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Characterization of a novel *Agrobacterium tumefaciens* Galactarolactone Cycloisomerase Enzyme for Direct Conversion of D-Galactarolactone to 3-Deoxy-2-keto-L-*threo*-hexarate*[S]

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Background: Earlier data suggest that in microbial oxidative pathways D-galacturonate is oxidized to D-galactarolactone, the lactone hydrolyzed to galactarate, which is further converted to 3-deoxy-2-keto-hexarate.
Results: A novel Galactarolactone cycloisomerase active on D-galactarolactone was found in *Agrobacterium tumefaciens*.
Conclusion: Galactarolactone cycloisomerase catalyzes direct conversion of D-galactarolactone to 3-deoxy-2-keto-hexarate.
Significance: The current study has identified a novel activity in the growing enolase superfamily.

Microorganisms use different pathways for D-galacturonate catabolism. In the known microbial oxidative pathway, D-galacturonate is oxidized to D-galactarolactone, the lactone hydrolyzed to galactarate, which is further converted to 3-deoxy-2-keto-hexarate and α-ketoglutarate. We have shown recently that *Agrobacterium tumefaciens* strain C58 contains an uronate dehydrogenase (*At* Udh) that oxidizes D-galacturonic acid to D-galactarolactone. Here we report identification of a novel enzyme from the same *A. tumefaciens* strain, which we named Galactarolactone cycloisomerase (*At* Gci) (E.C. 5.5.1.-), for the direct conversion of the D-galactarolactone to 3-deoxy-2-keto-hexarate. The *At* Gci enzyme is 378 amino acids long and belongs to the mandelate racemase subgroup in the enolase superfamily. *At* Gci was heterologously expressed in *Escherichia coli*, and the purified enzyme was found to exist as an octameric form. It is active both on D-galactarolactone and D-glucarolactone, but does not work on the corresponding linear hexaric acid forms. The details of the reaction mechanism were further studied by NMR and optical rotation demonstrating that the reaction product of *At* Gci from D-galactaro-1,4-lactone and D-glucaro-1,4-lactone conversion is in both cases the L-*threo* form of 3-deoxy-2-keto-hexarate.

Lignocellulosic biomass sugars are considered as an attractive renewable source to develop biotechnological processes for production of biofuels, chemicals, and materials. A major part of the work has so far focused on conversion of glucose to bioethanol and other useful platform chemicals. To create novel alternative products, other biomass waste stream sugars need to be considered. Pectin is present in primary cell walls of all higher terrestrial plants, and it is particularly abundant in agro wastes such as citrus peel, apple pomace, and sugar beet pulp. Pectin is largely composed of D-galacturonic acid, and the simplest form of pectin is homogalacturonan, a linear chain of α-(1,4)-linked D-galacturonic acid.

To be able to develop optimal processes for cost efficient microbial production of chemicals, a comprehensive understanding of the metabolic pathways is required. For the microbial catabolism of D-galacturonate, three different pathways have been described, (i) a reductive pathway, (ii) an isomerase pathway, and (iii) an oxidative pathway (1). The reductive pathway is used in eukaryotic microorganisms, while the two other pathways have been found in prokaryotic microorganisms. The oxidative pathway has been shown to be active in *Pseudomonas* species and in *Agrobacterium tumefaciens* (2–6). Here D-galacturonic acid is first oxidized to galactaric acid and in the following steps converted through 3-deoxy-2-keto-galactarate to α-keto-glutarate, which is a metabolite in the tricarboxylic acid (TCA)[2] cycle. We have recently identified and characterized the uronate dehydrogenase (EC 1.1.1.203, Udh) from *A. tumefaciens* C58 strain (7) and further determined the complex structure of the enzyme with NADH and product (8). The reaction product of *At* Udh from the oxidation of D-galacturonic acid (*i.e.* the pyranose ring form) is D-galactaro-1,5-lactone, which can rearrange non-enzymatically to a more stable 5-membered ring structure, D-galactaro-1,4-lactone, detectable in solution by NMR and MS analysis (7, 8). The following enzymatic steps in this oxidative pathway should include hydrolysis of the lactone ring either spontaneously or with the help of a lactonase to galactaric acid, followed by a dehydration step to convert galactaric acid to 3-deoxy-2-keto-hexarate (KDG) (Fig. 1). Dehydration of galactarate can lead to two isoforms of the 3-deoxy-2-keto-hexarate, the L-*threo* or the D-*threo*- form, but it is not known which one is produced in the oxidative D-galacturonic acid pathway.

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[S] This article contains supplemental Table S1 and Figs. S1 and S2.
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[2] The abbreviations used are: TCA, tricarboxylic acid; KDG, 3-deoxy-2-keto-hexarate; DEPT, distortionless enchancement by polarization transfer; DQFCOSY, double-quantum filtered correlated spectroscopy; HSQC, heteronuclear single-quantum correlation; Udh, uronate dehydrogenase; Gci, galactarolactone cycloisomerase.

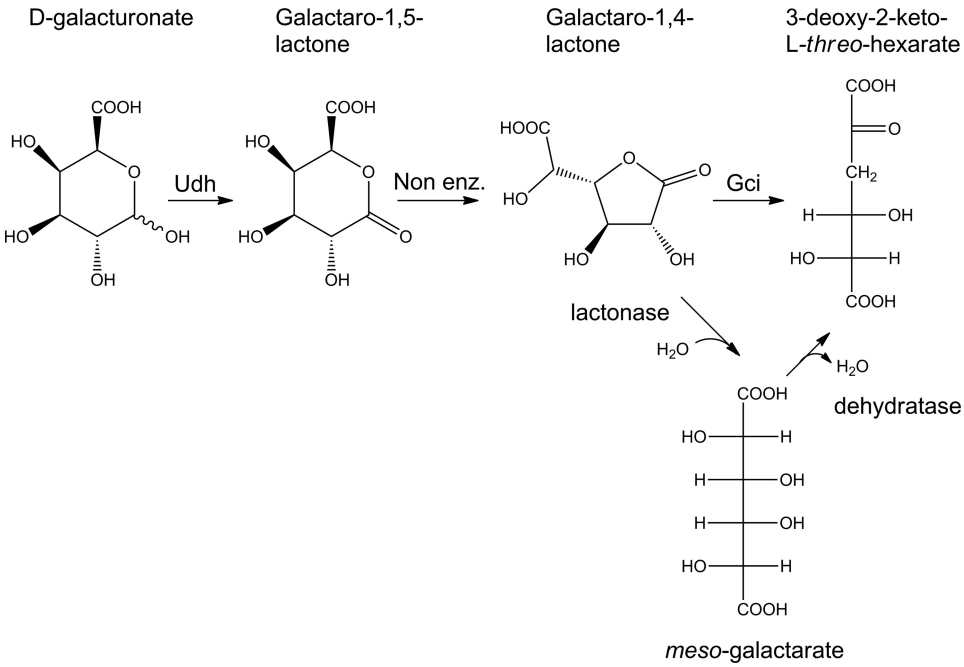

FIGURE 1. **Reaction scheme of the first steps of the oxidative pathway for D-galacturonate catabolism.** D-Galacturonate is oxidized by the uronate dehydrogenase, *At* Udh, to D-galactarolactone which can hydrolyze spontaneously or with the aid of a lactonase to *meso*-galactarate. Then a dehydratase converts galactarate to 3-deoxy-2-keto-hexarate. In the present work, we show that *A. tumefaciens* has an enzyme, *At* Gci, for the direct conversion of the D-galactarolactone to 3-deoxy-2-keto-L-*threo*-hexarate.

Here we describe a novel enzyme from *A. tumefaciens* C58 that we named a galactarolactone cycloisomerase (*At* Gci) as it can convert D-galactarolactone, produced by *At* Udh, directly to a linear 3-deoxy-2-keto-galactarate. In this reaction, the stoichiometry of the product does not change. *At* Gci belongs to the enolase superfamily and shows sequence homology with sugar acid dehydratases. Enolase superfamily enzymes catalyze different overall reactions, (9) but share some common features in their reaction mechanisms. The reaction is initiated by general-base abstraction of the α-proton of a carboxylic acid substrate that is coordinated to an essential Mg^{2+}. An enolic intermediate that is stabilized by the metal ion is formed, which is then directed into different products depending of the active site structure of the superfamily members. The members of the enolase superfamily also share a bi-domain structure with a $\alpha+\beta$ capping domain that contains the residues determining the substrate specificity, and a C-terminal $(\beta/\alpha)_7\beta$ (modified TIM-barrel) domain that contains the conserved ligands for the essential Mg^{2+} and the residues involved in the acid-base chemistry. The enzymes in the enolase superfamily can be divided into distinct subgroups based on the identity and position of the active site residues (9, 10). *At* Gci seems to belong to the mandelate racemase subgroup.

EXPERIMENTAL PROCEDURES

Purification and Identification of Galactarolactone Cycloisomerase, Gci, from A. tumefaciens—A. tumefaciens (*Rhizobium radiobacter*) strain C58 (ATCC 33970) was cultivated aerobically for 36 h at 28 °C in a mineral minimum medium containing 10 g l^{-1} D-galacturonic acid as a sole carbon source (4). The cells from 4 liters of culture were harvested by centrifugation at 4000 × *g* for 15 min, washed once with Milli-Q grade water, and suspended in 10 mM Tris-Cl, pH 7.5, 10 mM DTT supplemented with 1× Complete EDTA-free (Roche Applied Science) protease inhibitor. Using a MSE Soniprep 150, the cells were disrupted by sonication (6 × 15 s), and the cell debris was removed by centrifugation at 38,000 × *g* for 30 min at 4 °C. To the cell-free extract ammonium sulfate was added to 35% saturation. After 30 min of incubation on ice, the precipitate was removed by centrifugation (12,000 × *g*, 20 min. at 4 °C), and ammonium sulfate was added gradually to the supernatant with constant stirring, to attain 55% saturation. After another centrifugation, the pellet was suspended in 20 mM Tris-Cl, pH 7.5. For hydrophobic interaction chromatography the conductivity of the sample solution was adjusted with saturated ammonium sulfate solution to that of 1.5 M $(NH_4)_2SO_4$. The sample was applied to a phenyl-Sepharose FF column (V = 50 ml) equilibrated with 1.5 M $(NH_4)_2SO_4$ in 20 mM Tris-Cl, pH 7.5 and washed with the same buffer. Proteins were eluted using a reversed linear gradient of 1.5–0 M $(NH_4)_2SO_4$ in 20 mM Tris-Cl, pH 7.5. The fractions were tested for dehydratase activity toward galactaric acid or D-galactarolactone produced in a coupled assay with uronate dehydrogenase using 6 mM D-galacturonic acid and 5 mM NAD^+ as substrates (for further assay details, see below). Fractions active on D-galactarolactone were pooled, and the sample buffer of one-third of the pooled sample was changed to 20 mM Tris-Cl, pH 7.5 by gel filtration on a 250 ml Sephadex G-25 Coarse column. The sample was applied to a weak anion exchange column (DEAE-Sepharose FF, V = 7 ml) in 20 mM Tris-Cl, pH 7 and eluted with a 100-ml linear gradient from 0 to 250 mM NaCl. Fractions containing active enzyme were combined and the buffer was changed to 20 mM Tris-Cl, pH 7 by gel filtration on PD-10 columns (GE HealthCare). The

enzyme was further purified with a high-resolution anion exchange column (Resource Q, V = 1 ml). The bound proteins were eluted with a linear NaCl gradient (0–500 mM).

The active fractions were analyzed by SDS-PAGE (10% Criterion Stain Free gel, Bio-Rad), and the proteins were visualized using the Criterion Stain Free gel imaging system (Bio-Rad). Each active fraction contained at least three major, and several minor protein bands. By comparing the intensity of the stained protein bands with the activity of each fraction, a correlation between the intensity of an ~40 kDa band and the enzyme activity was found. The proteins were separated on a 12% SDS-PAGE gel, stained with GelCode Blue stain reagent (Thermo Scientific) followed by in-gel digestion with trypsin. The peptide extraction from gel and sample preparation was performed as described previously (7). The peptide masses were determined by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometer using a Bruker Autoflex II mass spectrometer (Bruker Daltonics, Bremen, Germany).

Enzyme Activity Measurements—The galactarolactone cycloisomerase activity was performed as a coupled assay with *A. tumefaciens* uronate dehydrogenase (*At* Udh). The recombinant *At* Udh was expressed in *S. cerevisiae* and purified as described before (7). The activity assay was conducted in 50 mM Tris-Cl buffer, pH 8, using 6 mM D-galacturonic acid, 5 mM NAD^+, 2 mM $MgCl_2$, ~1 μg uronate dehydrogenase, and 0.5–2 μg of galactarolactone cycloisomerase (*At* Gci) enzyme. The reaction in 100 μl was stopped typically after 20 min, if not otherwise indicated, by adding 12% (w/v) trichloroacetic acid. Galactaric acid dehydratase activity was assayed using 1–2 μg enzyme in 100 μl 50 mM Tris-Cl buffer, pH 8.0 containing 10 mM galactaric acid and 10 mM $MgCl_2$. The formation of 3-deoxy-2-keto-hexarate (KDG) was determined using the thiobarbituric acid (TBA) assay described by Buchanan *et al.* (ϵ = 67,800 M^{-1} cm^{-1}) (11), or alternatively the semicarbazide method described by MacGee and Doudoroff (12).

Cloning of the A. tumefaciens gci Gene—The open reading frame of the *gci* was amplified by PCR using primers containing NcoI and HindIII restriction sites and using genomic DNA isolated from *A. tumefaciens* as template. The PCRs were performed with Phusion thermostable polymerase (Finnzymes, Finland), in a reaction mixture recommended by the manufacturer and using the following primers, 5′-GAGGGGAGCCATGGACAAAATCACG-3′ (sense) and 5′-GTTTTCTGACGAGAAGCTTCATGGGTCTG-3′ (antisense). The N-terminal methionine was followed by an Asp, added to create a NcoI site. The PCR program had an initial denaturation step of 30 s at 98 °C, followed by 35 cycles of 10 s at 98 °C, 30 s at 62 °C, and 40 s at 72 °C. The annealing temperature was raised with 0.3 °C each cycle up to 72 °C, and the final step was elongation for 10 min at 72 °C. The amplified PCR product was digested with NcoI and HindIII and ligated to the *Escherichia coli* expression vector pBAT4 (13). The resulting pBAT4-Atu3139 vector was transformed to *E. coli* BL21(DE3) cells.

The *gci* was also tagged with an eight amino acid long Strep-tag II (Trp-Ser-His-Pro-Gln-Phe-Glu-Lys) at the N and C terminus. The Strep-tagged *gci* was amplified by PCR using the following primers; GACGCCATGGATTGGTCTCATCCGCAGTTTGAAAAAAAAATCACGGCGGTGCGC-3′(sense) and 5′-GAAACTCAAGCTTCATGGGTCTGGCATC-3′ (antisense), for the N-terminal Strep-tag, and 5′-GATATATCCATGGATAAAATCACGGC-3′(sense) and 5′-GTTTTCTGACGAGAAGCTTCATTTTTCAAACTGCGGATGAGACCAAGGGTCTGGCATC-3′ (antisense) for the C-terminal Strep-tag, ligated to the pBAT4 vector as described above and sequenced.

Production and Purification of the StrepII-tagged Galactarolactone Cycloisomerase—For protein purification of the Strep-tagged galactarolactone cycloisomerase (*At* Gci), 3 L of *E. coli* BL21(DE3) cells containing the plasmid described above were grown at 37 °C, 250 rpm in Luria Broth medium (14) supplemented with 100 μg/ml carbenicillin, until A_{600} = 0.5–0.8 was reached. After the addition of 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) to induce the expression of the Strep-tagged protein, the culture was further grown at 30 °C, 250 rpm overnight. Cells were harvested by centrifugation for 15 min at 4,000 × *g* at 4 °C, and suspended in ice-cold lysis buffer (50 mM sodium phosphate buffer, pH 8 containing 300 mM NaCl, 1 mM DTT, protease inhibitors (Complete EDTA-free, Roche), and lysozyme (Sigma-Aldrich), and incubated on ice for 30 to 60 min. The cells were disrupted by sonication (3 × 20 s) on ice, incubated in the presence of DNaseI and RNase A (Roche) for 30 min, and centrifuged for 30 min at 38,000 × *g* at 4 °C. The cell-free extract was loaded onto a 1-ml *Strep*-Tactin Superflow column (Qiagen) equilibrated with 50 mM sodium phosphate, pH 8, 300 mM NaCl buffer. After the column was washed with 20 ml of the equilibration buffer, the enzyme was eluted with 2.5 mM desthiobiotin in 50 mM sodium phosphate, pH 8, 300 mM NaCl buffer. Fractions of 0.8 ml were collected and *At* Gci activity was measured as described above. The purity of the protein was analyzed by SDS-PAGE analysis (10% Criterion SF gel, Bio-Rad), and the fractions containing *At* Gci activity were pooled and concentrated by Vivaspin 20 centrifugal concentrator (MWCO 10,000 Da, Sartorius AG, Goettingen, Germany). The buffer was changed to 50 mM Tris-Cl, pH 8 by gel filtration on a PD-10 column (GE Healthcare Life Science, Uppsala, Sweden).

Kinetic Analysis of the Galactarolactone Cycloisomerase—The kinetic behavior of the *At* Gci was determined using D-galactarolactone and D-glucarolactone as substrates at 22 °C. The two lactone substrates were produced by *At* Udh starting from D-galacturonic acid or D-glucuronic acid in a reaction containing 10 to 20 mM of the uronic acid, the same molar amount of NAD^+, 10 mM $MgCl_2$, 50 mM sodium phosphate buffer, pH 6.8 and 0.01 μg/μl *At* Udh in a total volume of 90 μl. After 1 h of incubation at 22 °C, the uronic acid was assumed to be completely converted by the dehydrogenase into D-galactaro- or D-glucarolactone. This reaction mix was diluted with sodium phosphate buffer, pH 6.8, 5 mM $MgCl_2$ to get various substrate concentrations. Fourteen different substrate concentrations (0.05–15 mM) were used, and the kinetic measurements were performed in triplicate. The reactions were started by adding 0.6 μg *At* Gci enzyme in 10 μl, and the formation of KDG was measured with the TBA assay after 30 min of incubation at 22 °C.

pH Optimum—For determination of the pH optimum, D-galactarolactone was first produced in a separate reaction as

described. The produced lactone was incubated with 0.6 μg of *At* Gci in McIlvaine buffer (pH 2.5–7.5), Tris-Cl buffer (pH 8–8.5), and in NaOH-glycine buffer (pH 9 and 10) in a total volume of 200 μl. The reaction was stopped by addition of 12% TCA, and the formed KDG was measured using the TBA assay. The pH optimum was also determined on D-glucarolactone produced similarly to the D-galactarolactone.

Circular Dichroism Spectroscopy—Circular dichroism (CD) spectra were recorded on a Chirascan CD spectrometer (Applied Photophysics) equipped with a peltier thermally controlled cuvette holder (PCS.3 single cell holder). Far-UV (190–240 nm) CD measurements were performed with 3 μM enzyme in 10 mM sodium phosphate buffer, pH 8, at 25 °C, in a 1 mm path length cuvette. Spectra were recorded using two scans, a bandwidth of 1 nm and a wavelength step of 0.5 nm, and the values were corrected for buffer contribution.

The protein concentration was calculated from A_{280} measured using a NanoDrop spectrophotometer (NanoDrop technologies, Wilmington, Delaware), using a theoretical extinction coefficient calculated by ProtParam ($\epsilon = 65{,}430\ \text{M}^{-1}\ \text{cm}^{-1}$).

Determination of the Protein Oligomeric State—Analysis of the oligomeric state of the active *At* Gci was performed at 22 °C using a Superdex 200 HR 10/30 gel filtration column (GE Healthcare). *At* Gci (26 μg in 250 μl) was injected onto the column equilibrated with the running buffer (50 mM sodium phosphate buffer, pH 8, 150 mM NaCl) in which the enzyme is known to be active. The eluate was monitored at 280 nm, and fractions of 0.5 ml were collected and analyzed by SDS-PAGE gel electrophoresis. The molecular mass was determined by loading known molecular mass standards in the running buffer; ferritin (440,000 Da), aldolase (158,000 Da), ovalbumin (44,000 Da), and ribonuclease (13,700 Da). The void volume of the column was determined by running thyroglobulin. A standard curve of molecular masses was generated by plotting elution volume against log MW and fitted to a linear equation.

Production of Reference Enzymes—The *E. coli glucD* gene (10) was amplified by PCR, ligated into pRSET A vector (Invitrogen) and transformed into *E. coli* BL21(DE3) cells. The *Salmonella typhimurium* LT2 L-talarate/galactarate dehydratase gene (*STM3697*) (15) optimized for *E. coli* expression was synthesized by GeneArt, and amplified with a Strep-tag by PCR, ligated into pBAT4 and transformed into BL21(DE3). For production of GarL (KDG aldolase) we amplified the *garL* from *E. coli* (10) by PCR, ligated it to the pBAT4 vector, and transformed it to BL21 (DE3). After IPTG induction of *E. coli* cells expressing *Ec* GlucD (glucarate dehydratase), *St* TalrD/GalrD (L-talarate/galactarate dehydratase), or *Ec* GarL (KDG aldolase), cell extracts were made as described above and used for the assays. For the NMR analysis, the Strep-tagged *St* TalrD/GalrD protein was purified from the cell extracts basically as described for purification of *At* Gci.

Reaction Product Analysis—3-Deoxy-2-keto hexarate was produced by incubating 2 g of D-glucaric acid with *Ec* GlucD cell extract or 2 g galactaric acid with *St* TalrD/GalrD cell extract in 50 mM Tris-Cl buffer, pH 8.0, 10 mM $MgCl_2$ at 22 °C for 16 h. 3-Deoxy-2-keto hexarate was also produced by *At* Gci in a coupled assay with *At* Udh using equimolar amount of galacturonic acid or D-glucuronic acid and NAD^+. To purify the 3-deoxy-2-keto hexarate, the reaction mix was loaded to a Dowex 1X8 (formic acid form) column and eluted with a linear gradient from 0 to 6 M formic acid. The fractions containing 3-deoxy-2-keto hexarate were identified with the TBA assay. Fractions containing KDG were pooled and freeze-dried. The optical rotation of the KDG samples was measured at the 589 nm sodium D-line by AUTOPOL IV automatic polarimeter (t = 25 °C) (Rudolph Research Analytical Company) using a 50 mm cuvette. To test whether the 3-deoxy-2-keto-hexarate was active with the *E. coli* GarL, the 3-deoxy-2-keto-hexarate was mixed with the *Ec* GarL preparation and the formation of pyruvate followed in a coupled enzyme assay as described by Hubbard *et al.* (10).

NMR Spectroscopy—NMR experiments in H_2O buffer were carried out at 12 or 22 °C on 500 MHz or 600 MHz Bruker Avance III NMR spectrometers and the experiments in D_2O buffer on a 600 MHz Varian Inova NMR spectrometer. The 600 MHz Bruker spectrometer was equipped with a QCI Cryoprobe, while in the other spectrometers inverse detection room temperature probes were used. The chemical shifts were referenced to TSP (3-(trimethylsilyl) propionic-2,2,3,3-d_4 acid). To follow the enzyme reaction in real time by NMR, a mixture of D-galactaro-1,4-lactone and galactaric acid was produced by heating galactaric acid at 95 °C for 3 h in 0.1 M HCl. Before the enzymatic reactions the pH of the substrate was adjusted with 0.5 M sodium phosphate buffer to ~6.8. The *At* Gci reactions were then carried out in 50 mM sodium phosphate buffer prepared either in H_2O or D_2O, and containing 2 mM $MgCl_2$. In the case of H_2O as the solvent, D_2O needed for the spectrometer lock was confined in a coaxially located glass capillary, so the samples were in a 100% H_2O environment. After acquiring a one-dimensional ^1H NMR spectrum, purified enzyme was added, and the reaction was followed by acquiring a series of ^1H NMR spectra over 1 h. For each spectrum, 16 scans were acquired with 2 s presaturation of the water signal and 5.5 s acquisition time. Thus, each spectrum is a sum of 16 scans that were recorded over 2 min.

For structural analysis of the reaction product, 1D ^{13}C spectrum and DEPT-135 were recorded with 1024 scans and 2-s relaxation delays. For DQFCOSY, TOCSY (mlev17) and HSQC spectra matrices of 2048 × 256 points were recorded, zero-filled once in F1, and multiplied by a cosine weighting function in both dimension prior to the Fourier transformation. The mixing time in TOCSY was 80 ms.

RESULTS

Identification of the A. tumefaciens gci Gene—The *A. tumefaciens* strain C58 was grown on D-galacturonic acid as a sole carbon source, and a cell extract was shown to have activity toward both the linear galactarate and the cyclic galactarolactone form to convert them both to KDG as judged by the TBA assay. D-Galactarolactone used for this test was produced using *A. tumefaciens* uronate dehydrogenase (*At* Udh), which oxidizes D-galacturonate in the presence of NAD^+ to D-galactarolactone (7, 8). In the cell-free enzyme preparation, the enzymatic activity with D-galactarolactone was higher than with linear galactarate and was purified from the crude cell extract using a hydrophobic interaction and two anion exchange chro-

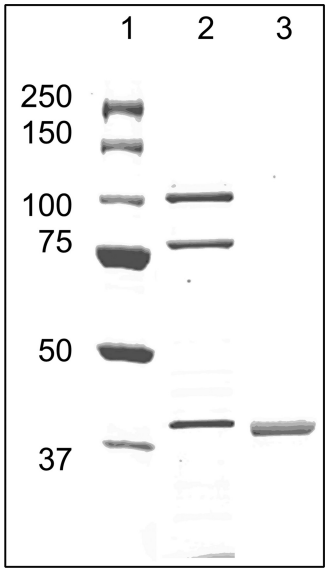

FIGURE 2. **SDS-PAGE of purified *At* Gci.** *Lane 1*, molecular weight marker proteins in kDa; *lane 2*, native *At* Gci partially purified from *A. tumefaciens* using HIC, DEAE and Resource Q chromatography; and *lane 3*, *At* Gci expressed and purified from *E. coli*. The proteins were visualized using the Criterion Stain Free gel imaging system (Bio-Rad).

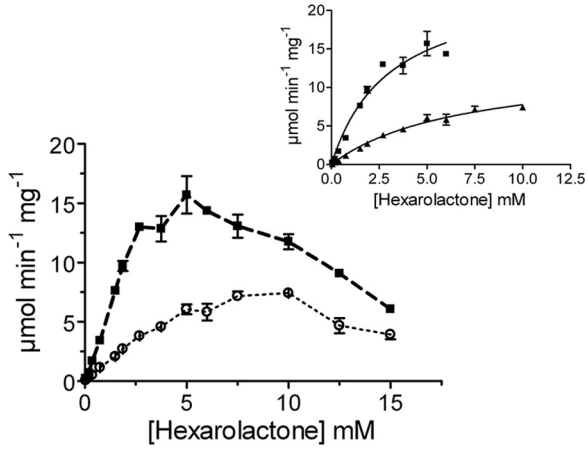

FIGURE 3. **Kinetic properties of the purified *At* Gci on D-galactaro- and D-glucarolactone.** Kinetic data were obtained from incubations of 0.6 μg of enzyme with D-galactaro- or D-glucarolactone (0.05–15 mM) in sodium phosphate buffer pH 6.8, 5 mM $MgCl_2$ at 22 °C. *Inset*: original data showing the measured initial rates in the substrate concentration area from 0- 10.0 mM. The apparent kinetic parameters were obtained by curve fitting analysis using Graphpad Prism software 4.01.

matography steps. After these three purification steps, the enzyme was not completely pure (Fig. 2); nevertheless, the partially purified enzyme showed activity only toward D-galactarolactone and not to the linear galactarate. An ~40 kDa band in the SDS-PAGE was identified since the enzyme activity correlated with this band intensity. The band was in-gel-trypsinated, and MALDI-TOF mass spectrometry analysis gave the following masses: 628.372, 771.334, 851.353, 1134.473, 1151.577, 1218.566, 1269.458, 1377.529, 1459.566, 1475.587, and 1632.655. Based on the genome sequence of *A. tumefaciens* strain C58, the Atu3139 gene was identified. As the gene was not annotated, we called it *gci* for galactarolactone cycloisomerase. To verify that we had identified the right gene, the *gci* (Atu3139) gene was expressed in the pBAT4 vector in *E. coli* BL21(DE3), and the crude cell extract was shown to have the galactarolactone cycloisomerase activity (0.3 ± 0.05 units per mg of extracted protein).

Purification and Characterization of the A. tumefaciens Galactarolactone Cycloisomerase—To facilitate the purification, an affinity tag (Strep II) was attached to the N or C terminus of *At* Gci, and the proteins were expressed under the control of the T7 promoter in *E. coli* BL21(DE3) cells. Only the *At* Gci with C-terminal Strep-tag bound to the Strep-tactin column and was therefore purified and used for the characterization work. Approximately 0.8 mg of protein from a 1-liter cell culture could be purified with estimated purity higher than 95%, as judged from SDS-PAGE (Fig. 2).

The enzyme activity was assayed by measuring the amount of KDG produced from D-galactarolactone, prepared enzymatically by *At* Udh and using the TBA assay. Besides D-galactarolactone, also the D-glucarolactone (produced with D-glucuronic acid, NAD^+ and *At* Udh) was a substrate of the purified *At* Gci, whereas linear galactaric acid was not. These results were confirmed by NMR analyses (see below). The activity was further shown to be dependent on the presence of exogenous Mg^{2+} ions. Addition of 1 mM EDTA followed by gel filtration in 50 mM Tris-Cl buffer, pH 8.0 to *At* Gci, resulted in the immediate loss of all activity, while addition of Mg^{2+} to the apoenzyme resulted in a complete restoration of the original activity. The optimal pH for conversion of D-galactaro- and D-glucarolactone was found to be at 8.5 for both substrates (data not shown).

The kinetic constants were determined on D-galactaro- and D-glucarolactone (produced in a coupled assay with *At* Udh). The reactions catalyzed by *At* Gci did not show a saturation profile when plotting the rate of product formation *versus* substrate concentration (Fig. 3). This could possibly be due to substrate inhibition at higher concentrations of substrate, although other reasons for this non Michaelis-Menten behavior cannot be excluded. From the profiles of the curves, it was evident that the maximum rate was higher on D-galactarolactone than on D-glucarolactone. Also the affinity seemed to be higher (*i.e.* K_m value lower) for D-galactarolactone, resulting in overall higher catalytic efficiency on this substrate. As can be seen from the inset in Fig. 3, catalytic efficiency (k_{cat}/K_m) of ~4× higher for D-galactarolactone (330×10^3 M^{-1} min^{-1}) than for D-glucarolactone (80×10^3 M^{-1} min^{-1}) was obtained. However, these values have to be taken as approximate.

SDS-PAGE analysis revealed that the *At* Gci subunit M_r was 41,000, which is in approximate agreement with the calculated M_r of 42,600. The oligomeric M_r was determined as 340,900 by gel filtration, suggesting an octameric species. The far-UV CD spectrum of *At* Gci measured at 25 °C was shown to have characteristic double minima at 208 and 223, consistent with an α-helical protein conformation (16) (data not shown).

NMR Analysis of the Products from D-Galactarolactone Conversion—A mixture of D-galactaro-1,4-lactone and linear galactaric acid was prepared by heating linear galactaric acid in 0.1 M HCl, and used in NMR experiments as a substrate for *At* Gci and the control enzyme *S. typhimurium* talarate/galactarate dehydratase (*St* TalrD/GalrD). The ^1H NMR spectra obtained after complete reactions (Fig. 4) verified that only the lactone is a substrate for *At* Gci and, on the other hand, only the

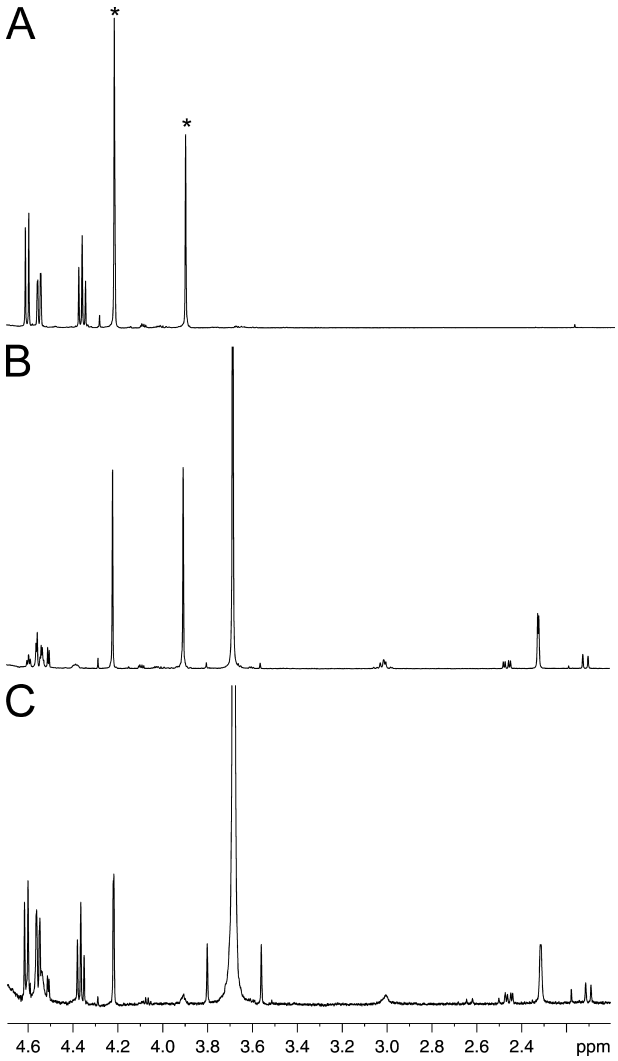

FIGURE 4. *A*, 600 MHz ^{1}H NMR spectrum of a mixture of galactaric acid and D-galactaro-1,4-lactone in H_2O at 22 °C. The galactaric acid signals at about 3.9 and 4.2 ppm are marked by *asterisks*, while the lactone signals (two doublets at 4.5–4.6 ppm, a triplet 4.35 ppm, and a narrow doublet overlapping with the galactaric acid signal at 4.2 ppm) are unmarked. *B*, ^{1}H NMR spectrum of a complete reaction of the mixture of galactaric acid and D-galactaro-1,4-lactone with *At* Gci. The lactone signals, which were unmarked in the *upper panel* have disappeared, while the *asterisk*-marked galactaric acid signals remain unchanged. New signals arising from the product KDG have appeared at 4.3–4.6 ppm, 3.9 ppm, 3.0 ppm, and between 2.1 and 2.5 ppm. The intensity of the product signals is low, because several different forms of the product are present. *C*, ^{1}H NMR spectrum of a complete reaction of the mixture of galactaric acid and D-galactaro-1,4-lactone with the control enzyme TalrD/GalrD. In this reaction, the galactaric acid signals have disappeared, while the lactone signals remained unchanged. The signals of the product are identical with both of the enzymes. The large signal at 3.9 ppm in *panels B* and *C* originates from the Tris buffer of the enzyme preparations.

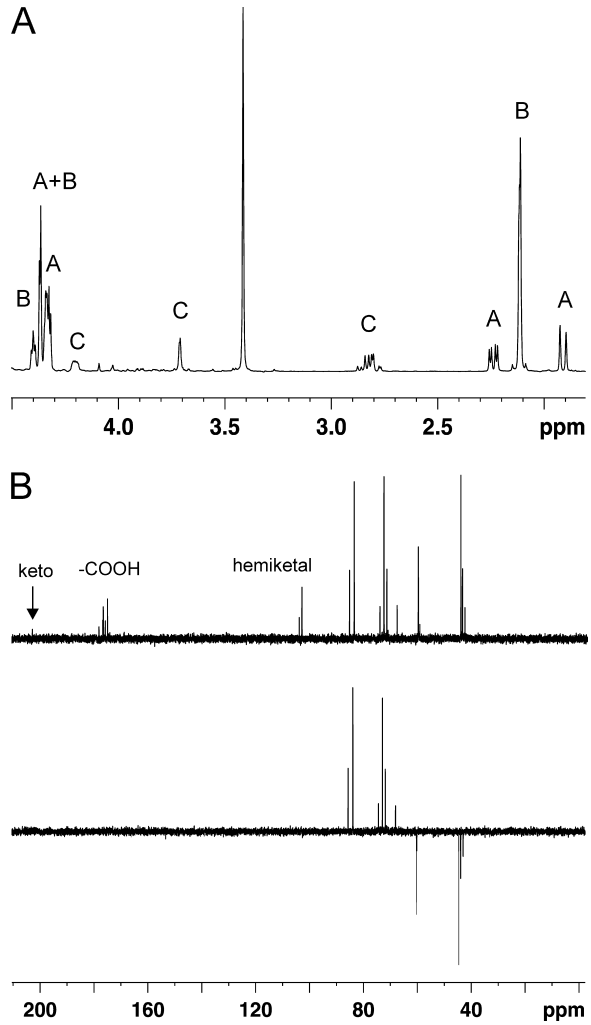

FIGURE 5. *A*, 500 MHz ^{1}H NMR spectrum of KDG. The *letters* above the spectrum indicate the three different spin-systems identified from a COSY spectrum. The presence of three distinct spin systems indicates that KDG exists in three different forms. *B*, ^{13}C NMR and DEPT135 spectra of 3-deoxy-2-keto-hexarate. In DEPT135 spectrum, CH_3 and CH carbons give positive signals, while CH_2 carbon signals are negative. Comparison of the ^{13}C spectrum with the DEPT spectrum was used to identify the signals of the quaternary carbons which are not present in the DEPT spectrum.

linear galactaric acid is a substrate for the control enzyme. The NMR spectra of the products of the two enzymes were identical. The product of the control enzyme has been identified earlier (15), but since our spectra had some differences from the published ones, we repeated the structural identification by NMR. The COSY spectra (not shown) revealed that there are three different spin systems (indicated on top of the ^{1}H spectrum in Fig. 5*A*). This is different than results published earlier, where only two spin systems were identified. Assignment of a DEPT-135 spectrum (Fig. 5*B*) from an HSQC spectrum (not shown) indicated that each spin system has one CH_2 group and two CH groups, which is consistent with the structure of the 3-deoxy-2-keto-hexarate (KDG). In addition, a ^{13}C spectrum (Fig. 5*B*) revealed the carboxyl carbons as well as two other quaternary carbons at about 104 ppm, which is a typical chemical shift for carbons in hemiacetal or hemiketal structures of sugar rings. This indicated that the carbonyl group of the KDG forms a hemiketal furanose ring having two anomers. The third form is the linear KDG, as indicated by a very weak quaternary carbon signal of the keto group at 203 ppm. The ^{1}H and ^{13}C chemical shifts of the three forms are given in supplemental Table S1. When the *At* Gci reaction is carried out in D_2O, one of the two protons in the CH_2 groups of the product is replaced by deuterium (supplemental Fig. S1). The replaced protons are the 3-pro-*R* protons as has been reported for the *St* TalrD/GalrD acting on the linear galactaric acid. Thus, the hydroxyl group of D-galactarolactone was replaced by deuterium with retention of the configuration.

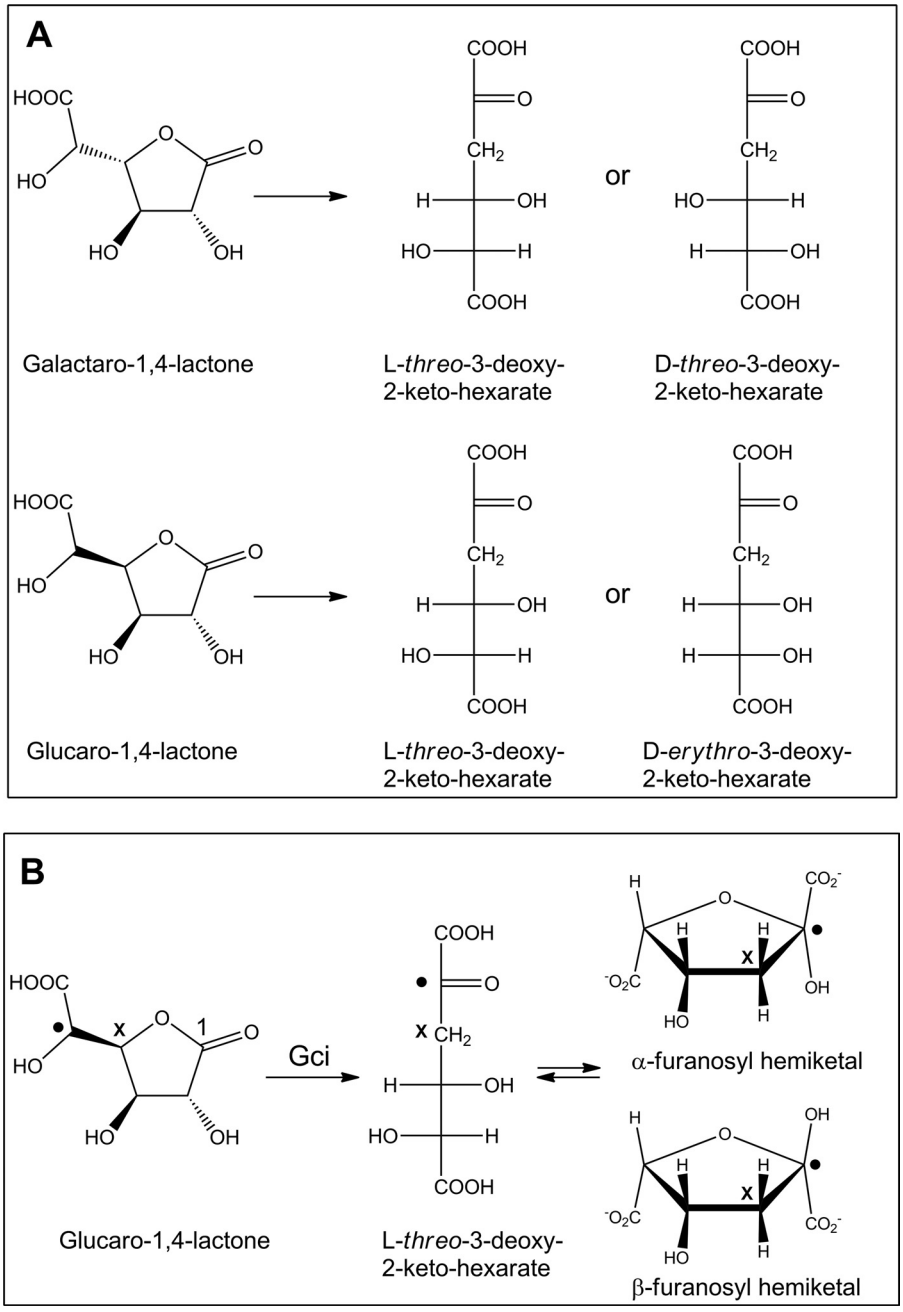

FIGURE 6. *A*, configuration of possible reaction products obtained by conversion of D-galactaro-1,4-lactone and D-glucaro-1,4-lactone by *At* Gci. *B*, configuration of the reaction products obtained in the reaction of *At* Gci with D-glucaro-1,4-lactone. The carbons involved in reaction are marked with a *filled circle* (●, C4 in the substrate) and a *cross* (x, C5 in the substrate). C3 in the product correspond to C4 in the substrate.

Configuration of the Product from D-Galactaro- and D-Glucarolactone—Both D-galactaro- and D-glucarolactone were substrates for *At* Gci and produced 3-deoxy-2-keto hexarate (KDG) as a product (see also below). The members of the enolase superfamily share a common initial reaction step in which the α-proton of a carboxylate substrate is abstracted to form an enediolate intermediate. Depending on whether the reaction takes place at positions 2 and 3, or 4 and 5 of the D-glucaro-1,4-lactone, D-*erythro* 3-deoxy-2-keto-hexarate or L-*threo* 3-deoxy-2-keto-hexarate, respectively, can be formed (Fig. 6*A*). To identify the reaction product, the partially purified product of *At* Gci, 3-deoxy-2-keto-hexarate was in both cases tested as a substrate for the reference enzyme *E. coli* KDG aldolase (*Ec* GarL), which is specific for 3-deoxy-2-keto-L-*threo*-hexarate (17). Should the *At* Gci produce either D-*threo* or D-*erythro* form of the 3-deoxy-2-keto-hexarate, this would not be converted to pyruvate and glyceraldehyde by the aldolase. However, *Ec* GarL was shown to be active on the product obtained by *At* Gci from conversion of D-galactarolactone and D-glucarolactone, suggesting that the reaction product is 3-deoxy-2-keto-L-*threo*-hexarate in both cases. We also measured the optical rotation of the purified 3-deoxy-2-keto-hexarate solutions and detected a positive rotation of the polarized light for the 3-deoxy-2-keto-hexarate obtained from D-galactarolactone and D-glucarolac-

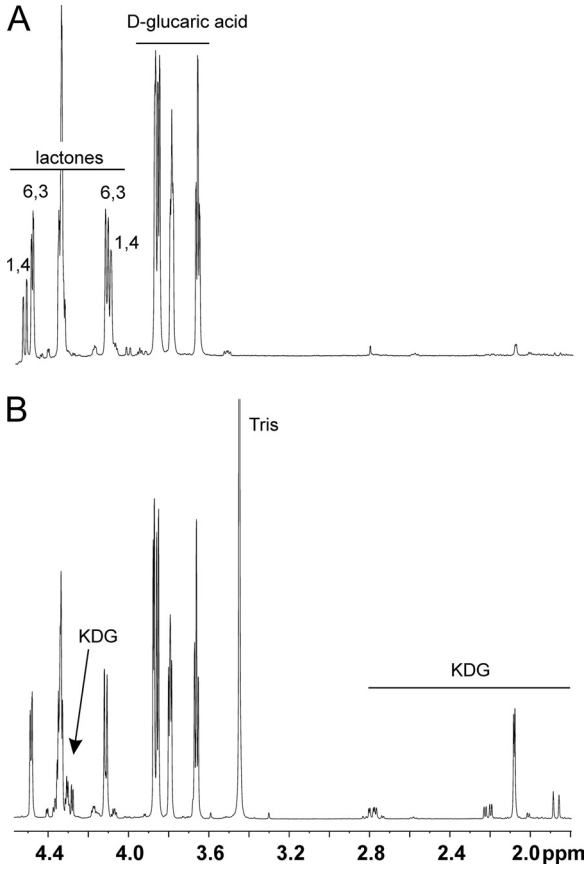

FIGURE 7. *A*, expansion of a 500 MHz ^{1}H NMR spectrum of a mixture of D-glucaric acid and its 1,4- and 6,3-lactones in H_2O at 12 °C. The D-glucaro-1,4- and 6,3-lactone signals from which the substrate specificity of *At* Gci was observed are labeled as 1,4 and 6,3, respectively. *B*, ^{1}H NMR spectrum of the same sample after a complete reaction with *At* Gci. The signals of the D-glucaro-1,4-lactone have disappeared, while the signals of D-glucaro-6,3-lactone and D-glucaric acid remain unchanged. This indicates that only the 1,4-lactone is a substrate of the enzyme. The product gives signals identical to the ones obtained from the reaction with D-galactaro-1,4-lactone (Fig. 4*B*), although the exact chemicals shifts are different because of the lower measurement temperature.

tone, providing further support that 3-deoxy-2-keto-L-*threo*-hexarate is produced. The 3-deoxy-2-keto-L-*threo*-hexarate causes a positive rotation and the D-*threo* form a negative rotation of polarized light (18).

Reaction Mechanism on D-Glucarolactone by At Gci Analyzed by NMR—To get deeper insights into the reaction mechanism, the reaction of *At* Gci with D-glucarolactone was also studied by NMR (Fig. 7). Unlike galactaric acid, D-glucaric acid is not a symmetrical molecule and therefore its different lactone forms (1,4 and 3,6 being the major stable forms) (19) as well as the potential products can be readily distinguished by NMR. A mixture of D-glucaric acid and its lactones was prepared by heating D-glucaric acid in 0.1 M HCl. The signals of different compounds of the neutralized mixture were identified by comparison to the spectra of D-glucaric acid and commercial D-glucaro-1,4-lactone (data not shown). The signals not matching the two model compounds were assigned to D-glucaro-6,3-lactone (19). In addition, small signals possibly representing a dilactone or six-membered lactone ring (l,5- or 6,2-lactone) were observed. When this mixture was incubated in an NMR tube with *At* Gci, the signals of the D-glucaro-1,4-lactone disappeared, and new signals identical to the KDG produced by *At* Gci from D-galactarolactone appeared (Fig. 7*B*). The signals of the 3,6-lactone as well as linear D-glucaric acid were unchanged, indicating that only the D-glucaro-1,4-lactone but not the 6,3-lactone or linear D-glucaric acid is a substrate for *At* Gci (data not shown). Furthermore, the lactones formed in the reaction where D-glucuronic acid was incubated with *At* Udh, were also analyzed by NMR. In this reaction, approximately half of the formed lactone was shown to exist as D-glucaro-1,4-lactone and the other half as D-glucaro-6,3-lactone. In this mixture again only the D-glucaro-1,4-lactone was utilized by *At* Gci (data not shown).

As explained in the previous section, from D-glucaro-1,4-lactone, either D-*erythro* 3-deoxy-2-keto-hexarate or L-*threo* 3-deoxy-2-keto-hexarate, can be formed (Fig. 6*A*), and our biochemical data suggested the formation of 3-deoxy-2-keto-L-*threo*-hexarate. NMR was used to give further support for this result. Taking together the NMR data, it could be demonstrated that the substrate for the *At* Gci is D-glucaro-1,4-lactone and that the reaction takes place at the carbons in positions 4 and 5 (not part of the lactone ring) (Fig. 6*B*).

Similar to the reaction of *At* Gci with D-galactarolactone, the reaction with D-glucarolactone was also followed in D_2O by NMR, and the hydrogen replaced by deuterium was again shown to be the 3-pro-*R* of the product 3-deoxy-2-keto-hexarate (see Fig. 6*B* for numbering). Thus, the reaction with D-glucaro-1,4-lactone occurs with inversion of configuration.

Sequence Analysis of At Gci—Amino acid sequence analysis revealed that *At* Gci belong to the mandelate racemase-subgroup (COG4948) of the large enolase superfamily. The multiple sequence alignment shown in supplemental Fig. S2 depicts highly conserved active site sequence motifs present in the members of the mandelate racemase-subgroup. The three conserved residues, Asp-194, Glu-220, and Glu-246, found in *At* Gci, are equivalent to the residues involved in the binding of an essential Mg^{2+} in mandelate racemase (20). From the sequence comparison *At* Gci is also suggested to possess the acid/base catalyst Lys-166, the His-296 –Asp-269 dyad, as well as Glu-329 suggested to act as a general acid catalyst in the 1,1-proton transfer reaction catalyzed by mandelate racemase (21).

Using the *At* Gci amino acid sequence for a BLASTP search in the NCBI data base, orthologs with high sequence identity, 77 and 72%, were found in *e.g. Xanthobacter autotrophicus* (Xaut_3370) and *Mesorhizobium* sp. BNC1 (Meso_1946), respectively. A further search against the Protein Data Bank revealed several homologues with ~30% identity, most of them having putative functions, but all belonging to the mandelate racemase-like family. The highest identity was found with a putative mandelate racemase from *Bordetella bronchiseptica* Rb50 (PDB 3H12), and a putative dehydratase from *Zymomonas mobilis* (PDB 2OX4). Of the known functional proteins deposited in PDB, the highest identity was found with *S. typhimurium* TalrD/GalrD (PDB 2PP0) (15), which was 32% identical to the sequence of *At* Gci. The sequence identity to galactarate dehydratase Ob2843 from *Oceanobacillus iheyensis* (PDB 2OQY) (18) was only 21%.

DISCUSSION

Chang and Feingold (4, 5) have reported earlier that galactarate dehydratase activity is present in the cell extract made from *A. tumefaciens* grown on D-galacturonic acid or galactaric acid as a sole carbon source. This galactarate dehydratase converted galactarate to 3-deoxy-2-keto-hexarate. The authors also noticed that the activity was more pronounced when D-galacturonate and NAD^+ were used as substrate instead of galactarate. Here we could confirm these earlier observations as the crude extract of *A. tumefaciens* strain C58 grown on D-galacturonic acid had the enzyme activity for the conversion of galactarate to 3-deoxy-2-keto-hexarate, and when NAD^+ and D-galacturonate were used as substrates, this activity was improved.

We have recently cloned and characterized the first enzyme in the oxidative galacturonic pathway of *A. tumefaciens,* the uronate dehydrogenase (*At* Udh), and also determined its three-dimensional structure in complex with the cofactor and product (7, 8). The *At* Udh enzyme is NAD^+ specific and produces from D-galacturonic acid D-galactaro-1,5-lactone, that rapidly rearranges to the D-galactaro-1,4-lactone, which is rather stable even at neutral pH of 7.0 as shown by NMR (7, 8). Galactarolactones are not commercially available, but with the help of the *At* Udh we could produce D-galactaro-1,4-lactone from D-galacturonate. This enabled here the purification of the novel *A. tumefaciens* enzyme activity for the conversion of D-galactarolactone to 3-deoxy-2-keto-hexarate. During the purification the galactarate dehydratase activity was lost, demonstrating that there are indeed two enzymes producing 3-deoxy-2-keto-hexarate. The corresponding gene for the conversion of D-galactarolactone to 3-deoxy-2-keto-hexarate was identified as Atu3139, which codes for a 378 amino acid enzyme. This enzyme is not a sugar acid dehydratase, since the substrate and the product have the same stoichiometry, $C_6H_8O_7$. We suggest calling it a galactarolactone 3-deoxy-2-keto-L-*threo*-hexarate isomerase, or shortly galactarolactone cycloisomerase (*At* Gci). It is an intramolecular lyase and belongs to the group of enzymes with the Enzyme Commission number EC 5.5.1.-.

The *At* Gci was produced in *E. coli* as a Strep-tagged version, purified and used for detailed characterization of the reaction mechanism. NMR analysis showed that the five-membered D-galactaro-1,4-lactone is the substrate for *At* Gci. As *At* Udh also accepts D-glucuronic acid as a substrate, it is reasonable to assume that similarly to the oxidation of the D-galacturonic acid, *At* Udh can oxidize D-glucuronic acid in its pyranose form to D-glucaro-1,5-lactone ring that is spontaneously rearranged to the D-glucaro-1,4-lactone. In solution D-glucarolactone can exist as a mixture of the monolactones, D-glucaro-1,4-lactone and D-glucaro-6,3-lactone, and the dilactone D-glucaro-1,4:6,3-dilactone (19), or as the six-membered glucaro-1,5- or 6,2-lactones Our NMR analysis showed that only the D-glucaro-1,4-lactone form was utilized by *At* Gci, consistent with the results obtained with the D-galactarolactone. Furthermore, we found *At* Gci to be more active on D-galactaro-1,4-lactone than on D-glucaro-1,4-lactone. Although we were unable to determine the kinetic constants for *At* Gci, the saturation curves indicated the catalytic efficiency to be ~3-fold higher on the D-galactaro-1,4-lactone than on the D-glucaro-1,4-lactone. The enzyme was also found to be inhibited by the hexarolactone substrates at concentrations above 5–10 mM. Since the two hexarolactones can exist in many different forms, most of which are not commercially available, or would be difficult to produce enzymatically or chemically in a stable form, it would be challenging to find out which form of the lactone inhibits the enzyme.

The NMR spectra of *At* Gci reaction products on D-galactaro-1,4-lactone and D-glucaro-1,4-lactone as a substrate were identical. These spectra were also identical with the spectra of the product of *St* TalrD/GalrD dehydratase on galactarate, which has been reported to be 3-deoxy-2-keto-L-*threo*-galactarate (Figs. 4 and 7). In the conversion of D-glucaro-1,4-lactone with *At* Gci it was clear that the reaction product is 3-deoxy-2-keto-L-*threo*-glucarate. But for the identification of the reaction product from D-galactaro-1,4-lactone other methods were needed, because the L-*threo* and the D-*threo* form of 3-deoxy-2-keto-hexarate would have identical NMR spectra. These further demonstrated that the product is indeed also 3-deoxy-2-keto-L-*threo*-hexarate produced from D-galactaro-1,4-lactone (Fig. 6).

The *At* Gci belongs to the large mechanistically diverse enolase superfamily, whose members share a common reaction step in which the α-proton of the substrate is abstracted from a carbon adjacent to a carboxylic acid to form an enediolate intermediate. Since *At* Gci catalyzes formation of 3-deoxy-2-keto-L-*threo*-hexarate from both D-galactaro- and D-glucaro-1,4-lactone (Fig. 6), the reaction has to be initiated by proton abstraction from C5 followed by leaving of the 4-OH group. The leaving group is the hydroxyl that has formed the lactone ring with the carboxyl group at C1. Reactions studied in D_2O further supported that the proton abstraction takes place next to the carboxylic acid pointing out from the lactone ring. Notably, superposition of the two substrates show the part involved in the lactone ring to be identical in D-glucaro-1,4- and D-galactaro-1,4-lactone, whereas the carbons C5-C6 left outside the ring are pointing up in the case of D-galactaro-1,4-lactone and down in the case of the D-glucaro-1,4-lactone ring. For the reaction to take place the substrate further needs to be bound to the essential Mg^{2+}. Although the two lactone substrates differ at the carbons C5 and C6, the carbons can be rotated in such a way that the carboxyl group at position 6 in both substrates would be positioned very close in the active site, thus enabling binding to Mg^{2+}. The initial proton abstraction from C5 in D-galactaro- and D-glucaro-1,4-lactone could possibly be catalyzed by distinct amino acids, since the hydroxyl groups at C5 in the two substrates are positioned far from each other. Because of the planar carbon-carbon double bond in the enolate intermediate, the 4-OH leaving group will be replaced by solvent deuterium in a reaction which is retaining for D-galactarolactone and inverting for D-glucarolactone. In the studied reaction mechanism of other mandelate racemase subgroup dehydratases, such as *St* TalrD/GalrD dehydrates, the proton abstractions from the two substrates, galactarate and L-talarate, are initiated by distinct residues in the active site. Lys197 is functioning as the base in the reaction with galactarate and His328 with the C2 epimer L-talarate. From both substrates the same reaction

product, 3-deoxy-2-keto-L-*threo*-hexarate is formed (15). For L-fuconate dehydratase L-fuconate, L-galactonate, and D-arabonate are substrates but also the C2 epimers of L-galactonate and D-arabonate namely L-talonate and D-ribonate (22). As for *St* TalrD/GalrD the initial proton abstraction is catalyzed by a different amino acid residue for the two groups of substrate epimers.

The mandelate racemase subgroup into which *At* Gci belongs hosts several distinct protein families in addition to the mandelate racemase protein family. At least seven families of sugar acid dehydratases are part of this subgroup, and for instance galactarate dehydratase from *S. typhimurium* is a member in this subgroup. The members of the enolase superfamily share a conserved bi-domain structure with an N-terminal $\alpha+\beta$ capping domain and a modified TIM barrel domain as a C-terminal domain. The CD spectra of *At* Gci showed α-helical character, consistent with a TIM barrel fold. In addition, sequence comparison of *At* Gci with members of the mandelate racemase family show that it contains the same conserved amino acid residues which can be found in the active site of the structurally characterized mandelate racemase and are positioned at the ends of the second, sixth, or seventh β-strands in the $(\beta/\alpha)_7\beta$-barrel domain (supplemental Fig. S2) (20). The ligands for the essential magnesium are also conserved in *At* Gci, and we also found the activity of *At* Gci to be dependent on the divalent cation Mg^{2+} as all members in the enolase superfamily. By gel filtration analysis the enzyme is suggested to exist as an octamer similarly to reported for *e.g. St* TalrD/GalrD galactarate dehydrates, and *P. putida* mandelate racemase and muconate lactonizing enzyme (15, 23).

Taken together, the sequence comparison, structural analysis, and biochemical data suggests *At* Gci to be a novel activity in the growing mandelate subgroup of the enolase superfamily. A detailed picture of all structural elements in catalysis and metal coordination will only be obtained by x-ray crystallographic analysis of the protein, and we are now in the process of solving the three-dimensional structure of this enzyme.

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