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Analysis of extracellular alginate lyase and its gene from a marine bacterial strain, *Pseudoalteromonas atlantica* AR06

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Abstract *Pseudoalteromonas atlantica* AR06 is a marine bacterial strain that can utilize alginate as a sole source of carbon and energy. The extracellular protein fraction prepared from the AR06 cultivation media exhibited alginate lyase activity to depolymerize the alginate molecules having homopolymeric and heteropolymeric forms of mannuronate and guluronate so as to mainly convert into the dimer to tetramer. A DNA fragment encoding a portion of alginate lyase was amplified from AR06 genomic DNA by PCR using a set of degenerated primers, and then the whole alginate lyase gene, named *alyA*, and its flanking regions were obtained from a cosmid library of AR06 genomic DNA. The *alyA* mutant of AR06 showed (1) the loss of alginate depolymerization activity on alginate agar plate and (2) significant growth defects in alginate minimal medium; these defects were complemented by the introduction of the *alyA* gene. Furthermore, zymography and biochemical analyses revealed that three extracellular protein bands of AR06 had alginate lyase activities and that all three protein bands were derived from the nascent *alyA* gene product. These results clearly indicated that the *alyA* gene greatly contributes to the assimilation of alginate in AR06. The transcription of the *alyA* gene was induced by the presence of alginate in minimal medium, but its obvious induction was not observed in rich medium even in the presence of alginate.

Keywords *Pseudoalteromonas atlantica* · Bifunctional alginate lyase · Extracellular enzyme · Expression of *alyA* gene

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Introduction

Alginates are abundant in the cell walls of brown algae and in bacterial exopolysaccharides, and these linear α-1,4-linked D-glycuronans are composed of residues of β-D-mannuronate (M) and/or its C-5 epimer α-L-guluronate (G) (Sutherland 1995). These residues are arranged in block structures such as homopolymeric [poly-M (PM) and poly-G (PG)] forms and heteropolymeric forms with random arrangements of M and G. Alginates from brown algae are widely used as viscosity improvers and gelators for foods, drugs, medicines, and cosmetics (Onsøyen 1996). In recent years, attention has also been paid to the oligoalginates because of their useful and biologically important functions such as the induction of cytokine production against lymphoma cells (Iwamoto et al. 2005), prebiotic effects (Murata et al. 1993), and plant root growth-promoting activities (Natsume et al. 1994).

Alginate lyases catalyze the depolymerization of alginate by eliminative cleavage of the glycosidic bonds between the uronate residues. These enzymes are produced by a

variety of organisms including algae, mollusks, fungi, soil and marine bacteria, viruses, and bacteriophages (Wong et al. 2000). Alginate lyases are classified into three groups on the basis of their substrate specificities. Two groups are PM lyases (EC 4.2.2.3) and PG lyases (EC 4.2.2.11) that depolymerize only PM and PG, respectively. The enzymes belonging to the last group, often designated bifunctional alginate lyases, are able to depolymerize PM, PG, and the heteropolymeric form. Although a number of PM and PG lyases have been reported, the bacterial bifunctional alginate lyases analyzed at their genetic level have been limited to those from *Pseudoalteromonas elyakovii* IAM 14594 (Sawabe et al. 2001), *Pseudoalteromonas* sp. strain 272 (Iwamoto et al. 2002) (Fig. 1b), and *Sphingomonas* sp. strain A1 (Murata et al. 1993). The alginate lyases from the former two strains are extracellular enzymes and those from the first and last strains are formed after the removal of N-terminal portions of their precursor proteins (Sawabe et al. 2001; Yoon et al. 2000). Although some strains express the alginate lyase activities constitutively (Kitamikado et al. 1989; Doubet and Quatrano 1982; Boyen et al. 1990), most bacterial extracellular alginate lyases exhibit their activities only when the host cells are cultivated in the presence of alginate (Wong et al. 2000; Kitamikado et al. 1989; Doubet and Quatrano 1982). The analysis of bacterial bifunctional alginate lyases has also been limited to their enzymatic properties for the depolymerization of the alginates (Sawabe et al. 2001; Iwamoto et al. 2002, 2003). The roles of their depolymerized products in the utilization by the host cells remain unclear, as does the regulatory expression of genes for the enzymes.

P. atlantica AR06 was isolated from a coastal water in Japan as a decomposer of seaweed brown alga (Uchida and Nakayama 1993; Uchida et al. 2002) and has been suggested to be a very efficient extracellular alginate lyase producer. However, the detailed mechanism underlying alginate decomposition by AR06 has remained unclear. In this paper, we established systems to manipulate the chromosomal genes of AR06, and these systems allowed us to clearly characterize the extracellular alginate lyase, its gene (*alyA*) and expression, and an *alyA* mutant of AR06.

Materials and methods

Bacterial strains, plasmids, primers, and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1, and the primers in Table 2. The

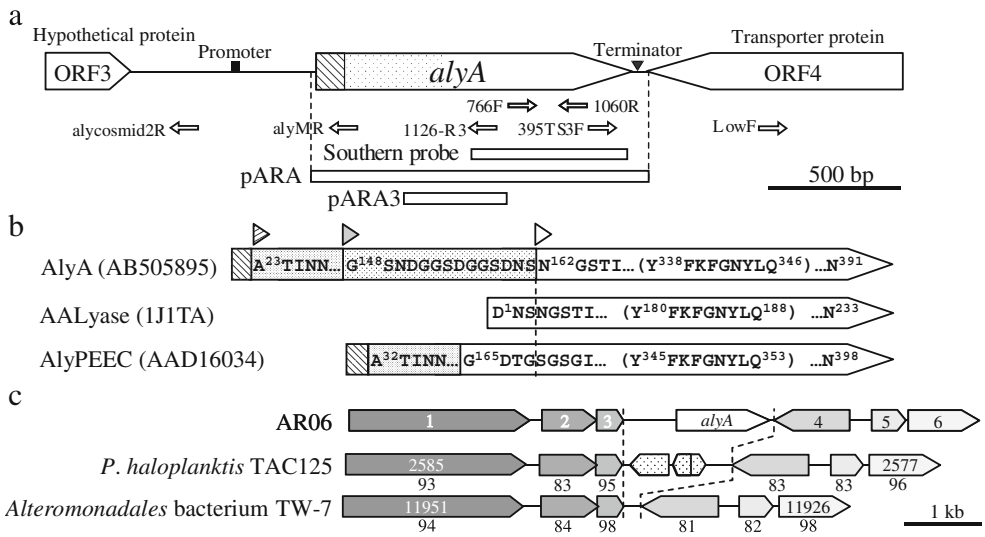

Fig. 1 AR06-specified *alyA* gene, its product, and their comparison with those from related strains. **a** Organization of *alyA* gene and its flanking regions in AR06. Pentagons indicate the sizes and orientations of ORFs. *Shaded and dotted boxes* in *alyA* correspond to the regions that encode the signal peptide and subsequently removed amino-acid sequences (carbohydrate-binding domain), respectively, in the nascent gene product. *Small solid and triangle boxes* indicate the putative promoter and terminator sequences, respectively. The directions of arrows represent the orientations of several primers in Table 2. *White boxes* indicate the DNA regions used for detailed analysis in this study. **b** Structure of nascent *alyA* product and its comparison with the related alginate lyases, AAlyase from *Alteromonas* sp. 272 and AlyPEEC from *P. elyakovii* IAM14594. The accession numbers of three proteins in the databases are indicated in *parentheses*. Three types of triangles show the cleavage positions in the nascent *alyA* product, and the cleavage at the *shaded*, *gray*, and *white triangle* positions gave rise to the 43-, 33-, and 30.5-kDa proteins, respectively, by the SDS-PAGE analysis. **c** Comparison of AR06-specified *alyA*-containing region with the corresponding regions on *P. haloplanktis* TAC125 chromosome I (locus tags from PSHAa2577 to PSHAa2585, CR954246) and contig 14 of *Alteromonadales* bacterium TW-7 (locus tags from ATW7_11926 to ATW7_11951 in AAVS01000014). The pentagons represent ORFs with their directions, and the ORFs encoding similar functions are displayed in the same darkness. See the text for details. The numbers in the pentagons are locus-tag numbers, and the three unique TAC125 ORFs (PSHAa2580 to PSHAa2582) with unknown functions are depicted by dotted pentagons. The number below the pentagon indicates the percent amino-acid identity to the corresponding gene product from AR06

Table 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
E. coli		
JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1Δ(lac-proAB)*/ F'[*traD36 proAB*$^+$ *lacI*^{q} *lacZΔM15*]	Toyobo
DH5α	F- Φ80d*lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1*	NBRP (NIG, Japan)
HB101	*supE44* Δ(*mcrC-mrr*) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 leuB6 thi-1*	Boyer and Roulland-Dussoix 1969
XL1-Blue MR	Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac*	Stratagene
Pseudoalteromonas atlantica		
AR06	*P. atlantica* wild-type strain, utilizer of alginate and agar as sole sources of carbon and energy	Uchida and Nakayama 1993; Uchida et al. 2002
ARA1	AR06 *alyA*::pARA3	This study
Plasmids		
pBluescriptII K(+)	Ap^r; cloning vector	Stratagene
pSuperCos1	Ap^r Km^r; cosmid vector	Stratagene
pNIT6012	Tc^r; broad-host-range expression vector	Heeb et al. 2000
pBBR1MCS	Cm^r; broad-host-range cloning vector	Kovach et al. 1994
pRK2013	Km^r; ColE1 derivative carrying all the conjugative transfer genes from a broad-host-range plasmid RP4	Figurski and Helinski 1979
pGreenTIR	Ap^r; clonig vector harboring a mutant *gfp* gene under *lac* promoter	NBRP (NIG, Japan)
pTn*Mod*-OCm	Cm^r; Tn*5*-derived transposable region carrying the Cm^r gene and *oriV* of pMB1; mobilizable by pRK2013	Dennis and Zylstra 1998
p333	SuperCos1 derivative with AR06 genomic DNA fragment containing the complete *alyA* gene	This study
pARA	pNIT6012 derivative carrying the complete *alyA* gene; Fig. 1	This study
pK18mob	Km^r; small mobilizable multipurpose cloning vector derived from *E. coli* plasmid pK18	NBRP (NIG, Japan)
pKARA	pK18mob derivative carrying a part of *alyA* gene	This study
pMobCm	pTn*Mod*-OCm derivative lacking transposase gene and one IR of Tn*5*	This study
pARA2	pMobCm derivative carrying a part of *alyA* gene	This study
pARA3	pARA2 derivative carrying pGreenTIR-derived *gpf* gene that is transcribed from pNIT6012-derived and constitutive promoter; Fig. 1	This study

NBRP National BioResource Project; *NIG* National Institute of Genetics

P. atlantica strain AR06 has been deposited in the National Institute of Agrobiological Sciences Genebank of Japan (http://www.gene.affrc.go.jp/databases_en.php) under the accession number MAFF 211893. *Escherichia coli* cells were grown at 37°C in Luria–Bertani (LB) broth (Sambrook et al. 1989), and *P. atlantica* cells at 20°C in Marine Broth 2216 (MB) (BD Difco; BD, Franklin Lakes, NJ, USA) or ASW (artificial seawater) minimal medium (NH_4NO_3 1.2 g, NaCl 24 g, KH_2PO_4 0.5 g, K_2HPO_4 1 g, KCl 0.3 g, $FeCl_2{\cdot}4H_2O$ 0.01 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, and $CaCl_2{\cdot}2H_2O$ 0.2 g) containing 0.3% glucose or 0.3% sodium alginate (500 centipoise grade; Nacalai Tesque, Kyoto, Japan) as a sole carbon and energy source. Solid media were prepared by the addition of 1.5% agar. Antibiotics were used at the following concentrations: 50 μg/ml for ampicillin (Ap) and kanamycin (Km), 30 μg/ml for chloramphenicol (Cm), and 20 μg/ml for tetracycline (Tc) for *E. coli*; 20 μg/ml for Cm and 10 μg/ml for Tc for *P. atlantica* in MB; and 10 μg/ml for Cm and 5 μg/ml for Tc for *P. atlantica* in ASW media. The growth of *P. atlantica* cells in liquid medium at a shaking speed of 30 rpm was monitored using Bio-Photorecorder TVS062CA (Advantec Toyo, Tokyo, Japan).

Preparation of proteins from culture supernatant

The cells cultivated in liquid medium were centrifuged at 12,000×*g* for 20 min at 4°C to recover the culture supernatant. The proteins in the supernatant were precipitated by the addition of ammonium sulfate at a final concentration of 75%. The precipitated proteins were dissolved in phosphate-buffered saline (PBS) and dialyzed against PBS buffer using Ultrafree-0.5 −10 kDa (Millipore, Billerica, MA, USA). A BCA Protein Assay Kit (Thermo Fisher Scientific,

Table 2 PCR primers used in this study

Primer	Sequence (5′ to 3′)	Use
Cloning and sequencing of *alyA*		
766F	CACCATGCTAGCGATAC	Amplification of a part of *alyA* gene
1060R	TATGGGTCTTGYGATTG	
395TS3F	ACTGTTTTGAGGATTTAGGCAT	Sequencing of *alyA* on cosmid p333
LowF	GGTTTCATCAAGCGTATCAAT	
1126-R3	GCGTAATATGTTGTGCCTCTA	
alyMR	GGTCGGTTTCGTTCCAGTTAC	
alycosmid2R	AATGCGCCACTACACTGAAA	
Southern analysis and RT-PCR analysis		
06alysouthF2	ACCTTAGTGTTTGTGCCGTTAGA	Amplification of a part of *alyA* gene
06alysouthR	CGCGTATAACTCACATTGGTCAT	
RT-PCR analysis		
27f	AGAGTTTGATCCTGGCTCAG	Amplification of a part of 16S rRNA gene
1492r	GGCTACCTTGTTACGACTT	
Expression of *alyA*		
AlyN-B	GCTAAGATCTGGTTTAAAGGAATAAAGATA	Amplification of the complete *alyA* gene
AlyC-E	CGAAGAATTCAATGGTTATAAAATAAAGTT	
Knockout of *alyA*		
KOalyN-H	AAAGAAGCTTAAACATTTACCTCAGCAAAC	Amplification of a part of *alyA* gene
KOalyC-B	TGTGGATCCGCCATCAGACATC	
NITGFP-F	GAACTAGTCGCCCCATCATCCAGCC	Cloning of *gfp* gene cassette
NITGFP-R	AATGCTATTTGTATAGTTCATCCATGCCAT	

The recognition site for restriction enzyme is underlined

Waltham, MA, USA) was used to determine the concentration of proteins.

Detection and measurement of alginate lyase activities

Alginate lyase activities of proteins from culture supernatant were examined using a substrate-containing gel overlay technique after SDS-PAGE electrophoresis and subsequent renaturation. We adopted the protocol described by Sawabe et al. (2001) with the following modifications. The proteins in the samples were size-fractionated by SDS-PAGE using 10% to 20% gradient precast gel (System Instruments, Tokyo, Japan). After electrophoresis, the proteins in the gel were renatured in a solution of 50 mM Tris–HCl (pH8.0), 2 mM EDTA, and 1% casein, and the gel was further rinsed twice in 2× PBS containing 50 mM $MgCl_2$. The treated gel was overlaid by 2× PBS-buffered and 0.5% alginate-containing 12.5% acrylamide gel and was incubated at 40°C for 4 h.

Alginate lyase activities in the solution were quantitatively determined by measuring the amount of reducing sugars generated from the alginate. This amount was assayed by the 2-cyanoacetamide reaction method using D-glucose as a standard (Bach and Schollmeyer 1992). Unless otherwise stated, a 10-μl aliquot of enzyme solution was added to the 90-μl solution that was composed of Tris–HCl 20 mM (pH7.4) and 0.1% alginate, and the mixture was incubated for 10 min at 40°C. The reaction was terminated by treatment at 98°C for 15 min, and the reaction mixture and 1% 2-cyanoacetamide solution were added to borate buffer. After the additional incubation for 10 min at 98°C, the solution was rapidly chilled to 4°C, and the amount of reduced form of 2-cyanoacetamide was spectrophotometrically measured. One unit of lyase activity was defined as the amount that generated reducing activity equal to 7.0×10^{-3} μmol of glucose in 1 min.

When the PM and PG blocks of alginate were used as the substrates, these blocks were prepared by mild hydrolysis of 1% sodium alginate according to the method of Haug et al. (1966). The enzyme activities were also examined at temperatures ranging from 15 to 55°C at 5°C intervals and at pH values ranging from 4.0 to 9.0. The alginate lyase activities were additionally investigated in the presence of biologically abundant ionic compounds (NaCl, KCl, $MgCl_2$, and $MgSO_4$) at concentrations of 10 to 50 mM and EDTA at 1 mM.

Thin-layer chromatography analysis of alginate lyase reaction products

The proteins from the culture supernatant were added to a reaction solution containing 1% alginate, 50 mM $MgCl_2$, 8.1 mM Na_2HPO_4, and 1.47 mM KH_2PO_4 and incubated

overnight at 37°C. Oligoalginate components of reaction products were developed on an HPTLC plate (Merck, Darmstadt, Germany) with a solvent of *n*-butanol/formic acid/water [4:6:1 (*v*/*v*)] (Zhang et al. 2006). A freshly prepared 50% ethanol solution containing 10% sulfuric acid and 0.1% 1,3-naphthalenediol was sprayed on the developed plate and treated for 10 min at 100°C for the color detection of oligoalginate components. The oligoalginate molecules were isolated by anion-exchange chromatography with Q-Sepharose (GE Healthcare, Chalfont St. Giles, UK) (Natsume et al. 1994). After the subsequent desalting, oligoalginate components were identified by MALDI-TOF MS (Momcilovic et al. 2003).

Amino sequence analysis

The proteins separated by SDS-PAGE were blotted on Sequi-Blot PVDF Membrane (Biorad Laboratories, Hercules, CA, USA). The extracted protein bands were analyzed with an ABI Procise 492cLC protein sequencer at Nippi Inc. (Tokyo, Japan).

DNA and RNA manipulations and construction of plasmids

Established methods were employed for the preparation of genomic and plasmid DNAs, digestion of DNA with restriction endonucleases, ligation, agarose gel electrophoresis, and transformation of *E. coli* cells (Sambrook et al. 1989). Southern blotting and subsequent detection of hybridized fragments (Sambrook et al. 1989) were carried out using the DIG system (Roche Diagnostics, Basel, Switzerland). The introduction of plasmids from *E. coli* to *P. atlantica* cells was carried out by triparental mating (Ruvkun and Ausubel 1981). The *E. coli* donor DH5α cells carrying the plasmid, the *E. coli* helper HB101(pRK2013) cells, and the recipient *P. atlantica* cells were mixed and incubated overnight at 30°C on MB agar plate. The transconjugants that received the plasmid were selected on ASW agar plate containing appropriate antibiotics. Unless otherwise stated, PCR was performed using *ExTaq* DNA polymerase (Takara, Kyoto, Japan). RNA was isolated using Sepasol-RNA I (Nacalai Tesque), and the TURBO DNA-free kit (Ambion, Tokyo, Japan) was used to remove the contaminated DNA. The resulting RNA sample was used to prepare the cDNA sample with Maxime RT PreMix Kit 25082 (iNtRON Biotechnology, Seongnam, Korea).

The *P. atlantica* AR06 genome was digested partially with *Sau*3AI, and the resulting DNA fragments were employed to construct the cosmid-based genomic library using the SuperCos1 Cosmid Vector Kit and Gigapack III Gold Packaging Extract (Stratagene, La Jolla, CA, USA). The in vitro packaged-cosmid library was introduced into *E. coli* XL1-Blue MR cells.

A DNA fragment containing the complete *alyA* gene was amplified with the primers AlyN-B and AlyC-E (Table 2) with the AR06 genomic DNA as the template. The amplicon was treated with *Bgl*II and *Eco*RI and inserted into the corresponding sites of pNIT6012 so that the cloned gene was transcribed from the vector-derived and constitutive promoter. The resulting plasmid was designated pARA (Fig. 1).

DNA sequence analysis

The nucleotide sequences were determined by ABI Prism Big Dye 3.1 (Applied Biosystems, Foster City, CA, USA) with an ABI 3100 automated sequencer (Applied Biosystems) and were analyzed using the Gene Runner program (http://www.generunner.net/). The promoter motifs and N-terminal signal peptide sequences were predicted by the BPROM (http://linux1.softberry.com/berry.phtml) and PSORT (http://psort.hgc.jp/) programs, respectively. Homology searches were performed using BLAST programs at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/BLAST/).

Construction of *alyA* mutant of AR06

A *Bgl*II fragment containing the transposase gene and one IR of Tn5 was removed from pTn*Mod*-OCm, a pMB1-derived and mobilizable plasmid, to obtain pMobCm. A part of the *alyA* gene was amplified by PCR using the primer set of KOalyN-H and KOalyC-B (Table 2) and the AR06 genomic DNA as the template. After digestion by *Hin*dIII and *Bam*HI, the amplicon was inserted into the corresponding sites of pK18mob. The resulting plasmid, pKARA, was linearized by *Hin*dIII digestion, blunt-ended by T4 DNA polymerase, and subsequently digested by *Kpn*I. This linearized and *alyA*-containing fragment was inserted between the *Hin*cII and *Kpn*I sites on pMobCm to obtain pARA2. A *gfp*-containing *Eco*RI fragment from pGreenTIR was inserted into an *Eco*RI site of pNIT6012, and the DNA fragment carrying the vector-derived and constitutive promoter for a *gfp* gene in the resulting plasmid was amplified by PCR with KOD Plus (Toyobo, Osaka, Japan) using the primer set of NITGFP-F and NITGFP-R (Table 2). The amplicon was digested with *Spe*I and ligated with the *Xba*I-digested, blunt-ended, and *Spe*I-digested pARA2 replicon to give rise to pARA3 (Figs. 1 and 2). A single-crossover-mediated homologous recombination strategy (Ruvkun and Ausubel 1981; Duilio et al. 2004) through the integration of pARA3 was employed to disrupt the chromosomal *alyA* gene in AR06 (Fig. 2). pARA3 was mobilized by triparental mating from *E. coli* DH5α to AR06, and the Cm^r transconjugants were selected on ASW agar plate. One of these transconjugants, designated ARA1,

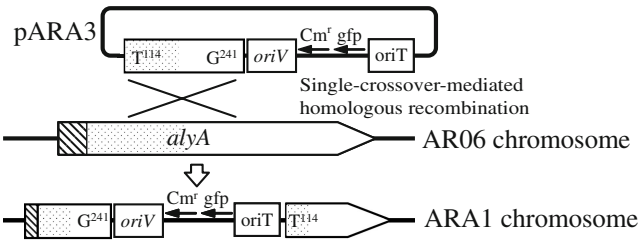

Fig. 2 Construction of chromosomal *alyA* mutant, ARA1. Homologous recombination was employed to disrupt the *alyA* gene of AR06. The scheme shows the integration of pARA3 into the AR06 genome through single crossover-mediated homologous recombination between the chromosomal and intact *alyA* gene and plasmid-specified and truncated *alyA* gene. The recombination event generated the ARA1 genome carrying incomplete *alyA* genes. oriT, RP4-derived origin of conjugal transfer. T^{114} and G^{241} indicate the 114th and 241st amino-acid residues, respectively, of the nascent *alyA* product, and pARA3 carried the part of *alyA* gene that specifies the peptide from the 114th to 241st residues

produced green fluorescence but did not show alginate lyase activity. Our PCR and subsequent sequencing analysis confirmed the expected integration of pARA3 into the AR06 chromosome by the single-crossover-mediated homologous recombination between the pARA3- and chromosome-specified *alyA* genes (data not shown).

Nucleotide sequence accession number

The nucleotide sequence determined in this study has been deposited in the DDBJ/EMBL/GenBank databases under the accession number AB505895.

Results

Properties of extracellular alginate lyase(s) from AR06

AR06 cells were cultivated in alginate-containing ASW liquid medium for 24 h, and the alginate lyase activities of the culture supernatant proteins (0.5 μg/μl) were measured under different temperatures, pH, and ionic conditions. The optimum reaction temperature was 40°C, and the enzyme activities at 20 and 50°C decreased to 50% and 30%, respectively, of that at 40°C. The maximum activity was observed at pH7.4. The addition of 50 mM Mg^{2+} and 1 mM EDTA led to a two-fold increase and a three-fold decrease, respectively, in activity levels, whereas the addition of K^+ and Na^+ ions at a concentration of 50 mM resulted in only a 10% increase (Table 3).

To know the substrate specificities of the AR06-derived extracellular alginate lyase(s), alginate and its PM and PG blocks were incubated with the supernatant proteins. The thin-layer chromatography (TLC) analysis (Fig. 3) revealed that all three substrates were depolymerized mainly into the dimer to tetramer forms. This indicated that AR06 has at least the alginate lyase able to depolymerize heteropolymeric alginate.

Table 3 Alginate lyase activities of culture supernatant proteins of AR06

Condition	Activity (U/mg protein)	Relative activity
Standard	$[2.00\pm0.05]\times10^2$	1.00
Standard+50 mM KCl	$[2.20\pm0.05]\times10^2$	1.10
Standard+50 mM NaCl	$[2.15\pm0.02]\times10^2$	1.07
Standard+50 mM $MgCl_2$	$[4.44\pm0.14]\times10^2$	2.22
Standard+50 mM $MgSO_4$	$[4.14\pm0.13]\times10^2$	2.07
Standard+1 mM EDTA	$[0.68\pm0.03]\times10^2$	0.34

The standard condition for the assay [in 20 mM Tris–HCl (pH7.4) containing 0.1% alginate at 40°C] is described in "Materials and methods." The values with their standard derivations were obtained from at least three independent experiments

Isolation and characterization of alginate lyase (*alyA*) gene from AR06

To identify the putative alginate lyase gene(s) from AR06, a set of primers, 766F and 1060R (Table 2 and Fig. 1a), was designed on the basis of alginate lyase genes from *Alteromonas* sp. strain 272 (Iwamoto et al. 2002) and *P. elyakovii* IAM14594 (Sawabe et al. 2001). The PCR amplification using the AR06 genomic DNA as a template led to the successful detection of a 431-bp fragment whose sequence was highly homologous to the internal parts of the two lyase genes described above. A cosmid library of AR06 genomic DNA was constructed, and the same set of primers was also used to identify the cosmids containing the putative alginate lyase gene. One cosmid, p333, carried the complete *alyA* gene (Fig. 1a). The genetic and biochemical analyses described below indicated that the *alyA* gene of AR06 indeed encoded the alginate lyase.

The *alyA* gene encoding a 391 amino-acid protein was monocistronic, and a canonical promoter sequence and a 17-bp palindromic terminator sequence were found to be located 340 bp upstream of the putative start codon and 8 bp downstream of the last codon, respectively. The first 22 amino-acid residues of the nascent gene product were predicted to be a signal peptide sequence. Computer analysis additionally predicted that this signal peptide would be followed by the carbohydrate-binding domain (CBM) [CBM_4_9 (pfam02018)] at the A^{23}–E^{145} region, and that the C-terminal (D^{177}–E^{376}) part of the gene product had the alginate lyase domain [Alginate_lyase2 (pfam08787)]. The AR06 *alyA* product showed 76% identity with the *P. elyakovii* IAM14594-specified *alyPEEC* product, a polysaccharide lyase family 18 extracellular alginate lyase (Sawabe et al. 2001). Furthermore, the C-terminal 233

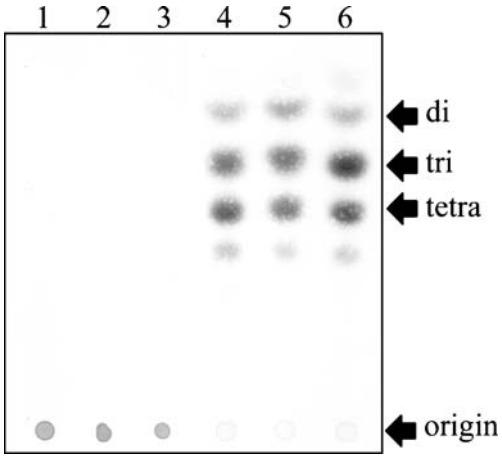

Fig. 3 TLC analysis of oligoalginates generated after reaction with culture supernatant proteins from AR06. Alginate, PM, and PG were reacted with the supernatant proteins, and the oligoalginates formed were separated by TLC and color-developed. *Lanes 4 to 6*: reaction products using alginate (*lane 4*), PG (*lane 5*), and PM (*lane 6*) as the substrates. *Lanes 1 to 3*; alginate, PG, and PM, respectively, with no reactions. The *arrows* represent the di-, tri-, and tetra-forms of oligoalginates, and "origin" indicates the substrate

amino acid sequence of the nascent AR06 *alyA* product was identical to that of alginate lyase (AALyase) from *Alteromonas* sp. 272 (Iwamoto et al. 2002) (Fig. 1b). These findings indicated that the *alyA* product belonged to the polysaccharide lyase family 18 [Carbohydrate-Active enZymes (CAZy) database, http://www.cazy.org/]. The *alyA* gene product had at its C-terminal portion a consensus amino-acid sequence motif, YFKhG+Y-Q (+, neutral; −, no consensus; and *h*, hydrophobic) (Fig. 1b), which is conserved among the 30 M_r class alginate lyases (Wong et al. 2000).

The AR06-derived 8-kb region covering the *alyA* gene additionally carried six open reading frames (ORFs), ORF1 to ORF6 (Fig. 1c), and all of their deduced products were not apparently involved in the depolymerization or assimilation of alginate [ORF1, GyrA (DNA topoisomerase IV subunit A); ORF2, putative acyltransferase; ORF3, hypothetical protein; ORF4, putative transporter protein; ORF5, hypothetical protein; and ORF6, RimK (ribosomal protein S6 modification protein)]. The ORF1 to ORF3 and ORF4 to ORF6 regions on the AR06 genome were highly conserved (more than 76% identities in nucleotide sequences) in the corresponding regions of the *P. haloplanktis* TAC125 and *Alteromonadales* bacterium TW-7 genomes with respect to the syntenies of genes and the significant (>81%) identities of gene products (Fig. 1c). However, the 1.9-kb *alyA*-containing region of AR06 was replaced by completely different 1,383- and 226-bp sequences in TAC125 and TW-7, respectively.

Southern hybridization analysis using a part of the *alyA* gene as a probe (Fig. 1a) revealed that the AR06 genome carried no structural homologs of *alyA* (data not shown).

Role of *alyA* in alginate assimilation in AR06

Cultivation of AR06 cells on alginate-containing ASW agar plate and subsequent flooding of 10% cetylpyridinium chloride (CPC) solution on the plate led to the formation of a clear zone around the colonies because of the alginate depolymerization by the secreted alginate lyase(s) (Fig. 4a, 1). ARA1, a chromosomal *alyA* knockout mutant of AR06 (Fig. 4a, 2), lost the ability to form such a clear zone, and this defect of ARA1 was complemented by the introduction of pARA, which can express the *alyA* gene from a vector-derived constitutive promoter (Fig. 4a, 3). Although the growth rates of ARA1 and AR06 in MB were similar (data not shown), the former, but not the latter, strain grew very slowly in the ASW liquid medium supplemented with 0.3% alginate (Fig. 4b). This growth defect of ARA1 was also restored by the introduction of pARA. These findings indicated that the *alyA* gene played an important role in the assimilation of alginate in AR06.

Alginate lyase activities of culture supernatant proteins

The AR06 cells were cultivated in ASW liquid medium containing 0.3% alginate, and the culture supernatant proteins were analyzed by SDS-PAGE and activity staining. Three protein bands with apparent sizes of 43, 33, and 30.5 kDa exhibited enzyme activities (Fig. 5a, b). ARA1 grew very slowly in the ASW medium supplemented with 0.3%

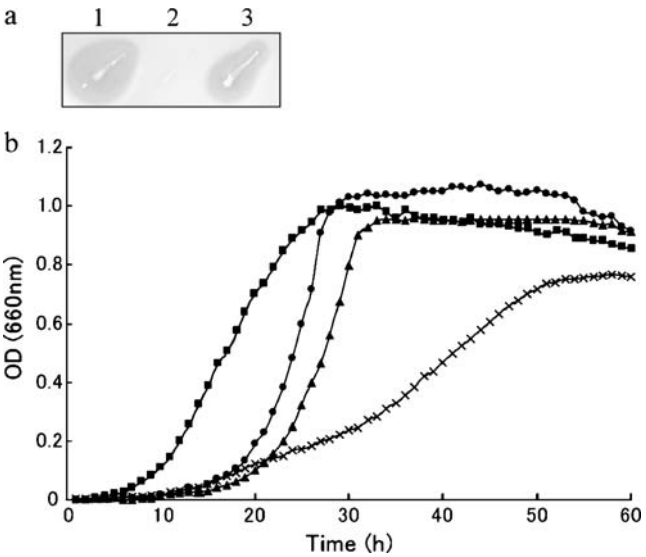

Fig. 4 Properties of AR06 and ARA1. **a** Detection of alginate lyase activities after cultivation on ASW alginate agar plate and subsequent flooding of CPC. *1* AR06(pBBR1MCS)(pNIT6012); *2* ARA1 (pNIT6012); *3* ARA1(pARA). **b** Growth curves at 20°C of AR06 and its derivatives in 0.3% alginate-containing ASW liquid media with or without the addition of Cm and Tc. *Filled squares* AR06; *filled circles* AR06(pBBR1MCS)(pNIT6012); *filled triangles* ARA1(pARA); and *ex marks* ARA1(pNIT6012). AR06 cells was cultivated without the two antibiotics and the remaining three strains with the two antibiotics

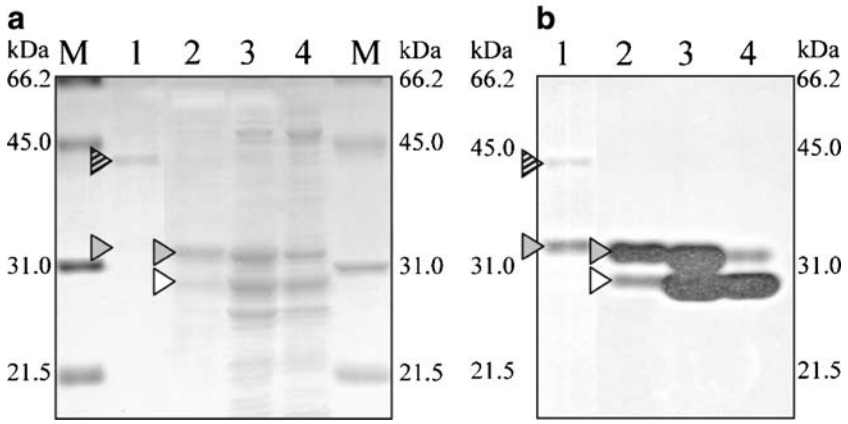

Fig. 5 SDS-PAGE and activity staining analyses of alginate lyases from supernatant proteins. **a**, **b** The AR06 cells were cultivated in 0.3% aliginate-containing ASW liquid medium, and the AR06 (pBBR1MCS)(pNIT6012) cells in the same medium with Cm and Tc. The culture supernatant proteins prepared at different periods after the cultivation were analyzed by SDS-PAGE and subsequent activity staining. **a** SDS-PAGE with CBB staining; **b** activity staining against alginate lyase. *Lane M*, protein markers (serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; and trypsin inhibitor, 21.5 kDa); *lane 1* sample prepared 14 h after the cultivation of AR06 (pBBR1MCS)(pNIT6012) (3 μg proteins); *lane 2* sample prepared 14 h after the cultivation of AR06 (3 μg proteins); *lane 3* sample prepared 24 h after the cultivation of AR06 (10 μg proteins); and *lane 4* sample prepared 38 h after the cultivation of AR06 (9 μg proteins). *Three triangles* correspond to the cleavage products of the nascent *alyA* gene product (see Fig. 1b)

alginate (see above), and no alginate lyase activity was detected at all in the supernatant (data not shown), indicating the involvement of the *alyA* gene in the activities. The three proteins described above had the N-terminal amino-acid sequences of [ATINNAGFESGFSNW], [GSNDG], and [NGSTIPS] residues, respectively. These results indicated that (1) the 43-kDa protein was the derivative of the nascent *alyA* gene product lacking the N-terminal 22 amino-acid signal peptide and (2) the 33- and 30.5-kDa proteins were the C-terminal portions of the nascent *alyA* gene products that started from the amino-acid residue positions 148 and 162, respectively (Fig. 1b). The weak enzymatic activity of the 43-kD band (Fig. 5a, b, lane 1) was observed only at the very early growth phase of AR06(pBBR1MCS)(pNIT6012) (14 h after the cultivation, see Fig. 4). The 33-kDa band showed enzymatic activity between 14 and 38 h of cultivation, albeit with relatively low activity after 38 h of cultivation. In contrast, the enzymatic activity of the 30.5-kDa band was weak at 14 h of cultivation, and such activity was relatively high from 24 and 38 h of cultivation. The semiqualitative comparison of intensities of CBB-stained bands with those of their activity bands suggested that the 33- and 30.5-kDa proteins shared similar specific enzymatic activities.

Expression of alginate lyase under different culture conditions

The transcription of *alyA* in AR06 was analyzed by reverse transcription-PCR (Fig. 6). No *alyA* transcription was detected in AR06 cells grown in MB or ASW glucose medium, and the addition of alginate to the ASW medium resulted in the induction of transcription. However, the addition of alginate to MB did not allow the detection of *alyA* transcription. These results indicated that the substrate, as well as nutritional conditions, regulated the *alyA* transcription.

Discussion

Our biochemical and genetic analyses in this study clearly revealed that AR06 produced extracellular and bifunctional alginate lyases encoded by a single gene, *alyA*. The three alginate lyases with apparent sizes of 43, 33, and 30.5 kDa started from the amino-acid residue positions, A^{23}, G^{149}, and N^{162}, respectively, of the nascent *alyA* gene product (Fig. 1b). Our activity staining experiments with culture supernatant proteins of AR06 prepared at different growth phases (Fig. 5a, b) suggested the formation of three bands by site-specific and probably three-step cleavage of the nascent product. The nascent product of the *alyPEEC* gene has also been reported to be processed at its N-terminal

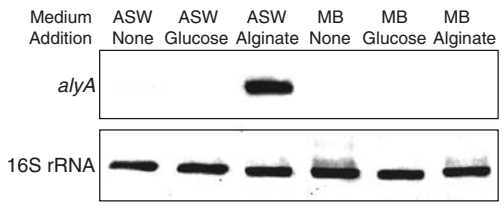

Fig. 6 Reverse transcription-PCR analysis of *alyA* expression. The AR06 cells were cultivated for 90 min in MB or ASW minimal liquid medium with or without the addition of 0.3% glucose or 0.3% sodium alginate, and the total RNA fractions prepared from the cells were used for reverse transcription-PCR analysis to examine the expression of *alyA*. The 16S rRNA sample was used as a control

positions (Fig. 1b) (Sawabe et al. 2001), and the processed AlyPEEC form lacks the carbohydrate-binding domain but still exhibits bifunctional alginate lyase activity (Sawabe et al. 2001). This was also indeed the cases with the 33- and 30.5-kDa forms in this study, confirming that such a domain was not necessary for the catalytic activity. In addition, the lack of any apparent differences in enzymatic activities between the 33- and 30.5-kDa forms suggests that the presence of the first 14 amino-acid residues in the 33-kDa form did not drastically affect the activity.

The dimer to tetramer forms of oligoalginates produced by AlyA were efficiently used as sole sources of carbon and energy for the AR06 and ARA1 cells (data not shown), indicating that the proteins other than AlyA are involved in the utilization of oligoalginates in AR06. However, it is unknown at present whether or not the extracellular low-molecular-weight oligoalginates were further metabolized in the periplasm and how the oligoalginates and/or their metabolites are transported into the cytoplasm. The oligoalginate formation by AR06 is apparently different from the alginate utilization mechanism of *Sphingomonas* sp. A1 (Hashimoto et al. 2001) since the high-molecular-weight alginate is directly incorporated into the A1 cytoplasm by a mouth-like pit formed on the cell surface, and the incorporated substrate is thereafter converted to oligoalginates and their monomer forms by three types of alginate lyases and one oligoalginate lyase, respectively, that are all present in the cytoplasm. Even if AR06 has this type of alginate utilization system, it is very inefficient since ARA1 grew much more slowly in the ASW alginate medium than did AR06 (Fig. 4b).

The *alyA* gene was not physically associated with its functionally related ones in the AR06 genome (Fig. 1c). The *alyA*-flanking regions of AR06 were well conserved in two marine bacterial strains, TAC125 and TW-7, and a 1.9-kb *alyA*-containing DNA fragment in AR06 was replaced by 1.4- and 0.2-kb DNA fragments in the latter two strains, respectively (Fig. 1c). Therefore, it is possible that the three different DNA fragments in the three strains might have been acquired by horizontal gene transfer events from other environmental bacteria.

Two apparently different types of transcriptional regulation of *alyA* were found in this study (Fig. 6). One was the induction of *alyA* transcription in the presence of alginate. Specific detection of extracellular alginate lyase activities by other bacterial strains in the presence of alginate (Wong et al. 2000; Kitamikado et al. 1989; Doubet and Quatrano 1982) might be, similar to the case with AR06, explained by the transcriptional induction of their structural genes. For transcriptional induction in AR06, alginate per se or its metabolite(s) might be the inducer(s). The clarification of the molecular mechanism underlying induction as well as the identification of actual inducer(s) is our important research topics in the near future. The other regulation of *alyA* transcription was its repression in MB even in the presence of alginate (Fig. 6). This kind of repression of alginate lyase genes in rich media has not been reported in bacterial strains other than AR06. It is therefore of interest to investigate (1) the chemical compound(s) necessary for repression, (2) the molecular mechanism of repression, and (3) the interaction between the repression and induction mechanisms for *alyA* transcription.

Unlike most other alginate lyase producers, strain AR06 is unique in its ability to use alginate as a sole source of carbon and energy. The *alyA*-specific extracellular alginate lyases greatly contributed to such ability since the *alyA* mutation caused very slow growth of its host cells in the alginate-containing ASW minimal medium (Fig. 4b). However, the residual growth of the *alyA* mutant indicated that the wild-type gene products are not essential for the utilization of alginate and implied the possibility that AR06 has one or more alternative but inefficient systems for the utilization of alginate. More detailed elucidation of alginate lyases of AR06 from genetic as well as biochemical standpoints is important for our understanding of the ocean carbon cycle and for the efficient utilization of sea biomass.

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