SUPPORTING INFORMATION

Galactaro δ Lactone Isomerase:

Lactone Isomerization by a Member of the Amidohydrolase Superfamily

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MATERIALS AND METHODS

Cloning of Atu3138 from Agrobacterium tumefaciens strain C58. Atu3138 was PCR amplified from genomic DNA using Phusion DNA polymerase (New England Biolabs). The PCR reaction (50 µL) contained 1 ng of genomic DNA, 10 µL of 5X GC buffer, 1.5 µL of DMSO, 0.4 mM dNTPs, 20 pmol of each primer (forward primer 5'-CTGAATTCAGGATGCCAGACATATGAGCGAACTCGTCAGAAAAC -3', reverse primer 5'-GGGGCCTCCCATTCCCAACCATCTCGAGTAAAACTAGGTCGCC -3'), and 1 unit of Phusion DNA

polymerase. The amplification used a PTC-200 gradient cycler (MJ Research) with the following parameters: 98 °C for 4 minutes followed by 35 total cycles of 98 °C for 20 seconds, 55 °C for 20 seconds, 72 °C for 30 seconds, and ending with a final extension at 72 °C for 7 minutes. The amplified gene was cloned into pET-15b vector.

Expression and purification of A9CEQ7. Protein was expressed in an 8 L culture of *E. coli* strain BL21-DE3 cells. Expression consisted of growth in LB broth (supplemented with 100 μg/mL ampicillin) at 37 °C and 220 RPM for 18 hours. IPTG was not needed to induce expression. Cells were harvested by centrifugation (4650 x g, 4 °C) and resuspended in 30-40 mL of binding buffer (20 mM Tris pH 7.9, 0.2 M NaCl, and 5 mM $MgCl_2$). All cells were lysed by sonication, and the resulting lysate was pelleted to remove cell debris by centrifugation (31,000 x g, 4 °C). The clarified supernatant containing the His-tagged protein was then loaded onto a 5 mL chelating Sepharose Fast Flow column charged with nickel and equilibrated with 10 column volumes of binding buffer. The column was washed with 100 mL of 15% elution buffer (0.5 M imidazole, 20 mM Tris pH 7.9, 0.2 M NaCl, and 5 mM $MgCl_2$) containing 85% binding buffer and eluted over a 150 mL linear gradient of 15 to 100% elution buffer with an additional 100 mL at 100% elution buffer. Purity was checked by SDS-PAGE gel electrophoresis. Fractions containing protein were then pooled and dialyzed against a solution of 20 mM HEPES pH 7.9, 5 mM $MgCl_2$, 0.1 M NaCl, and 10% glycerol. The mass of the polypeptide was confirmed by mass spectrometry (Figure S1). Protein was flash frozen drop wise into liquid nitrogen, and the beads were transferred to a cryogenic vial and stored at -80 °C.

Construction, expression, and purification of the N240D mutant. Asn 240 was mutated to aspartate using the following 50 µL reaction: 5 µL of 10X *Pfx* Amplification Buffer, 0.3 mM dNTP mixture, 1 mM $MgSO_4$, 0.25 µM of each primer (forward primer 5'-CATCGTCTGGGGCACCGACTGGCCGCATAATTC -3', reverse primer 5'-GAATTATGCGGCCAGTCGGTGCCCCAGACGATG -3'), 1.25 units of Platinum® *Pfx* Platinum DNA Polymerase (Invitrogen™), in H_2O with varying amounts (6.25, 12.5, 25, and 50 ng) of template pET-15b with the gene encoding A9CEQ7. The amplification was performed using a PTC-200 gradient cycler (MJ Research) with the following cycling profile: 94 °C for 5 minutes; followed by 12 cycles of 94 °C for 30 seconds, 55 °C for 1 minute, and 68 °C for 7 minutes and 30 seconds. When complete, the reactions were pooled, 40 units of DpnI were added, and the mixture was incubated at 37 °C for one hour. Next the amplified product was gel extracted and electroporated into *E. coli* XL1-Blue cells. Single colonies were used to inoculate an overnight culture from which plasmid was isolated, and the correct sequence confirmed by sequencing. Protein was expressed in a 6 L culture of *E. coli* BL21-DE3 cells. Expression consisted of growth in LB broth (supplemented with 100 µg/mL ampicillin) at 16 °C and 220 RPM for 48 hours. IPTG was not needed to induce expression. Cells were harvested by centrifugation (5000 x g, 4 °C) and resuspended in 75 mL of binding buffer (20 mM Tris pH 7.9, 0.5 M NaCl, and 5 mM $MgCl_2$). All cells were lysed by sonication, and the resulting lysate was pelleted to remove cell debris by centrifugation (30,000 x g, 4 °C). The clarified supernatant containing the His-tagged protein was then loaded onto a 50 mL chelating Sepharose Fast Flow column charged with nickel and equilibrated with 10 column volumes of binding buffer. The protein was eluted over a 1200 ml linear gradient of 0 to 25% elution buffer (1 M imidazole, 20 mM Tris pH 7.9, 0.5 M NaCl, and 5

mM $MgCl_2$). Purity was checked by SDS-PAGE gel electrophoresis. Fractions containing protein were then pooled and dialyzed against a solution of 20 mM Tris pH 7.9, 5 mM $MgCl_2$, and 0.15 M NaCl. The mass of the polypeptide was confirmed by mass spectrometry (Figure S2). Protein was flash frozen drop wise into liquid nitrogen, and the beads were transferred to a cryogenic vial and stored at -80 °C.

Preparation of D-galactaro-1,5-lactone by bromine oxidation. D-Galactaro-1,5-lactone (δ-lactone) was prepared from the sodium salt of D-galacturonic acid using the procedure described by Isbell and Frush (1) with minor modifications: at ½ the scale in D_2O, 2X bromine, a reaction time of 22 minutes and 30 seconds, and separation in a separatory funnel at 4 °C . The final product was stored at -80 °C (Table S1 and Figure S3A).

Udh assay. To confirm that the ^1H NMR spectrum of the product from the bromine oxidation was indistinguishable from the ^1H NMR spectrum of the product from the Udh reaction, the spectra were compared (Figures S3A and S3B). To obtain a spectrum of the product of the Udh reaction, 5 µM Udh was added to a reaction mixture containing 50 mM sodium phosphate buffer, 2 mM $MgCl_2$, 5 mM D-galacturonate, and 6 mM NAD^+ at pD 6.4 in a final volume of 500 µL. After a one minute incubation at room temperature the reaction was stopped by decreasing the pD to 4 with the addition of DCl (2). The reaction mixture then was mixed with 2% (w/v) activated charcoal (100 mesh) for one minute to remove the NAD^+/NADH and then centrifuged for 30 seconds in a microcentrifuge. The supernatant was filtered through

a syringe filter to remove any additional charcoal; 300 µL was added to a 5 mm Shigemi NMR tube matched to D_2O. All NMR spectra were collected on an Agilent 600MHz spectrometer.

Activity assay by ^{1}H-NMR spectroscopy. To determine if D-galactaro-1,5-lactone is a substrate for A9CEQ7, the conversion of D-galactaro-1,5-lactone to D-galactaro-1,4-lactone (γ-lactone) was monitored by ^{1}H-NMR spectroscopy. A 650 µL reaction contained 6 mM D-galactaro-1,5-lactone, 10 nM enzyme, 50 mM sodium phosphate buffer, and 2 mM $MgCl_2$, at pD 6.4 in D_2O. The spectrum of D-galactaro-1,5-lactone was acquired, and then 10 nM A9CEQ7 was added. A spectrum was recorded every two minutes at room temperature over the course of one hour. All NMR spectra were collected on an Agilent 600MHz spectrometer.

Metal testing. A continuous polarimetric assay was used to determine the effect of added divalent cations on A9CEQ7's activity. The change in optical rotation was monitored using a polarimeter (Jasco P-1010) and a mercury line filter (405 nm). A 1300 µL reaction containing 100 nM enzyme, 50 mM MES buffer, 2 mM D-galactaro-1,5-lactone, and 2 mM of Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, or Zn^{2+} at pD 6.4 in D_2O was monitored for 10 minutes at room temperature.

Kinetic assay by polarimetry. A continuous polarimetric assay was used to determine the kinetic parameters of A9CEQ7 using a Jasco P-1010 as described above. A 1300 µL reaction containing 100 nM enzyme and 50 mM sodium phosphate buffer at pD 6.4 in D_2O was monitored for one minute at room temperature with varying substrate concentrations (0.24

mM – 5.85 mM). The rates of the uncatalyzed reaction and the reaction catalyzed by the N240D mutant enzyme were determined using the same reaction conditions and monitoring for an additional one minute and 30 seconds. All enzymes were exchanged into 50 mM sodium phosphate buffer at pD 7.7 in D_2O prior to kinetic assays being performed. Data were fit to the Michaelis-Menten equation in Sigma Plot using the Enzyme Kinetics Module.

Crystallization and Structure Determination. A9CEQ7 was crystallized by sitting drop vapor diffusion using 96-well Intelliplates (Douglas Instruments). Crystallization drops were assembled by combining 0.5 µl of protein (10 mg/mL in 20 mM Tris pH 7.9, 0.15 M NaCl, 5 mM $MgCl_2$) with 0.5 µl of the reservoir (70 µl of 0.1 M HEPES pH 7.5, 20% (w/v) PEG 4000, 10% (w/v) isopropanol). Crystals grew as intertwined rods over 7 days with dimensions of 50 x 50 x 200 µm. A single crystal was extracted, transferred to the reservoir condition with 20% (w/v) isopropanol, and then vitrified by plunging in liquid nitrogen. Data were collected at the Advanced Photon Source (Beamline 31-ID of Argonne National Laboratories) using a wavelength of 0.9788 Å and a Rayonix 225 HE detector (Rayonix, Llc). Data were integrated in MOSFLM (3) and scaled using SCALA (4). An ensemble model was generated from a structurally unique set of structures available from IPR006992 (PDB IDs: 2ffi, 4do7, 4i6k, 4dia, 4di8) and used in molecular replacement. The program PHASER (5) within PHENIX (6) located the three molecules within the asymmetric unit and the final model was built with iterative cycles of fitting to electron density maps within the molecular graphics program COOT (7), followed by refinement within PHENIX. The quality and stereochemistry of the final structure were verified using MOLPROBITY (8) (refinement statistics in Table S2).

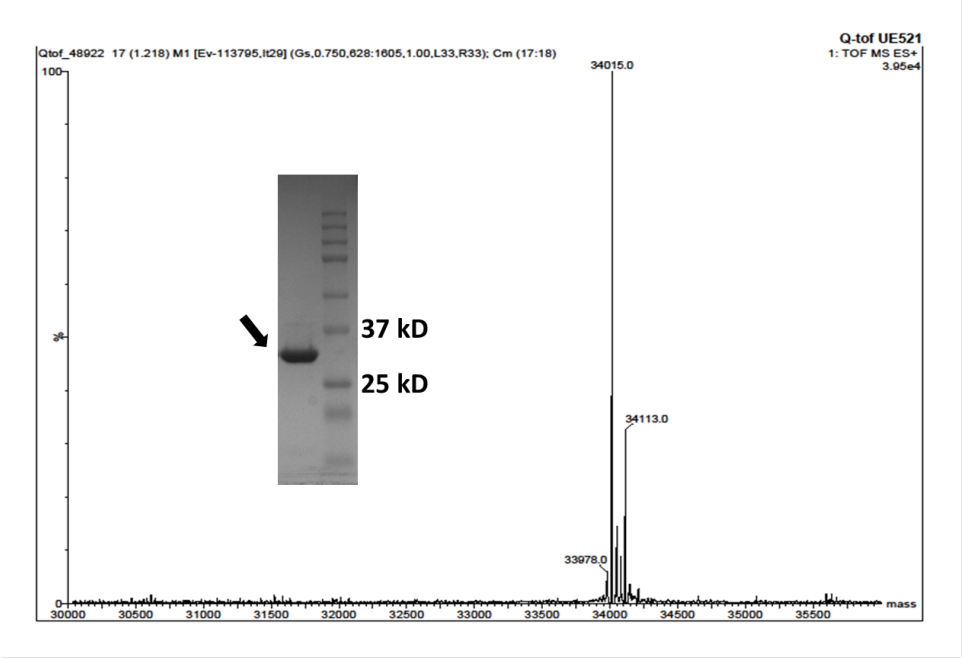


Figure S1. Grayscale photo of an SDS-PAGE gel and mass spectrometry chromatogram for purified A9CEQ7. The expected mass of the his-tagged protein is 34016 daltons. Protein (arrow) in lane 1 and protein ladder in lane 2.

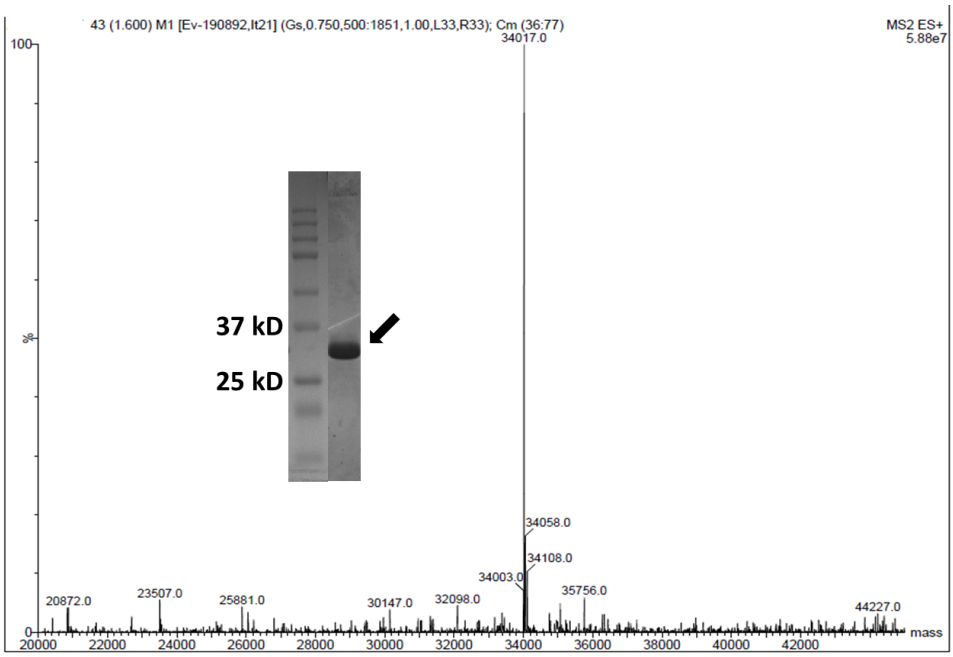
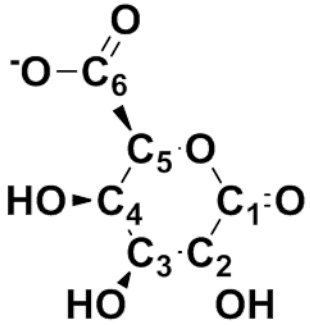


Figure S2. Grayscale photo of an SDS-PAGE gel and mass spectrometry chromatogram for purified N240D mutant. The expected mass of the his-tagged protein is 34017 daltons. Protein ladder in lane 1 and protein (arrow) in lane 2.

Compound	H	δ (PPM)		J
1) D-Galactaro-δ-Lactone	2	4.32	d	10.62
	3	4.00	dd	2.76, 10.56
	4	4.40	t	2.31
	5	4.71	d	1.98
2) D-Galactaro-γ-Lactone	2	4.11	d	1.74
	3	4.47	dd	1.74, 8.52
	4	4.28	t	8.82
	5	4.52	d	9.24

1)



2)

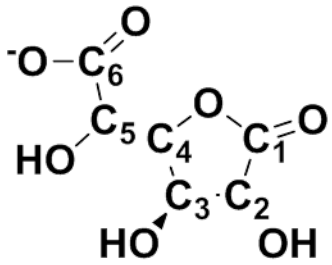


Table S1. ^{1}H-NMR chemical shifts for D-galactaro-δ-lactone and D-galactaro-γ-lactone at pD 4.0.

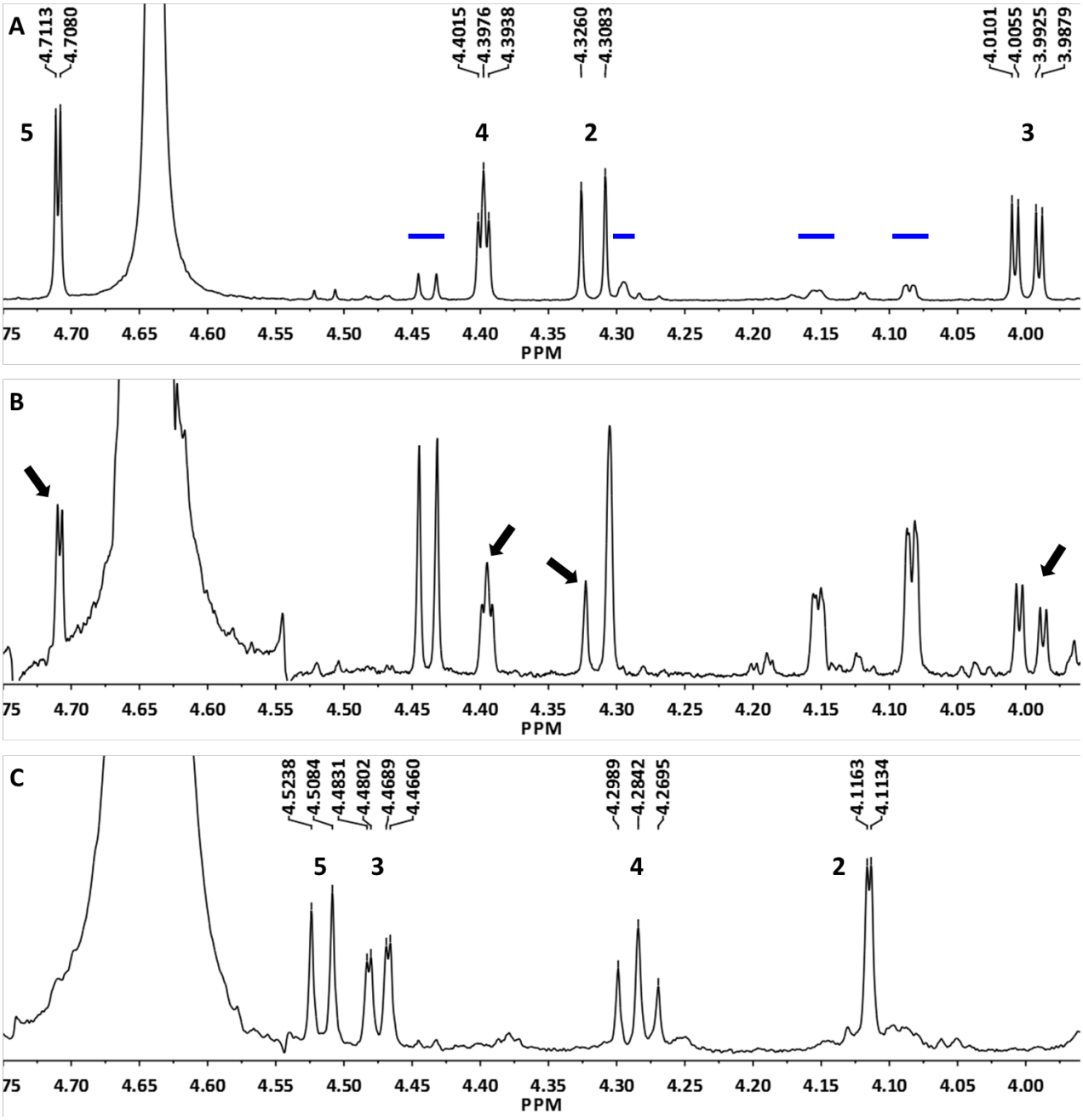


Figure S3. ^{1}H-NMR spectra for (A) D-galactaro-δ-lactone, (B) Udh reaction, and (C) D-galactaro-γ-lactone at pD 4.0. (A) Blue bars denote D-galacturonate. Numbers correspond to the hydrogen attached to the carbon as numbered in structure number one of Table S1. (B) Arrows point to the δ-lactone after acid quenching and removal of NAD^{+}/NADH from the oxidation of

D-galacturonate by the Udh. (C) Numbers correspond to the hydrogen attached to the carbon as numbered in structure number two of Table S1.

Table S2. Data Collection and Refinement Statistics.[a]

DATASET STATISTICS	
Space Group	$P2_1$
Unit Cell (Å , °)	*a*=53.2, *b*=77.6, *c*=99.7, β=95.5
Resolution (Å)	31.6-1.6 (1.69-1.60)
Completeness (%)	97.1 (100.0)
Redundancy	3.6 (3.5)
Mean(I)/sd(I)	8.5 (2.0)
Rsym	0.095 (0.541)
STRUCTURE STATISTICS	
Resolution (Å)	31.6-1.6 (1.62-1.60)
Unique reflections	103114 (3564)
Rcryst (%)	14.7 (21.8)
Rfree (%, 5% of data)	18.1 (23.4)
Residues In Model [Expected]	A6-289, B4-289, C12-44, C46-246, C252-289 [1-292]
Residues / Waters / Atoms total	842 / 1073 / 8205
Average B-factor ($Å^2$)	
Protein/Waters	17.4 / 30.3
RMSD	
Bond Lengths (Å) / Angles (degrees)	0.009 / 1.276
MOLPROBITY STATISTICS	
Ramachandran Favored / Outliers (%)	98.6 (0.0)
Clashscore [b]	1.78 (99^{th} pctl.)
Overall score [b]	0.94 (100^{th} pctl.)

[a] Statistics in parenthesis are for the highest resolution bin
[b] Scores are ranked according to structures of similar resolution as formulated in MOLPROBITY

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