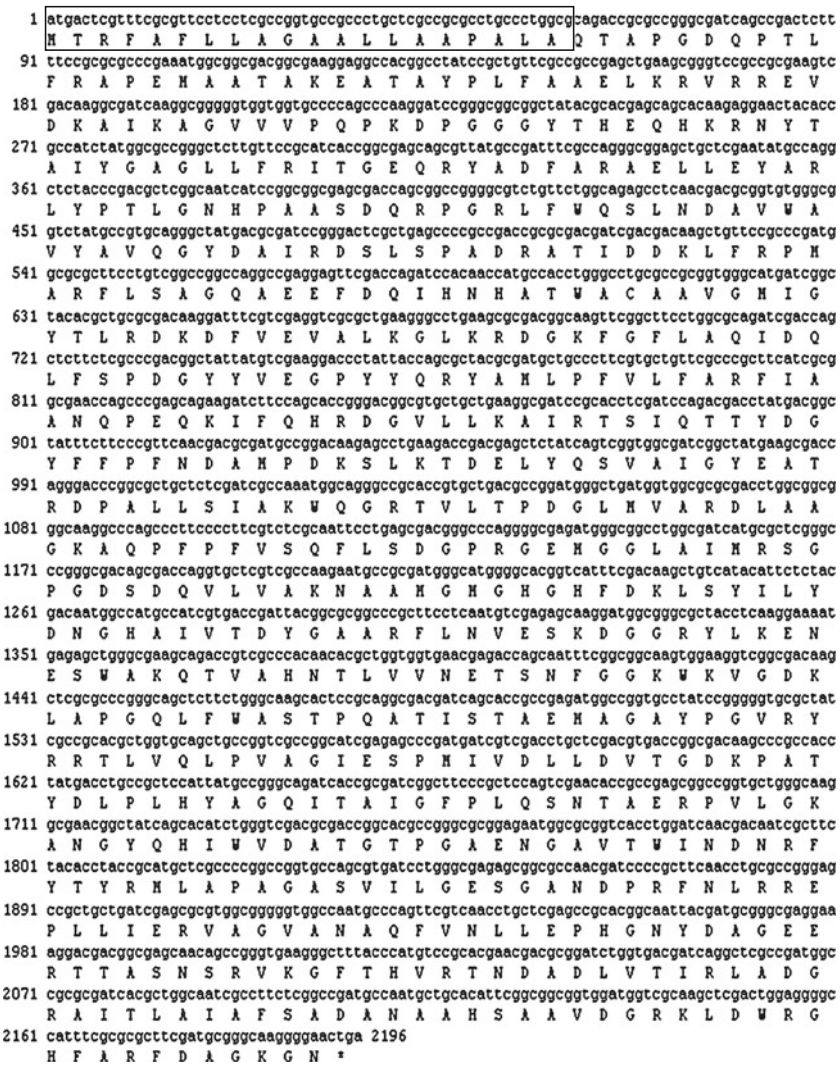

Fig. 5 Nucleotide sequences and deduced amino acid sequences of alginate lyase gene from *Sphingomonas* sp. MJ-3. A *box* and *asterisk* indicate a signal peptide sequence and a stop codon, respectively

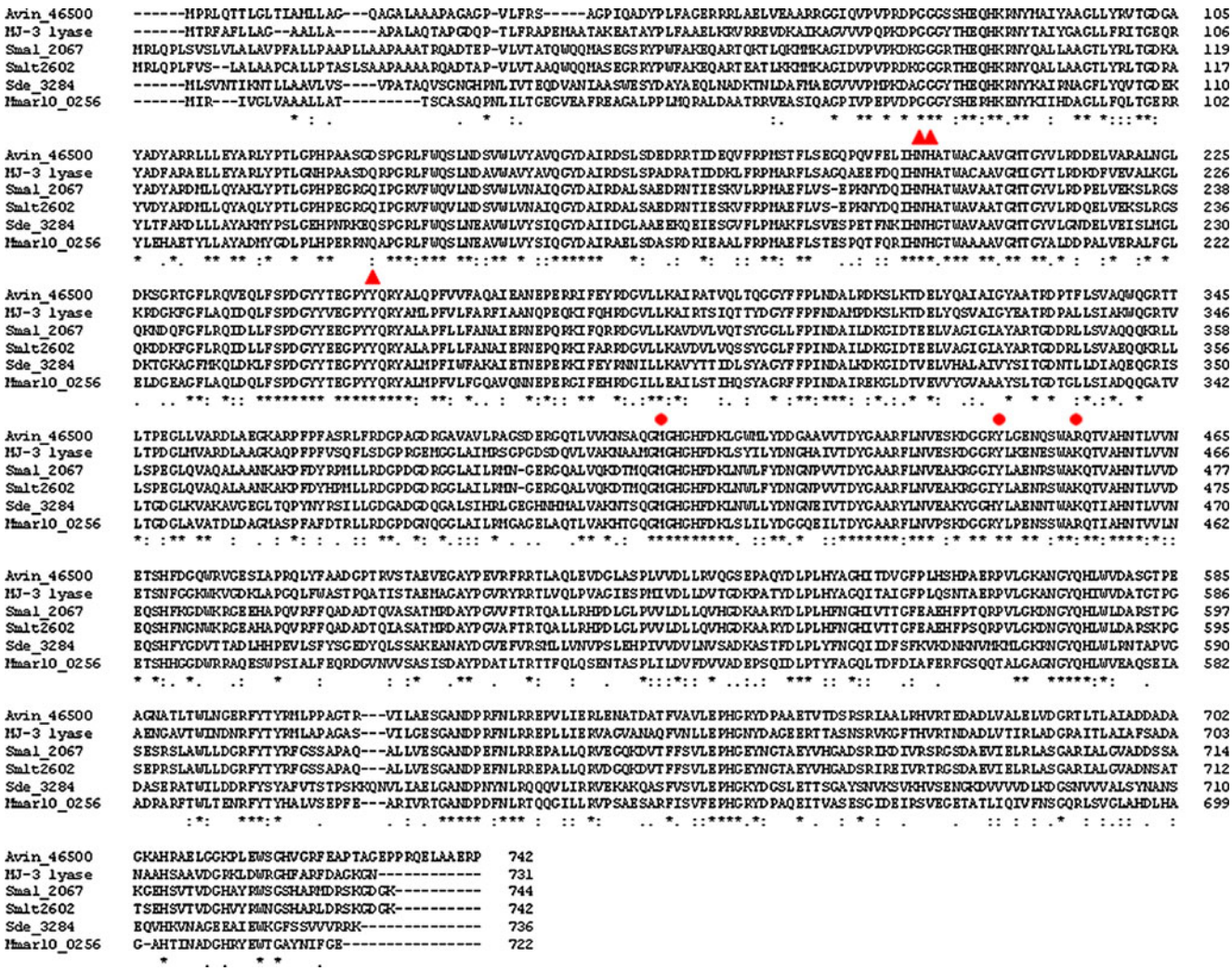

Fig. 6 Multiple sequence alignment analysis of MJ-3 alginate lyase with other alginate lyases and heparinase II/III family proteins. Protein sequences of Avin_46500 (*Azotobacter vinelandii* DJ alginate lyase, AC080755), Smlt2067 (*S. maltophilia* K279a putative alginate lyase protein, CAQ46078), Sde_3264 (*S. degradans* 2–40 hypothetical protein, YP_528751), Mmar10_0256 (*Maricaulis maris* MCS10 Heparinase II/III family protein, ABI64549), and BresuDRAFT_1835 (*B. subvibrioides* ATCC 15264 heparinase II/III family protein, YP_003819826) were retrieved from GenBank Genome Project of NCBI. Identical and similar amino acid residues in the six proteins are denoted by *asterisks* and *dots*, respectively. The *triangle and circle symbols* on the sequences indicate the possible catalytic sites of algL domain and oligoalginate lyase domain, respectively

(Avin_46500, 64% identity) from *A. vinelandii* DJ. When the amino acid sequence of MJ-3 alginate lyase was compared with those of the other deduced proteins, relatively high similarity was shown with putative alginate lyase protein of *S. maltophilia* K279a (Smlt2602, 53% identity), heparinase II/III family protein of *B. subvibrioides* ATCC 15264 (BresuDraft_1835, 53% identity), heparinase II/III family protein of *M. maris* MCS10 (Mmar10_0256, 49% identity), and hypothetical protein of *S. degradans* 2-40 (Sde_3284, 48% identity). When the amino acid sequence of MJ-3 alginate lyase was compared with those of the exotype alginate lyases of *Sphingomonas* sp. A1 (A1-IV) and *A. tumefaciens* strain C58 (atu3025 protein), which belong to polysaccharide-family 15 (PL-15) (http://www.cazy.org/), the partial N-terminal amino acid sequence (from aa 1 to aa 350) of MJ-3 alginate lyase showed <27.0% and 28.6% to the sequences of the front fragments (α/α barrel region) of A1-IV and atu3025, respectively. The sequence identity between A1-IV and atu3025 were about 56.8%. This indicates that the primary structure of MJ-3 alginate lyase is somewhat deviated from other exolytic oligoalginate lyases.

When the AlgL-like conserved domain of MJ-3 alginate lyase was compared with A1-III (endolytic poly-M lyase from *Sphingomonas* sp. strain A1), sequence identity was <27.9%, but catalytically important amino acids such as ^{207}Asn, ^{208}His, and ^{254}Tyr were highly conserved (indicated as triangle in Fig. 6), indicating that MJ-3 alginate lyase might

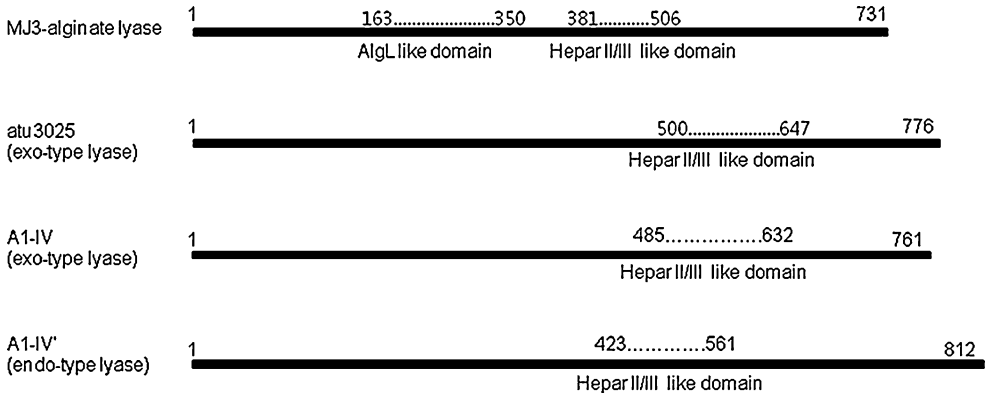

Fig. 7 Conserved domains in the protein sequences of MJ-3 alginate lyase and other PL-15 alginate lyases. Protein sequences were analyzed using Conserved Domain Search Service of NCBI (http://www.ncbi.nlm.gov/Structure/cdd/wrpsb.cgi). GenBank accession numbers of atu3025 (exotype oligoalginate lyase of *Agrobacterium tumefaciens* strain C58), A1-IV (exotype oligoalginate lyase of *Sphingomonas* sp. A1), and A1-IV′ (alginate lyase of *Sphingomonas* sp. A1) are NP_357573, BAD90006, and BAB03319, respectively

also possess the active site similar to the endolytic poly-M lyase. When heparinase-like protein domain (residue from 350 to 731) of MJ-3 alginate lyase was homology-modeled base on 2fuq.pdb (heparinase protein from *Pedobacter heparinus*), ^{409}His, ^{446}Tyr, and ^{445}Lys residues were conserved (indicated as circle in Fig. 6). These residues are expected to be the key amino acids for binding site of unsaturated heparine dimer (Shaya et al. 2006). When the protein sequence of MJ-3 alginate lyase was submitted to NCBI for searching conserved domain, AlgL domain (from aa 163 to aa 350) and heparinase II/III-like domain (from aa 381 to aa 506) appeared to be conserved, as shown in Fig. 7.

Heterologous Expression of the Recombinant Alginate Lyase of *Sphingomonas* sp. MJ-3

The alginate lyase gene from *Sphingomonas* sp. MJ-3 was expressed in *E. coli* BL21 (DE3), and the resulting proteins were analyzed by SDS-PAGE and immunoblotting (Fig. 8). The alginate lyase gene of *Sphingomonas* sp. MJ-3 encoded

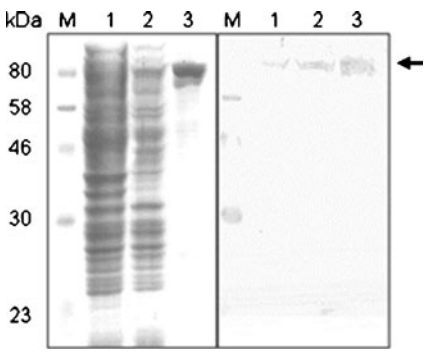

Fig. 8 SDS-PAGE and immunoblotting profile of the recombinant MJ-3 alginate lyase proteins. The recombinant BL21 cells containing pET21b(+)/MJ-3 alginate lyase gene were cultured at 15°C for 24 h with or without 1 mM IPTG. The cell lysate or purified protein was loaded on each well. *Lane 1* Cultured without IPTG (15 μg protein loading), *lane 2* cultured with IPTG (15 μg protein loading), *lane 3* purified enzyme (3 μg protein loading). *Left and right panels* indicate CBB staining and immunoblotting of the gel, respectively

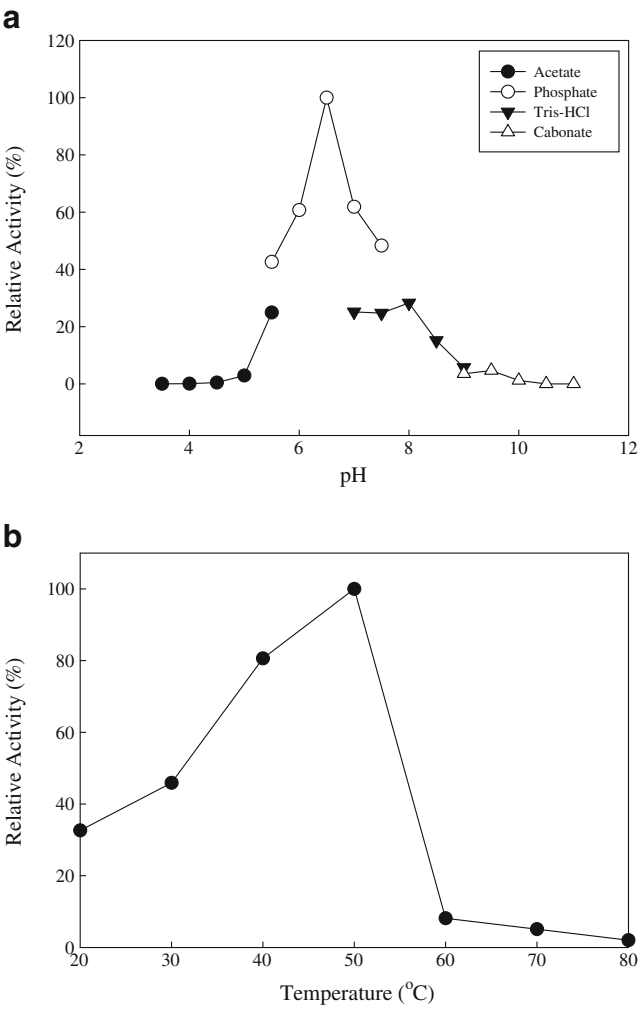

Fig. 9 Effects of pH and temperature on the activity of the recombinant MJ-3 alginate lyase. **a**, **b** Temperature and pH dependence, respectively

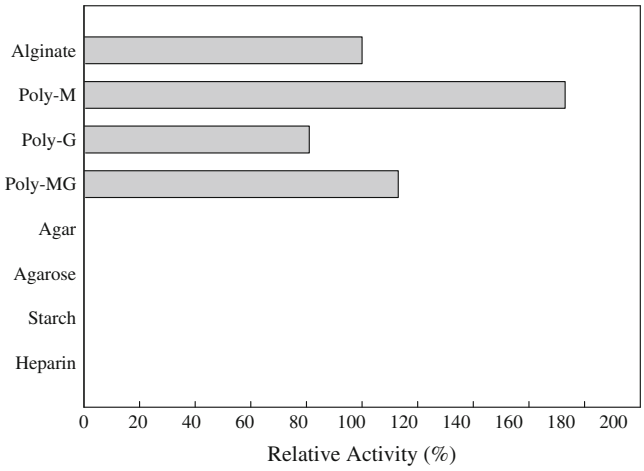

Fig. 10 Analysis of substrate specificity of the recombinant alginate lyase of *Sphingomonas* sp. MJ-3

731 amino acid residues with the calculated molecular mass of 79.9 kDa. In the case of the recombinant alginate lyase without signal peptide, total amino acid residues were expected to be 711 with the calculated molecular mass of 78.0 kDa. We expressed the alginate lyase without signal peptide because recombinant alginate lyase without signal peptide showed higher activity than that with signal peptide in the other alginate lyase previously examined (Kim et al. 2009). When the recombinant protein fused with $(His)_6$-tag was expressed, the molecular mass of MJ-3 alginate lyase protein would be 79.6 kDa with 724 amino acids. As shown in Fig. 8, a band corresponding to the molecular mass of MJ-3 alginate-lyase, i.e., 79.6 kDa, appeared in the SDS-PAGE.

Characterization of the Recombinant Alginate Lyase of *Sphingomonas* sp. MJ-3

In order to characterize the recombinant MJ-3 alginate lyase, the enzyme was purified to homogeneity by the N-Sepharose column chromatography. The pH and temperature dependencies of the recombinant alginate lyase of *Sphingomonas* sp. MJ-3 were investigated. The recombinant

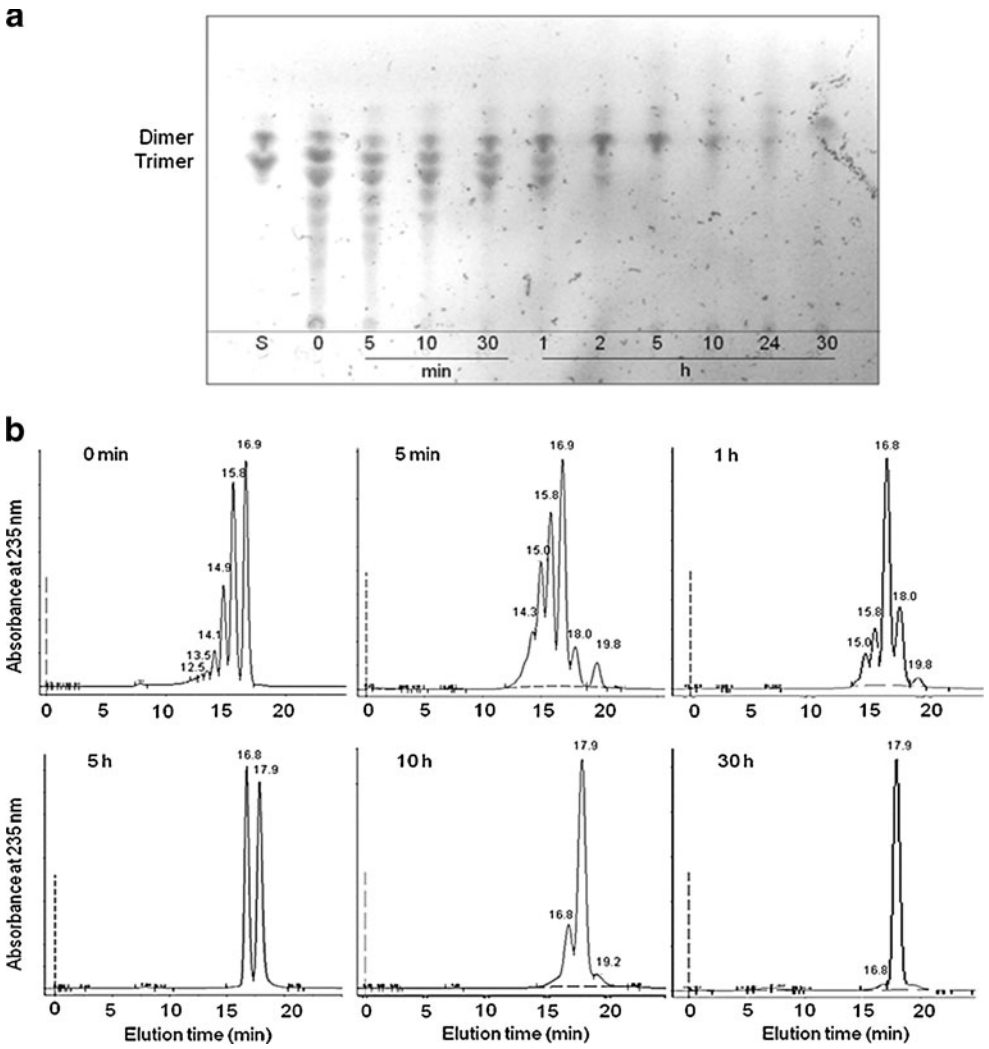

Fig. 11 TLC and FPLC analyses for the degradation products produced by the recombinant MJ-3 alginate lyase. **a** TLC analysis of the degraded alginate oligosaccharides by the recombinant MJ-3 alginate lyase. A 5% (*w*/*v*) alginate oligosaccharide in 20 mM phosphate buffer (pH 7.2) was degraded by the recombinant MJ-3 alginate lyase at 30°C, and 30 μl of the reaction mixture was periodically withdrawn for TLC analysis. The degraded alginate oligosaccharides were visualized by dipping TLC plate into 10% (*w*/*v*) sulfuric acid/ethanol with vanillin. *Lane S* The purified dimer and trimer standards prepared from enzymatic degradation of alginate. **b** FPLC chromatogram of the reaction products. The *vertical dot line* in the chromatogram indicates the injection time. The elution times of unsaturated monomer, dimer, trimer, tetramer, and pentamer are 17.9, 16.8, 15.8, 15.0, and 14.3 min, respectively. The substance eluted at 19.8 min has not been determined

MJ-3 alginate lyase showed alginate-degrading activity between pH 5 and 9 with the optimal activity at pH 6.5 (Fig. 9a). With respect to temperature, the recombinant MJ-3 alginate lyase could exhibit activity in the range from 20°C up to 80°C with an optimal temperature of 50°C when the reaction was kept for 10 min (Fig. 9b).

We analyzed the substrate specificity of the recombinant MJ-3 alginate lyase. We used poly-M block (M/G= 84.6:15.4), poly-G block (M/G=15.2:84.8), poly-MG block (M/G=69.3:30.7), which were prepared by the method of Haug et al. (1966) and sodium alginate (M/G=54.9:45.1) as the substrates. As shown in Fig. 10, the recombinant MJ-3 alginate lyase exhibited the highest activity toward poly-M block on the basis of relative activity scale. It also showed degradation activities toward poly-MG, poly-G block, and sodium alginate. Interestingly, although MJ-3 alginate lyase possessed heparinase II/III-like domain, it could not degrade heparin.

Analysis for Degradation Products of Oligoalginate Produced by the Recombinant MJ-3 Alginate Lyase

We tested whether the recombinant MJ-3 alginate lyase can degrade unsaturated alginate oligosaccharide or not. If the recombinant MJ-3 alginate lyase possesses oligoalginate lyase activity, it can completely degrade alginate oligosaccharides into alginate monosaccharides. The alginate oligosaccharides were prepared using the recombinant endolytic alginate lyase from *Streptomyces* sp. ALG-5 (Kim et al. 2009). The alginate oligosaccharides mainly consisted of dimer, trimer, and tetramers. The final degradation product mixture of alginate oligosaccharides by the recombinant MJ-3 alginate lyase was analyzed by TLC (Fig. 11a), FPLC (Fig. 11b), and LC-MS, to identify the products. After finishing the degradation reaction of alginate oligosaccharides mixture, the peaks at dimer, trimer, and tetramer disappeared (Fig. 11a). We analyzed the reaction products using FPLC and LC-MS to confirm the formation of alginate monosaccharides because the momomeric alginate could hardly be visualized on TLC plate by sulfuric-acid staining. The FPLC elution chromatogram showed almost one single peak at 17.9 min (Fig. 11b, sixth panel). The peaks at the elution time of 15.7, 16.8, and 17.9 min were subsequently analyzed by electrospray ionization MS (ESI-MS). When ESI-MS data for peaks were analyzed with negative mode ($[M–H]^-$), the main peaks of *m*/*z* were 527 (elution time=15.7 min, trimer), 351 (elution time=16.7 min, dimer), and 175 (elution time=17.9 min, monomer), respectively. The last peak was confirmed to be unsaturated monosaccharide since the molecular mass of unsaturated monosaccharide is 176 Da. All these results clearly indicated that the recombinant MJ-3 alginate lyase possesses the activity for complete degradation of alginate oligosaccharides into monosaccharides. Based on analysis for substrate specificity, degradation product, and multiple sequence alignment of conserved domains, the recombinant alginate lyase of *Sphingomonas* sp. MJ-3 was identified as an oligoalginate lyase that possesses lyase activities toward alginate, alginate oligosaccharides, poly-M block, poly-G block, and poly-MG block.

As mentioned in the section of multiple sequence alignment, the sequence identity of the recombinant MJ-3 alginate lyase with other exotype alginate lyases was very low. Therefore, the oligoalginate lyase of *Sphingomonas* sp. MJ-3 can possess a 3D structure, which is distinct from those of the other exotype alginate lyases. The recombinant MJ-3 alginate lyase can be used as a biocatalyst for saccharification of alginate since it can efficiently degrade poly-M block, poly-G block, poly-MG block, alginate oligosaccharides, and alginate into alginate monosaccharides.

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