Identification in the Mold *Hypocrea jecorina* of the First Fungal D-Galacturonic Acid Reductase†

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ABSTRACT: A D-galacturonic acid reductase and the corresponding gene were identified from the mold *Hypocrea jecorina* (*Trichoderma reesei*). We hypothesize that the enzyme is part of a fungal D-galacturonic acid catabolic pathway which has not been described previously and which is distinctly different from the bacterial pathway. *H. jecorina* grown on D-galacturonic acid exhibits an NADPH-dependent D-galacturonic acid reductase activity. This activity is absent when the mold is grown on other carbon sources. The D-galacturonic acid reductase was purified, and tryptic digests of the purified protein were sequenced. The open reading frame of the corresponding gene was then cloned from a cDNA library. The open reading frame was functionally expressed in the yeast *Saccharomyces cerevisiae*. A histidine-tagged protein was purified, and the enzyme kinetics were characterized. The enzyme converts in a reversible reaction from D-galacturonic acid and NADPH to L-galactonic acid and NADP. The enzyme also exhibits activity with D-glucuronic acid and DL-glyceraldehyde.

Since D-galacturonic acid is enriched in pectin-containing biomass, it is a potential carbon source for microorganisms living on decaying plant material. For bacteria, a pathway is known to consist of five enzymes converting D-galacturonic acid (D-galacturonate) to pyruvate and D-glyceraldehyde 3-phosphate. The intermediate metabolites are D-tagaturonate, D-altronate, 2-dehydro-3-deoxy-D-gluconate, and 2-dehydro-3-deoxy-D-gluconate 6-phosphate. The enzymes are uronate isomerase (EC 5.3.1.12) (*1*), an NADH-utilizing D-tagaturonate reductase (EC 1.1.1.5) (*2*), altronate dehydratase (EC 4.2.1.7) (*3*), 2-dehydro-3-deoxy-D-gluconatekinase (EC 2.7.1.45) (*4*), and 2-dehydro-3-deoxy-D-gluconate-6-phosphate aldolase (EC 4.1.2.14) (*5*) as shown in Figure 1. This pathway was only described for prokaryotic organisms. There is only limited knowledge about the metabolic route for D-galacturonic acid catabolism in eukaryotic organisms; however, such a metabolic route exists since many species of yeast and mold are able to utilize and grow on D-galacturonic acid. There are no reports about genes which are similar to the genes of the bacterial D-galacturonic acid pathway in the genome of any eukaryotic microorganism of which the genome was sequenced. This suggests that there is a eukaryotic path for the catabolism of D-galacturonic acid which is different from the bacterial path.

There are a few studies on the D-galacturonic acid path in the mold *Aspergillus nidulans*. Mutants which were not able to utilize D-galacturonate were identified as transaldolase and pyruvate dehydrogenase mutants (*6*). In a later work, it was shown that a mutant defective in pyruvate dehydrogenase could not grow on D-galacturonic acid whereas a mutant defective in pyruvate kinase could grow. This was interpreted that D-galacturonate is converted to pyruvate but not through phosphoenolpyruvate (*7*). It was also shown that an NADP-dependent glycerol dehydrogenase was induced on D-galacturonic acid (*8*).

In *Aspergillus niger*, an NADP-dependent glycerol dehydrogenase is also induced on D-galacturonic acid, and this enzyme was shown to be active with D-glyceraldehyde in the reverse reaction and not with dihydroxyacetone (*9*). Visser et al. (*10*) concluded that D-galacturonic acid is metabolized through glyceraldehyde and pyruvate with glyceraldehyde being metabolized through glycerol.

This already suggests that there is a eukaryotic path different from the bacterial path since glyceraldehyde and not glyceraldehyde 3-phosphate is an intermediate.

A D-galacturonic acid reductase activity has not been previously described in fungi. However, it has been reported in some other eukaryotic organisms, for example, in plant. In ripe strawberry fruit, the NADPH-dependent D-galacturonic acid reductase activity was observed and the corresponding gene identified (*11*); however, here the enzyme is not part of a catabolic pathway but was suggested to be part of the synthetic pathway for ascorbic acid. In rat kidney, a glucuronolactone reductase (EC 1.1.1.20) was described. This NADPH-dependent enzyme also exhibited activity with D-galacturonic acid (*12*).

MATERIALS AND METHODS

Strains and Growth Conditions. Escherichia coli strain DH5α was used in the cloning procedures. It was grown in Luria-Bertani medium with ampicillin at 37 °C. The *Hypo-*

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crea jecorina (*Trichoderma reesei*) strain used in all experiments was Rut C-30. It was grown in a medium containing 20 g/L D-galacturonic acid/sodium D-galacturonate (pH 7.0) (or another carbon source when specified), 2 g/L proteose peptone, 15 g/L KH_2PO_4, 5 g/L $(NH_4)_2SO_4$, 0.6 g/L $MgSO_4 \cdot 7H_2O$, 0.8 g/L $CaCl_2 \cdot 2H_2O$, and trace elements (*13*) at 28 °C. *Saccharomyces cerevisiae* strain CEN.PK2-1B (VW1b) was used. It was grown in synthetic complete (SC) medium (*14*) which lacked uracil when required for selection.

Enzyme Activity Measurements. In the standard procedure for measuring D-galacturonic acid reductase activity, 25 μL of the enzyme preparation, or cell extract, was added to 200 μL of buffer containing 100 mM sodium phosphate (pH 7.0) and 0.25 mM NADPH. The reaction was started by adding 25 μL of D-galacturonic acid to a final concentration of 94 mM. To measure the Michaelis−Menten constants for D-galacturonic acid, D-glucuronic acid, and DL-glyceraldehyde, their concentrations were varied and the NADPH concentration was 0.3 mM; when the constants were measured for NADPH, its concentration was varied and the D-galacturonic acid concentration was 50 mM. The activities were calculated from the decrease in NADPH absorbance at 340 nm. Linear L-galactonic acid and L-gulonic acid were obtained by dissolving L-galactonic acid γ-lactone and L-gulonic acid γ-lactone, respectively, in water, heating, and adding sodium hydroxide until the pH stopped changing and could be set to pH 6 at room temperature. To measure the Michaelis−Menten constants for L-galactonic acid, L-gulonic acid, and glycerol, their concentration was varied and the NADP concentration was 0.2 mM; when the constants were measured for NADP, its concentration was varied and the L-galactonic acid concentration was 30 mM. The following substrates were also tested with NADPH as a cofactor: D-glucose (20 g/L), D-fructose (20 g/L), D-xylose (20 g/L), D-galactose (20 g/L), L-arabinose (20 g/L), and D-mannose (20 g/L). In the reverse direction with NADP, we tested L-gluconic acid (20 g/L), glycerol (22 g/L), D-arabitol (20 g/L), L-arabitol (15 g/L), xylitol (20 g/L), galactitol (2.5 g/L), and ribitol (adonitol) (20 g/L).

All the enzymatic assays were conducted with a Cobas Mira automated analyzer (Roche) at 30 °C. All activities were given in units of activity (U) which is defined as the activity that converts 1 μmol of substrate/min. The amount of protein was determined with the Bio-Rad protein assay using γ-globulin as the standard.

Purification of the D-Galacturonic Acid Reductase. H. jecorina (Rut C-30) was grown in a medium containing 20 g/L D-galacturonic acid as a carbon source. Four grams of mycelia was harvested by filtration. The lysis and all the purification steps were performed at 4 °C; 150 mg of mycelia, 600 mg of glass beads (0.5 mm diameter), and 450 μL of buffer containing 100 mM sodium phosphate (pH 7.0), 0.5 mM EDTA, 5 mM DTT, and Complete protease inhibitor (Roche) were vortexed in a Mini Bead Beater (Stratech Scientific) two times for 1 min. The tubes were centrifuged in a minicentrifuge (Eppendorf) at full speed for 25 min and the supernatants collected. The crude extract had a protein content of 6 g/L and a D-galacturonic acid reductase activity of 0.2 U/mg of extracted protein. Ten milliliters of this crude cell extract was desalted by gel filtration with PD10 columns (Pharmacia) which were equilibrated with a buffer containing 5 mM sodium phosphate (pH 7.0), 0.5 mM EDTA, and 5 mM DTT; 12 mL of desalted cell extract was loaded onto a column with 15 mL of Fractogel TSK DEAE-650 (M) (Merck) and the enzyme activity eluted with a linear gradient from 0 to 300 mM NaCl in a buffer containing 5 mM sodium phosphate (pH 7.0), 0.5 mM EDTA, and 5 mM DTT. The highest activity (2.5 U/mg, 1 g/L) eluted at ~50 mM NaCl. The active fraction was then concentrated 4-fold with Amicon Centricon centrifugation tubes (Millipore) and run by native PAGE (12% acrylamide, Bio-Rad). The gel was then stained by Zymogram staining as described previously (*15*) but modified for the D-galacturonic acid reductase. In this staining method, L-galactonic acid and NADP produced NADPH which developed a blue color in the presence of nitroblue tetrazolium and phenazine methosulfate. The gel was soaked in 200 mM Tris-HCl buffer (pH 8.1) containing 0.5 mM NADP, 0.2% L-galactonic acid, 0.25 mM nitroblue tetrazolium, and 0.06 mM phenazine methosulfate. The blue band which appeared in the staining was cut out and eluted by overnight incubation in 1 mL of 200 mM Tris-HCl (pH 8.1) and 0.1% SDS. It was then concentrated to ~70 μL in an Amicon Centricon centrifugation tube (Millipore). This gave an enzyme preparation with a major band at ~40 kDa in SDS−PAGE.

The 40 kDa protein band was cut out from the SDS−PAGE gel and the protein in gel alkylated with iodoacetamide and digested with trypsin. The peptides resulting from trypsin cleavage were extracted, and the quality of the generated peptide mass fingerprint was determined by MALDI-TOF mass spectrometry. Partial sequences of individual peptides were then determined by liquid chromatography/electrospray tandem mass spectrometry (LC−ESI-MS/MS). The details of these procedures have been described elsewhere (*16*).

Identification and Cloning of the Open Reading Frame. After the amino acid sequences KGISPTTVLLSYHVNR and YVYPPFGIDFGFPFPDKS were obtained, the DNA sequence encoding these amino acid sequences was identified as *H. jecorina* cDNA clone tric026xe09 (NCBI GenBank accession number CB901350). This DNA sequence did not cover the complete open reading frame. The part encoding the N-terminus of the protein was missing. The missing sequence for the open reading frame was identified by PCR using a *H. jecorina* cDNA library in a yeast expression vector (*17*) as a template. In the antisense direction, the primer (5′-GGATCCCTATGATTTATCAGGGAAGC-3′) was used. Both primers contained an additional *Bam*HI restriction site (underlined). In the sense direction, six different primers containing a start codon were chosen from the upstream sequence, as obtained from the JGI *T. reesei* database. With two different primers, a PCR product of ~1 kb was obtained. The slightly larger fragment was amplified when using the primer (5′-GGATCCAAAATGGTCGCTACTTCGTT-3′) in the sense direction. The PCR product was cloned into a TOPO vector (Invitrogen) and sequenced.

Expression the D-Galacturonic Acid Reductase Gene. The vector containing the *gar1* gene was digested with *Bam*HI and the resulting 1 kb fragment ligated to the *Bam*HI site of a multicopy yeast expression vector having the *TPI1* promoter and terminator and *URA3* selection marker. The expression vector was derived from the pYX212 plasmid by digesting it with *Eco*RI and *Xho*I to remove the ATG and the HA tag from the multiple cloning site and introducing

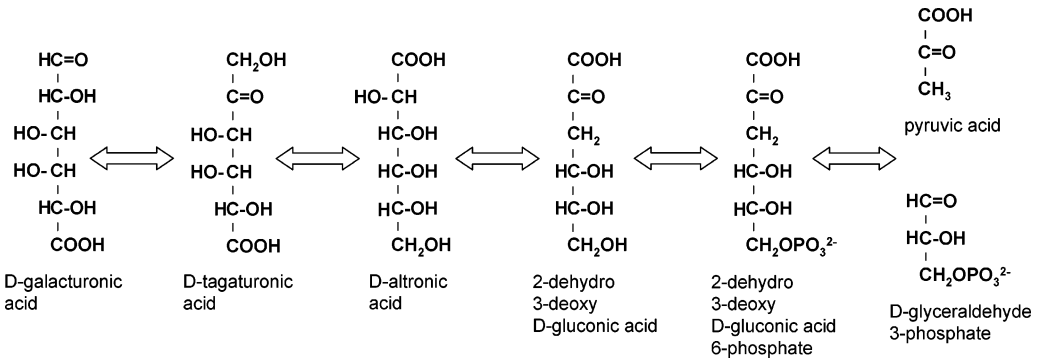

FIGURE 1: Bacterial pathway for D-galacturonic acid catabolism. The metabolites are represented in Fischer projection. In this representation, C6 of the D-galacturonic acid and the D-tagaturonic acid becomes C1 of D-altronic acid and the two following metabolites. The enzymes of this pathway are uronate isomerase (EC 5.3.1.12), NADH-utilizing D-tagaturonate reductase (EC 1.1.1.58), altronate dehydratase (EC 4.2.1.7), 2-dehydro-3-deoxy-D-gluconatekinase (EC 2.7.1.45), and 2-dehydro-3-deoxy-D-gluconate-6-phosphate aldolase (EC 4.1.2.14).

a *Bam*HI restriction site into the cloning site by inserting a *Eco*RI- and *Sal*I-cut fragment from the pUC19 plasmid (*18*).

A histidine-tagged D-galacturonic acid reductase with six histidines at the C-terminus was obtained by applying the protocol described above but using a different primer (5′-GGATCCCTAGTGGTGATGATGGTGATGTGATTT-ATCAGGGAAGC-3′) in the antisense direction. The resulting open reading frame was also ligated to the modified pYX212 vector as described above.

Expression in S. *cerevisiae and Purification of the Histidine-Tagged Protein. S. cerevisiae* strain CEN.PK2-1B was used for expressing the open reading frame for the D-galacturonic acid reductase. The CEN.PK2-1B strain was transformed with the yeast plasmids and grown on selective medium. Yeast cell extract was obtained by vortexing yeast cells with glass beads, and the cell extract was assayed for D-galacturonic acid reductase activity. The histidine-tagged protein was purified with a nickel−nitrilotriacetic acid column (Qiagen) according to the manufacturer's instructions.

Northern Blot Analysis. H. jecorina was grown on carbon sources D-glucose, D-fructose, D-xylose, lactose, galactose, glycerol, D-mannose, and D-galacturonic acid. The mycelium was collected by filtration, frozen in liquid nitrogen, and ground with a pestle and mortar. Total RNA was isolated with the Trizol reagent kit (Invitrogen); 10 mg of RNA was glyoxylated, separated in a 1% agarose gel, and transferred to Hybond N nylon filter (Amersham). The Northern blot analysis was conducted using standard procedures. The probe was a 406 bp fragment obtained with *Aat*II−*Bam*HI digestion from a TOPO vector carrying the *gar1* gene. To normalize the signals, a PCR fragment of the gene for actin, *act1*, was also used as a probe. The intensity of the signals was measured using a Typhoon 8600 instrument (Amersham).

RESULTS

D-Galacturonic Acid Reductase Activity. In filamentous fungus *H. jecorina* (*T. reesei*), D-galacturonic acid reductase is active during growth on D-galacturonic acid. In crude extracts of *H. jecorina* strain Rut C-30, the D-galacturonic acid reductase activity was on average 0.25 ± 0.1 U/mg of extracted protein when the mycelia were grown on D-galacturonic acid. No D-galacturonic acid reductase activity was observed when *H. jecorina* was grown on D-glucose, D-fructose, D-galactose, D-xylose, or glycerol.

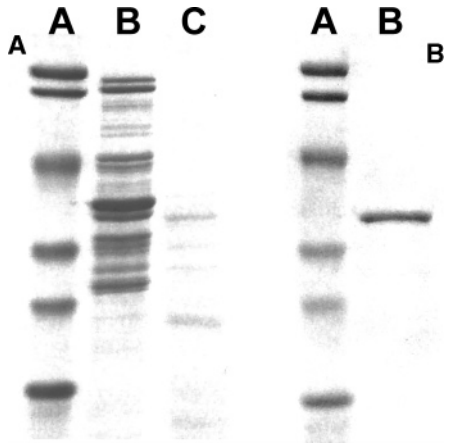

FIGURE 2: (A and B) SDS−PAGE of the purified and histidine-tagged protein. In panel A, lane A contained molecular mass markers (from top to bottom, 108, 90, 50.7, 35.5, 28.6, and 21.2 kDa), lane B the active fraction after the DEAE column, and lane C the fraction after zymogram staining. Panel B had molecular mass markers in lane A (as in lane A of panel A) and the histidine-tagged purified protein in lane B.

Purification and Characterization of the Enzyme. H. jecorina strain Rut C-30 was grown on D-galacturonic acid. The mycelium was extracted by vortexing with glass beads. The crude extract which was obtained in this way had a D-galacturonic acid activity of 0.33 U/mg of extracted protein. The crude extract was then desalted by gel filtration, and subsequently, the enzyme activity was bound to a DEAE column. It was eluted with a salt gradient. The active fraction had an activity of 2.5 U/mg of protein. This partly purified protein was specific for NADPH, and no activity was observed when NADH was used as a cofactor. L-Galactonic acid and NADP also exhibited activity, suggesting that the enzyme is a reversible enzyme converting D-galacturonic acid and NADPH to L-galactonic acid and NADP. We noticed that activity was only observed with L-galactonic acid and not with L-galactonic acid γ-lactone.

The active fraction was concentrated ~4-fold before it was separated by native PAGE. The PAGE gel was then stained by zymogram staining. The stained bands were then cut out from the gel, and the enzyme was eluted. This purified enzyme had an estimated molecular mass of 40 kDa as estimated by SDS−PAGE (Figure 2A).

Amino Acid Sequence, Cloning of the Open Reading Frame, and Heterologous Expression. The purified enzyme

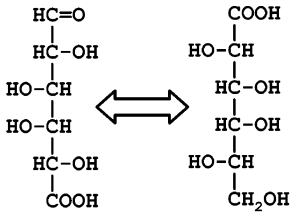

FIGURE 3: Fischer projection of the substrate and product of the D-galacturonic acid reductase. C1 of the D-galacturonic acid is the reduced C6 of the L-galactonic acid.

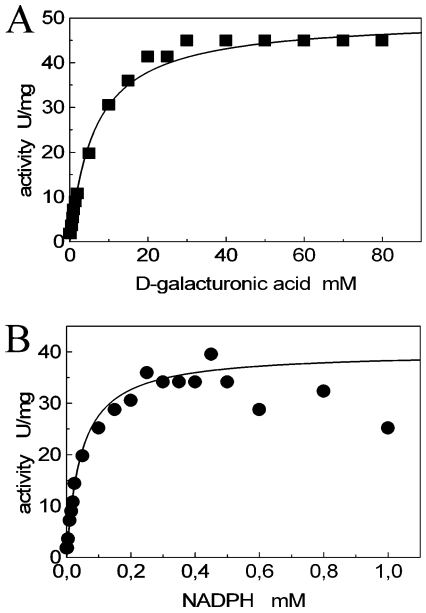

FIGURE 4: Kinetics of the purified histidine-tagged protein. (A) The NADPH concentration is 200 μM. (B) The concentration of D-galacturonic acid is 480 mM. The curves are calculated Michaelis−Menten kinetics using the constants as indicated. For D-galacturonic acid, K_m = 4.4 mM and V_{max} = 50 U/mg. For NADPH, K_m = 45 μM and V_{max} = 40 U/mg.

was digested in gel with trypsin, and the amino acid sequences of two fragments were obtained. The sequences were KGISPTTVLLSYHVNR and YVYPPFGIDFGFPFP-DKS. The DNA sequence encoding these amino acid sequences was identified as *H. jecorina* cDNA clone tric026xe09 (NCBI GenBank accession number CB901350).

The DNA sequence showed homology to genes encoding aldo-keto reductases; however, comparison to other proteins of the aldo-keto reductase family suggested that the DNA sequence for the N-terminal part of the protein was missing.

To identify the missing part of the open reading frame, a cDNA library in a yeast expression vector was screened. PCR primers were chosen from the genomic sequence received from the JGI *T. reesei* database, and the missing part of the open reading frame was identified. The sequence revealed an open reading frame encoding a protein with 309 amino acids and a molecular mass of 33 940 Da. We called the gene *gar1* for D-galacturonic acid reductase. The sequence for the open reading frame was deposited in NCBI GenBank as accession number AY862503. Comparing the open reading from the cDNA library with the sequence of the genome revealed five introns (Figure 5).

The open reading frame was then expressed in *S. cerevisiae*, on a plasmid with a constitutive promoter. An activity of 0.15 U/mg of extracted protein was found in the cell extract. In the control where the gene was not expressed, no activity was detected. The protein was also tagged with a histidine tag and then expressed in *S. cerevisiae*. The tagged protein had an activity (0.21 U/mg of protein) in the crude extract similar to that in nontagged protein, indicating that tag was not affecting the activity. The tagged protein was then purified and used for kinetic analysis. The histidine-tagged protein had a molecular mass of ~40 kDa as estimated by SDS−PAGE (Figure 2B). The reason for the difference from the calculated molecular mass is not known.

In the forward direction with D-galacturonic acid and NADPH as substrates, we found the Michaelis−Menten constants for D-galacturonic acid (K_m = 6 mM) and for NADPH (K_m = 30 μM and V_{max} ~ 40 U/mg of protein) (Figure 4). The D-galacturonic acid reductase also exhibited activity with D-glucuronic acid and DL-glyceraldehyde. The Michaelis−Menten constants for them were as follows: K_m = 11 mM and V_{max} = 25 U/mg of protein and K_m = 6 mM and V_{max} = 7 U/mg of protein, respectively. No activity was observed with D-glucose, D-fructose, D-xylose, D-galactose, L-arabinose, or D-mannose. Activity was seen only with NADPH and not with NADH.

We tested the enzyme activity also in the reverse direction with NADP as a substrate. We assumed that the reaction product is L-galactonic acid. Since L-galactonic acid is not commercially available, it had to be derived from L-galactono-1,4-lactone. L-Galactono-1,4-lactone hydrolyzes spontaneously in water, but the released proton has to be neutralized to favor the formation of the hydrolyzed form. A fresh solution of L-galactono-1,4-lactone which had not been hydrolyzed to L-galactonic acid did not serve as a substrate. When the L-galactono-1,4-lactone had been hydrolyzed, as verified by following the pH, it served as a substrate. For L-galactonic acid, we estimated a K_m of 4 mM and for NADP a K_m of 1 μM and a V_{max} of ~2 U/mg of protein. No backward reaction was observed with L-gulonic acid, glycerol, D-arabitol, L-arabitol, xylitol, galactitol, or ribitol (adonitol).

Northern Blot Analysis. The expression of D-galacturonic acid dehydratase in *H. jecorina* on different carbon sources was examined by Northern blot analysis. The gene was expressed on all the studied carbon sources, but a difference could be seen when the expression level of D-galacturonic acid reductase was compared to the expression level of actin. The ratio was the same in *H. jecorina* grown on D-fructose, D-xylose, lactose, galactose, glycerol, and D-mannose. Compared to this reference level, the expression level on D-glucose was 3 times greater and on D-galacturonic acid 8 times greater. The size of the messenger RNA is ~1.2 kb.

DISCUSSION

The metabolic route for D-galacturonic acid catabolism in eukaryotic organisms is not known; however, such a metabolic route exists since many species of yeast and mold are able to utilize D-galacturonic acid. We choose the mold *H. jecorina* (*T. reesei*) to study the eukaryotic D-galacturonic acid pathway because *H. jecorina* can grow on this carbon source and the genome sequence is available. *H. jecorina* is widely used for industrial enzyme production.

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1      ATGGTCGCTACTTCGTTCAAGCTCAACAATGGCCTGGAGATTCCAGCTGTTGGTCTCggt      1
1       M  V  A  T  S  F  K  L  N  N  G  L  E  I  P  A  V  G  L

       atgtctcaatcagtctccctcctgtgaggggggaggcagagttgccaatggcgggggagg      61

       ggcaatttcgatgtgcgcgcaaagcaagcaaagcaaagcaaagcaaaggaaatcaaagca     121

       aaggaaatcaaagcaaagaaaatcaaagcaaagaaaatcaaaaagtgccatcgatcaaca     181

58     tgactgattttgaacttttgcaGGAACATGGCAGTCCAAAGCCGGCGAGGTCAAGGCGGC     241
20                          G  T  W  Q  S  K  A  G  E  V  K  A  A

96     CGTCTCCTACGCGCTTCAGATCGGCTACAAGCTCATCGACGGAGCTTACTGCTACGGCAA     301
33      V  S  Y  A  L  Q  I  G  Y  K  L  I  D  G  A  Y  C  Y  G  N

156    CGAGGACGAGGTGGGCGAGGGCCTCAAGGAGGCTTTTGCTGCGGGGGTGAAGCGCGAGGA     361
53      E  D  E  V  G  E  G  L  K  E  A  F  A  A  G  V  K  R  E  D

216    CATTTTCGTCGTGACCAAGATCTGGGCTACGTACAACACGCGGGTTGTGCTGGGCCTGGA     421
73      I  F  V  V  T  K  I  W  A  T  Y  N  T  R  V  V  L  G  L  D

276    TAAGAGCTTGAGAAGCCTGGGGCTGGACTATGTCGACTTGCTGCTGGTGgtgagtacgga     481
93      K  S  L  R  S  L  G  L  D  Y  V  D  L  L  L  V

       cctggacctggcacctgggttgcctacatattcgtgagcagcatgctgacgagttattga     541

325    caactgcagCATTGGCCAGTTCTGCTGAACCCTGAAggtaagagatatgacatgtttttt     601
109             H  W  P  V  L  L  N  P  E

352    atcgacgagacgccttcaatcagatcgtctaactccttactcacaGGCAATCACGACAAG     661
118                                                 G  N  H  D  K

367    TTCCCTACCCTGCCCGACGGCAAGCGAGACGTCATCTGGGACTACAATCACGTCGACGGC     721
123     F  P  T  L  P  D  G  K  R  D  V  I  W  D  Y  N  H  V  D  G

427    TGGAAGCAGATGGAGGCTGTTCTCGCGACGGGCAAGACCAAGTCCATTGGCGTCAGCAAC     781
143     W  K  Q  M  E  A  V  L  A  T  G  K  T  K  S  I  G  V  S  N

       gtatgtcttggagcatctcatactgctgtccgacagcctctaacataatgacatggtgta     841

487    gTACAGCAAGAAGTATCTCGAGCAGCTTCTCCCTCACGCCACCGTTATCCCCGCCGTCAA     901
163     Y  S  K  K  Y  L  E  Q  L  L  P  H  A  T  V  I  P  A  V  N

546    CCAGATTGAGAACCACCCCAGCCTGCCCCAGCAGGAGATTGTCGATTTCTGCAAGGAAAA     961
183     Q  I  E  N  H  P  S  L  P  Q  Q  E  I  V  D  F  C  K  E  K

606    GGGCATCCACATCATGGCCTACAGCCCCCTGGGCAGCACGGGCAGCCCGCTGATGAGCGC    1021
203     G  I  H  I  M  A  Y  S  P  L  G  S  T  G  S  P  L  M  S  A

666    CGATCCCGTGGTCAAGATTGCCGAGAAGAAGGGGATTTCACCCACCACTGTCCTGTTGAG    1081
223     D  P  V  V  K  I  A  E  K  K  G  I  S  P  T  T  V  L  L  S

726    CTATCACggtacgcttcttgttcctctacttgagcatgcctctcatcggactacacatac    1141
243     Y  H

733    ttacattctgttttgaataGTGAACCGTGGCAGCACCGTGCTGGCAAAGTCGGTTACGCC    1201
245                        V  N  R  G  S  T  V  L  A  K  S  V  T  P

774    CGCTCGCATCAAGGCCAACCTGGAGATTGTCGACCTGGACGACGAGGACATGAAGCTGCT    1261
259     A  R  I  K  A  N  L  E  I  V  D  L  D  D  E  D  M  K  L  L

834    GAATGATTACTCAAACGACCTGGCGAGCAAGGGCGAGCTGAAGCGCTACGTCTACCCTCC    1321
279     N  D  Y  S  N  D  L  A  S  K  G  E  L  K  R  Y  V  Y  P  P

894    GTTCGGCATCGATTTTGGCTTCCCTGATAAATCATAG                          1381
299     F  G  I  D  F  G  F  P  D  K  S  *
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FIGURE 5: DNA and amino acid sequence of GAR1 gene. The coding sequence is in uppercase letters, and the intron sequences are in lowercase letters. The amino acid sequence is under the coding sequence, and the fragments which have been sequenced are underlined. The cDNA sequence is deposited in GenBank as accession number AY862503.

It was suggested that in *A. niger* D-galacturonic acid is metabolized through pyruvate and glyceraldehyde (*10*). This already suggests that the eukaryotic path is different from the bacterial path since glyceraldehyde and not glyceraldehyde 3-phosphate is an intermediate. Genes which would be similar to the genes of the bacterial D-galacturonic acid pathway have not been found in any eukaryotic microorganism, and there are also no direct indications that mold would have the enzymes of the bacterial pathway. We have tested *H. jecorina* for such activities but could not detect them (data not shown). However, we identified an NADPH-dependent D-galacturonic acid reductase activity. This activity is induced during growth on D-galacturonic acid, and the activity is absent when the mold is grown on a different carbon source; i.e., it is likely that this enzyme is involved in the catabolism of D-galacturonic acid. Also in Northern analysis, we could

show that the D-galacturonic acid reductase gene is most strongly expressed in *H. jecorina* when grown on D-galacturonic acid.

We have purified the enzyme and cloned the corresponding gene. Expression of the gene in the heterologous host *S. cerevisiae* resulted in an active enzyme. The activity was similar to the activity in the *H. jecorina* crude cell extract. *S. cerevisiae* does not have any background D-galacturonic acid reductase activity. The enzyme remained active after a histidine tag was added to the C-terminal end of the protein; the activity in the *S. cerevisiae* crude cell extract was similar with or without the histidine tag. We purified the histidine-tagged enzyme and used it for kinetic analysis. We found activity with D-galacturonic acid, D-glucuronic acid, and DL-glyceraldehyde. For the cofactor, the enzyme is specific for NADPH. We found no activity with NADH.

In the reverse direction, we found activity with NADP and L-galactonic acid but not with L-galactonic acid γ-lactone (L-galactono-1,4-lactone). This suggests that in the forward direction with D-galacturonic acid as the substrate, the reaction product is L-galactonic acid and not the lactone. In aqueous solution, the lactone is generally believed to have a higher energy; i.e., the thermodynamic equilibrium is on the side of the L-galactonic acid. In case the L-galactonic acid would be converted to L-galactonic acid γ-lactone, this could be the substrate for an L-galactono-1,4-lactone oxidase to form L-ascorbic acid. Such an oxidase activity is present in yeast (*19*, *20*). In the absence of a lactone-forming step, the catabolism is probably not proceeding through L-ascorbic acid.

It is not unusual that carbohydrates are metabolized through different pathways in prokaryotic bacteria and in eukaryotic microorganisms. D-Xylose for example is catabolized in bacteria through a path consisting of xylose isomerase and xylulokinase (*21*), whereas in fungi, the path consists of xylose reductase, xylitol dehydrogenase, and xylulokinase (*22*). Another example is L-arabinose catabolism. Also here, a bacterial path exists which is completely different from the fungal path (*23*, *24*).

Apparently, the catabolism of D-galacturonic acid also follows distinctly different routes in bacteria and eukaryotic microorganisms. The first step in the eukaryotic route is an NADPH-dependent D-galacturonic reductase forming L-galactonic acid.

A possible route for the further catabolism of L-galactonic acid would be the route which was suggested for the catabolism of myoinositol (*25*). In this route, L-galactonic acid is metabolized through the intermediates 3-ketogulanate, L-xylulose, xylitol, D-xylulose, and xylulose 5-phosphate. This pathway is established from L-xylulose to xylulose 5-phosphate (*18*, *24*).

A D-galacturonic acid reductase was identified previously (*11*) in a plant. The two enzymes, from plant and mold, are similar in size (~310 amino acids) and have similar sequences; in the amino acid sequence, the level of identity is ~30%. In plant, the enzyme activity was shown and the corresponding gene was cloned. This enzyme was suggested to be part of the L-ascorbic acid synthetic pathway. The enzyme is also NADPH-dependent. The difference is that the plant enzyme is more specific for D-galacturonic acid as the substrate. The enzyme from *H. jecorina* has activity with D-galacturonic acid, D-glucuronic acid, and DL-glyceraldehyde. The enzyme activity of a D-galacturonic acid reductase was also shown in rat kidney (*12*). Here the enzyme activity was shown, but the corresponding gene was not identified. This enzyme is also NADPH-dependent. It also has a molecular mass of ~40 kDa and uses D-galacturonic acid, D-glucuronic acid, and DL-glyceraldehyde as substrates. It is not clear if the kidney enzyme is involved in the catabolism of the hexuronic acids.

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