*Biosci. Biotechnol. Biochem.*, **73** (7), 1505–1512, 2009

# Molecular Cloning and Functional Expression of a New Amylosucrase from *Alteromonas macleodii*

Suk-Jin HA,[1] Dong-Ho SEO,[1] Jong-Hyun JUNG,[1] Jaeho CHA,[2] Tae-Jip KIM,[3] Young-Wan KIM,[4] and Cheon-Seok PARK[1,†]

[1]*Graduate School of Biotechnology and Institute of Life Science and Resources, Kyung Hee University, Yongin 446-701, Korea*
[2]*Department of Microbiology, Pusan National University, Busan 609-735, Korea*
[3]*Department of Food Science and Technology, Chungbuk National University, Cheongju 361-763, Korea*
[4]*Department of Food and Biotechnology, Korea University, Yeongi 339-700, Korea*

Received December 15, 2008; Accepted March 20, 2009; Online Publication, July 7, 2009
[doi:10.1271/bbb.80891]

**The presence of amylosucrase in 12 *Alteromonas* and *Pseudoalteromonas* strains was examined. Two *Alteromonas* species (*Alteromonas addita* KCTC 12195 and *Alteromonas macleodii* KCTC 2957) possessed genes that had high sequence homology to known amylosucrases. Genomic clones containing the ASase analogs were obtained from *A. addita* and *A. macleodii*, and the deduced amino acid sequences of the corresponding genes (*aaas* and *amas*, respectively) revealed that they were highly similar to the ASases of *Neisseria polysaccharea*, *Deinococcus radiodurans*, and *Deinococcus geothermalis*. Functional expression of *amas* in *Escherichia coli* was successful, and typical ASase activity was detected in purified recombinant AMAS, whereas the purified recombinant AAAS was nonfunctional. Although maximum total activity of AMAS was observed at 45 °C, the ratio of transglycosylation to total activity increased as the temperature decreased from 55 to 25 °C. These results imply that transglycosylation occurs preferentially at lower temperatures while hydrolysis is predominant at higher temperatures.**

**Key words:** *Alteromonas macleodii*; amylosucrase; transglycosylation

Amylosucrase (ASase, EC 2.4.1.4) is a glycosyltransferase that belongs to family 13 of the glycoside hydrolases, which contains a variety of known polysaccharide-degrading enzymes (*e.g.*, $\alpha$-amylase).[1)] This enzyme transfers a D-glucopyranosyl moiety from sucrose to maltooligosaccharides, producing an insoluble $\alpha$-1,4-linked glucan polymer.[2,3)] The most interesting aspect of synthesizing this amylose-like polymer is the use of sucrose as the sole substrate, unlike starch or glycogen synthases, which normally require an expensive nucleotide-activated sugar (*e.g.*, ADP- or UDP-glucose) as a donor.[3–5)] It is known that ASase catalyzes sucrose hydrolysis, sucrose isomerization, and polymer synthesis (soluble malto-oligosaccharides and insoluble $\alpha$-1,4-linked glucan).[1,3)]

Although the presence of ASase in microorganisms was first found in cultures of *Neisseria perflava*,[6)] most enzymatic and biochemical studies of ASase have been conducted in *Neisseria polysaccharea*.[3,7,8)] In *N. polysaccharea*, ASase is secreted and synthesizes an amylose-like polysaccharide outside the cells. The *N. polysaccharea* ASase gene, *npas*, was cloned and the functional protein was recombinantly produced in *Escherichia coli*. Using the full coding region of NPAS, including the N-terminal signal peptide, ASase was isolated from culture supernatants of transformed *E. coli* and was able to synthesize linear $\alpha$-1,4-glucans from sucrose.[7)] Recently, the presence of ASase genes in various microorganisms other than *Neisseria* genus has been described.[9,10)] These genera include *Alteromonas macleodii*, *Caulobacter crescentus*, *Deinococcus radiodurans*, *Rhodopirellula baltica* SH1, and *Xanthomonas campestris*. The putative ASase gene of *D. radiodurans (dras)* was actively expressed in *Escherichia coli*, and the purified recombinant enzyme catalyzed insoluble amylose polymer formation together with side-reactions of sucrose hydrolysis, sucrose isomerization, and soluble maltooligosaccharide formation.[9)] Recently, an ASase gene from the moderately thermostable bacterium *Deinococcus geothermalis (dgas)* was also cloned and functionally expressed, and its recombinant protein was characterized.[10)] The recombinant DGAS had exceptional thermostable characteristics, as demonstrated by a half-life of 6.8 h at 55 °C, in addition to typical ASase enzymatic properties, as found in DRAS.[10)]

*Alteromonas* is a Gram-negative, mesophilic, heterotrophic bacterium. It belongs to the genus of Proteobacteria and is usually found in seawater, either in the open ocean or on the coast. *Alteromonas macleodii* is an obligate marine bacterium isolated from both surface and deep seawater and is often found with particulate matter. *Alteromonas macleodii* secretes a high-molecular-weight polysaccharide in culture.[11)] This exopolysaccharide has similarities to xanthan, a bacterial polysaccharide used as a food additive and a rheology modifier in industry. The backbone of xanthan consists of two $\beta$-D-glucose units linked through the 1 and 4 positions.[12–14)] The side chain consists of two mannoses and one glucuronic acid. Thus the chain consists of repeating modules of 5 sugar units. The genome of *A. macleodii* Deep ecotype, an obligate marine bacte-

† To whom correspondence should be addressed. Tel: +82-31-201-2631; Fax: +82-31-204-8116; E-mail: cspark@khu.ac.kr

rium isolated at a depth of 3,500 meters in the Urania Basin, has been sequenced and made available at the National Center for Biotechnology Information (NCBI).[15] A putative protein tagged MADE_00676, which is significantly homologous to known ASases, is located in the genome of the *A. macleodii* Deep ecotype. Based on the fact that *A. macleodii* produces a xanthan-like exopolysaccharide and ASase forms an $\alpha$-1,4-linked glucan polysaccharide, which has a similar backbone to xanthan polymers, we hypothesized that the putative ASase homolog of *A. macleodii* is a functional protein with a role in the biosynthesis of exopolysaccharides. In this study, we sought homologs of *Neisseria* and *Deinococcus* ASase in various *Alteromonas* strains and cloned the potential ASase gene from *A. macleodii (amas)*. We further expressed *amas* in *E. coli* and characterized the enzymatic properties of the recombinant protein.

## Materials and Methods

*Bacterial strains, media, and plasmids.* Twelve strains belonging to the genera *Alteromonas* and *Pseudoalteromonas* were obtained from the Korean Collection for Type Cultures (KCTC). These strains were *Alteromonas addita* KCTC 12195, *Alteromonas macleodii* KCTC 2957, *Pseudoalteromonas byunsanensis* KCTC 12274, *Pseudoalteromonas issachenkonii* KCTC 12958, *Pseudoalteromonas maricaloris* KCTC 12960, *Pseudoalteromonas marina* KCTC 12241, *Pseudoalteromonas phenolica* KCTC 12086, *Pseudoalteromonas ruthenica* KCTC 12959, *Pseudoalteromonas* sp. KCTC 12273, *Pseudoalteromonas* sp. KCTC 12275, *Pseudoalteromonas ulvae* KCTC 12940, and *Pseudoalteromonas undina* KCTC 12423. They were grown in Marine broth or on Marine agar (Difco Laboratories, Detroit, MI) at 25–30 °C for 48 h and used as the source of chromosomal DNA. *E. coli* DH5$\alpha$ [F$^-$ $\Phi$80*lac*Z $\Delta$M15 $\Delta$(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17($r_k^+$, $m_k^+$) *pho*A *sup*E44 *thi*-1 *gyr*A96 *rel*A1$\lambda^-$] and *E. coli* BL21 [F$^-$ *ompT* *hsdS$_B$* ($r_B^-$, $m_B^-$) *gal dcm* $\lambda$(DE3) pLysS T1R] were used as hosts in DNA manipulation and for gene expression respectively. The selection of recombinant *E. coli* clones was performed on LB agar plates supplemented with 100 µg/ml ampicillin, 0.5 mM of isopropyl-$\beta$-D-thiogalactopyranoside (IPTG), and 40 µg/ml of 5-bromo-4-chloro-3-indolyl-$\beta$-D-galactopyranoside (Xgal). pGEM-T Easy plasmid (Promega, Madison, WI) and the pGEX-4T-1 vector (Amersham Biosciences, Buckinghamshire, UK) were utilized in the cloning of PCR products and the construction of the expression vector respectively.

*Enzymes and chemicals.* Restriction endonucleases and other DNA modifying enzymes, such as T4 DNA ligase and *Pfu* DNA polymerase, were purchased from New England Biolabs (Beverly, MA) and Stratagene (La Jolla, CA). The genomic DNAs of *Alteromonas* and *Pseudoalteromonas* strains was isolated and purified with the Genomic DNA Prep Kit for Bacteria (Solgent, Seoul, Korea). The purification of PCR products and DNA restriction fragments was performed using the QIAquick gel extraction kit from Qiagen (Valencia, CA). All other chemicals used were of reagent-grade quality and were obtained from Sigma Chemical (St. Louis, MO).

*Design of degenerate primers specific to the ASase gene and detection of it.* To detect potential ASase genes from the *Alteromonas* and *Pseudoalteromonas* strains, a degenerate ASase primer set was developed (Fig. 1). The primer set AS-N1 and AS-C1, specific to the ASase gene, was designed based on conserved regions of known ASases of *N. polysaccharea*, *D. radiodurans*, and *D. geothermalis* (Fig. 2). The presence of an ASase was detected by PCR amplification using *Taq* polymerase (Promega). Standard PCR reactions were carried out in a 20 µl volume at the following final concentrations: 50 mM KCl, 10 mM Tris–HCl (pH 9.0 at 25 °C), 0.1% Triton X-100, 1.5 mM $MgCl_2$, 100 mM of each dNTP, 0.5 mM of each primer, and 0.5 U *Taq* DNA polymerase. Thirty nanograms of genomic DNA was used as a template. The PCR cycling conditions were as follows: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and an extension at 72 °C for 1 min, with an additional extension at 72 °C for 5 min during the final cycle. After amplification, the PCR products in each reaction were analyzed by agarose gel electrophoresis. The amplified PCR products were then purified using the QIAquick gel extraction kit and were cloned into the pGEM-T Easy vector. The nucleotide sequence of the subcloned PCR products was determined using the BigDye Terminator Cycle Sequencing Kit for an ABI377 PRISM (PE Applied Biosystems, Boston, MA). The resulting DNA sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) server available at NCBI (Bethesda, MD, http://blast.ncbi.nlm.nih.gov/Blast.cgi).

*Cloning and analysis of the* amas *gene.* Southern blot analysis was performed with *A. macleodii* KCTC 2957 genomic DNA. Genomic DNA was isolated from *A. macleodii* KCTC 2957 and digested with *Bam*HI, *Eco*RI, *Hind*III, *Sph*I, *Xba*I, or *Xho*I. The DNA digests were separated on 0.6% agarose gels and then transferred by capillary-blotting onto Hybond-$N^+$ nylon membranes (GE Healthcare, Buckinghamshire, UK). Membranes were hybridized with digoxigenin (DIG)-labeled probe, which was generated by PCR with the AS-N1 and AS-C1 primer set. Detection was performed with a chemiluminescent detection system according to the manufacturer's protocols (Roche Applied Science, Indianapolis, IN). Restriction endonuclease mapping localized the *amas* gene within a 7.5 kb fragment spanning two *Sph*I sites. Colony hybridization was used to screen a mini-library made from 7–8 kb *Sph*I digests of *A. macleodii* KCTC 2957 genomic DNA. A positive clone was identified with the DIG-labeled probe used in Southern blot analysis. An isolated positive clone was designated pGEM-Sph7.5. The insert was fully sequenced by the dideoxy chain termination method, using T7 or SP6 sequencing primers and the BigDye Terminator Cycle Sequencing Kit for the ABI 377 PRISM (PE Applied Biosystems). The DNA and amino acid sequences were subsequently analyzed with DNASIS version 2.1 (Hitachi Software, Yokohama, Japan) and the BLAST server at NCBI.

*Phylogenetic analysis.* Known ASase sequences of *N. polysaccharea*, *D. geothermalis*, and *D. radiodurans* and the complete amino acid sequences of AAAS and AMAS were aligned with the default parameters of the Clustal X (version 1.81) multiple sequence alignment algorithm.[16] A phylogenetic tree was constructed using the Neighbor-joining (NJ) method, version 3.6b of PHYLIP software. The phylogenetic tree was evaluated by a bootstrap test on 1,000 replicates.[17]

*Nucleotide sequence accession numbers.* The AAAS and AMAS nucleotide sequences were submitted to GenBank (accession nos. AB469415 and AB469558 respectively).

*Expression and purification of AMAS.* The putative AMAS coding region was amplified by PCR from pGEM-Sph7.5 with primers AMASNBamHI (5′-GGA TCC ATG AGC TAT GCT GCT GAC-3′) and AMASCXhoI (5′-CTC GAG TTA ATC AGC AAG AAG CCA-3′). The introduced *Bam*HI and *Xho*I restriction sites of each primer are underlined. The amplified products were cloned into pGEX-4T-1 vector (Amersham Biosciences, Piscataway, NJ, USA) to create pGEX-AMAS, in which the glutathione S-transferase (GST) was fused to AMAS. Cultures of recombinant *E. coli* BL21(DE3)/pGEX-AMAS were grown in 1 liter of LB medium supplemented with 0.1 mg/ml of ampicillin at 37 °C with agitation. When the optical density at 600 nm reached 0.5 to 0.6, IPTG was added to a final concentration of 1 mM, and the cells were grown for 3 h to induce expression of the *amas* gene. After induction, the cells were harvested by centrifugation at 5,000 × *g* for 20 min at 4 °C. The pellet was resuspended in 5 ml of phosphate-buffered saline (PBS) buffer (140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, and 1.8 mM $KH_2PO_4$, pH 7.3) per g (wet weight) of cells. The resuspended cells were incubated on ice for 30 min after adding 1 mg/ml lysozyme and were disrupted by sonication (Sonifier 450, Branson, Danbury, CT; output 4, 6 times for 10 s, constant duty) in an ice bath. The cell lysate was centrifuged at 10,000 × *g* for 10 min at 4 °C. The clarified supernatant was applied directly onto a Glutathione-Sepharose™ high performance affinity column (Amersham Biosciences) that was pre-equilibrated with PBS. The column was washed with

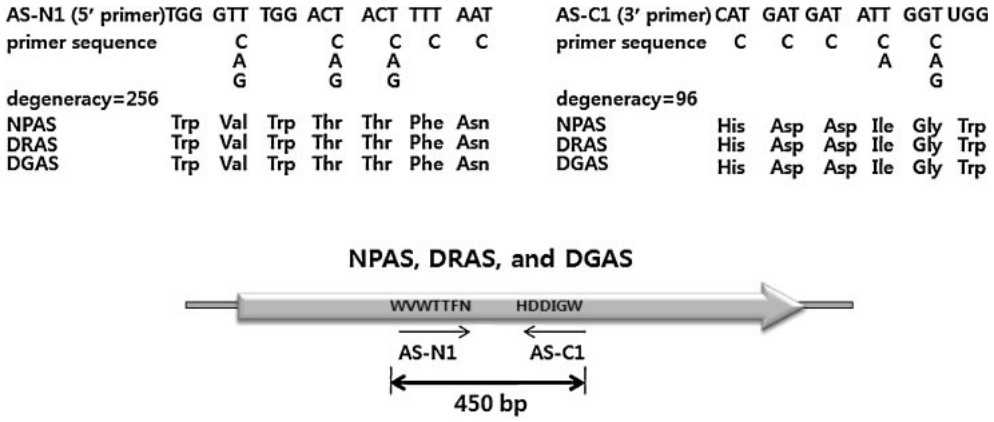


**Fig. 1.** The Degenerate Primer Set Specific to ASase Genes.
The degenerate primer set was designed from two highly conserved regions from the *N. polysaccharea*, *D. geothermalis*, and *D. radiodurans* ASase genes.

2 volumes of PBS. Bound recombinant AMAS was eluted with 50 mM Tris–HCl (pH 8.0) containing 30 mM of reduced glutathione. The size and purity of isolated recombinant AMAS were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and Coomassie brilliant blue staining (Bio-Rad, Hercules, CA). The fused GST protein was removed from the purified recombinant AMAS by treatment with thrombin as suggested by the manufacturer (Amersham Biosciences).

*Enzymatic assay of AMAS.* The enzymatic activity of recombinant AMAS was assayed at 45 °C with 50 g/l sucrose in 50 mM Tris–HCl (pH 8.0) for 10 min. Since the formation of fructose reflects total consumption of sucrose, total activity was defined as the amount of enzyme needed to catalyze the production of 1 µmole of fructose per min under the assay conditions. Hydrolysis activity was defined as the amount of enzyme required to catalyze the production of 1 µmole of glucose per min. The release of glucose results from sucrose hydrolysis when water is used as an acceptor. Transglycosylation activity was calculated by subtracting the amount of glucose from the amount of fructose. The effect of pH on enzymatic activity was investigated within a range of pH 5.0 to pH 10.0 (0.1 M sodium citrate for pH 5.0, 6.0, and 7.0, 0.1 M Tris–HCl for pH 7.0, 8.0, and 9.0, and 0.1 M glycine-NaOH for pH 9.0 and 10.0) at 45 °C. The effect of temperature on the activity was studied between 25 °C and 55 °C at pH 8.0. The thermostability of purified AMAS was observed by pre-incubating the enzyme without substrate at 35 °C, 40 °C, 45 °C, and 50 °C. After various durations, the residual activity was measured under standard assay conditions.

*Analysis of reaction products.* The quantification of the released fructose and glucose was performed using a D-glucose/D-fructose kit (Roche Diagnostics) and high performance anion exchange chromatography (HPAEC) analysis. HPAEC analysis was performed with an analytical column for carbohydrate detection (CarboPac PA100, Dionex, Sunnyvale, CA) and an electrochemical detector (ED40, Dionex). Filtered samples were eluted with a linear gradient from 100% buffer A (100 mM NaOH in water) to 60% buffer B (500 mM of sodium acetate in buffer A) over 70 min. The flow rate of the mobile phase was maintained at 1.0 ml/min. Soluble maltooligosaccharides were quantified using the linear relationship existing between the detector response per mole $\alpha$-1,4-glucan chains and the degree of polymerization, as described by Pizzut-Serin.[14)]

## Results

### Alteromonas *sp. ASase*

The existence of the ASase gene in the *Alteromonas* and *Pseudoalteromonas* strains was investigated by PCR using a degenerate primer set designed from $^{253}$WVWTTFN$^{259}$ (AS-N1) and $^{400}$HDDIGW$^{405}$ (AS-C1) (numbers are from the NPAS amino acid sequence), which are two highly conserved sequences among the ASase genes from various microbial sources (Figs. 1 and 2). When PCR was performed with genomic DNA from 12 *Alteromonas* and *Pseudoalteromonas* strains using AS-N1 and AS-C1, two *Alteromonas* strains (*A. addita* KCTC 12195 and *A. macleodii* KCTC 2957) displayed distinct bands around 450 bp (Fig. 3), although a few non-specific minor bands were noticed in some of the *Pseudoalteromonas* strains. Based on a preliminary BLAST sequence alignment, both cloned PCR products had strong homology with known ASases, NPAS, DRAS, and DGAS. The deduced amino acid sequence of the *A. addita* KCTC 12195 PCR product was 64% identical (97 amino acids out of 150 amino acids) to that of NPAS, 47% (74/155) to that of DGAS, and 47% (73/155) to that of DRAS, whereas the amino acid sequence of *A. macleodii* KCTC 2957 was 66% identical (101 amino acids out of 153 amino acids) to NPAS, 49% (77/157) identical to DGAS, and 49% (77/157) identical to DRAS. This suggests that the cloned PCR products were newly-described ASase genes in *A. addita* KCTC 12195 and *A. macleodii* KCTC 2957.

### *Cloning and analysis of two* Alteromonas *ASase genes*

The *A. addita* KCTC 12195 and *A. macleodii* KCTC 2957 ASase genes were cloned from genomic DNA of the two strains. The *A. macleodii* KCTC 2957 ASase gene (*amas*) was located in a 7.5 kb *Sph*I fragment, and that of *A. addita* KCTC 12195 (*aaas*) was located in a 6.5 kb *Eco*RI fragment. DNA sequence analysis of *aaas* identified an ORF of 1,950 nucleotides that encoded a protein of 649 amino acids with a calculated molecular mass of 73,735 Da and an isoelectric point (pI) of 5.3. Similarly, *amas* sequence analysis revealed an ORF of 1,950 nucleotides that translated to a protein of 649 amino acids with an estimated molecular mass of 73,863 Da and an isoelectric point (pI) of 6.1. The deduced amino acid sequences of *aaas* and *amas* had relatively strong homology to ASases from various microorganisms (Fig. 2). The predicted amino acid sequences of *aaas* and *amas* were 48% (301/623) and 49% (311/629) similar to NPAS, 40% (260/644) and 41% (263/628) similar to DRAS, and 41% (261/627)

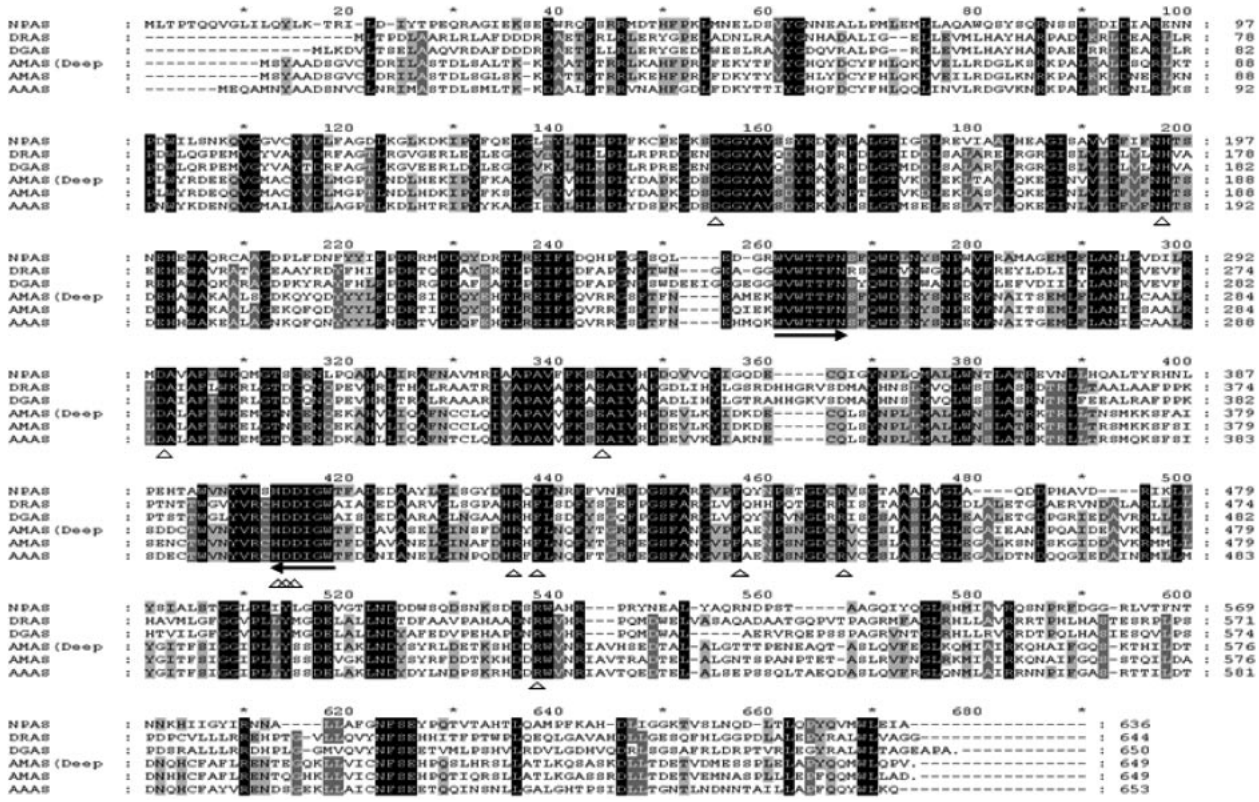

**Fig. 2.** Amino Acid Sequence Alignment of *Alteromonas* sp. ASases with Known ASases.

*Alteromonas* sp. ASases are AAAS (accession no. BAG82877) and AMAS (accession no. BAG82876) and known ASases from various microorganisms include NPAS (accession no. AAM51153), DRAS (accession no. NP_294657), DGAS (accession no. ABF44874), and AMAS (Deep ecotype) (accession no. YP_002124988). Amino acid sequences from the indicated sources were aligned by CLUSTALW. The numbers on the right refer to the amino acid sequence position. Black boxes highlight residues identical in all sequences, whereas gray boxes indicate regions where a majority of the residues matched. Open triangles indicate amino acid residues required for catalytic activity. The two arrows are the regions against which the degenerate primer sets were designed.

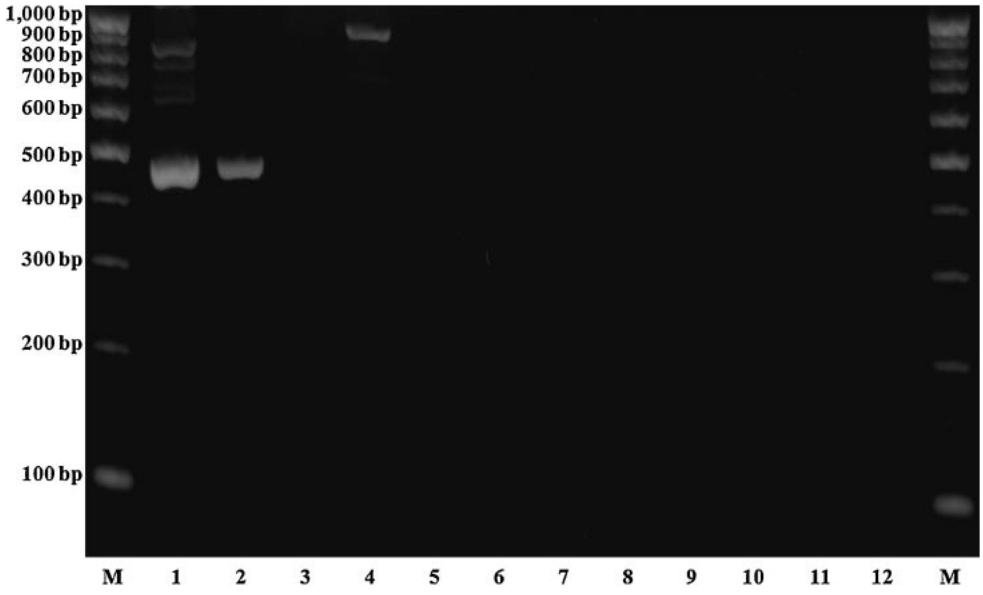


**Fig. 3.** Agarose Gel Electrophoresis of PCR-Amplified Products with ASases-Specific Degenerate Primers.

M, 100 bp plus marker (Solgent): 1, *Alteromonas addita* KCTC 12195; 2, *Alteromonas macleodii* KCTC 2957; 3, *Pseudoalteromonas byunsanensis* KCTC 12274; 4, *Pseudoalteromonas issachenkonii* KCTC 12958; 5, *Pseudoalteromonas maricaloris* KCTC 12960; 6, *Pseudoalteromonas marina* KCTC 12241; 7, *Pseudoalteromonas phenolica* KCTC 12086; 8, *Pseudoalteromonas ruthenica* KCTC 12959; 9, *Pseudoalteromonas* sp. KCTC 12273; 10, *Pseudoalteromonas* sp. KCTC 12275; 11, *Pseudoalteromonas ulvae* KCTC 12940; 12, *Pseudoalteromonas vndina* KCTC 12423.

and 41% (259/631) similar to DGAS respectively. Both ASases (AAAS and AMAS) were highly homologous [76% (497/648) and 87% (563/646)] to a putative ASase from *A. macleodii* Deep ecotype. AAAS and AMAS displayed 77% (501/647) homology to each other. Phylogenetic analysis demonstrated that the ASases of

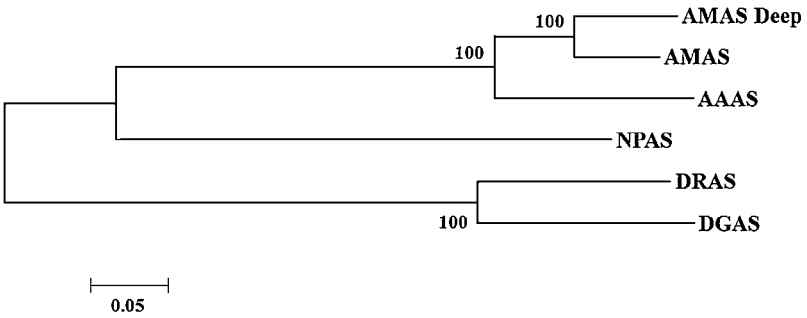


**Fig. 4.** Phylogenetic Tree of Six ASases.

Phylogenetic tree of six ASases (NPAS, DGAS, DRAS, AMAS-Deep ecotype, AMAS, and AAAS) was constructed by the neighbor-joining in PHYLIP based on a CLUSTALW version 1.81 alignment with default parameters. The scale bar represents an amount of evolutionary change corresponding to an expected 0.05 changes per site.

the *Alteromonas* strains were more closely related to those of *Neisseria* than to those of *Deinococcus* (Fig. 4). These results indicate that the newly-discovered ASases of *Alteromonas* strains must be characterized to define their activities and function.

*Expression and characterization of AMAS*

Both ASase genes were expressed in *E. coli* using the pGEX expression system, in which the recombinant protein is fused to glutathione-S-transferase (GST). The vectors for *aaas* and *amas* expression were designated pGEX-AAAS and pGEX-AMAS respectively. The recombinant proteins were purified with Glutathione-Sepharose™ high performance affinity columns and the GST-tags were removed, as shown in Fig. 5. The apparent molecular mass of the recombinant proteins was calculated to be about 70 kDa by SDS–PAGE, close to the estimated molecular masses of AAAS and AMAS (73,735 Da and 73,863 Da respectively).

Enzymatic activity was determined by incubating a sucrose solution with a crude cell extract prepared from recombinant *E. coli* strains harboring pGEX-AAAS or pGEX-AMAS. Cleavage of sucrose to both glucose and fructose was detected with the AMAS crude cell extract but not with that of AAAS. These results did not change even when purified AAAS was used in lieu of the crude cell extract. It will be very interesting to investigate how sequence differences in AAAS render it non-functional as compared to NPAS, DGAS, and DRAS. However, in this study, we focused on detailed enzymatic characterization of AMAS.

The optimum temperature for hydrolysis and transglycosylation by recombinant AMAS was found to be between 25 and 55 °C (Fig. 6). Hydrolysis and transglycosylation activities were highest at 45 °C (432 U/mg of protein and 304 U/mg of protein respectively), and decreased drastically as the temperature increased to 55 °C. As the temperature decreased, from 45 °C to 25 °C, hydrolysis also decreased, from 432 to 138 U/mg of protein, whereas transglycosylation was not significantly affected (254–304 U/mg of protein). The ratio of transglycosylation/hydrolysis (T/H) increased as the reaction temperature decreased, and was highest (1.86) at 25 °C. In contrast, hydrolysis correlated directly with temperature, and as a result, the lowest ratio of T/H (0.79) was observed at 55 °C. These data imply that there are two different catalytic activities of AMAS that can be modulated by regulating the reaction temperature.

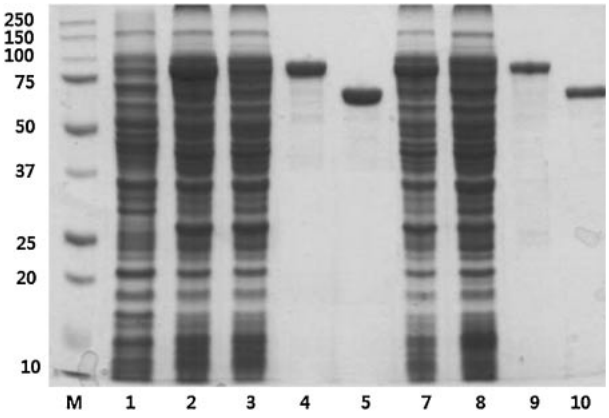


**Fig. 5.** SDS–PAGE Analysis of Recombinant AMAS and AAAS Expressed in *E. coli* BL21.

M, standard size markers; lane 1, cell extract of *E. coli* BL21 (control); lanes 2 and 7, cell extract of *E. coli* BL21 harboring pGEX-AMAS and pGEX-AAAS, respectively; lanes 3 and 8, cell extract of *E. coli* BL21 harboring pGEX-AMAS and pGEX-AAAS respectively, after adsorption to a Glutathione-Sepharose™ high performance affinity column; lanes 4 and 9, purified recombinant AMAS and AAAS tagged with GST respectively; lanes 5 and 10, purified recombinant AMAS and AAAS after GST-tags were removed by thrombin treatment.

The optimum pH of recombinant AMAS for hydrolysis and transglycosylation was determined using various buffers, including sodium citrate (pH 5.0, 6.0, and 7.0), Tris–HCl (pH 7.0, 8.0, and 9.0), and glycine-NaOH (pH 9.0 and 10.0) (Fig. 6). Hydrolysis and transglycosylation were highest at pH 7.0 (481 U/mg protein) and pH 8.0 (399 U/mg protein) respectively. As the pH decreased from pH 8.0 to pH 5.0, hydrolysis changed little and ranged from 425–481 U/mg protein; however, transglycosylation declined to below 116 U/mg of protein. In all pH ranges except pH 8.0 and 9.0 (Tris–HCl), the T/H ratio was below 0.25, implying that hydrolysis is dominant over transglycosylation. However, transglycosylation is relatively preferred at pH 8.0 and 9.0 (Tris–HCl) with T/H ratios of 0.89 and 1.27 respectively. The maximum hydrolysis and transglycosylation activities of recombinant AMAS were observed at pH 7.0 (sodium citrate) and pH 8.0 (Tris–HCl) respectively.

*Thermostability and polymerization activity*

The thermal stability of recombinant AMAS was investigated by incubating the enzyme in 50 mM Tris–HCl (pH 8.0) at various temperatures (35, 40, 45,

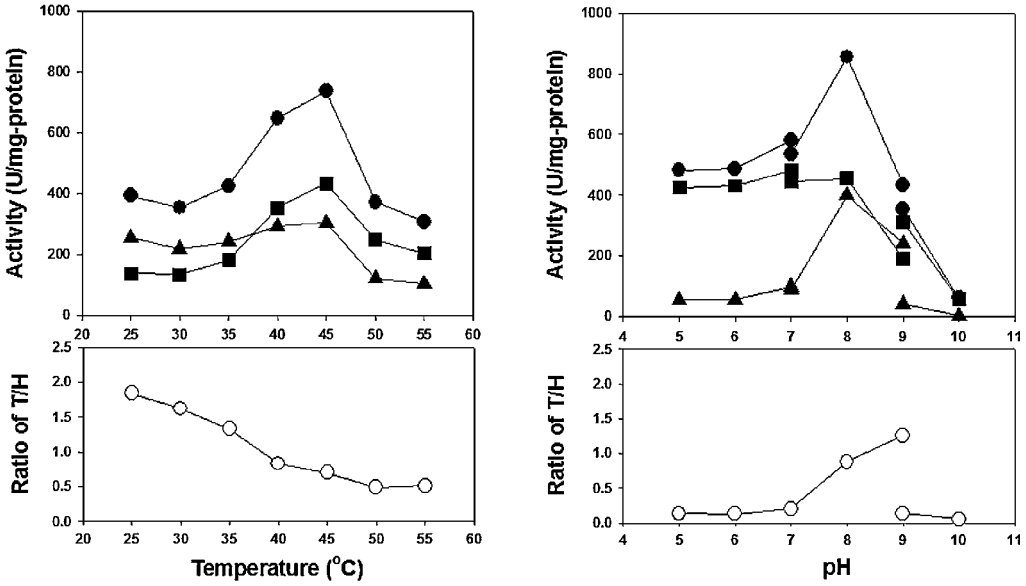


**Fig. 6.** Effects of Temperature and pH on Hydrolysis Activity, Transglycosylation Activity, and the Ratio of Transglycosylation/Hydrolysis (T/H) of Recombinant AMAS.

The reactions were performed at various temperatures and pH levels under standard assay conditions. Total activity (●), hydrolysis activity (■), transglycosylation activity (▲), the ratio of hydrolysis/total activity (□), and the ratio of transglycosylation/total activity (△) are described in the figure. Sodium-citrate (pH 5.0, 6.0, and 7.0), Tris–HCl (pH 7.0, 8.0, and 9.0), and glycine-NaOH (pH 9.0 and 10.0) buffers were used in the experiment.

and 50 °C). As shown in Fig. 7, hydrolysis and transglycosylation were relatively stable below 40 °C, but decreased significantly above 45 °C. The half-life of the transglycosylation activity was 20.2 min and 9.1 min at 45 °C and 50 °C respectively. However, the half-life of the hydrolysis activity was 33.3 min and 14.3 min at 45 °C and 50 °C respectively, implying that transglycosylation is more sensitive to temperature than hydrolysis.

Glucan polysaccharide synthesis was analyzed by HPAEC to determine the dependence of the transglycosylation activity of AMAS on temperature. As shown in Fig. 8, recombinant AMAS exhibited strong glucan polysaccharide synthesis activity at 35 °C as compared to 30 °C and 40 °C. HPAEC analysis revealed that the reaction mixture included various maltooligosaccharides, from maltotriose (G3) to glucan polymers longer than G35 at 35 °C. More glucan polymers were formed at 30 °C than at 40 °C. The yields of soluble maltooligosaccharides and insoluble glucan were 16.9% and 40.7% (w/w%) respectively. But they were much lower at 30 °C and 40 °C.

## Discussion

Until now, only three ASases from two microbial genera (*Neisseria* and *Deinococcus*) have been reported, although the presence of ASase homologs is expected based on whole microbial genome sequence projects.[1,3,9] The function of the ASase secreted from *N. polysaccharea* may be to produce insoluble amylose-like polymers, whereas the function of the ASases of *Deinococcus* is not understood.[18,19] Recently, the whole genome of *A. macleodii* Deep ecotype was sequenced, revealing a putative ASase homolog.[15] The putative protein has 49% identity (307/622) with NPAS, 40% identity (255/627) with DGAS, and 40% identity (253/627) with DRAS. Our investigation of the 12 different *Alteromonas* and related *Pseudoalteromonas* strains

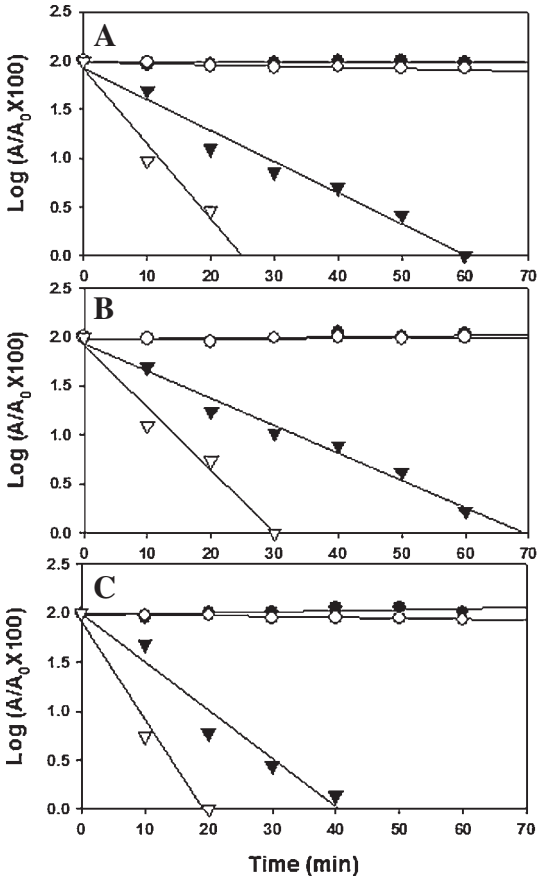


**Fig. 7.** Thermostability of the Total (A), Hydrolysis (B), and Transglycosylation (C) Activity of Recombinant AMAS.

The enzyme in 50 mM Tris–HCl buffer (pH 8.0) was incubated at 35 °C (●), 40 °C (○), 45 °C (▼), and 50 °C (▽).

available in our laboratory demonstrated that two *Alteromonas* strains (*A. addita* and *A. macleodii*) had genes highly homologous to the putative ASase gene of the *A. macleodii* Deep ecotype [76% (497/648) se-

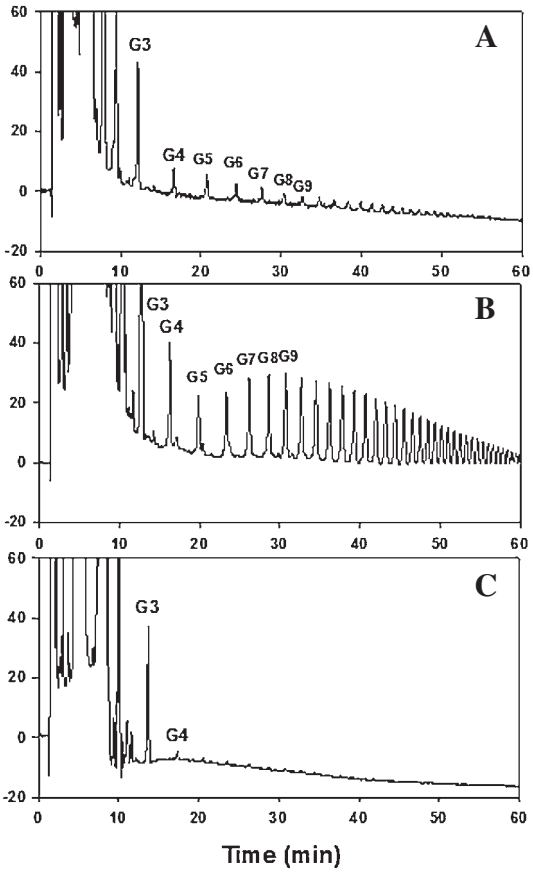


**Fig. 8.** HPAEC Analysis of Maltooligosaccharides Synthesized by AMAS.

Reactions were performed for 45 h at 30 °C (A), 35 °C (B), and 40 °C (C) by recombinant AMAS. Samples were diluted 10-fold and filtered before injection.

quence identity to *A. addita* KCTC 12195 and 87% (563/646) sequence identity to *A. macleodii* KCTC 2957]. Expression of the putative ASase of *A. macleodii* KCTC 2957 indicated that this gene encodes an ASase; the recombinant gene product had all the activities (hydrolysis, isomerization, and polymerization) typical of ASases. Curiously, however, the gene product obtained from *E. coli* expressing a putative ASase of *A. addita* KCTC 12195 did not display ASase activity, although it was successfully purified from the cells. All amino acids known to be important are well conserved in the *aaas* gene. However, there is still a possibility of a point mutation located in an amino acid, not proved but critical to the enzymatic activity in the *aaas* gene product. In-depth studies are needed to determine the relationship between critical amino acid residues and the enzymatic activities of AAAS to determine why AAAS is non-functional.

There are few publications describing the exopolysaccharides produced by *Alteromonas* species.[20–22] A subspecies of *Alteromonas macleodii* is reported to secrete an extremely viscous polysaccharide in culture,[11,23] with rheological and chemical properties similar to xanthan, a commercial polysaccharide. The backbone of xanthan consists of two $\beta$-D-glucose units linked through the 1 and 4 positions, and the side chain is composed of two mannoses and one glucuronic acid.[12,14] Xanthan biosynthetic genes are clustered in the genomes of *Xanthomonas campestris* pv. *campestris* strains. We analyzed the locations of ASases of *Alteromonas* species within the genome to identify possible involvement in the synthesis of exopolysaccharides. In the *A. macleodii* Deep ecotype, the ASase gene is not located in any exopolysaccharide biosynthetic gene cluster, based on the fact that there are no putative homologs to the genes involved in exopolysaccharide biosynthesis near the ASase gene (Fig. 9). This is also true of *A. addita* KCTC 12195 and *A. macleodii* KCTC 2957. The deduced amino acids of the genes near *aaas* and *amas* did not align significantly with any exopolysaccharide-related proteins, indicating that the ASases of *Alteromonas* species are not involved in the biosynthesis of exopolysaccharides.

The hydrolysis and transglycosylation activities of AMAS were substantially dependent on temperature. The highest activities for both hydrolysis and transglycosylation were observed at 45 °C. However, as reaction temperature increased from 25 to 55 °C, hydrolysis was favored over transglycosylation. The ratio of T/H was 1.86 at 25 °C, whereas it was 0.79 at 55 °C. Temperature dependence of hydrolysis and transglycosylation reactions has been reported for other enzymes. A trans-sialidase expressed by *Trypanosoma cruzi*, the causative agent of Chagas disease, exhibits maximal transglycosylation at 13 °C, while the hydrolysis rate increases for temperatures up to 35 °C.[24] We noted that as the rate of hydrolysis decreased, the rate of transglycosylation increased. This result was explained by differences in the optimal temperatures of hydrolysis and transglycosylation resulting from the differential binding of the acceptor to the enzyme. With increased reaction temperatures the affinity for the acceptor decreases, resulting in a simultaneous increase in the rate of transfer to water, which in turn is suppressed by increasing the acceptor concentration. In a thermostable $\beta$-glycosidase of *Thermus thermophilus*, however, both hydrolysis and transglycosylation increase as temperature is elevated.[25] A cyclodextrin glucanotransferase from *Bacillus macerans* synthesized cyclic $\alpha$-1,4-glucan products (transglycosylation) more rapidly at 60 °C compared to 40 °C, whereas the opposite was found for the coupling reaction, resulting in hydrolysis mainly of the larger cyclic $\alpha$-1,4-glucans.[13] These results imply that the effect of temperature on hydrolysis and transglycosylation cannot be generalized, and that it is an intrinsic characteristic of each enzyme.

HPAEC analysis revealed that significantly more transglycosylation products were produced at 35 °C than at 30 and 40 °C, although maximum transglycosylation activity was observed at 45 °C and the highest T/H at 25 °C. At 45 °C, no noticeable transglycosylation products were detected, most likely due to its low thermostability. In fact, AMAS was extremely unstable at 45 °C, with a half-life ($t_{1/2}$) of 30 min. At 40 °C, some G3 was produced, but there was markedly little of the longer maltooligosacchrides; this may be because the reaction proceeded for 48 h and the enzyme activity might have been limited as a result of relatively low thermostability ($t_{1/2}$ at 40 °C = 11.8 h). In contrast, considerably more maltooligosaccharides were detected at 30 °C than at 40 °C, consistently with the observation that AMAS transglycosylation was more active at 30 °C than at 40 °C. These data indicate that there are two

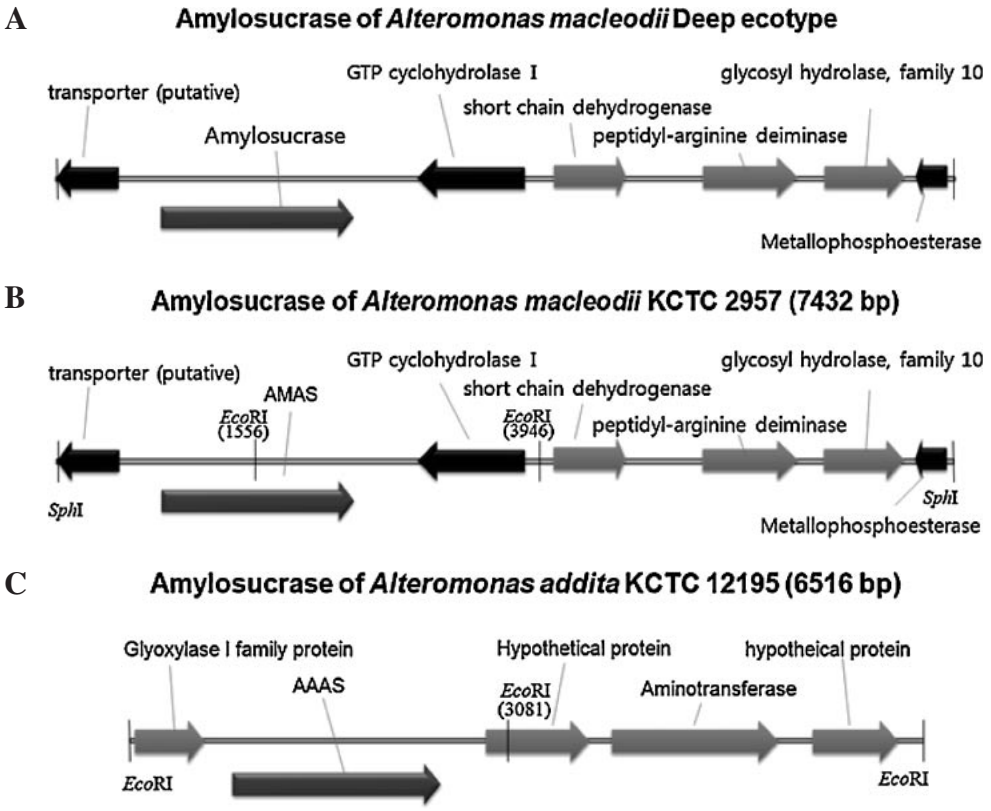


**Fig. 9.** Schematic Gene Organization of the Genomic Regions Containing the ASases.
A, Gene organization of *Alteromonas macleodii* (Deep ecotype); B, gene organization of *Alteromonas macleodii* KCTC 2957; and C, gene organization of *Alteromonas addita* KCTC 12195. Arrows indicate transcriptional orientation. Potential functions based on homologies are indicated.

catalytic activities of AMAS that can be modulated by adjusting the reaction temperature.

## Acknowledgment

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund) (KRF-2008-313-F00145).

## References

1) Skov LK, Mirza O, Henriksen A, De Montalk GP, Remaud-Simeon M, Sarcabal P, Willemot RM, Monsan P, and Gajhede M, *J. Biol. Chem.*, **276**, 25273–25278 (2001).
2) Buttcher V, Welsh T, Willmitzer L, and Kossmann J, *J. Bacteriol.*, **179**, 3324–3330 (1997).
3) Potocki de Montalk G, Remaud-Simeon M, Willemot RM, Sarcabal P, Planchot V, and Monsan P, *FEBS Lett.*, **471**, 219–223 (2000).
4) Okada G and Hehre EJ, *J. Biol. Chem.*, **249**, 126–135 (1974).
5) van der Veen BA, Potocki-Veronese G, Albenne C, Joucla G, Monsan P, and Remaud-Simeon M, *FEBS Lett.*, **560**, 91–97 (2004).
6) Tao BY, Reilly PJ, and Robyt JF, *Carbohydr. Res.*, **181**, 163–174 (1988).
7) De Montalk GP, Remaud-Simeon M, Willemot RM, Planchot V, and Monsan P, *J. Bacteriol.*, **181**, 375–381 (1999).
8) Potocki de Montalk G, Remaud-Simeon M, Willemot RM, and Monsan P, *FEMS Microbiol. Lett.*, **186**, 103–108 (2000).
9) Pizzut-Serin S, Potocki-Veronese G, van der Veen BA, Albenne C, Monsan P, and Remaud-Simeon M, *FEBS Lett.*, **579**, 1405–1410 (2005).
10) Seo DH, Jung JH, Ha SJ, Yoo SH, Kim TJ, Cha J, and Park CS, "Carbohydrate-active Enzyme Structure, Function and Application," ed. Park KH, CRC press, Boca Raton, pp. 125–140 (2008).
11) Raguenes G, Cambon-Bonavita MA, Lohier JF, Boisset C, and Guezennec J, *Curr. Microbiol.*, **46**, 448–452 (2003).
12) Garcia-Ochoa F, Santos VE, Casas JA, and Gomez E, *Biotechnol. Adv.*, **18**, 549–579 (2000).
13) Qi Q, She X, Endo T, and Zimmermann W, *Tetrahedron*, **60**, 799–806 (2004).
14) Becker A, Katzen F, Puhler A, and Ielpi L, *Appl. Microbiol. Biotechnol.*, **50**, 145–152 (1998).
15) Ivars-Martinez E, Martin-Cuadrado AB, D'Auria G, Mira A, Ferriera S, Johnson J, Friedman R, and Rodriguez-Valera F, *ISME J.*, **2**, 1194–1212 (2008).
16) Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, and Higgins DG, *Nucleic Acids Res.*, **25**, 4876–4882 (1997).
17) Efron B, Halloran E, and Holmes S, *Proc. Natl. Acad. Sci. USA*, **93**, 13429–13434 (1996).
18) Albenne C, Skov LK, Mirza O, Gajhede M, Feller G, D'Amico S, Andre G, Potocki-Veronese G, van der Veen BA, Monsan P, and Remaud-Simeon M, *J. Biol. Chem.*, **279**, 726–734 (2004).
19) MacKenzie CR, Johnson KG, and McDonald IJ, *J. Microbiol.*, **23**, 1303–1307 (1977).
20) Guezennec J, *J. Ind. Microbiol. Biotechnol.*, **29**, 204–208 (2002).
21) Bozzi L, Milas M, and Rinaudo M, *Int. J. Biol. Macromol.*, **18**, 9–17 (1996).
22) Colliec Jouault S, Chevolot L, Helley D, Ratiskol J, Bros A, Sinquin C, Roger O, and Fischer AM, *Biochim. Biophys. Acta*, **1528**, 141–151 (2001).
23) Raguenes G, Pignet P, Gauthier G, Peres A, Christen R, Rougeaux H, Barbier G, and Guezennec J, *Appl. Environ. Microbiol.*, **62**, 67–73 (1996).
24) Ribeirao M, Pereira-Chioccola VL, Eichinger D, Rodrigues MM, and Schenkman S, *Glycobiology*, **7**, 1237–1246 (1997).
25) Fourage L, Dion M, and Colas B, *Glycoconj. J.*, **17**, 377–383 (2000).