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Characterization of a laminaribiose phosphorylase from *Acholeplasma laidlawii* PG-8A and production of 1,3-β-D-glucosyl disaccharides

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ABSTRACT

We identified a glycoside hydrolase family 94 homolog (ACL0729) from *Acholeplasma laidlawii* PG-8A as a laminaribiose (1,3-β-D-glucobiose) phosphorylase (EC 2.4.1.31). The recombinant ACL0729 produced in *Escherichia coli* catalyzed phosphorolysis of laminaribiose with inversion of the anomeric configuration in a typical sequential bi bi mechanism releasing α-D-glucose 1-phosphate and D-glucose. Laminaritriose (1,3-β-D-glucotriose) was not an efficient substrate for ACL0729. The phosphorolysis is reversible, enabling synthesis of 1,3-β-D-glucosyl disaccharides by reverse phosphorolysis with strict regioselectivity from α-D-glucose 1-phosphate as the donor and suitable monosaccharide acceptors (D-glucose, 2-deoxy-D-*arabino*-hexopyranose, D-xylose, D-glucuronic acid, 1,5-anhydro-D-glucitol, and D-mannose) with C-3 and C-4 equatorial hydroxyl groups. The D-glucose and 2-deoxy-D-arabino-hexopyranose caused significantly strong competitive substrate inhibition compared with other glucobiose phosphorylases reported, in which the acceptor competitively inhibited the binding of the donor substrate. By contrast, none of the examined disaccharides served as acceptor in the synthetic reaction.

1. Introduction

Phosphorylases catalyze phosphorolysis of particular glycoside to produce monosaccharide 1-phosphate with strict substrate specificity.[1,2] The phosphorolysis is reversible, enabling the synthesis of various oligosaccharides from monosaccharide 1-phosphate as the donor substrate and several suitable carbohydrate acceptors by the reverse phosphorolysis with strict regioselectivity.[1,2] In addition, the reversibility makes it possible to synthesize oligosaccharides practically from abundantly available natural sugar by using single phosphorylase[2–4] or by combined reaction of two phosphorylases that share the same monosaccharide 1-phosphate.[5–13] The phosphorylases have been classified as members of glycoside hydrolase families (GH) 13, 65, 94, 112, 130 and glycosyl transferase families (GT) 4 and 35 on Carbohydrate-Active Enzymes database (http://www.cazy.org/) based on the sequence similarity.[14]

Laminaribiose phosphorylase (EC 2.4.1.31) has been assigned as the GH94 member that mainly phosphorolyzes β-D-glucosides with inversion of the anomeric configuration at the C-1 to give α-D-glucose 1-phosphate, together with cellobiose phosphorylase (EC 2.4.1.20),[15–20] cellodextrin phosphorylase (EC 2.4.1.49),[16,20] and *N,N'*-diacetylchitobiose phosphorylase (EC 2.4.1.280).[21,22] Laminaribiose phosphorylase was first described in 1963 from *Euglena gracilis*, which possesses a metabolism of paramylon, intracellular storage β-1,3-glucan, within Class Phytomastigophora[23] and it was reported to catalyze reversible phosphorolysis of laminarioligosaccharides (different degrees of polymerization: DP = 2–7).[24,25] Recently, a bacterial laminaribiose phosphorylase was isolated from *Paenibacillus* sp. YM-1, catalyzing reversible phosphorolysis of laminaribiose and weakly phosphorolyzing laminaritriose and higher laminarioligosaccharide.[26] We here describe another bacterial laminaribiose phosphorylase from phytopathogenic *Acholeplasma laidlawii* PG-8A, cell wall-less bacterium within the Class Mollicutes,[27] which is proposed to be involved in a metabolism of extracellular β-1,3-glucan. In addition, we report that six 1,3-β-D-glucosyl disaccharides synthesized from α-D-glucose 1-phosphate as the donor and suitable monosaccharide acceptors by the reverse phosphorolysis with strict β-1,3-regioselectivity were first identified.

2. Results and discussion

2.1. Gene cloning, expression and purification of recombinant ACL0729

The genomic sequence of *A. laidlawii* PG-8A (GenBank ID: CP000 896)[28] has revealed that it possesses three genes encoding possible GH 94 phosphorylases (http://www.cazy.org/GH94_bacteria.html).

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A GH94 homologous protein encoded by *acl0729* (GenBank ID: ABX81345.1) exhibits 38% sequence identity and 55% similarity with laminaribiose phosphorylase from *Paenibacillus* sp.[26] and the phylogenetic tree analysis also categorizes it as laminaribiose phosphorylase (Fig. 1), whereas the other homologous proteins encoded by *acl0039* and *acl1061* have been annotated to be cellobiose phosphorylases.[28] The amino acid sequence of ACL0729 shows no predicted N-terminal signal peptide based on a SignalP 4.0 analysis (http://www.cbs.dtu.dk/services/SignalP/),[29,30] suggesting that it is located in the cytoplasm. In addition, the *acl0729* belongs to a gene cluster in the genome of *A. laidlawii*, which is proposed to be involved in the metabolism of β-1,3-glucan. The gene cluster encodes extracellular and intracellular putative *endo*-1,3-β-glucanases (*acl0717* and *acl0727*, respectively), ATP-binding cassette-type sugar transporter (*acl0718* to *acl0725*), and possible laminaribiose phosphorylase (*acl0729*), suggesting that ACL0729 plays a necessary role to phosphorolyze the intra-cellular laminaribiose or short-chained laminarioligosaccharides. In this study, the *acl0729* cloned from the genomic DNA of *A. laidlawii* PG-8A was expressed as a His_6-tag fusion protein in *Escherichia coli* BL21 (DE3) to investigate its enzymatic properties, resulting that approximately 4 mg of purified protein was obtained from cell lysate of 200 mL culture. The purified ACL0729 migrated in SDS polyacrylamide-gel electrophoresis as a single protein band with an estimated size of 96 kDa in agreement with the theoretical molecular mass of 97,158.

2.2. Basic properties of ACL0729

ACL0729 was stable up to 35 °C during 15 min incubation (Fig. 2A). The optimum temperature for phosphorolytic activity was 40 °C (Fig. 2B). This corresponds to the optimum growth temperature of *A. laidlawii*.[27] ACL0729 was stable at 4 °C for 24 h in the range of pH 5.5–10.5 (Fig. 2C) and showed highest apparent phosphorolytic activity on laminaribiose at pH 7.5, whereas the reverse phosphorolytic reaction using α-D-glucose 1-phosphate as the donor and D-glucose as the acceptor has optimum at pH 6.0 (Fig. 2D). The preference of phosphorolysis at the neutral pH suggests that ACL0729 plays a role to phosphorolyze laminaribiose in the cytosol of *A. laidlawii* as the enzyme was predicted to be located in the cytoplasm by the primary sequence analysis (see Section 2.1).

2.3. Substrate specificity of ACL0729 in phosphorolysis

The phosphorolytic activity of ACL0729 on several β-linked glucobioses such as sophorose (β-1,2), laminaribiose (β-1,3), cellobiose (β-1,4), and gentiobiose (β-1,6) was examined. In the presence of inorganic phosphate, ACL0729 phosphorolyzed only laminaribiose with inversion of the anomeric configuration releasing α-D-glucose 1-phosphate and D-glucose (Scheme 1). The specific activity on laminaribiose was 4.0 U/mg. In addition, ACL0729 did not cleave the laminaribiose in the absence of inorganic phosphate. These results indicate that ACL0729 is a typical laminaribiose phosphorylase, as with those from *E. gracilis* and *Paenibacillus* sp.[25,26] On the other hand, ACL0729 weakly phosphorolyzed a laminaritriose with a specific activity of 0.2 U/mg, which is approximately 5% compared to that of laminaribiose, indicating that ACL0729 shows strong preference for laminaribiose. The chain-length dependence of ACL0729 was similar to that of laminaribiose phosphorylase from *Paenibacillus* sp. (laminaribiose/laminaritriose, 100:3.9)[26] rather than that of the enzyme from *E. gracilis* (laminaribiose/laminaritriose, 100:12).[25,26]

The double reciprocal plots of initial velocities against various initial concentrations of laminaribiose and phosphate gave a series of lines intersecting at a point (Fig. S1). This indicates that the phosphorolytic reaction follows a sequential bi bi mechanism, as reported for other inverting phosphorylases[2] including laminaribiose phosphorylases from *E. gracilis* and *Paenibacillus* sp.[25,26] Kinetic parameters that appear in Eq. 1 for the sequential bi bi mechanism (see Section 3.7) were calculated to be $k_{cat} = 6.5\ s^{-1}$, $K_{mA} = 2.7$ mM, $K_{mB} = 0.4$ mM, and $K_{iA} = 4.4$ mM, where A and B

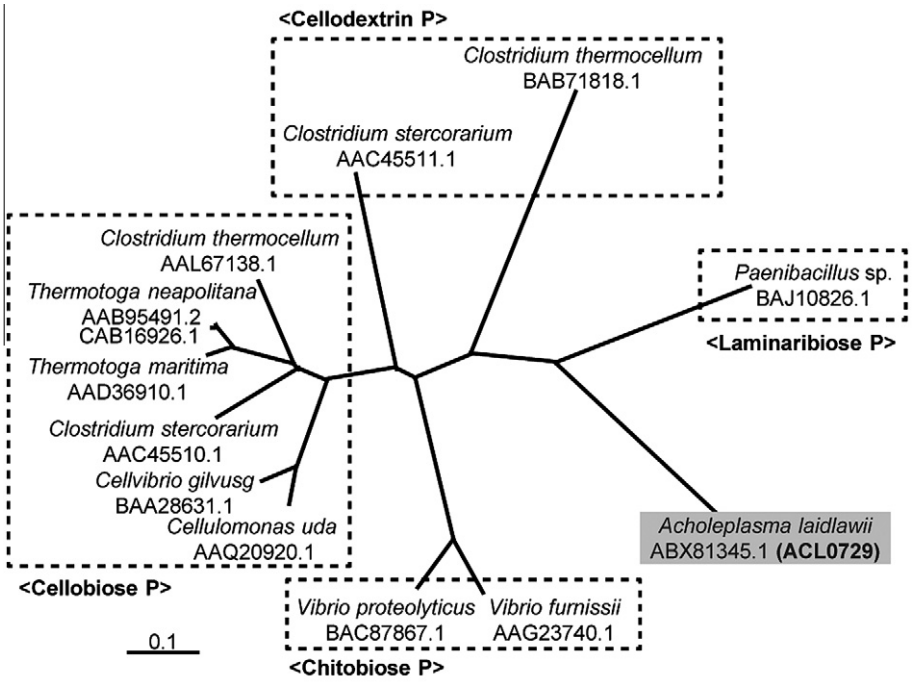


Figure 1. Phylogenetic tree of characterized GH94 enzymes. Multiple alignments were performed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). A phylogenetic tree was constructed using TreeView version 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/rod.html). The gene cloned in this study is represented by the organism name and GenBank™ accession number with a gray background. The other genes encoding characterized GH94 enzymes (http://www.cazy.org/GH94.html), represented by the organism names and GenBank™ accession numbers, are categorized in boxes framed with broken lines according to their substrate specificities.

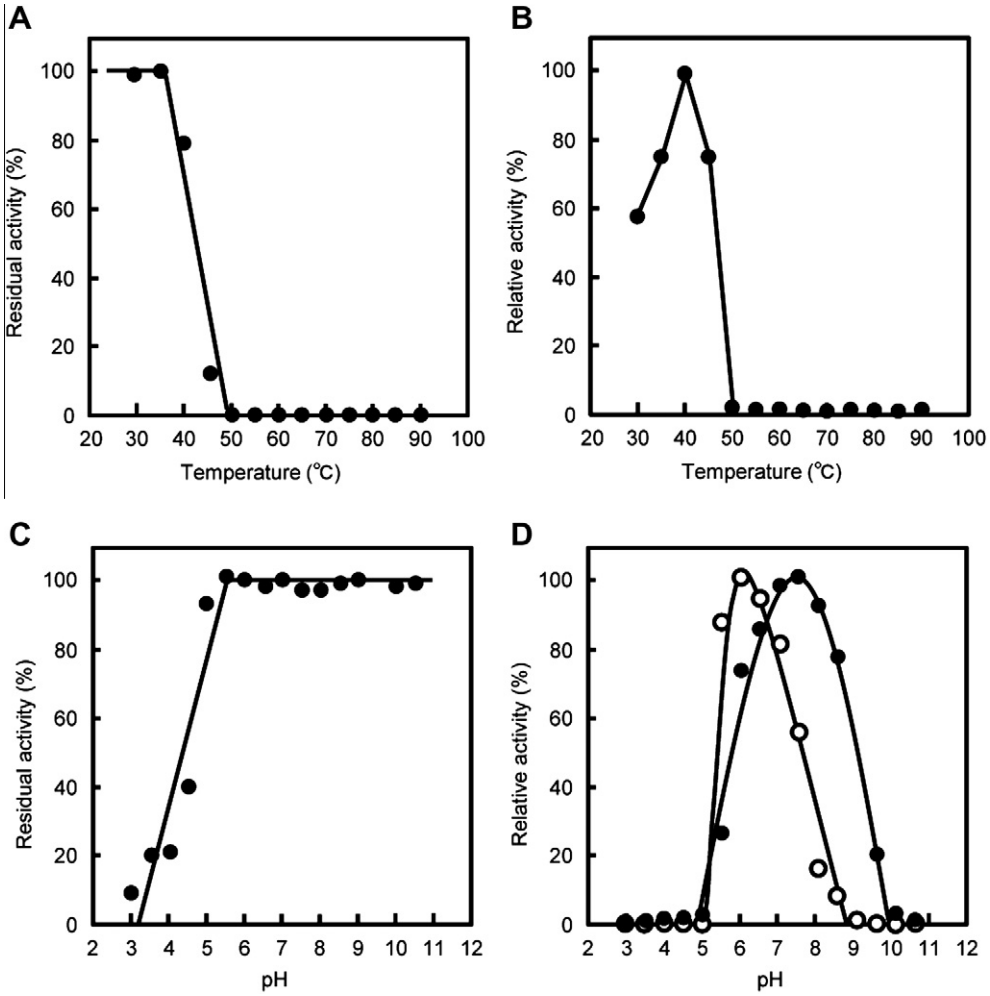
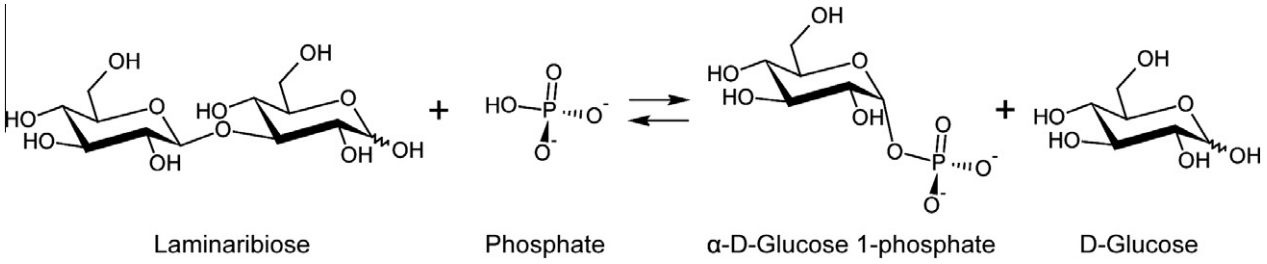


Figure 2. Effect of pH and temperature on the activity and stability of ACL0729. (A) Stability of 774 nM ACL0729 at the temperature range 30–90 °C for 15 min. (B) Temperature activity dependence for phosphorolysis of laminaribiose by 155 nM ACL0729 with 10 min incubation. (C) pH stability of 774 nM ACL0729 at 4 °C for 24 h and (D) pH activity dependence for phosphorolysis and synthesis of laminaribiose by 155 nM ACL0729 in 40 mM sodium citrate (pH 3.0–5.5), Bis(2-hydroxyethyl)imino-tris(hydroxymethyl)methane-HCl (pH 5.5–7.0), HEPES-NaOH (pH 7.0–8.5), and glycine-NaOH (pH 8.5–10.5). Closed and open symbols represent phosphorolytic and synthetic activities, respectively.



Scheme 1. Schematic representation of the reversible phosphorolysis catalyzed by laminaribiose phosphorylase.

represent laminaribiose and phosphate, respectively. Although the k_{cat} value of ACL0729 is slightly smaller than those of laminaribiose phosphorylases from *E. gracilis* and *Paenibacillus* sp. (29 s^{-1} and 18 s^{-1}, respectively),[25,26] the kinetic parameters are in the same range as other inverting phosphorylases,[31–38] assuming that laminaribiose is the substrate for ACL0729.

2.4. Acceptor specificity of ACL0729 in reverse phosphorolysis

In the reverse phosphorolysis using various carbohydrate acceptors (see Section 3.6) together with α-D-glucose 1-phosphate as the donor, ACL0729 utilized D-glucose, 2-deoxy-D-*arabino*-hexopyranose (2-deoxy-D-glucose), D-xylose, D-glucuronic acid, 1,5-anhydro-D-glucitol, and D-mannose having C-3 and C-4 equatorial hydroxyl groups as the acceptors. In addition, none of the examined disaccharides including laminaribiose (see Section 3.6) served as the acceptor in the reverse phosphorolysis. Each reverse reaction gave a single disaccharide product except for the reaction with D-xylose, in which two disaccharide peaks appeared on HPLC analysis. The products from D-glucose, 2-deoxy-D-glucose, D-glucuronic acid, 1,5-anhydro-D-glucitol, and D-mannose were identified by ^{1}H and ^{13}C NMR spectroscopic analysis to be the corresponding 1,3-β-D-glucosyl disaccharides: laminaribiose (Fig. S2), 3-*O*-β-D-glucopyranosyl-2-deoxy-D-*arabino*-hexose (Fig. S3), 3-*O*-β-D-glucopyranosyl-D-glucuronic acid

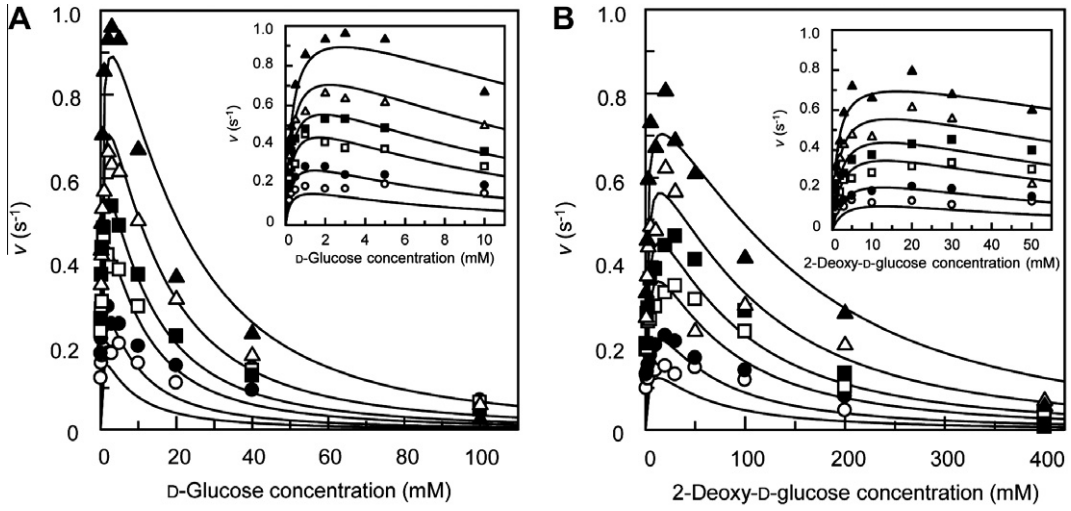


Figure 3. [Acceptor]-*v* plots of the reverse phosphorolysis catalyzed by ACL0729 at different concentrations of α-D-glucose 1-phosphate. (A) Various concentrations of D-glucose (0.2–100 mM) were used as the acceptor. (B) Various concentrations of 2-deoxy-D-glucose (1.0–400 mM) were used as the acceptor. The concentrations of α-D-glucose 1-phosphate are as follows: Open circle: 0.5 mM; closed circle: 1 mM; open square: 2 mM; closed square: 3 mM; open triangle: 5 mM; closed triangle: 10 mM. The [acceptor]-*v* plots at low concentrations of D-glucose (0.2–10 mM) and 2-deoxy-D-glucose (1.0–50 mM) are shown as insets.

(Fig. S4), 3-*O*-β-D-glucopyranosyl-1,5-anhydro-D-glucitol (Fig. S5), and 3-*O*-β-D-glucopyranosyl-D-mannose (Fig. S6), respectively. The major product (83%) in the reaction with D-xylose was 3-*O*-β-D-glucopyranosyl-D-xylose (Fig. S7) and the other fraction (17%) contained 2-*O*-β-D-glucopyranosyl-D-xylose as the major component (Fig. S8). In addition, it should be noted that the purification of 3-*O*-β-D-glucopyranosyl-2-deoxy-D-*arabino*-hexose using HPLC with an amino-polymer column was not successful probably due to the alkaline instability of 3-linked free 2-deoxy sugars.[39]

2.5. Kinetic analysis on reverse phosphorolysis

In the reverse phosphorolysis, the D-glucose caused competitive substrate inhibition, in which the D-glucose as the acceptor substrate competitively inhibited the binding of the α-D-glucose 1-phosphate as the donor substrate (Fig. 3A), as often described with GH94 phosphorylases including laminaribiose[25,26] and cellobiose phosphorylase.[19,28,40,41] The kinetic parameters that appear in Eq. 2 (see Section 3.7) were calculated to be $k_{cat} = 1.4\ s^{-1}$, $K_{mQ} = 0.05$ mM, $K_{mP} = 0.35$ mM, $K_{iQ} = 1.8$ mM, $K_{I1} = 0.23$ mM, and $K_{I2} = 0.08$ mM, where Q and P represent α-D-glucose 1-phosphate and D-glucose, respectively. The K_m values of ACL0729 for α-D-glucose 1-phosphate and D-glucose are approximately 10 times smaller than those of the laminaribiose phosphorylases from *E. gracilis* and *Paenibacillus* sp.[25,26] Noticeably, ACL0729 exhibited highest activity at the concentration of 1 mM to 3 mM D-glucose (Fig. 3A) and showed strongest competitive substrate inhibition in comparison with other glucobiose phosphorylases reported.[25,26,31,40,41]

The high concentration of 2-deoxy-D-glucose also caused the competitive substrate inhibition (Fig. 3B). The kinetic parameters that appear in Eq. 2 were calculated to be $k_{cat} = 0.98\ s^{-1}$, $K_{mQ} = 0.11$ mM, $K_{mP} = 0.72$ mM, $K_{iQ} = 12$ mM, $K_{I1} = 4.3$ mM, and $K_{I2} = 0.01$ mM, indicating that 2-deoxy-D-glucose causes weaker inhibition in comparison with Dglucose. The inhibition has been not observed in the reported laminaribiose phosphorylases.[24–26] The reason is that k_{cat}/K_m value of 2-deoxy-D-glucose in ACL0729 is approximately 40% of that of D-glucose (Table 1), which is 5–27 times higher compared to the reported ones.[24–26]

On the other hand, Michaelis–Menten equation curve fit the experimental data for the other acceptors (D-xylose, D-glucuronic acid, 1,5-anhydro-D-glucitol, and D-mannose; Table 1). The kinetic parameters suggest that ACL0729 recognizes the D-glucose derivatives with C-3 and C-4 equatorial hydroxyl groups at C-1 (1,5-anhydro-D-glucitol), C-2 (2-deoxy-D-glucose and D-mannose), and C-6 (D-xylose and D-glucuronic acid) positions.

3. Experimental

3.1. Construction of expression plasmid

The *acl0729* (GenBank ID: ABX81345.1) was amplified by PCR from genomic DNA of *A. laidlawii* PG-8A as the template using KOD-plus DNA polymerase (Toyobo, Osaka, Japan) with the following oligonucleotides based on the genomic sequence (GenBank ID: CP000896)[28]: 5′-aaaccatgggcaaaaaaaagcaatcaattaag-3′ as the forward primer containing an *Nco*I site (underlined) and 5′-tttctcgagtaattcatctagatatac-3′ as the reverse primer containing a *Xho*I site (underlined). The amplified *acl0729* was purified with a High Pure PCR Purification Kit (Roche Applied Science, Mannheim, Germany), digested by *Nco*I and *Xho*I (New England Biolabs, Beverly, MA, USA), and inserted into pET28a (+) (Novagen, Madison, WI, USA) to add a His_6-tag at the C-terminal of the recombinant protein. The expression plasmid *acl0729*/pET28a was propagated in *E. coli* JM109 (Toyobo, Osaka, Japan), purified by High Pure Plasmid Isolation Kit (Roche Applied Science), and verified by sequencing (Operon Biotechnologies, Tokyo, Japan).

3.2. Recombinant ACL0729 preparation

An *E. coli* BL21 (DE3) (Novagen) transformant harboring *acl0729*/pET28a was grown at 37 °C in 200 mL Luria–Bertani medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) containing 50 μg/mL of kanamycin up to an absorbance of 0.6 at 600 nm. Expression was induced by 0.1 mM isopropyl β-D-thiogalactopyranoside and continued at 18 °C for 24 h. The cells harvested by centrifugation at 10,000*g* for 20 min were suspended in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-NaOH (pH 7.5) containing 500 mM NaCl (buffer A). The suspended cells were disrupted by sonication, and the supernatant collected by centrifugation at 20,000*g* for 20 min was applied to a HisTrap HP column (GE Healthcare, Buckinghamshire, UK) equilibrated with buffer A containing 10 mM imidazole using a ÄKTA prime (GE Healthcare). After a wash with buffer A containing 22 mM imidazole, followed by elution using a 22–400 mM imidazole linear

Table 1
Kinetic parameters for the reverse phosphorolysis catalyzed by ACL0729

Acceptor	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m (mM^{-1} s^{-1})
D-Glucose[a]	0.4 ± 0.1	1.4 ± 0.1	3.5
2-deoxy-D-glucose[a]	0.7 ± 0.5	1.0 ± 0.1	1.4
D-xylose[b]	—	—	0.064
D-glucuronic acid[b]	—	—	0.024
1,5-anhydro-D-glucitol[b]	—	—	0.024
D-mannose[b]	—	—	0.009

[a] Calculated by curve-fitting with the curve of Eq. 2 (see Section 3.7)
[b] Determined from the slope of the linear [acceptor]- v plots

gradient in buffer A, fractions containing ACL0729 were pooled, dialyzed against 10 mM HEPES-NaOH (pH 7.5), and concentrated (AMICON Ultra; Millipore Co., Billerica, MA, USA). The protein concentration was determined spectrophotometrically at 280 nm using a theoretical extinction coefficient of $\varepsilon = 111{,}050\ cm^{-1}\ M^{-1}$, based on the amino acid sequence.[42] SDS polyacrylamide-gel electrophoresis was performed using Mini-PROTEAN Tetra electrophoresis system (Bio-Rad Laboratories, Inc., Hercules, CA, USA) according to the manufacturer's protocol.

3.3. Measurement of enzymatic activity

Phosphorolytic activity was routinely determined by quantifying the α-D-glucose 1-phosphate or D-glucose released during the enzyme reaction in 50 mM HEPES-NaOH (pH 7.5), 10 mM substrates, and 10 mM phosphate at 30 °C by phosphoglucomutase–glucose 6-phosphate dehydrogenase method[43] or glucose oxidase–peroxidase method,[44] respectively, as described previously.[26,37] One unit of the phosphorolytic activity was defined as the amount of enzyme that liberated 1 μmol α-D-glucose 1-phosphate or D-glucose from the substrate per min under the above conditions.

Reverse phosphorolytic activity was routinely determined by measuring the increase in phosphate in the reaction mixture containing 10 mM α-D-glucose 1-phosphate and 10 mM D-glucose in 50 mM MES-NaOH (pH 6.0) at 30 °C by following the method of Lowry and Lopez[45] as described previously.[37]

3.4. Substrate specificity analysis

Phosphorolytic activities on β-linked glucobioses (sophorose, laminaribiose, cellobiose, and gentiobiose) were determined under the standard conditions using 155 nM ACL0729 by quantifying the liberated D-glucose. The phosphorolytic activities on laminaribiose and laminaritriose were determined by quantifying the released α-D-glucose 1-phosphate under the standard conditions using 35 and 175 nM ACL0729, respectively.

3.5. Temperature and pH profile

The effects of pH on the enzymatic activity for phosphorolysis and synthesis of laminaribiose using 155 nM ACL0729 were measured under the standard conditions by substituting 50 mM HEPES-NaOH (pH 7.5) into the following 40 mM buffers: sodium citrate (pH 3.0–5.5), bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane-HCl (pH 5.5–7.0), HEPES-NaOH (pH 7.0–8.5), and glycine–NaOH (pH 8.5–10.5). Similarly, the effect of temperature on the phosphorolytic activity was measured under the standard conditions at various temperatures for 10 min. The thermal and pH stabilities were evaluated by measuring the residual phosphorolytic activity on laminaribiose under the standard conditions after incubation of ACL0729 (774 nM) at the temperature range 30–90 °C for 15 min in 50 mM HEPES-NaOH (pH 7.5) and in the various pH values at 4 °C for 24 h, respectively.

3.6. Acceptor specificity analysis

Reverse phosphorolysis to investigate the acceptor specificity of ACL0729 (5.2 μM) was performed under the standard conditions by substituting D-glucose into putative carbohydrate acceptors [D-mannose, D-allose, D-galactose, D-xylose, D-arabinose, L-arabinose, D-lyxose, L-fucose, L-rhamnose, D-fructose, 2-deoxy-D-glucose, D-glucal, D-glucosamine, D-galactosamine, D-mannosamine, α-D-glucose 1-phosphate, β-D-glucose 1-phosphate, D-glucose 6-phosphate, D-glucuronic acid, D-galacturonic acid, methyl α-D-glucoside, methyl β-D-glucoside, 1,5-anhydro-D-glucitol, 3-*O*-methyl-D-glucose, 2-acetamido-2-deoxy-D-glucose, 2-acetamido-2-deoxy-D-galactose, 2-acetamido-2-deoxy-D-mannose, trehalose, kojibiose, nigerose, maltose, isomaltose, sophorose, laminaribiose, cellobiose, gentiobiose, xylobiose, melibiose, lactose, lacturose, 1,4-β-D-manno-biose, sucrose, and *N*,*N*′-diacetylchitobiose] for 2 h. The reaction mixture (1 μL) was spotted on a thin layer chromatography (TLC) plate (Kieselgel 60 F_{254}; Merck, Darmstadt, Germany), and the sample was developed with a solution of acetonitrile–water (4:1, v/v). The TLC plates were soaked in 5% sulfuric acid–methanol solution and heated in an oven until bands were sufficiently visible.

3.7. Kinetic analysis

The initial rates on the phosphorolytic reaction were determined under the standard conditions with 347 nM ACL0729 and a combination of initial concentrations of laminaribiose (0.50, 1.0, 2.0, 3.0, 5.0, and 10 mM) and phosphate (0.25, 0.5, 1.0, 2.0, 3.0, and 5.0 mM). The kinetic parameters were calculated by curve-fitting the experimental data with the curve of Eq. 1 for the sequential bi bi mechanism using GraFit version 7.0.2 (Erithacus Software Ltd, London, UK).

$$v = V_{max}[A][B]/(K_{iA}K_{mB} + K_{mB}[A] + K_{mA}[B] + [A][B]) \quad (A = \text{laminaribiose}, B = \text{phosphate}) \tag{1}$$

Kinetic analysis of the reverse phosphorolysis was carried out under the standard conditions with 347 nM ACL0729 and various concentrations of the suitable acceptors. In the case of D-glucose or 2-deoxy-D-glucose as the acceptor, initial rates of the release of phosphate were measured with a combination of the initial concentrations of α-D-glucose 1-phosphate (0.5, 1.0, 2.0, 3.0, 5.0, and 10 mM) and D-glucose (0.2, 0.3, 0.5, 1.0, 2.0, 3.0, 5.0, 10, 20, 40, and 100 mM) or 2-deoxy-D-glucose (1.0, 2.0, 3.0, 5.0, 10, 20, 30, 50, 100, 200, and 400 mM). The experimental data were fitted with the curve of Eq. 2 of competitive substrate inhibition by D-glucose or 2-deoxy-D-glucose using GraFit Ver. 7.0.2.

$$v = V_{max}[Q][P]/\{K_{iQ}K_{mP} + (K_{mQ} + K_{iQ}K_{mP}/K_{I1})[P] + K_{mP}[Q] + [Q][P] + (K_{iQ}K_{mP}/K_{I1}K_{I2} + K_{mQ}/K_{I1})[P]^2 + K_{mQ}/K_{I1}K_{I2}[P]^3\} \quad (Q = \alpha\text{-D-glucose1-phosphate}, P = \text{glucose}) \tag{2}$$

In cases of the other acceptors (D-xylose, D-glucuronic acid, 1,5-anhydro-D-glucitol, and D-mannose), initial rates of the release of phosphate were measured with various concentration of acceptors (1.0, 2.0, 3.0, 5.0, 10, 20, and 40 mM) and 10 mM α-D-glucose 1-phosphate as the donor. The experimental data were fitted with the curve of Michaelis–Menten equation $\{v = k_{cat}\ [E]_0\ [S]/(K_m + [S])\}$ and the values of k_{cat}/K_m were calculated from the slope of a linear fit of initial rates versus acceptor concentrations.

3.8. Structural determination

Products for structural determination were generated in reaction mixture containing 1.55 μM ACL0729, 50 mM α-D-glucose 1-phosphate, and 50 mM suitable acceptor (D-glucose, 2-deoxy-D-glucose, D-xylose, D-glucuronic acid, 1,5-anhydro-D-glucitol, and D-mannose) in a final volume of 500 μL of 10 mM MES-NaOH (pH 6.0) incubated at 30 °C for 70 h. In cases where D-glucose, D-xylose, 1,5-anhydro-D-glucitol, and D-mannose were used as the acceptors, the reaction mixtures were desalted using Amberlite MB-3 (Organo, Tokyo, Japan), and the products were purified by a high performance liquid chromatography system (Prominence, Shimadzu, Kyoto, Japan) equipped with a Shodex Asahipak NH2P-50 4E column (4.6 mm φ × 250 mm; Showa Denko K.K., Tokyo, Japan) at 30 °C under a constant flow (1.0 mL/min) of mobile phase (acetonitrile/water v/v, 75/25). In cases of 2-deoxy-D-glucose and D-glucuronic acid as the acceptors, the reaction mixtures were neutralized by addition of 1 M NaOH and treated with a recombinant α-D-glucose 1-phosphatase from *E. coli*[46] at 30 °C for 12 h, and the products were separated on a Toyopearl HW-40S column (26 mm φ × 650 mm; TOSOH, Tokyo, Japan) equilibrated with distilled water at a flow rate of 0.5 mL/min. Fractions containing the reaction products were collected, followed by lyophilization. The amounts of products obtained were 5.5, 4.9, 5.9 (4.9 mg of main product and 1.0 mg of by-product), 4.0, 5.0, and 6.0 mg from D-glucose, 2-deoxy-D-glucose, D-xylose, D-glucuronic acid, 1,5-anhydro-D-glucitol, and D-mannose as the acceptor, respectively. The one-dimensional (^{1}H and ^{13}C) and two-dimensional [double-quantum-filtered correlation spectroscopy (DQF-COSY), heteronuclear single-quantum coherence (HSQC), and heteronuclear multiple-bond correlation (HMBC)] nuclear magnetic resonance (NMR) spectra of the product were taken in D_2O with 2-methyl-2-propanol as an internal standard using Bruker Avance 500 or Bruker Avance 800 spectrometer (Bruker Biospin, Rheinstetten, Germany). Proton signals were assigned based on DQF-COSY spectra. ^{13}C signals were assigned with HSQC spectra, based on the assignment of proton signals. The linkage position in each disaccharide was determined by detecting inter-ring cross peaks in each HMBC spectrum.

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Supplementary data

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