Biotechnology and Bioprocess Engineering 19: 866-878 (2014)
DOI 10.1007/s12257-014-0622-3

RESEARCH PAPER

Metabolic Pathway of 3,6-anhydro-L-galactose in Agar-degrading Microorganisms

Sun Bok Lee, Sun Ja Cho, Jeong Ah Kim, Shin Youp Lee, Suk Min Kim, and Hyun Seung Lim

Received: 16 July 2014 / Revised: 30 July 2014 / Accepted: 31 July 2014

Abstract Recently, agarose-containing macroalgae have gained attention as possible renewable sources for bioethanol production because of their high polysaccharide content. Complete hydrolysis of agarose produces two monomers, D-galactose (D-Gal) and 3,6-anhydro-L-galactose (L-AnG). However, at present, bioethanol yield from agarophyte macroalgae is low due to the inability of bioethanol-producing microorganisms to convert non-fermentable sugars, such as L-AnG, to bioethanol. Therefore, to increase the bioethanol productivity of agarophytes, it is necessary to determine how agar-degrading microorganisms metabolize L-AnG, and accordingly, construct recombinant microorganisms that can utilize both D-Gal and L-AnG. Previously, we isolated a novel microorganism belonging to a new genus, *Postechiella marina* M091, which hydrolyzes and metabolizes agar as the carbon and energy source. Here, we report a comparative genomic analysis of *P. marina* M091, *Pseudoalteromonas atlantica* T6c, and *Streptomyces coelicolor* A3(2), of the classes Flavobacteria, Gammaproteobacteria, and Actinobacteria, respectively. In this bioinformatic analysis of these agarolytic bacteria, we found candidate common genes that were believed to be involved in L-AnG metabolism. We then experimentally confirmed the enzymatic function of each gene product in the L-AnG cluster. The formation of two key intermediates, 2-keto-3-deoxy-L-galactonate and 2-keto-3-deoxy-D-gluconate, was also verified using enzymes that utilize these molecules as substrates. Combining bioinformatic analysis and experimental data, we showed that L-AnG is metabolized to pyruvate and D-glyceraldehyde-3-phosphate *via* six enzyme-catalyzed reactions in the following reaction sequence: 3,6-anhydro-L-galactose → 3,6-anhydro-L-galactonate → 2-keto-3-deoxy-L-galactonate → 2,5-diketo-3-deoxy-L-galactonate → 2-keto-3-deoxy-D-gluconate → 2-keto-3-deoxy-6-phospho-D-gluconate → pyruvate + D-glyceraldehyde-3-phosphate. To our knowledge, this is the first report on the metabolic pathway of L-AnG degradation.

Keywords: 3,6-anhydro-L-galactose, novel metabolic pathway, agarophyte macroalgae, *Postechiella marina*, *Pseudoalteromonas atlantica*, *Streptomyces coelicolor*

1. Introduction

Recently, there has been increasing interest in next-generation biofuels made from marine macroalgae (also called seaweeds) such as *Gelidium*, *Ulva*, and *Laminaria* [1-7]. The advantages of using marine macroalgae over terrestrial biomass include higher production yields per unit area, higher carbon dioxide fixation rates, and easier depolymerization and pretreatment processes due to the lack of hemicellulose and lignin in algae [8]. Marine macroalgae, which are classified as red, green, and brown macroalgae based on their characteristic phytopigment, are known to contain various sugars that can be used as substrates for fermentation [9]. Compared to green and brown macroalgae, red macroalgae are considered to be more attractive as an alternative biofuel feedstock due to their high polysaccharide content with fewer sugar types.

The main components that make up red macroalgae are

Sun Bok Lee, Sun Ja Cho, Jeong Ah Kim, Shin Youp Lee, Suk Min Kim, Hyun Seung Lim
Department of Chemical Engineering, Pohang University of Science and Technology, Pohang 790-784, Korea

Sun Bok Lee*
Graduate School of Engineering Mastership, Pohang University of Science and Technology, Pohang 790-784, Korea
Tel: +82-54-279-2268; Fax: +82-54-279-2699
E-mail: sblee@postech.ac.kr

galactans (*e.g.*, agaroses, porphyrans, and carrageenans), which are comprised of D-galactose (D-Gal) and modified D-Gal or L-Gal units [10]. Agarose, which is an important polysaccharide found in agarophyte macroalgae, is composed of repeating units of a disaccharide, made up of D-Gal and 3,6-anhydro-L-galactose (L-AnG), linked by α-1,3- and β-1,4-glycosidic bonds. Enzymatic depolymerization of agarose by β-agarase cleaves the β-1,4-glycosidic bonds and forms a disaccharide called α-neoagarobiose (α-L-AnG-β-D-Gal) [11-13]. Subsequent hydrolysis of α-neoagarobiose by α-neoagarobiose hydrolase cleaves the α-1,3-glycosidic bonds to yield two monomers, D-Gal and L-AnG [13,14]. The D-Gal and L-AnG content in agar is 51 ~ 54% and 38 ~ 44%, respectively [15,16]. D-Gal is a fermentable sugar that can be used by yeasts; however, L-AnG is a non-fermentable sugar that cannot be used as a carbon source by bioethanol-producing microorganisms.

Agarose-containing red macroalgae such as *Gelidium*, *Gracilaria*, and *Gracilariopsis* have been explored as potential sources for bioethanol production due to the high galactan content in their polysaccharides [4,17,18]. However, the bioethanol-producing microorganisms currently used can only utilize fermentable sugars, such as D-Gal and D-glucose. Due to their inability to convert non-fermentable sugars, such as L-AnG, to bioethanol, the yield from agarophyte macroalgae is low. If we can identify the genes that encode the enzymes involved in L-AnG catabolism, it might be possible to construct recombinant microorganisms that utilize both D-Gal and L-AnG. Therefore, to increase bioethanol productivity, we need to determine how agar-degrading microorganisms metabolize L-AnG.

Although many microorganisms are capable of utilizing agarose as a carbon source and numerous agarases have been identified in microorganisms of various genera, the L-AnG metabolic pathways in these microorganisms have not yet been elucidated. Since little is known about L-AnG metabolism, we initiated a study to determine the genes encoding the enzymes that are involved in the utilization of L-AnG. To this end, microorganisms that hydrolyze and metabolize agar as a carbon and energy source were screened from seawater. Recently, we reported the characterization of a novel microorganism belonging to a new genus, *Postechiella marina* M091, which was isolated from a seawater sample obtained at Damupo beach in Pohang, Korea [19]. We also carried out a genomic analysis of this microorganism to search for genes involved in L-AnG metabolism (unpublished results).

The phylogenetic analysis of the 16S rRNA of *P. marina* M091 showed that it belongs to the class Flavobacteria in the phylum Bacteroidetes. For the comparative genomic analysis of *P. marina* M091, we selected two agar-degrading microorganisms that are distantly related to *P. marina* M091, *Pseudoalteromonas atlantica* T6c and *Streptomyces coelicolor* A3(2). *P. atlantica* T6c, which is a gram-negative, aerobic marine bacterium that has been widely used as a source of β-agarases [20,21], belongs to the class Gammaproteobacteria in the phylum Proteobacteria. Unlike *P. marina* and *P. atlantica*, which are gram-negative marine bacteria, *S. coelicolor* A3(2), which can degrade and utilize agar as the sole carbon source [22], is a gram-positive soil bacterium of the class Actinobacteria in the phylum Actinobacteria.

In this study, we investigated the metabolic pathway of L-AnG degradation by comparative genomic analysis of *P. marina* M091, *P. atlantica* T6c and *S. coelicolor* A3(2), which represent the classes Flavobacteria, Gammaproteobacteria, and Actinobacteria, respectively. Through systematic genomic comparisons of these bacteria, we found common genes clustered with genes encoding agarolytic enzymes, such as β-agarases and α-neoagarobiose hydrolases. Based on bioinformatic analysis and experimental data, we determined the function of each enzyme as well as the reaction sequences of L-AnG metabolism in these agar-degrading microorganisms. To our knowledge, this is the first report on the metabolic pathway of L-AnG degradation.

2. Materials and Methods

2.1. Microbial strains and culture medium

Postechiella marina M091 (KCTC 23537) was cultivated using previously described methods [19]. *Pseudoalteromonas atlantica* T6c (ATCC BAA-1087) was cultivated at 26°C in marine broth 2216 (Difco, Detroit, MI, USA). *Streptomyces coelicolor* A3(2) (ATCC BAA-471) was cultivated at 28°C in growth medium containing (per L) 5.0 g of tryptone and 3.0 g of yeast extract (pH 7.0). *Aspergillus niger* (CBS 513.88) was cultured in potato dextrose medium at 28°C. *Thermoplasma acidophilum* (JCM 9062) was cultivated as described previously [23]. *E. coli* DH5α was used as a host strain for the construction of recombinant plasmids. *E. coli* BL21 (DE3), *E. coli* BL21 CodonPlus (DE3)-RIL, and *E. coli* Rosetta (DE3) were used as host strains for the expression of recombinant plasmids. *E. coli* DH5α and *E. coli* Rosetta (DE3) strains harboring plasmids were grown at 30°C in LB medium with ampicillin (100 μg/mL), unless otherwise stated. Recombinant *E. coli* BL21 (DE3) cells harboring pHR plasmids were cultivated in LB medium supplemented with ampicillin (100 μg/mL) and chloramphenicol (34 μg/mL). When required, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the medium.

2.2. Molecular cloning and protein expression

Genomic DNA preparation and recombinant DNA techniques were performed according to standard procedures [24]. Target genes were amplified from genomic DNA by PCR. The primers used are listed in Table S1 in the supplementary materials. All genomic DNA sequences, except *P. marina* M091, are available in the NCBI database (the DNA sequence of *P. marina* M091 will be provided upon request). The genes were amplified by PCR using *Taq* DNA polymerase (Takara, Otsu, Japan). The amplified fragments were cloned into the corresponding site of the pQE-80L vector (Quiagen, Valencia, CA, USA), unless otherwise stated, and then transformed into *E. coli* DH5α, BL21 (DE3), or Rosetta (DE3). The recombinant cells were grown at 37°C to an optical density of cultures at 600 nm (OD_{600}) of 0.4 ~ 0.6, and then 0.5 mM IPTG was added to induce expression of the recombinant protein. After incubation at 20 ~ 30°C for 10 ~ 20 h, the cells were harvested (optimal induction temperature and incubation period varied depending on the genes cloned). The cells were resuspended in 50 mM sodium phosphate buffer (pH 8) and disrupted by sonication. The unbroken cells and cell debris were removed by centrifugation (50,000 × *g*, 1 h, 4°C). The clarified cell lysate was loaded onto a Ni-NTA agarose (Qiagen) column to purify the His-tagged proteins by chromatography. The proteins were eluted by a stepwise increase in the imidazole concentration (0 ~ 250 mM). Protein concentration was measured using the Bradford assay [25] and bovine serum albumin as the standard. The crude extracts and purified proteins were separated by SDS-PAGE using standard procedures [24].

2.3. Enzymatic production of 3,6-anhydro-L-galactose (L-AnG)

To produce L-AnG, agarose (Promega, Madison, WI, USA) was hydrolyzed by two enzymes, a neoagarobiose-producing exo-type β-agarase (Patl_1971), which produced neoagarobiose from agarose, and a neoagarobiose hydrolase (SCO3481), which produced L-AnG from neoagarobiose (Fig. S1). These two enzymes were chosen after testing various combinations of endo- and exo-type β-agarases and neoagarobiose hydrolases obtained from *P. atlantica* T6c, *S. coelicolor* A3(2) and *P. marina* M091. The genes encoding the two enzymes, Patl_1971 and SCO3481, were amplified from *P. atlantica* and *S. coelicolor* A3(2) genomic DNA, respectively, using the primers shown in Table S1. The Patl_1971 PCR product was digested with the designated restriction enzyme and ligated into pHR, which was constructed by replacing the pBR322 origin in pQE-80L with the p15A origin from pACYC184. The SCO3481 PCR product was digested with the designated restriction enzyme and ligated into pQE-80L. The recombinant cells were cultivated as described above, and recombinant enzymes were purified from the cell lysates by Ni-NTA chromatography.

Agarose was hydrolyzed sequentially: neoagarobiose production from agarose by β-agarase and L-AnG production from neoagarobiose by α-neoagarobiose hydrolase. Agarose was melted by heating to 95°C and was used as a substrate in a liquid state. For the enzymatic hydrolysis, 1% agarose (w/v) was hydrolyzed by 700 mg of β-agarase (Patl_1971) in 100 mL of 50 mM MES buffer (pH 6.5) at 37°C for 12 h in a water bath shaking at 150 rpm. Then, the reaction mixture was centrifuged, and the supernatant was hydrolyzed with 1 mg of α-neoagarobiose hydrolase (SCO3481) in a water bath at 30°C for 1 h with shaking at 150 rpm. The enzymes were removed by spin filtration (Viva Spin 30), and the reaction product was concentrated using a rotary vacuum evaporator at 30°C. L-AnG was purified from the concentrated sample using silica gel chromatography as described in [26]. Briefly, the sample was applied to a column (1 × 50 cm) packed with silica gel 60 (70-230 mesh; Merck) and eluted with a solvent composed of chloroform/methanol/water (70:28:2, by volume). Fractions containing L-AnG were collected after TLC analysis of each sample. The TLC plate was developed with *n*-butanol/acetic acid/water (2:1:1, by volume) for 1 h, and dried and visualized using a solution composed of 10% (v/v) sulfuric acid and 0.2% (w/v) naphthoresorcinol (Sigma–Aldrich, St. Louis, USA) in ethanol [27]. The L-AnG-containing fraction was then concentrated and dried to a powder in a rotary vacuum evaporator at 40°C. The L-AnG obtained in this way was almost 100% pure (Fig. S2) when the purity was analyzed by HPLC (9600L, Younglin, Korea) equipped with a refractive index detector (RI750F, Younglin, Korea) using Aminex HPX-87H column (300 mm × 7.8 mm; Bio-Rad) at 35°C (mobile phase, 5 mM H_2SO_4; flow rate, 0.6 mL/min). The purity of L-AnG was determined using commercial 3,6-anhydro-D-galactose (Dextra Laboratories, Berkshire, UK) as a reference.

2.4. Enzyme activity measurements

The enzyme activity of L-AnG dehydrogenase was determined spectrophotometrically. Reaction mixtures (total volume, 0.5 mL) containing 1 mM L-AnG, 0.2 mM $NAD(P)^+$, and appropriate amounts of enzyme in 50 mM MES (pH 6.5) were incubated at 25°C for 10 min. The increase in absorption at 340 nm (A_{340}) due to reduction of $NAD(P)^+$ was monitored in a spectrophotometer (UV-1700, Shimadzu, Kyoto, Japan).

The enzyme activity of 3,6-anhydro-L-galactonate cycloisomerase was determined by the thiobarbituric acid (TBA) assay [28]. The reaction mixture (total volume, 250 μL) containing the preceding enzyme reaction mixture

was incubated with appropriate amounts of enzyme at 25°C in 50 mM sodium phosphate (pH 8.0). After reaction, 25 μL of a 12% TCA solution was added, and then the mixture was centrifuged. To the supernatant (50 μL), 25 mM periodic acid/0.25 M H_2SO_4 (126 μL) was added, and the mixture was incubated at room temperature for 20 min. Then, 2% sodium arsenite/0.5 M HCl (250 μL) was added. Finally, the mixture was incubated with 0.3% (w/v) TBA in a boiling water bath for 10 min. After cooling at room temperature, the absorbance of the pink chromogen was measured at 549 nm.

The enzyme activity of 2-keto-3-deoxy-L-galactonate dehydrogenase was determined spectrophotometrically. Reaction mixtures (total volume, 0.5 mL) containing the products obtained from the preceding enzyme reaction were mixed with 0.2 mM $NAD(P)^+$ and appropriate amounts of enzyme in 50 mM sodium phosphate (pH 8.0), and incubated at 25°C for 20 min. The A_{340} was monitored with a spectrophotometer for increases due to the reduction of $NAD(P)^+$.

The enzyme activity of 2,5-diketo-3-deoxy-L-galactonate reductase (2-keto-3-deoxy-D-gluconate dehydrogenase) was also determined spectrophotometrically. Reaction mixtures (total volume, 0.5 mL) containing the preceding enzyme reaction mixture, 0.2 mM NAD(P)H and appropriate amounts of enzyme in 50 mM sodium phosphate (pH 8.0) were incubated at 25°C for 90 min. The A_{340} was monitored in a spectrophotometer for decreases due to the oxidation of NAD(P)H.

The enzyme activity of 2-keto-3-deoxy-D-gluconate kinase was determined at 25°C by enzyme-coupled assay, measuring the amount of ADP generated by the kinase as previously described [29,30]. The enzyme activity of 2-keto-3-deoxy-6-phspho-D-gluconate (KDPG) aldolase was determined by the aldol condensation reaction of pyruvate and D-glyceraldehyde-3-phosphate [31,32]. The enzyme reaction was carried out at 25°C in 50 mM sodium phosphate (pH 8.0) with pyruvate (5 mM) and D-glyceraldehyde-3-phosphate (10 mM) as substrates. The formation of KDPG was measured using the TBA assay method described above.

2.5. Enzymatic confirmation of 2-keto-3-deoxy-aldonate formation

To confirm the formation of 2-keto-3-deoxy-L-galactonate (L-KDGal) and 2-keto-3-deoxy-D-gluconate (KDG) experimentally, the recombinant aldolase enzymes specific for these compounds were produced in *E. coli*. The two genes, An02g07720 and Ta0619, which respectively encode L-KDGal aldolase and KDG aldolase, were amplified from *A. niger* (CBS 513.88) and *T. acidophilum* (JCM 9062) genomic DNA, respectively, using the primers listed in Table S1 and cloned into *E. coli*. An02g07720 was codon optimized by changing the original codons to those used in the genes encoding glycolytic enzymes in *E. coli* [33]. The PCR product containing codon-optimized An02g07720 was digested with the designated restriction enzyme, ligated into pQE-80L, and then transformed into *E. coli* BL21 (DE3), and the gene product was overexpressed. The Ta0619 PCR product was digested with the designated restriction enzyme, ligated into pRSET B, and then transformed into *E. coli* BL21 CodonPlus (DE3)-RIL, and the gene product was overexpressed as described above. Recombinant enzymes were purified from the cell lysates by Ni-NTA chromatography.

The reaction products obtained in the second and fourth steps were incubated with An02g07720 and Ta0619 aldolase, respectively. The L-KDGal aldolase reaction (total volume, 250 μL) was carried out at 25°C in 50 mM MES buffer (pH 6.5), containing An02g07720 (100 μg), 1 mM $MgCl_2$, and the reaction products obtained after the reactions of Patl_2553 and Patl_2550. The KDG aldolase reaction(total volume, 250 μL) was carried out at 50°C in 50 mM MES buffer (pH 6.5), containing Ta0619 (200 μg), 1 mM $MgCl_2$, and the reaction products obtained after the reactions of Patl_2553, Patl_2550, Patl_2551, and Patl_2552. Low aldolase activities, presumably due to low expression levels of eukaryal and archaeal genes in *E. coli*, resulted in a longer reaction time. The cleavage of aldonate by aldolase was measured using the TBA assay method described above.

The production of L-glyceraldehyde by L-KDGal aldolase (An02g07720) was determined spectrophotometrically. Reaction mixtures (total volume, 0.5 mL), which contained the reaction products of L-KDGal aldolase (50 μL), 0.2 mM NAD^+, and appropriate amounts of enzyme (SCO3486) in 50 mM MES buffer (pH 6.5), were incubated for 20 min at 30°C, and the A_{340} was monitored in a spectrophotometer. The production of D-glyceraldehyde by KDG aldolase (Ta0619) was also determined spectrophotometrically. Reaction mixtures (total volume, 0.5 mL), containing the reaction products of KDG aldolase (50 μL), 0.20 mM $NADP^+$, and appropriate amounts of enzyme (Ta0809) in 50 mM MES buffer (pH 6.5), were incubated for 60 min at 50°C, and the A_{340} was monitored in a spectrophotometer.

2.6. Bioinformatics tools

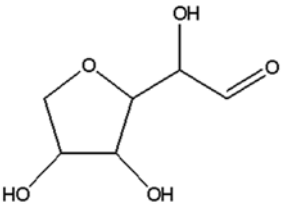
Searches for amino acid sequence homologs and multiple sequence alignments were performed using BLAST and ClustalW, respectively. Conserved domains (CDs) and clusters of orthologous groups of proteins (COGs) were analyzed using the CD-Search tool at the NCBI website. The phylogenetic tree analysis was conducted based on the neighbor-joining method in MEGA 6.0 [34] using the bootstrap test of phylogeny with 1,000 replicates.

3. Results and Discussion

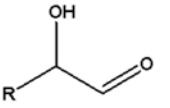
3.1. Identification of L-AnG metabolism gene clusters

As mentioned above, we previously isolated a novel agar-degrading bacterium, *P. marina* M091, from seawater and the whole-genome sequence of this new isolate was determined. One possible approach to search for relevant candidate genes involved in L-AnG metabolism is to identify the first enzyme in the metabolic pathway. Since the first enzyme would use L-AnG as a substrate, we searched various databases, such as KEGG, PubChem, and ChEBI for the information on L-AnG. However, we could only find information about 3,6-anhydro-D-galactose (D-AnG). Structural data in the database (KEGG C06474) showed that, unlike other hexoses, D-AnG has an open-chain aldehyde structure that resembles α-hydroxyaldehydes such as glycolaldehyde, glyceraldehyde, and lactaldehyde (Fig. 1). Since L-AnG is an optical isomer of D-AnG, we expected that L-AnG would have the same open-chain aldehyde structure. In the literature, there were ^{1}H NMR data that supported the expected structure; when agarose (a polysaccharide composed of L-AnG and D-Gal) and κ-carrageenan (a polysaccharide composed of D-AnG and D-galactose-4-sulfate) were completely depolymerized by mercaptolysis, aldehyde derivatives of L-AnG and D-AnG were produced [35]. Thus, we reasoned that the first step in the L-AnG degradation pathway would involve an enzyme that catalyzes aldehyde transformation.

By analyzing the genomic sequence of *P. marina* M091, we were able to find a putative aldehyde dehydrogenase gene (M091_0723; GenBank accession number KJ646024) that was clustered with the gene encoding α-neoagarobiose hydrolase (M091_0719). From our comparative genomic analysis of *P. marina* M091, *P. atlantica* T6c, and *S. coelicolor* A3(2), we found four common genes that are clustered in the genomes and organized in operon-like arrangements (colored genes in Fig. 2). As shown in Fig. 2, the gene arrangement in these three agar-degrading microorganisms is quite different. However, despite their differences in phylogenetic classification and habitats, all three microorganisms contained four core genes. The list of the genes predicted to be involved in the metabolism of L-AnG in *P. marina* M091, *P. atlantica* T6c, and *S. coelicolor* A3(2) are summarized in Table 1. In *P. marina* M091, there are additional genes in the gene cluster, such as aldose epimerase (M091_0721) and sulfatase (M091_0724) that are absent from *P. atlantica* T6c and *S. coelicolor* A3(2). This suggests that *P. marina* M091 may also express the genes required for the utilization of the agaropectin, which contains L-galactose-6-sulfate, when the L-AnG metabolizing genes are induced.



3,6-anhydrogalactose



glycolaldehyde R = H
glyceraldehyde R = CH_2OH
lactaldehyde R = CH_3

Fig. 1. Chemical structure of 3,6-anhydrogalactose, glycolaldehyde, glyceraldehyde, and lactaldehyde.

3.2. Bioinformatic analysis of L-AnG metabolizing enzymes

To predict the putative functions of the *P. marina* M091 gene products, we performed a bioinformatic analysis of L-AnG metabolizing enzymes, which included a BLAST search, multiple sequence alignment, CD search, and phylogenetic tree analysis (see Materials and Methods). Using these bioinformatics tools, we assigned a COG number to each protein and find homologs of the *P. marina* M091 proteins in *E. coli* and *S. typhimurium*, which are the best characterized microorganisms (Table 1).

As shown in the third column of Table 1, the COG numbers of the four core proteins were COG4948 (M091_0722), COG1012 (M091_0723), COG1028 (M091_0725), and COG1063 (M091_0726). Once the COG number is known, it is easier to predict the function of unknown proteins because proteins with the same COG number exhibit similar cellular function. Previously, we studied the non-phosphorylated metabolic pathways (NMPs) in thermoacidophilic archaea, such as *Thermoplasma acidophilum* and *Sulfolobus solfataricus*, and characterized several NMP enzymes in these microorganisms, including D-gluconate dehydratase [36], D-glyceraldehyde dehydrogenase [37], L-rhamnose dehydrogenase [22], 2-keto-3-deoxy-D-gluconate (KDG) kinase [29,38] and 2-keto-3-deoxy-6-phospho-D-gluconate (KDPG) aldolase [32], which belongs to COG4948, COG1012, COG1028, COG0524, and COG0800, respectively. We also studied the L-rhamnose NMP in the thermoacidophilic bacterium *Sulfobacillus thermosulfidooxidans* and found that the enzyme catalyzing the conversion of 2-keto-3-deoxy-L-rhamnonate to 2,5-diketo-3-deoxy-L-

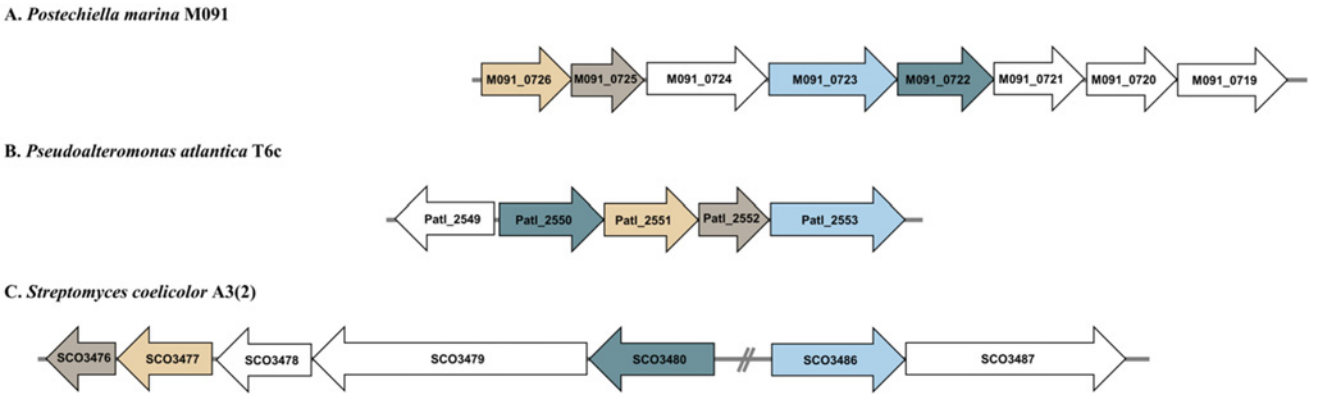


Fig. 2. Schematic diagram of the L-AnG metabolism gene clusters in three agar-degrading microorganisms. (A) *Postechiella marina* M091 (Flavobacteria), (B) *Pseudoalteromonas atlantica* T6c (Gammaproteobacteria), and (C) *Streptomyces coelicolor* A3(2) (Actinobacteria). The four common genes involved in L-AnG metabolism are shown in color (genes with the same functions are denoted by the same color). Annotations are listed in Table 1.

Table 1. List of genes in the L-AnG gene cluster[a]

Protein function	*P. marina* M091 (Flavobacteria)		*P. atlantica* T6c (Gammaproteobacteria)		*S. coelicolor* A3(2) (Actinobacteria)		Closest homolog in *E. coli* or *S. typhimurium* (% identity to *P. atlantica* protein)
	Locus tag	COG number	Locus tag	Annotation	Locus tag	Annotation	
α-neoagarobiose hydrolase	M091_0719	COG2152	Patl_1968	hypothetical protein	SCO3481	hypothetical protein	
transporter	M091_0720	COG0477					
aldose epimerase	M091_0721	COG2017					
3,6-anhydro-L-galactonate cycloisomerase	M091_0722	COG4948	Patl_2550	mandelate racemase/muconate lactonizing enzyme-like	SCO3480	racemase	*S. typhimurium* STM3697 L-talarate dehydratase (29.9%)
3,6-anhydro-L-galactose dehydrogenase	M091_0723	COG1012	Patl_2553	betaine-aldehyde dehydrogenase	SCO3486	aldehyde dehydrogenase	*E. coli* aldA L-lactaldehyde dehydrogenase (42.6%)
sulfatase	M091_0724	COG3119					
2,5-diketo-3-deoxy-L-galactonate 5-reductase	M091_0725	COG1028	Patl_2552	short-chain dehydrogenase/ reductase SDR	SCO3476	short-chain dehydrogenase	*E. coli* kduD 2-keto-3-deoxy-D-gluconate 5-dehydrogenase (51.4%)
2-keto-3-deoxy-L-galactonate 5-dehydrogenase	M091_0726	COG1063	Patl_2551	alcohol dehydrogenase GroES-like	SCO3477	dehydrogenase	*E. coli* yjjN L-galactonate 5-dehydrogenase (25.9%)

[a]Proteins whose function was experimentally confirmed in the present study are shown in bold.

rhamnonate belongs to COG1063 (details will be described elsewhere). Our previous experiences in NMPs allowed us to putatively predict the functions of the L-AnG metabolizing enzymes as aldonate dehydratase (COG4948), aldehyde dehydrogenase (COG1012), 2-keto-3-deoxy-L-aldonate 5-dehydrogenase (COG1063), KDG kinase (COG0524) and KDPG aldolase (COG0800). COG1028 protein was predicted to be a KDG 5-dehydrogenase (KduD), an enzyme which converts 2,5-diketo-3-deoxy-D-gluconate to KDG [39], as described below.

For a more precise prediction of enzyme function, we also searched for homologs of *P. marina* M091 proteins in *E. coli* and *S. typhimurium* (last column in Table 1). A careful examination of *E. coli* and *S. typhimurium* homologs

suggested that M091_0722, M091_0723, and M091_0726 were homologs of L-talarate dehydratase, L-lactaldehyde dehydrogenase, and L-galactonate 5-dehydrogenase, respectively and that M091_0725 is a homolog of KDG 5-dehydrogenase. This indicated that three enzymes (M091_0722, M091_0723, M091_0726) are involved in the transformation of the L-form substrate and one enzyme (M091_0725) is involved in the transformation of the D-form compound.

To test the predictions based on our bioinformatic analysis, we must determine the sequence of the reactions. Since the amino acid sequence homology between Patl_2552 and *E. coli* KduD (KDG 5-dehydrogenase) is very high (51.4% identity; Table 1), it is very likely that the last reaction product is KDG. If a protein has greater than 40% sequence identity to a protein whose biochemical function is known, it is generally regarded that the two proteins have a common biochemical function [40]. As mentioned earlier, the first step in the L-AnG pathway would be catalyzed by an enzyme that uses L-form aldehyde as a substrate. Therefore, we presumed that the first enzyme of the L-AnG pathway would be M091_0725 followed by enzymes that utilize L-form substrate, and the last one being to M091_0725, which convert the L-form compound to KDG. The enzymes in the second- and third-steps were expected to be M091_0722 (COG4948) and M091_0726 (COG1063) because COG1063 is an enzyme whose substrate is 2-keto-3-deoxy-L-aldonate, which can be produced from L-aldonate by aldonate dehydratase (COG4948). Based on this, the order of the enzymes in the pathway was reasoned to be M091_0723 (COG1012), M091_0722 (COG4948), M091_0726 (COG1063), and M091_0725 (COG1028).

The predicted end product of the L-AnG cluster, KDG, prompted us to search for genes that encode KDG metabolizing enzymes in the genomes of agar-degrading microorganisms. It is well known that KDG is converted to pyruvate and D-glyceraldehyde-3-phosphate by two enzymes, KDG kinase (KdgK) and KDPG aldolase (Eda), which are a part of the Entner-Doudoroff (ED) pathway in *E. coli* [41]. KdgK is an enzyme that phosphorylates KDG to KDPG, and Eda is an enzyme that breaks down KDPG into pyruvate and D-glyceraldehyde-3-phosphate *via* an aldol cleavage reaction [42]. A BLAST search of the genomes of agar-degrading microorganisms revealed multiple genes encoding KdgK and Eda in *P. marina* M091, *P. atlantica* T6c, and *S. coelicolor* A3(2). If KdgK and Eda are essential for the utilization of L-AnG, these two genes would be induced when agar-degrading enzymes are induced. Thus, we narrowed down the gene search and examined whether any KdgK and Eda genes were clustered with a β-agarase gene. As shown in Table 2, there was one gene cluster near the β-agarase gene of all three microorganisms. In *S. coelicolor* A3(2), the KDG gene cluster was linked to the L-AnG gene cluster, whereas in *P. marina* M091 and *P. atlantica* T6c, the KDG gene cluster was separated from the L-AnG gene cluster. The genes in the L-AnG cluster and the KDG cluster are summarized in Tables 1 and 2, respectively. The proposed enzyme names, which are derived from a combination of the substrate name and type of reaction, are also shown.

3.3. Functional analysis of L-AnG-metabolizing enzymes

To experimentally confirm the predicted function of L-AnG-metabolizing enzymes, the genes listed in Tables 1 and 2 were expressed in *E. coli*. Due to differences in codon usage among the three agar-degrading microorganisms, the protein expression levels and enzyme activities varied, depending on the genes cloned. When compared, *P. atlantica*

Table 2. List of genes in the KDG gene cluster[a,b]

Protein function	*P. marina* (Flavobacteria)		*P. atlantica* (Gammaproteobacteria)		*S. coelicolor* (Actinobacteria)		Closest homolog in *E. coli* (% identity to *P. atlantica* protein)
	Locus tag	COG number	Locus tag	Annotation	Locus tag	Annotation	
KDG kinase	M091_1651	COG0524	Patl_1969	PfkB	SCO3474	sugar kinase	*E. coli* kdgK KDG kinase (32.7%)
KDPG aldolase	M091_1649	COG0800	Patl_0974	2-dehydro-3-deoxyphosphogluconate aldolase/4-hydroxy-2-oxoglutarate aldolase	SCO3473	aldolase	*E. coli* eda KDPG aldolase (65.7%)
β-agarase	M091_1661	COG2273	Patl_1971	agarase	SCO3471	extracellular agarase precursor	

[a]Proteins whose function was experimentally confirmed in the present study are shown in bold.
[b]KDG: 2-keto-3-deoxy-D-gluconate, KDPG: 2-keto-3-deoxy- 6-phospho-D-gluconate.

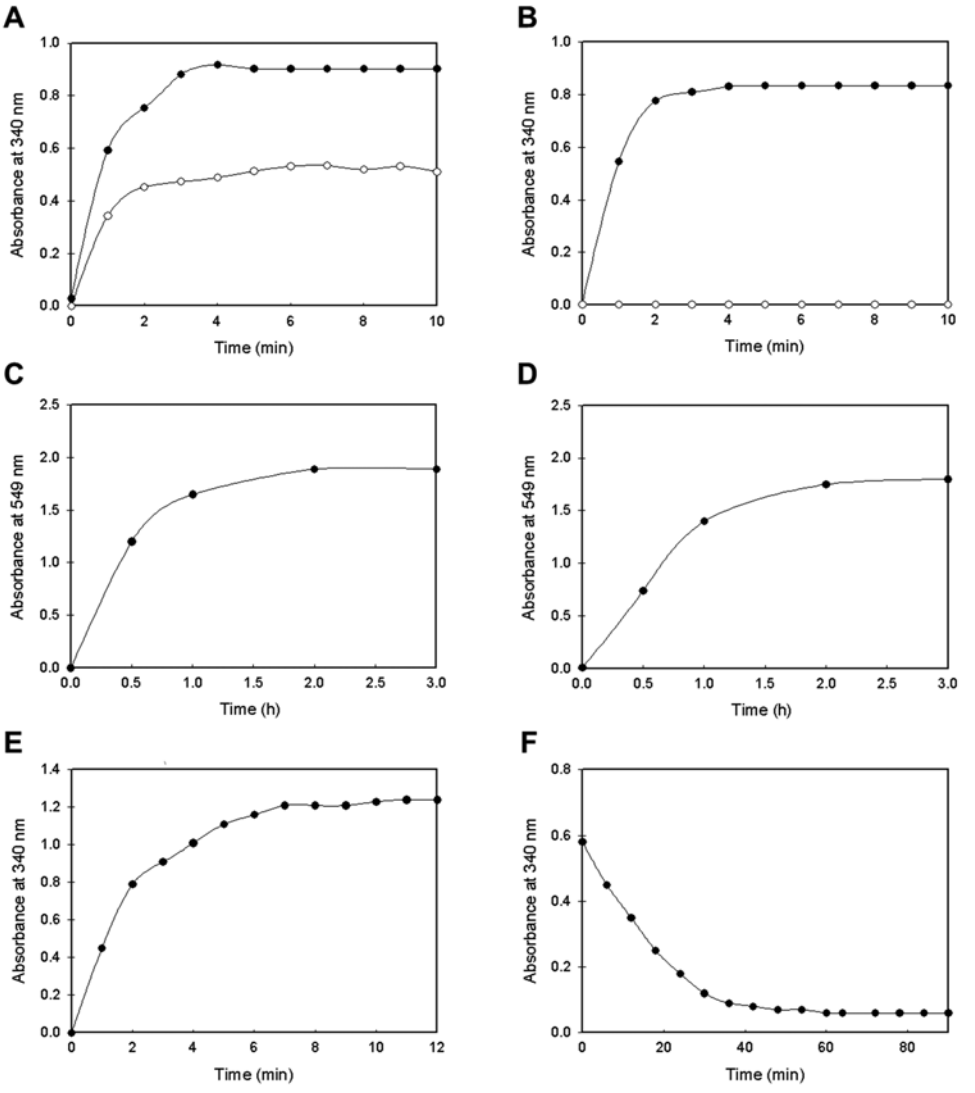


Fig. 3. Experimental verification of the predicted function of L-AnG-metabolizing enzymes. First-step reaction catalyzed by Patl_2553 (A) and M091_0723 (B) in the presence of $NADP^+$ (closed circles) or NAD^+ (open circles). Second-step reaction catalyzed by Patl_2550 (C) and M091_0722 (D). (E) Third-step reaction catalyzed by Patl_2551 in the presence of NAD^+. (F) Fourth-step reaction catalyzed by Patl_2552 in the presence of NADH.

showed the highest expression level, followed by *P. marina* and *S. coelicolor*. This may be because *P. atlantica* and *E. coli* are in the same class of Proteobacteria. However, we had to use *E. coli* Rosetta (DE3) as a host strain even for *P. atlantica* genes due to the presence of rare codons. Because of this, we tested Patl_2553, M091_0723, and SCO3486 for the first-step reaction, Patl_2550 and M091_0722 for the second-step reaction, and Patl_2551 and Patl_2552 for the third- and fourth-step reactions, respectively.

Experimental data for all proteins, except SCO3486, are shown in Fig. 3. The substrate and cofactor specificity of SCO3486 was different from that of Patl_2553 and M091_0723; therefore, we conducted further detailed investigation of SCO3486, and this will be described in a future study. If the predicted enzyme functions and sequence of reactions are correct, then the enzyme activities should be detectable. Fig. 3 shows data that support our bioinformatics-based predictions. In all cases, the enzymes showed activities towards the compound generated in the preceding step.

The first-step of the L-AnG metabolic pathway was predicted to be catalyzed by an aldehyde dehydrogenase possessing substrate specificity towards L-AnG. The data in Figs. 3A and 3B show that both Patl_2553 and M091_0723 are L-AnG dehydrogenases with different cofactor specificities. Patl_2553 utilized both NAD^+ and $NADP^+$, with a preference for $NADP^+$, whereas M091_0723

only used $NADP^+$.

The second-step of the L-AnG metabolic pathway was predicted to produce 2-ketoaldonate, which can be measured by the TBA assay, as is true for aldonate dehydratases such as D-gluconate dehydratase [36,43]. The data in Figs. 3C and 3D show that both Patl_2550 and M091_0722 produced chromogenic compounds from 3,6-anhydro-L-galactonate. Furthermore, as described in the next section, the product was cleaved by 2-keto-3-deoxy-L-galactonate (L-KDGal) aldolase, supporting the formation of L-KDGal in the second-step reaction. Because the enzyme catalyzed the conversion of a cyclic compound (3,6-anhydro-L-galactonate) into an acyclic isomer (L-KDGal), it was named 3,6-anhydro-L-galactonate cycloisomerase, for its functional similarity to D-galactarolactone cycloisomerase, which converts a cyclic compound (D-galactarolactone) into an acyclic isomer (2-keto-3-deoxy-D-galactarate) [44].

The enzyme involved in the third-step of the L-AnG metabolic pathway was predicted to be similar to *E. coli* YjjN (L-galactonate 5-dehydrogenase) with low sequence identity (25.9%; Table 1). Using the product of the second-step reaction, the dehydrogenase activity of Patl_2551 was assessed by measuring the reduction of $NAD(P)^+$. As shown in Fig. 3E, Patl_2551 activity was observed when NAD^+ was used as a cofactor. Since the compound in the second-step reaction was L-KDGal, the expected product of the third-step reaction was 2,5-diketo-3-deoxy-L-galactonate (L-DDGal). The third-step enzyme was named as 2-keto-3-deoxy-L-galactonate 5-dehydrogenase.

The fourth-step reaction was predicted to be a KDG 5-dehydrogenase due to the high sequence identity of Patl_2552 with *E. coli* KduD (51.4%; Table 1). Patl_2552 activity using the third-step reaction mixture was tested by measuring the oxidation of NAD(P)H as the decrease in A_{340}. As shown in Fig. 3F, Patl_2552 activity was observed when NADH was used as a cofactor. The reduction of L-DDGal at the C5 position with NADH converts an L-form compound (L-DDGal) to a molecule that has a D-form configuration (KDG). The fourth-step enzyme was called 2,5-diketo-3-deoxy-L-galactonate 5-reductase, the function of which is identical to that of KDG 5-dehydrogenase (2,5-diketo-3-deoxy-L-galactonate and 2,5-diketo-3-deoxy-D-gluconate are the same molecule).

The fifth- and sixth-step enzymes were predicted to be KDG kinase and KDPG aldolase, respectively. The amino acid sequence identities of Patl_1969 and Patl_0974 with *E. coli* KdgK and Eda were 32.7 and 65.7%, respectively (Table 2). To experimentally confirm the predicted enzyme functions, we measured the enzyme activities of Patl_1969 and Patl_0974 using previously described methods [29,32]. As mentioned earlier, if two proteins share greater than 40% sequence identity, they usually have the same biochemical function [40]. As expected, we were able to show that Patl_1969 and Patl_0974 possess KDG kinase and KDPG aldolase activity, respectively (Fig. S3).

3.4. Enzymatic confirmation 2-keto-3-deoxy-aldonate formation

To experimentally confirm the formation of two key metabolites, L-KDGal and KDG, we employed two aldolase enzymes that are specific to these metabolites. It has been reported that *Hypocrea jecorina* (*Trichoderma reesei*) produces L-KDGal aldolase [45], which converts L-KDGal to pyruvate and L-glyceraldehyde. It is also known that the KDG aldolases from *S. solfataricus* and *T. acidophilum* convert KDG to pyruvate and D-glyceraldehyde [31,32]. In this study, *A. niger* An02g0772, which is a homolog of *H. jecorina lga 1* (GenBank EF203091), and *T. acidiphilum* Ta0619 were used as genes encoding L-KDGal aldolase and KDG aldolase, respectively. The enzymes were tested for the formation of 2-keto-3-deoxy-aldonates using the reaction products obtained after the second- and fourth-step reactions. As shown in Fig. 4A, the addition of L-KDGal aldolase to the reaction products obtained after the reaction with Patl_2553 and Patl_2550 reduced its concentration. Similar responses were observed when the KDG aldolase was added to the reaction products obtained after the reaction with Patl_2553, Patl_2550, Patl_2551, and Patl_2552 (Fig. 4C).

Next, we further examined the products of the aldolase reactions. In samples containing L-KDGal and KDG, the expected products after aldolase cleavage reaction by aldolase are L-glyceraldehyde and D-glyceraldehyde, respectively. Recently, we found that SCO3486, which encodes an L-AnG dehydrogenase, has enzyme activity towards L-glyceraldehyde (35% L-AnG) in the presence of NAD^+. The D-glyceraldehyde dehydrogenase activity of Ta0809 has been studied in our laboratory [37]. Using these two dehydrogenases, the formation of L-glyceraldehyde or D-glyceraldehyde was assessed by measuring A_{340} in a spectrophotometer. The results show that the L-form and D-form of glyceraldehyde were indeed produced by L-KDGal and KDG aldolases, respectively (Figs. 4B and 4D). On the basis of the experimental data in Fig. 4, we concluded that the reaction products of the second- and fourth-step reactions are L-KDGal and KDG, respectively.

3.5. The L-AnG metabolic pathway

By combining our bioinformatic analysis results and experimental data, we were able to construct the pathway of L-AnG metabolism in agar-degrading microorganisms. The overall pathway from L-AnG to pyruvate and D-

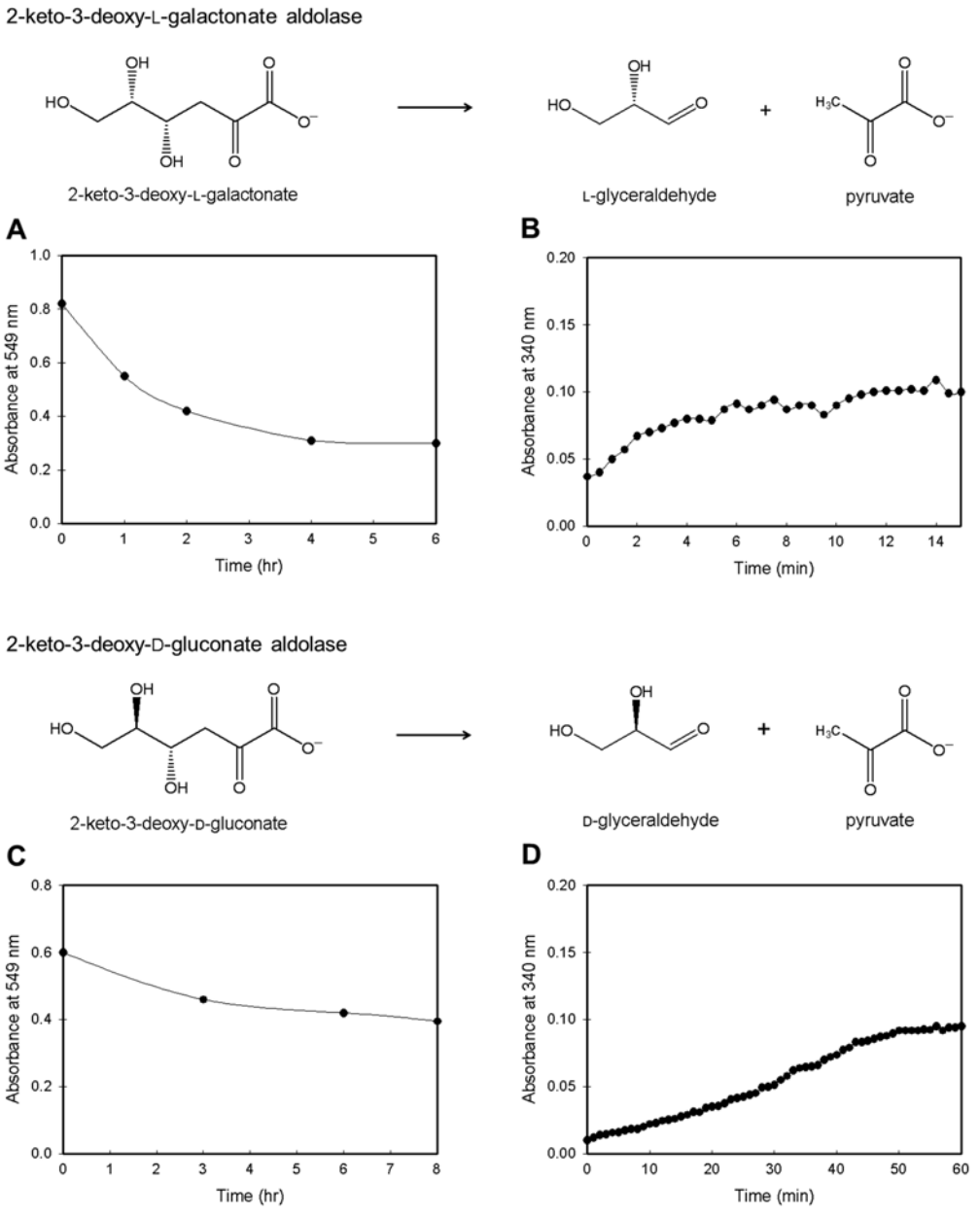
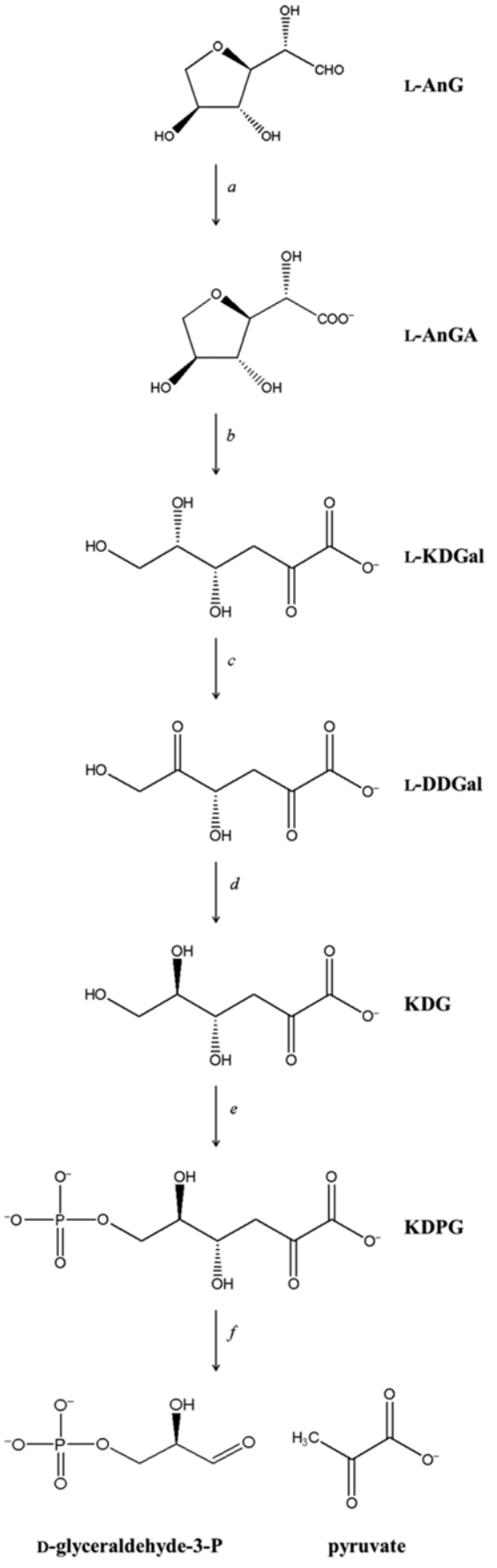


Fig. 4. Enzymatic confirmation of 2-keto-3-deoxy-aldonate formation. Time course profiles of the reaction catalyzed by (A) An02g0772 (L-KDGal aldolase), (B) SCO3486 (L-glyceraldehyde dehydrogenase), (C) Ta0619 (KDG aldolase), and (D) Ta0809 (D-glyceraldehyde dehydrogenase) using the reaction products of the second-step reaction, An02g0772 aldolase reaction, fourth-step reaction, and Ta0619 aldolase reaction, respectively,

glyceraldehyde-3-phosphate is shown in Fig. 5, and the names of each enzyme with the reaction stoichiometry are shown in the figure legend.

The L-AnG pathway in agar-degrading microorganisms can be divided into two parts. The first part of the pathway is the conversion of the L-form sugar (L-AnG) to a D-form sugar acid (KDG) by four successive reactions: (a) oxidation of L-AnG to 3,6-anhydro-L-galactonate, (b) isomerization of 3,6-anhydro-L-galactonate to 2-keto-3-deoxy-L-galactonate, (c) oxidation of 2-keto-3-deoxy-L-galactonate to 2,5-diketo-3-deoxy-L-galactonate (2,5-diketo-3-deoxy-D-gluconate), and (d) reduction of 2,5-diketo-3-deoxy-L-galactonate (2,5-diketo-3-deoxy-D-gluconate) to KDG. The second part of the pathway, which is identical to the downstream reactions of the well-known ED pathway in *E. coli*, is conversion of the D-form sugar acid (KDG) to two glycolysis pathway intermediates, pyruvate and D-glyceraldehyde-3-phosphate, by two successive enzyme reactions: (e) phosphorylation of KDG to KDPG and (f) aldol cleavage of KDPG to pyruvate and D-glyceraldehyde-3-phosphate.



In conclusion, agar-degrading microorganisms transform L-AnG in red macroalgae into glycolysis intermediates *via* six enzyme-catalyzed reactions using the metabolic route illustrated in Fig. 5.

4. Conclusion

Understanding the metabolism of L-AnG in microorganisms is important for the utilization of agar-containing macroalgae as an alternative biomass for bioethanol production. L-AnG metabolism is also of particular interest for studying the carbon cycle in oceans and seaweed-microbe interactions. In this study, we established the metabolic pathway of L-AnG by comparative genomic analysis of three agar-degrading microorganisms *P. marina* M091, *P. atlantica* T6c, and *S. coelicolor* A3(2). From this analysis, we were able to find common genes predicted to encode enzymes involved in L-AnG metabolism. We experimentally confirmed the predicted enzyme function of four core genes in the L-AnG gene cluster, and finally, we showed that the pathway of L-AnG degradation is composed of two parts: transformation of the L-form sugar L-AnG to a D-form sugar acid KDG, an ED pathway intermediate, *via* four enzyme reactions, and breakdown of a C6 compound to two C3 glycolysis intermediates *via* two enzyme reactions. The methods used in this study (bioinformatics-based prediction and experimental verification) may be applicable to the elucidation of other unknown metabolic pathways in microorganisms. In addition, the end products of the L-AnG pathway, pyruvate and D-glyceraldehyde-3-phosphate, can be readily converted to bioethanol in ethanologenic microorganisms such as *E. coli* KO11 and *Saccharomyces cerevisiae*. Since we now know the metabolic pathway of L-AnG, a compound previously regarded as a useless, non-

Fig. 5. Metabolic pathway of L-AnG breakdown in agar-degrading microorganisms. L-AnG, 3,6-anhydro-L-galactose; L-AnGA, 3,6-anhydro-L-galactonate; L-KDGal, 2-keto-3-deoxy-L-galactonate; L-DDGal, 2,5-diketo-3-deoxy-L-galactonate; KDG, 2-keto-3-deoxy-D-gluconate; KDPG, 2-keto-3-deoxy-6-phospho-D-gluconate. The enzyme names and reactions catalyzed are as follows: (*a*) 3,6-anhydro-L-galactose dehydrogenase: 3,6-anhydro-L-galactose + $NAD(P)^+$ + H_2O → 3,6-anhydro-L-galactonate + NAD(P)H + H^+; (*b*) 3,6-anhydro-L-galactonate cycloisomerase: 3,6-anhydro-L-galactonate → 2-keto-3-deoxy-L-galactonate; (*c*) 2-keto-3-deoxy-L-galactonate 5-dehydrogenase: 2-keto-3-deoxy-L-galactonate + $NAD(P)^+$ → 2,5-diketo-3-deoxy-L-galactonate + NAD(P)H + H^+; (*d*) 2,5-diketo-3-deoxy-L-galactonate 5-reductase (2-keto-3-deoxy-D-gluconate 5-dehydrogenase): 2,5-diketo-3-deoxy-L-galactonate + NAD(P)H + H^+ → 2-keto-3-deoxy-D-gluconate; (*e*) 2-keto-3-deoxy-D-gluconate kinase: 2-keto-3-deoxy-D-gluconate + ATP → 2-keto-3-deoxy-6-phospho-D-gluconate + ADP; (*f*) 2-keto-3-deoxy-6-phospho-D-gluconate aldolase: 2-keto-3-deoxy-6-phospho-D-gluconate → pyruvate + D-glyceraldehyde-3-phosphate.

fermentable sugar, we may be able to construct recombinant microorganisms that can produce bioethanol from L-AnG. We are currently exploring this possibility.

Acknowledgements

This work was supported by POSCO. The authors would like to thank Sung Han Lim, Hye Ran Chang, Cheong Byeol Shin, and Kelly Sooyeun Song for their assistance with the experimental work.

References

1. Roesijadi, G., S. B. Jones, L. J. Snowden-Swan, and Y. Zhu (2010) *Macroalgae as A Biomass Feedstock: A Preliminary Analysis*. Pacific Northwest National Laboratory.
2. Meinita, M. D. N., B. Marhaeni, T. Winanto, G. T. Jeong, M. N. A. Khan, and Y. K. Hong (2013) Comparison of agarophytes (*Gelidium*, *Gracilaria*, and *Gracilariopsis*) as potential resources for bioethanol production. *J. Appl. Phycol*. 25: 1957-1961.
3. Trivedi, N., V. Gupta, C. R. Reddy, and B. Jha (2013) Enzymatic hydrolysis and production of bioethanol from common macrophytic green alga *Ulva fasciata* Delile. *Bioresour. Technol.* 150: 106-112.
4. Choi, W. Y., D. -H. Kang, and H. -Y. Lee (2013) Enhancement of the saccharification yields of *Ulva pertusa* Kjellmann and rape stems by the high-pressure steam pretreatment process. *Biotechnol. Bioproc. Eng.* 18: 728-735.
5. Wargacki, A. J., E. Leonard, M. N. Win, D. D. Regitsky, C. N. Santos, P. B. Kim, S. R. Cooper, R. M. Raisner, A. Herman, A. B. Sivitz, A. Lakshmanaswamy, Y. Kashiyama, D. Baker, and Y. Yoshikuni (2012) An engineered microbial platform for direct biofuel production from brown macroalgae. *Science* 335: 308-313.
6. Enquist-Newman, M., A. M. Faust, D. D. Bravo, C. N. Santos, R. M. Raisner, A. Hanel, P. Sarvabhowman, C. R. Le, D. D., S. R. Cooper, L. Peereboom, A. Clark, Y. Martinez, J. Goldsmith, M. Y. Cho, P. D. Donohoue, L. Luo, B. Lamberson, P. Tamrakar, E. J. Kim, J. L. Villari, A. Gill, S. A. Tripathi, P. Karamchedu, C. J. Paredes, V. Rajgarhia, H. K. Kotlar, R. B. Bailey, D. J. Miller, N. L. Ohler, C. Swimmer, and Y. Yoshikuni (2014) Efficient ethanol production from brown macroalgae sugars by a synthetic yeast platform. *Nature* 505: 239-243.
7. Ra, C. H. and S. -K. Kim (2013) Optimization of pretreatment conditions and use of a two-stage fermentation process for the production of ethanol from seaweed, *Saccharina japonica*. *Biotechnol. Bioproc. Eng.* 18: 715-720.
8. Wei, N., J. Quarterman, and Y. -S. Jin (2013) Marine macroalgae: An untapped resource for producing fuels and chemicals. *Trends Biotechnol.* 31: 70-77.
9. Hwang, H. J., S. Y. Lee, S. M. Kim, and S. B. Lee (2011) Fermentation of seaweed sugars by *Lactobacillus* species and the potential of seaweed as a biomass feedstock. *Biotechnol. Bioproc. Eng.* 16: 1231-1239.
10. Cole, K. M. and R. G. Sheath (1990) *Biology of the Red Algae*. Cambridge University Press, NY, USA.
11. Araki, C. (1956) Structure of the agarose constituent of agar-agar. *Bull. Chem. Soc. Japan* 29: 543-544.
12. Hamer, G. K., S. S. Bhattacharjee, and W. Yaphe (1977) Analysis of the enzymic hydrolysis products of agarose by ^{13}C-n.m.r. spectroscopy. *Carbohydr. Res.* 54: C7-C10.
13. Chi, W. J., Y. K. Chang, and S. K. Hong (2012) Agar degradation by microorganisms and agar-degrading enzymes. *Appl. Microbiol. Biotechnol.* 94: 917-930.
14. Day, D. F. and W. Yaphe (1975) Enzymatic hydrolysis of agar: purification and characterization of neoagarobiose hydrolase and *p*-nitrophenyl α-galactoside hydrolase. *Can. J. Microbiol.* 21: 1512-1518.
15. O'Nell, A. N. and D. K. R. Stewart (1956) On the structure of agar from *Gelidium cartilaginium*. *Can. J. Chem*. 34: 1700-1703.
16. Siegel, B. Z. and S. M. Siegel (1973) The chemical composition of algal cell walls. *Crit. Rev. Microbiol.* 3: 1-26.
17. Park, J. H., J. Y. Hong, H. C. Jang, S. G. Oh, S. H. Kim, J. J. Yoon, and Y. J. Kim (2012) Use of *Gelidium amansii* as a promising resource for bioethanol: a practical approach for continuous dilute-acid hydrolysis and fermentation. *Bioresour. Technol.* 108: 83-88.
18. Kumar, S., R. Gupta, G. Kumar, D. Sahoo, and R. C. Kuhad (2013) Bioethanol production from *Gracilaria verrucosa*, a red alga, in a biorefinery approach. *Bioresour. Technol.* 135: 150-156.
19. Lee, D. H., S. J. Cho, S. M. Kim, and S. B. Lee (2012) *Postechiella marina* gen. nov., sp. nov., isolated from seawater. *Int. J. Syst. Evol. Microbiol.* 62: 1528-1535.
20. Yaphe, W. (1957) The use of agarase from *Pseudomonas atlantica* in the identification of agar in marine algae (Rhodophyceae) *Can. J. Microbiol*. 3: 987-993.
21. Morrice, L. M., M. W. McLean, F. B. Williamson, and W. F. Long (1983) β-Agarases I and II from *Pseudomonas atlantica*: Purifications and some properties. *Eur. J. Biochem*. 135: 553-558.
22. Stanier, R. Y. (1942) Agar-decomposing strains of the *Actinomyces coelicolor* species-group. *J. Bacteriol.* 44: 555-570.
23. Kim, S. M., K. H. Paek, and S. B. Lee (2012) Characterization of $NADP^+$-specific L-rhamnose dehydrogenase from the thermoacidophilic Archaeon *Thermoplasma acidophilum*. *Extremophiles* 16: 447-454
24. Sambrook, J. and D. W. Russell (2001) *Molecular Cloning: A Laboratory Manual*. 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.
25. Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254.
26. Sugano, Y., H. Kodama, I. Terada, Y. Yamazaki, and M. Noma (1994) Purification and characterization of a novel enzyme, α-neoagarooligosaccharide hydrolase (α-NAOS hydrolase), from a marine bacterium, *Vibrio* sp. strain JT0107. *J. Bacteriol.* 176: 6812-6818.
27. Duckworth, M. and W. Yaphe (1970) Thin-layer chromatographic analysis of enzymic hydrolysates of agar. *J. Chromatogr.* 49: 482-487.
28. Skoza, L. and S. Mohos (1976) Stable thiobarbituric acid chromophore with dimethyl sulphoxide. Application to sialic acid assay in analytical de-*O*-acetylation. *Biochem. J*. 159: 457-462.
29. Kim, S. and S. B. Lee (2006) Characterization of *Sulfolobus solfataricus* 2-keto-3-deoxy-D-gluconate kinase in the modified Entner-Doudoroff pathway. *Biosci. Biotechnol. Biochem.* 70: 1308-1316.
30. Noh, M., J. H. Jung, and S. B. Lee (2006) Purification and characterization of glycerate kinase from the thermoacidophilic archaeon *Thermoplasma acidophium*: an enzyme belonging to the second glycerate kinase family. *Biotechnol. Bioproc. Eng.* 11: 344-350.
31. Buchanan, C. L., H. Connaris, M. J. Danson, C. D. Reeve, and D. W. Hough (1999) An extremely thermostable aldolase from *Sulfolobus solfataricus* with specificity for non-phosphorylated substrates. *Biochem. J.* 343: 563-570.
32. Lim, S. H. (2008) *Thermostable aldolases from Thermoplasma acidophilum*. Master Thesis, POSTECH, Korea.
33. Karlin, S., J. Mrazek, A. Campbell, and D. Kaiser (2001) Characterizations of highly expressed genes of four fast-growing bacteria. *J. Bacteriol*. 183: 5025-5040.
34. Kumar, S., M. Nei, J. Dudley, and K. Tamura (2008) MEGA: A biologist-centric software for evolutionary analysis of DNA and

protein sequences. *Brief Bioinform.* 9: 299-306.
35. Ducatti, D. R., A. Massi, M. D. Noseda, M. E. Duarte, and A. Dondoni (2009) Production of carbohydrate building blocks from red seaweed polysaccharides. Efficient conversion of galactans into C-glycosyl aldehydes. *Org. Biomol. Chem.* 7: 576-588.
36. Kim, S. and S. B. Lee (2005) Identification and characterization of *Sulfolobus solfataricus* D-gluconate dehydratase: A key enzyme in the non-phosphorylated Entner–Doudoroff pathway. *Biochem. J.* 387: 271-280.
37. Jung, J. H. and S. B. Lee (2006) Identification and characterization of *Thermoplasma acidophilum* glyceraldehyde dehydrogenase: A new class of $NADP^+$-specific aldehyde dehydrogenase. *Biochem. J.* 397: 131-138.
38. Jung, J. H. and S. B. Lee (2005) Identification and characterization of *Thermoplasma acidophilum* 2-keto-deoxy-D-gluconate kinase: a new class of sugar kinases. *Biotechnol. Bioproc. Eng.* 10: 535-539.
39. Condemine, G., N. Hugouvieuxcottepattat, and J. Robert-Baudouy (1984) An enzyme in the pectinolytic pathway of *Erwinia chrysanthemi*: 2-keto-3-deoxygluconate oxidoreductase. *J. Gen. Microbiol*. 130: 2839-2844.
40. Petsko, G. A. and D. Ringe (2004) *Protein Structure and Function*. p. 136. New Science Press, London.
41. Entner, E. and M. Doudoroff (1952) Glucose and gluconic acid oxidation of *Pseudomonas saccharophila*. *J. Biol. Chem*. 196: 853-862.
42. Peekhaus N. and T. Conway (1998) What's for dinner?: Entner-Doudoroff metabolism in *Escherichia coli*. *J. Bacteriol.* 180: 3495-3502.
43. Kim, S. and S. B. Lee (2008) Identification and characterization of the bacterial D-gluconate dehydratase in *Achromobacter xylosoxidans*. *Biotechnol. Bioproc. Eng.* 13: 436-444.
44. Andberg, M., H. Maaheimo, H. Boer, M. Penttilä, A. Koivula, and P. Richard (2012) Characterization of a novel *Agrobacterium tumefaciens* galactarolactone cycloisomerase enzyme for direct conversion of D-galactarolactone to 3-deoxy-2-keto-L-threo-hexarate. *J. Biol. Chem*. 287: 17662-17671.
45. Hilditch, S., S. Berghall, N. Kalkkinen, M. Penttila, and P. Richard (2007) The missing link in the fungal D-galacturonate pathway: Identification of the L-threo-3-deoxy-hexulosonate aldolase. *J. Biol. Chem.* 282: 26195-26201.