Dissertation

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Satu Hilditch

Identification of the fungal catabolic D-galacturonate pathway

VTT

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Abstract

Pectin is a natural polymer consisting mainly of D-galacturonic acid monomers. Microorganisms living on decaying plant material can use D-galacturonic acid for growth. Although bacterial pathways for D-galacturonate catabolism had been described previously, no eukaryotic pathway for D-galacturonate catabolism was known at the beginning of this work. The aim of this work was to identify such a pathway.

In this thesis the pathway for D-galacturonate catabolism was identified in the filamentous fungus *Trichoderma reesei*. The pathway consisted of four enzymes: NADPH-dependent D-galacturonate reductase (GAR1), L-galactonate dehydratase (LGD1), L-threo-3-deoxy-hexulosonate aldolase (LGA1) and NADPH-dependent glyceraldehyde reductase (GLD1). In this pathway D-galacturonate was converted to pyruvate and glycerol via L-galactonate, L-threo-3-deoxy-hexulosonate and L-glyceraldehyde.

The enzyme activities of GAR1, LGD1 and LGA1 were present in crude mycelial extract only when *T. reesei* was grown on D-galacturonate. The activity of GLD1 was equally present on all the tested carbon sources. The corresponding genes were identified either by purifying and sequencing the enzyme or by expressing genes with homology to other similar enzymes in a heterologous host and testing the activities. The new genes that were identified were expressed in *Saccharomyces cerevisiae* and resulted in active enzymes. The GAR1, LGA1 and GLD1 were also produced in *S. cerevisiae* as active enzymes with a polyhistidine-tag, and purified and characterised. GAR1 and LGA1 catalysed reversible reactions, whereas only the forward reactions were observed for LGD1 and GLD1. When *gar1*, *lgd1* or *lga1* was deleted in *T. reesei* the deletion strain was unable to grow with D-galacturonate as the only carbon

source, demonstrating that all the corresponding enzymes were essential for D-galacturonate catabolism and that no alternative D-galacturonate pathway exists in *T. reesei.*

A challenge for biotechnology is to convert cheap raw materials to useful and more valuable products. Filamentous fungi are especially useful for the conversion of pectin, since they are efficient producers of pectinases. Identification of the fungal D-galacturonate pathway is of fundamental importance for the utilisation of pectin and its conversion to useful products.

Preface

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Espoo, May 2010

Satu Hilditch

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List of publications

I Kuorelahti, S., Kalkkinen, N., Penttilä, M., Londesborough, J. and Richard, P. (2005). Identification in the mold *Hypocrea jecorina* of the first fungal D-galacturonic acid reductase. *Biochemistry* 44, 11234–11240.

II Kuorelahti, S., Jouhten, P., Maaheimo, H., Penttilä, M. and Richard, P. (2006). L-Galactonate dehydratase is part of the fungal path for D-galacturonic acid catabolism. *Mol. Microbiol.* 61, 1060–1068.

III Liepins, J., Kuorelahti, S., Penttilä, M. and Richard, P. (2006). Enzymes for the NADPH-dependent reduction of dihydroxyacetone and D-glyceraldehyde and L-glyceraldehyde in the mould *Hypocrea jecorina*. *FEBS J.* 273, 4229–4235.

IV Hilditch, S., Berghäll, S., Kalkkinen, N., Penttilä, M. and Richard, P. (2007). The missing link in the fungal D-galacturonate pathway: identification of the L-threo-3-deoxy-hexulosonate aldolase. *J. Biol. Chem.* 282, 26195–26201.

List of symbols

DHA	dihydroxyacetone
DHAP	dihydroxyacetone phosphate
DHDPS	dihydrodipicolinate synthase
DNS	3,5-dinitrosalicylate
EC	Enzyme Commission
ESI-MS	electrospray ionisation mass spectrometry
FucD	L-fuconate dehydratase of *Xanthomonas campestris*
gaaA	D-galacturonate reductase-encoding gene of *Aspergillus niger*
GAAA	D-galacturonate reductase of *A. niger* or *Aspergillus nidulans*
GAAB	L-galactonate dehydratase of *A. niger*
gaaC	2-keto-3-deoxy-L-galactonate aldolase-encoding gene of *A. niger*
GAAC	2-keto-3-deoxy-L-galactonate aldolase of *A. niger*
gaaD	glyceraldehyde reductase-encoding gene of *A. niger*
GAAD	glyceraldehyde reductase of *A. niger*
gar1	D-galacturonate reductase-encoding gene of *Trichoderma reesei*
GAR1	D-galacturonate reductase of *T. reesei*
gar2	a putative D-galacturonate reductase-encoding gene of *T. reesei*
GAR2	a putative D-galacturonate reductase of *T. reesei*
Gcy1p	glyceraldehyde reductase of *Saccharomyces cerevisiae*
gld1	glyceraldehyde reductase-encoding gene of *T. reesei*

GLD1	glyceraldehyde reductase of *T. reesei*
Gld1	DHA reductase of *Trichoderma atroviride*
gld2	DHA reductase-encoding gene of *T. reesei*
GLD2	DHA reductase of *T. reesei*
GldB	DHA reductase of *A. nidulans*
HPLC	high performance liquid chromatography
KER	alkyl 4-halo-3-oxobutyrate reductase of *Penicillium citrinum*
lga1	L-threo-3-deoxy-hexulosonate aldolase-encoding gene of *T. reesei*
LGA1	L-threo-3-deoxy-hexulosonate aldolase of *T. reesei*
lgd1	L-galactonate dehydratase-encoding gene of *T. reesei*
LGD1	L-galactonate dehydratase of *T. reesei*
Mbp	mega base pairs
MR	mandelate racemase of *Pseudomonas putida*
MS-MS	tandem mass spectrometry
NCBI	National Center for Biotechnology Information
NMR	nuclear magnetic resonance
TBA	thiobarbituric acid
TIM	triose phosphate isomerase
Ypr1p	glyceraldehyde reductase of *S. cerevisiae*

1. Introduction

Mould fungi, yeast fungi and mushrooms are the most commonly known members of the fungal kingdom. Characteristic to fungi is their ability efficiently to degrade organic materials. They secrete a wide range of extracellular enzymes to break down complex substrates into simple components such as sugars and amino acids that can then be taken up by the fungus and further metabolised by numerous specialised pathways for growth and energy. This osmotrophic growth habit has enabled fungi to compete successfully with other organisms and to colonise diverse habitats. For example, they are important in degrading plant debris and in that way recycle carbon and other elements in the environment. On the other hand they also contaminate crops and cause many diseases in both plants and animals.

The filamentous fungus *Trichoderma reesei* belongs to the subphylum *Pezizomycotina* of the phylum *Ascomycota* (NCBI Taxonomy Browser: http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi). It is non-pathogenic and has a long history of being safe to use for enzyme production in industrial scale (Nevalainen *et al.*, 1994). It has an exceptionally efficient protein secretion system and it is one of the most important industrial producers of cellulases and hemicellulases that are used for hydrolysis of plant cell wall polysaccharides. It was one of the first fungi for which the entire genome was sequenced. The seven chromosomes of *T. reesei* comprise a genome of 34 Mbp. Although *T. reesei* has the lowest number of cellulase and hemicellulase genes of all sequenced plant cell wall-degrading fungi, it breaks down cellulose and hemicellulose efficiently. It has also very few pectinase genes in its genome and therefore it has been suggested that for pectin degradation *T. reesei* may largely depend on other fungi and bacteria in the soil (Martinez *et al.*, 2008). *T. reesei* was originally discovered in the Solomon Islands during World War II, where it was found to be responsible for the rapid decomposition of US Army military

uniforms and cotton canvas tents (Reese, 1976). All *T. reesei* strains used nowadays in biotechnology and basic research have been derived from that single isolate QM6a (Reese *et al.*, 1950). *T. reesei* was long believed to propagate only asexually and was shown to be an anamorph derived from the sexual (teleomorphic) ancestor *Hypocrea jecorina* (Kuhls *et al.*, 1996). Recently, *T. reesei* QM6a was found to carry only the mating type *MAT1-2*, whereas some other *H. jecorina* isolates from different geographical locations contained only the opposite mating type *MAT1-1*. Heterothallic fungi require both types to be present for sexual reproduction. In mating experiments between strains QM6a and *H. jecorina MAT1-1*, sexual reproduction could be induced (Seidl *et al.*, 2009).

Pectin is a common biopolymer in nature. Its backbone is a linear chain of D-galacturonate molecules. Many bacterial species are able to utilise D-galacturonate, and some bacterial catabolic pathways are known. Several fungal species, such as *T. reesei*, are able to grow using D-galacturonate as the only carbon and energy source. It was well established that fungi do not have the same set of enzymes to break down D-galacturonate as bacteria. However, it was not known how eukaryotic species catabolise D-galacturonate. In this thesis the first eukaryotic pathway for D-galacturonate catabolism was identified using *T. reesei* as the model organism.

1.1 Pectin and D-galacturonate

Pectin is a common component of the primary cell wall and middle lamella of all higher plants. The primary cell wall may contain approximately 35% pectin, 30% cellulose, 25% hemicellulose and 10% structural protein, but the proportions vary greatly depending on the species and cell type (Brownleader *et al.*, 1999). The amounts of pectin in some fruit and vegetable tissues are listed in Table 1.

Table 1. Pectin contents of some fruit and vegetable tissues. The amount of pectin is approximately tenfold higher in the dry matter compared to the fresh substance. Adapted from Jayani *et al.*, 2005; Prasanna *et al.*, 2007.

Fruit/vegetable	**Tissue**	**Substance**	**Pectin content (%)**
African Mango	Pulp	Fresh	0.72
Apple	Pulp	Fresh	0.5–1.6
Apple	Pomace	Fresh	1.5–2.5
Avocado	Pulp	Fresh	0.73
Banana	Pulp	Fresh	0.7–1.2
Cashew	Pomace	Fresh	1.28
Cherry	Pulp	Fresh	0.24–0.54
Guava	Pulp	Fresh	0.26–1.2
Lemon	Pulp	Fresh	2.5–4.0
Lemon	Peel	Fresh	5.0
Litchi	Pulp	Fresh	0.42
Mango	Pulp	Fresh	0.66–1.5
Orange	Pulp	Fresh	1.35
Orange	Peel	Fresh	3.5–5.5
Papaya	Pericarp	Fresh	0.66–1.0
Passion fruit	Pulp	Fresh	0.5
Passion fruit	Rind	Fresh	2.1–3.0
Pea	Pulp	Fresh	0.9–1.4
Peach	Pulp	Fresh	0.1–0.9
Pineapple	Pulp	Fresh	0.04–0.13
Strawberry	Pulp	Fresh	0.14–0.7
Tomato	Pulp	Fresh	0.2–0.6
Carrot	Pulp	Dry matter	6.9–18.6
Orange	Pulp	Dry matter	12.4–28.0
Potato	Pulp	Dry matter	1.8–3.3
Tomato	Pulp	Dry matter	2.4–4.6
Sugar beet	Pulp	Dry matter	10.0–30.0

Pectin is a general name for a family of complex, highly heterogeneous polysaccharides with molecular masses ranging from 25 to 360 kDa (Jayani *et al.*, 2005). Homogalacturonan, rhamnogalacturonan I, rhamnogalacturonan II and xylogalacturonan are some of the structural domains that form pectic

polysaccharides and may be covalently joined to each other (Perez *et al.*, 2003). Their structures are presented in Figure 1. Approximately 70% of pectin is composed of D-galacturonic acid residues which are mainly found in the backbone of pectin (Mohnen, 2008).

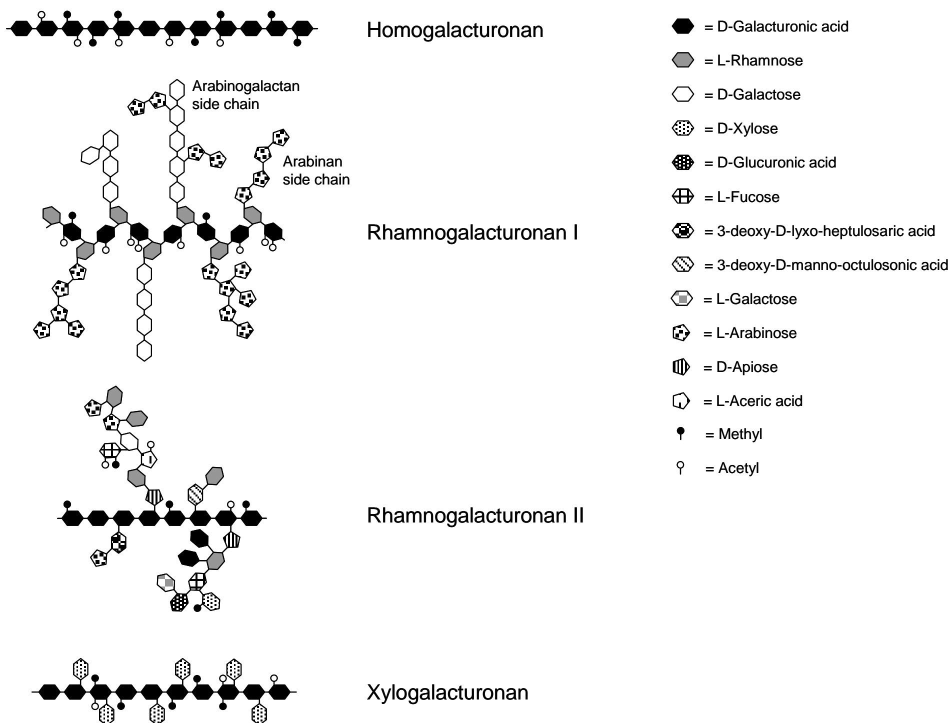


Figure 1. The structures of homogalacturonan, rhamnogalacturonan I, rhamnogalacturonan II and xylogalacturonan.

In the classical pectin structure long “smooth” regions may occasionally be interrupted by “hairy” regions. The “smooth” regions are composed of homogalacturonan, in which about 100 α-(1->4)-linked D-galacturonic acid residues form linear chains. In some plants the hydroxyl groups on C2 and C3 of the D-galacturonic acid residues may carry acetyl groups (Renard *et al.*, 1995). The carboxyl groups at C6 of D-galacturonic acids can be methyl-esterified, which removes the negative charge and makes the homogalacturonan more hydrophobic. In native pectins the homogalacturonan is highly methyl-esterified,

with up to 70% of the carboxyl groups carrying a methyl group (Ralet *et al.*, 2001). Homogalacturonan with a low degree of methyl-esters is referred to as pectic acid or pectate. The negative charge of un-esterified carboxyl groups in pectin tends to expand the structure or interact through divalent cations. In the so called "egg-box model" (Figure 2) two or more antiparallel homogalacturonan chains are bound together via Ca^{2+} ions and form a gel (Grant *et al.*, 1973). The pattern and degree of methyl-esterification is associated with the compressive strength, elasticity, water holding capacity and the porosity of the formed gels and hence also plays a role *in vivo* in determining the cell wall matrix properties. In addition, the ability of different pectinolytic enzymes to cleave pectin is strongly influenced by the degree of methyl- and acetyl-esterification (Willats *et al.*, 2001b).

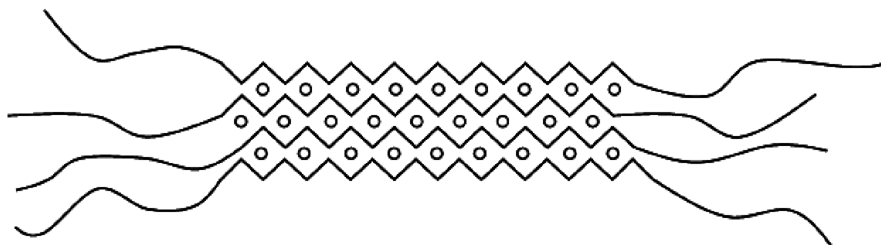


Figure 2. In the "egg-box model" antiparallel homogalacturonan chains are bound via Ca^{2+} ions.

Rhamnogalacturonan I, rhamnogalacturonan II and xylogalacturonan are structural domains that often have abundant and branched side chains and therefore are called the "hairy" regions of pectin. The backbone of rhamnogalacturonan I is composed of repeating (1->4)-α-D-galacturonic acid-(1->2)-α-L-rhamnose disaccharide units. Neutral sugars, mainly L-arabinose and D-galactose, are present as side chains in the form of arabinan, galactan or two types of arabinogalactan, connected to the backbone through the C4 position of rhamnose (Carpita and Gibeaut, 1993). Pectin may also contain complex rhamnogalacturonan II, in which side chains are attached to a backbone of at least eight α-(1->4)-linked D-galacturonic acids and contain rare sugar residues such as L-fucose, D-glucuronic acid, D-apiose, 3-deoxy-D-manno-2-octulosonic acid and 3-deoxy-D-lyxo-2-heptulosonic acid (Vidal *et al.*, 2000). In xylogalacturonan the α-(1->4)-linked D-galacturonic acid backbone is highly substituted at C3 with β-D-xylose (Zandleven *et al.*, 2005). The chemical

composition, structure and amount of pectin vary depending on the plant material. Pectin has various functions in plants, for example in physiology, growth, development and defence mechanisms. Pectin is by no means an inert substance but is dynamically synthesised, modified and degraded during different development stages of the plant. Pectin is the major adhesive material between the plant cells and is for example involved in the control of cell wall porosity. When plant pathogens degrade pectin the released oligosaccharides may function as signalling molecules for the plant's defence mechanisms. The rare sugars in the pectin structure may also have a defensive role against pathogens because they might make pectin more difficult to degrade (Willats *et al.*, 2001a).

1.2 Pectin degradation and D-galacturonate utilisation

In order to gain access to the significant carbon and energy supply stored in pectin, organisms must first break it down to its components. A variety of pectinolytic enzymes such as pectin methyl-esterases, polygalacturonases, endo-pectate lyases, endo-pectin lyases, rhamnogalacturonases, arabinases and galactanases are required to biodegrade completely the complex and heterogeneous structure of pectin. Pectinolytic activity is mainly found in mould fungi and bacteria. *Aspergillus niger* is one of the best producers of pectinases and almost all the commercially available pectin-degrading enzymes are produced by this mould fungus (Zandleven *et al.*, 2005). Among the other extensively studied pectin-degrading mould fungi are the saprotrophs *Aspergillus nidulans* and *T. reesei* and the plant pathogens *Botrytis cinerea*, *Fusarium oxysporum* and *Sclerotium sclerotiorum* (Gamauf *et al.*, 2007). Some *Erwinia* and *Bacillus* species are examples of bacteria with high pectinolytic activity (Ried and Collmer, 1986; Soares *et al.*, 1999). Some yeast fungi, for example *Cryptococcus albidus*, *Kluyveromyces marxianus* and some strains of *Saccharomyces cerevisiae* also produce exocellular polygalacturonases and are therefore capable of degrading pectin (Blanco *et al.*, 1999). In addition to bacteria and fungi, pectinolytic enzymes are also found in plants. For example the degradation of pectin is responsible for tissue softening of many fruits during the ripening process. For a review of pectinolytic enzymes see Jayani *et al.* (2005).

D-Galacturonate is the main component of pectin and is therefore an important carbon source for many pectin-degrading microorganisms. However, the ability to

degrade pectin does not always mean that the organism is able to catabolise D-galacturonate. Depolymerising pectin might aim only at exposing other cell wall polymers to degradation and utilisation, or at using some monosaccharides of pectin other than D-galacturonate as the carbon source. On the other hand, the coexistence of different species on pectic substances may explain why some species do not have strong pectinolytic activity but can catabolise D-galacturonate.

Many bacterial and fungal species are able to grow using D-galacturonate as the only carbon and energy source. Different species have different kinds of catabolic D-galacturonate pathways. Furthermore, some plant and animal cells are able to metabolise D-galacturonate but their pathways are not catabolic but lead to production of L-ascorbate (vitamin C). These pathways are discussed below.

1.2.1 Bacterial D-galacturonate pathways

Two different catabolic D-galacturonate pathways have been found in bacteria. One has an isomerisation step and the other an oxidation step as the initial reaction. The isomerisation pathway was first reported in *Escherichia coli* (Ashwell *et al.*, 1958). After that it was also found in several other bacteria including *Erwinia carotovora* (Kilgore and Starr, 1959b) and *Erwinia chrysanthemi* (Hugouvieux-Cotte-Pattat and Robert-Baudouy, 1987). This pathway is presented in Figure 3. In the pathway, D-galacturonate is first isomerised to form D-tagaturonate by a uronate isomerase (EC 5.3.1.12). D-Tagaturonate is reduced to D-altronate by an NADH-dependent D-tagaturonate reductase (EC 1.1.1.58). A D-altronate dehydratase (EC 4.2.1.7) converts D-altronate in an irreversible reaction to D-erythro-3-deoxy-hexulosonate, which is then phosphorylated by a 2-keto-3-deoxy-D-gluconate kinase (EC 2.7.1.45) to D-erythro-3-deoxy-hexu-losonate-6-phosphate. This is cleaved by a 2-keto-3-deoxy-6-phosphogluconate aldolase (EC 4.1.2.14) to form pyruvate and D-glyceraldehyde-3-phosphate (Ashwell *et al.*, 1960; Hickman and Ashwell, 1960; Smiley and Ashwell, 1960; Cynkin and Ashwell, 1960; Meloche and Wood, 1964). The end-products of this pathway thus link D-galacturonate metabolism to the glycolytic path.

An alternative, the oxidative pathway is presented in Figure 4. It was observed in *Pseudomonas syringae* and *Agrobacterium tumefaciens*, in which the initial step was an NAD-dependent oxidation of D-galacturonate to galactarate (mucate) (Kilgore and Starr, 1959a; Zajic, 1959). In physiological conditions the reaction was irreversible and the reaction was proposed to occur via

galactarolactone that is converted to galactarate by a spontaneous hydration step (Chang and Feingold, 1969; Wagner and Hollmann, 1976). Galactarate is more stable than its lactone form in neutral pH and the reverse reaction of the D-galacturonate dehydrogenase (EC 1.1.1.203) was only observed at acidic pH, when some of the galactarate was in the lactone form (Wagner and Hollmann, 1976). The D-galacturonate dehydrogenase was induced when cells were grown on D-galacturonate but not when grown on D-glucose (Chang and Feingold, 1969; Bateman *et al.*, 1970). In the following steps a dehydratase (EC 4.2.1.42) converted galactarate to 5-dehydro-4-deoxy-D-glucarate, then via the action of dehydratase-decarboxylase (EC 4.2.1.41) to α-ketoglutarate semialdehyde, possibly via a 2,5-diketoadipic acid intermediate and finally an NAD(P)-dependent dehydrogenase (EC 1.2.1.26) completed the conversion to α-ketoglutarate (Dagley and Trudgill, 1965; Chang and Feingold, 1969; Chang and Feingold, 1970). α-Ketoglutarate is an intermediate of the citric acid cycle. *Azospirillum brasilense* also has another NAD-preferring α-ketoglutarate semialdehyde dehydrogenase, the expression of which was induced during growth on galactarate (Watanabe *et al.*, 2006b).

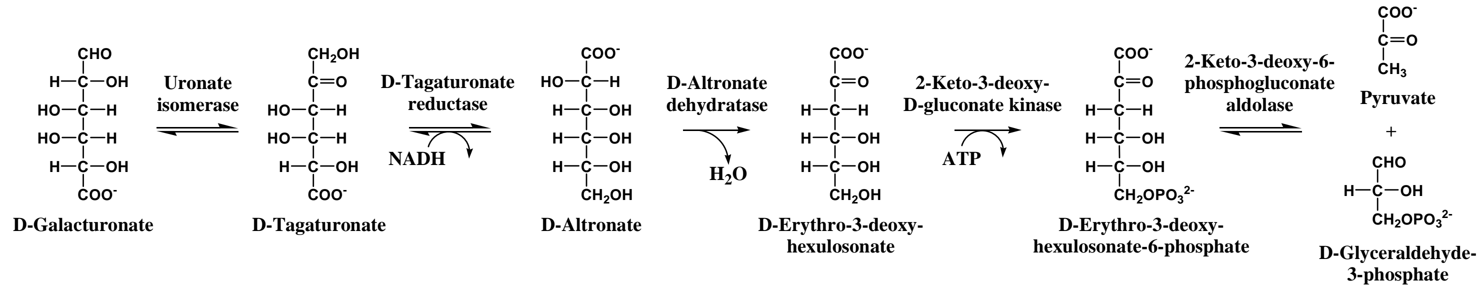


Figure 3. The D-galacturonate catabolic pathway in *E. coli.*

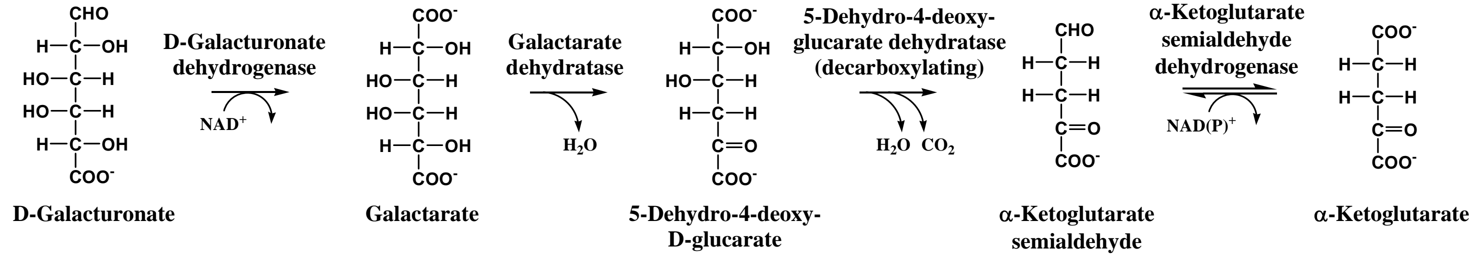


Figure 4. The D-galacturonate catabolic pathway in *A. tumefaciens*.

It was observed in polygalacturonate-adapted Pseudomonas that the degradation of polygalacturonate resulted in the monosaccharides D-galacturonate and 4-deoxy-5-keto-L-threo-hexuronate. This catabolic route is presented in Figure 5. Polygalacturonate was first cleaved to oligosaccharides by a polygalacturonase enzyme (EC 4.2.2.2). Hydrolysis of the saturated oligosaccharide produced free D-galacturonate. This was further catabolised to pyruvate and D-glyceraldehyde-3-phosphate via D-tagaturonate as in *E. coli* in Figure 3. Elimination of the unsaturated oligosaccharide produced 4-deoxy-5-keto-L-threo-hexuronate. The free D-galacturonate was not an intermediate in the formation of 4-deoxy-5-keto-L-threo-hexuronate. The 4-deoxy-5-keto-L-threo-hexuronate was converted to 3-deoxy-D-glycero-2,5-hexodiulosonate by an isomerase (EC 5.3.1.17) and further to D-erythro-3-deoxy-hexulosonate by an NAD(P)H-dependent dehydrogenase (EC 1.1.1.127). The D-erythro-3-deoxy-hexulosonate was then phosphorylated to D-erythro-3-deoxy-hexulosonate-6-phosphate and cleaved by an aldolase to pyruvate and D-glyceraldehyde-3-phosphate. The last two reactions are the same as in the D-galacturonate pathway of *E. coli* (Figure 3). All the enzymes of both these pathways were induced when cells were grown on polygalacturonate, and D-galacturonate dehydrogenase activity was also present in the cell extract (Preiss and Ashwell, 1963a; Preiss and Ashwell, 1963b).

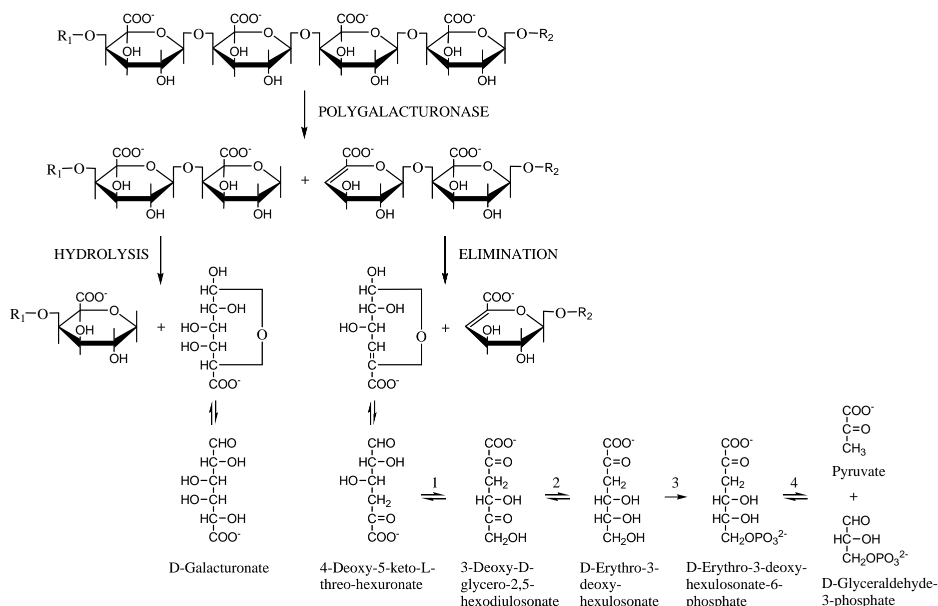
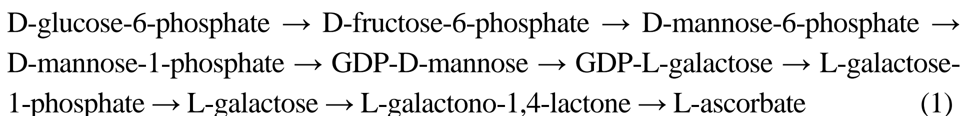


Figure 5. Polygalacturonate degradation in polygalacturonate-adapted *Pseudomonas*. The residues of the saturated oligosaccharide do not have double bonds, whereas the non-reducing terminal residue of the unsaturated oligosaccharide has a double bond between C4 and C5. *1* 4-Deoxy-5-keto-uronate isomerase; *2* 2-dehydro-3-deoxy-D-gluconate 5-dehydrogenase; *3* 2-keto-3-deoxy-D-gluconate kinase; *4* 2-keto-3-deoxy-6-phosphogluconate aldolase. Revised from the figure of Preiss and Ashwell 1963a.

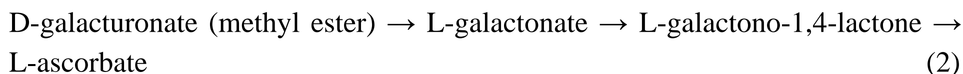
1.2.2 Metabolism of D-galacturonate in plants and animals

Higher plants produce L-ascorbate mainly from D-glucose-6-phosphate via a pathway that has GDP-D-mannose and GDP-L-galactose as two of the intermediates (Pathway 1) (Wheeler *et al.*, 1998):


D-glucose-6-phosphate → D-fructose-6-phosphate → D-mannose-6-phosphate → D-mannose-1-phosphate → GDP-D-mannose → GDP-L-galactose → L-galactose-1-phosphate → L-galactose → L-galactono-1,4-lactone → L-ascorbate (1)

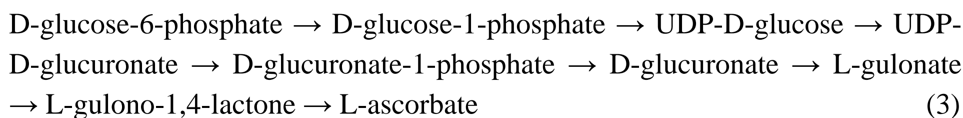
However, there is evidence for alternative biosynthetic L-ascorbate pathways, one of which has D-galacturonate as an intermediate. A correlation between fruit softening and ripening, disassembling of pectin in the cell walls and elevation of the L-ascorbate content has been shown for example in strawberry fruit

(Medina-Escobar *et al.*, 1997; Agius *et al.*, 2003). It has been suggested that the D-galacturonate derived from pectin in the fruit ripening process is converted to L-ascorbate (Pathway 2). The enzymes of the pathway are D-galacturonate reductase, aldono-lactonase and L-galactono-1,4-lactone dehydrogenase (Davey *et al.*, 1999; Agius *et al.*, 2003). The conversion of D-galacturonate to L-ascorbate in ripening strawberry has been confirmed by studying the metabolism of radiolabelled D-galacturonate-1-C^{14} (Loewus and Kelly, 1961). By contrast, studies with *Arabidopsis thaliana* showed that D-galacturonate itself did not serve as a substrate but only its non-ionic derivative, the D-galacturonate methyl ester, was converted to L-ascorbate (Davey *et al.*, 1999):


D-galacturonate (methyl ester) → L-galactonate → L-galactono-1,4-lactone → L-ascorbate (2)

It has also been suggested that plants may utilise some of the free D-galacturonate for energy production. *Phaseolus aureus* (mung bean) seedlings contain an enzyme system capable of oxidising D-galacturonate to galactarate (Kessler *et al.*, 1961). A partially purified enzyme from *Citrus sinensis* leaves was also able to catalyse oxidation of D-galacturonate in the presence of molecular oxygen to galactarate and H_2O_2. This enzyme had highest activity with D-galacturonate as substrate (Riov, 1975). Bacterial pathways are known in which galactarate is converted to α-ketoglutarate (Figure 4) (Dagley and Trudgill, 1965) or to pyruvate and D-glycerate (Hubbard *et al.*, 1998). It is not certain whether galactarate catabolism is similar in eukaryotes.

Those animals that do not require dietary L-ascorbate synthesise it from D-glucose-6-phosphate in the liver or kidneys (Pathway 3). The last three enzyme reactions resemble the alternative L-ascorbate synthesis pathway in plants (Pathway 2) (Agius *et al.*, 2003). In fact, when either D-glucuronate or D-galacturonate was incubated with a cell-free preparation of rat liver, L-ascorbate was produced (Ul Hassan and Lehninger, 1956).


D-glucose-6-phosphate → D-glucose-1-phosphate → UDP-D-glucose → UDP-D-glucuronate → D-glucuronate-1-phosphate → D-glucuronate → L-gulonate → L-gulono-1,4-lactone → L-ascorbate (3)

1.2.3 Earlier observations concerning D-galacturonate catabolism in mould fungi

D-Galacturonate catabolism in the filamentous fungus *A. nidulans* was concluded to be different from that in *E. coli* because the enzyme activities of the bacterial pathway were not found in this mould fungus after growing on D-galacturonate. Furthermore, *A. nidulans* does not catabolise D-galacturonate through one of the main carbohydrate degradation pathways, which was shown by growing several D-galacturonate non-utilising mutant strains on different carbon sources. For example on D-glucose and D-xylose these mutants grew normally (Uitzetter *et al.*, 1986). The pentose phosphate pathway appeared to have some role in D-galacturonate catabolism because an *A. nidulans* mutant with an inoperative transaldolase showed almost no growth on D-galacturonate. Mutants of *A. nidulans* with inoperative pyruvate carboxylase or pyruvate dehydrogenase had poor or no growth on D-galacturonate. Therefore pyruvate was predicted to be one of the degradation products of D-galacturonate. A mutant lacking pyruvate kinase activity grew on D-galacturonate, which indicated that pyruvate was not produced from D-galacturonate via phosphoenol pyruvate (Uitzetter *et al.*, 1982). The relationship between these enzyme reactions is presented in Figure 6.

In addition, the *A. nidulans* mutants that were unable to utilise glycerol were also unable to grow on D-galacturonate (Uitzetter *et al.*, 1982). Furthermore, when a wild-type strain of *A. nidulans* was transferred from D-fructose to glycerol or D-galacturonate medium the glycerol kinase and FAD-dependent mitochondrial glycerol-3-phosphate dehydrogenase activities were induced. Induction of NADH-dependent D-glyceraldehyde reductase and alcohol dehydrogenase activities was also observed. However, it was suggested that the alcohol dehydrogenase is responsible for both these activities. After transfer to D-galacturonate, NAD-dependent cytosolic glycerol-3-phosphate dehydrogenase and weak dihydroxyacetone (DHA) kinase and D-glyceraldehyde kinase activities were also induced (Hondmann *et al.*, 1991). These results indicated that the glycerol and D-galacturonate pathways are linked in some way. It was suggested that D-glyceraldehyde is the common intermediate. It was also predicted that in *A. nidulans* D-galacturonate is metabolised through a non-phosphorylating pathway, because glyceraldehyde-3-phosphate did not appear to be one of the end products (Uitzetter *et al.*, 1986). Studies with *A. niger*, a related filamentous fungus species, supported the suggested connection between

the glycerol and D-galacturonate pathways. A glycerol kinase mutant strain of *A. niger* grew poorly on glycerol and on D-galacturonate. When the mutant strain was transferred from D-glucose to D-galacturonate it accumulated glycerol in the culture. Therefore, it was concluded that in this filamentous fungus D-galacturonate is converted to glycerol, which cannot be further metabolised by the glycerol kinase mutant strain.

It was proposed that the D-galacturonate catabolic pathway in *A. nidulans* contains at least two enzymes. This was because two separate gene sites with point mutations produced strains that were unable to grow specifically on D-galacturonate (Uitzetter *et al.*, 1986). It was not until 22 years later that these genes were reported to code for D-galacturonate reductase and L-galactonate dehydratase (Martens-Uzunova and Schaap, 2008). A microarray analysis some years earlier had shown that when *A. niger* was grown on D-galacturonate the genes possibly coding for an aldo-keto reductase, a racemase and an aldolase were co-expressed (Martens-Uzunova *et al.*, 2005). The degradation of D-galacturonate was proposed also to involve an $NADP^+$-dependent glycerol dehydrogenase-catalysed reaction because this activity was induced on D-galacturonate. The enzyme was proposed to reduce D-glyceraldehyde in the reverse reaction but not to use DHA as a substrate (Witteveen *et al.*, 1990). An $NADP^+$-dependent glycerol dehydrogenase activity was also specifically induced in *A. nidulans* during growth on D-galacturonate (Sealy-Lewis and Fairhurst, 1992)

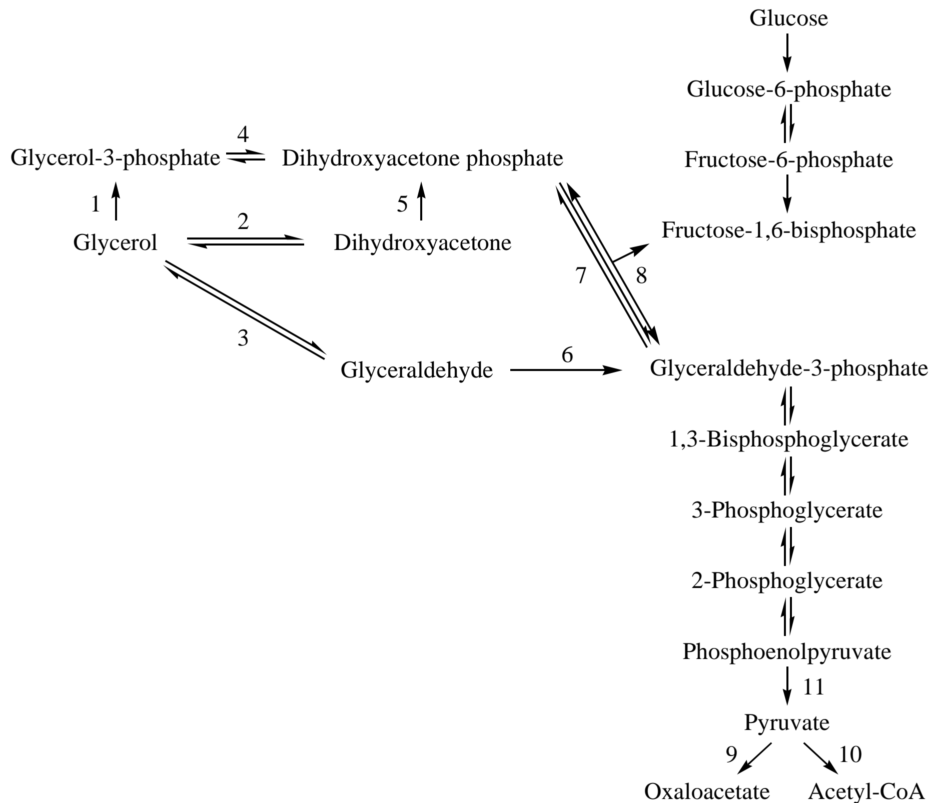


Figure 6. Relationship between different enzyme reactions that may be involved in D-galacturonate catabolism in mould fungi (adapted from Tom *et al.*, 1978). *1* Glycerol kinase; *2* NAD^+- or $NADP^+$-dependent glycerol dehydrogenase; *3* alcohol dehydrogenase/NADH-dependent D-glyceraldehyde reductase or $NADP^+$-dependent glycerol dehydrogenase; *4* NAD^+- or FAD-dependent glycerol-3-phosphate dehydrogenase; *5* DHA kinase; *6* D-glyceraldehyde kinase; *7* triose phosphate isomerase; *8* aldolase; *9* pyruvate carboxylase; *10* pyruvate dehydrogenase; *11* pyruvate kinase.

1.3 Examples of non-phosphorylated catabolic pathways

Some known catabolic pathways in which no phosphorylation step is involved and in which a 2-keto-3-deoxy sugar acid is one of the intermediates are presented in Figure 7. *A. niger* converts D-galactose to pyruvate and D-glyceraldehyde via a non-phosphorylated route. In this pathway D-galactose is first oxidised to D-galactono-1,4-lactone in the presence of NAD^+ by a D-galactose dehydrogenase (EC 1.1.1.48). The D-galactono-1,4-lactone is then linearised by a lactonase (EC 3.1.1.25) to D-galactonate. An inducible D-galactonate dehydratase (EC 4.2.1.6) was proposed to convert the D-galactonate to 2-keto-3-deoxy-D-galactonate, after which a constitutive 2-keto-3-deoxy-D-galactonate aldolase converts this metabolite further to pyruvate and D-glyceraldehyde (Elshafei and Abdel-Fatah, 2001). The last two reactions had also been detected in *Aspergillus terreus* (Elshafei and Abdel-Fatah, 1991).

A. niger is also able to catabolise D-glucose to D-glyceraldehyde and pyruvate through a similar pathway. *A. niger* oxidises D-glucose to D-glucono-1,5-lactone by a glucose oxidase (EC 1.1.3.4) in the presence of molecular oxygen. This filamentous fungus also has a gluconolactonase enzyme (EC 3.1.1.17) that converts D-glucono-1,5-lactone to D-gluconate (Ogawa *et al.*, 2002). D-Gluconate is dehydrated to 2-keto-3-deoxy-D-gluconate by a D-gluconate dehydratase (EC 4.2.1.39). This is further cleaved to D-glyceraldehyde and pyruvate by a 2-keto-3-deoxy-D-gluconate aldolase (EC 4.1.2.20) (Elzainy *et al.*, 1973). A similar pathway, the non-phosphorylated Entner-Doudoroff pathway, also operates in thermophilic archaea (Buchanan *et al.*, 1999; Siebers and Schönheit, 2005). Here the only difference is in the first reaction from D-glucose to D-glucono-1,5-lactone, which is catalysed by a glucose dehydrogenase (EC 1.1.1.47) instead of a glucose oxidase.

L-Rhamnose catabolism in the yeast fungus *Pichia stipitis* and the bacterium *Azotobacter vinelandii* also involves non-phosphorylated intermediates. The enzymes of the pathway are $NAD(P)^+$-dependent L-rhamnose dehydrogenase (EC 1.1.1.173), L-rhamnono-1,4-lactonase (EC 3.1.1.65), L-rhamnonate dehydratase (EC 4.2.1.90), 2-keto-3-deoxy-L-rhamnonate aldolase and $NAD(P)^+$-dependent L-lactaldehyde dehydrogenase (EC 1.2.1.22). These enzymes convert L-rhamnose to pyruvate and L-lactate via the intermediates L-rhamnono-1,4-lactone, L-rhamnonate, 2-keto-3-deoxy-L-rhamnonate and L-lactaldehyde (Twerdochlib *et al.*, 1994; Watanabe *et al.*, 2008a, Watanabe *et al.*, 2008b).

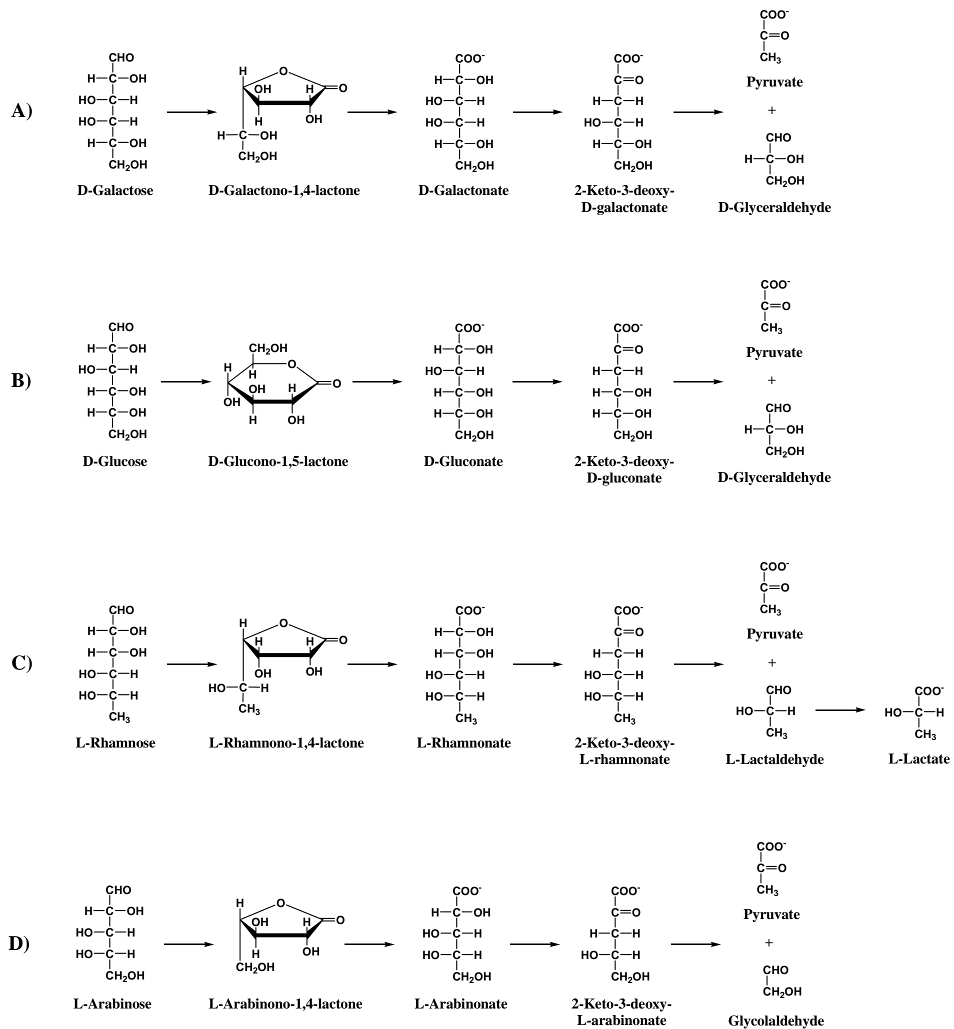


Figure 7. Non-phosphorylated catabolism of A) D-galactose, B) D-glucose, C) L-rhamnose and D) L-arabinose in different organisms.

In some bacteria L-arabinose is also believed to be catabolised via a non-phosphorylated pathway. L-Arabinose is oxidised to L-arabinono-1,4-lactone by an NAD(P)-dependent dehydrogenase (EC 1.1.1.46) and the lactone is cleaved by a lactonase (EC 3.1.1.15) to L-arabinonate. Then a dehydratase (EC 4.2.1.25) converts L-arabinonate to 2-keto-3-deoxy-L-arabinonate (Watanabe *et al.*, 2006a; Watanabe *et al.*, 2006c). In *Pseudomonas* strain MSU-1, 2-keto-3-deoxy-

L-arabinonate is cleaved to pyruvate and glycolaldehyde by an aldolase (EC 4.1.2.18) (Dahms and Anderson, 1969).

1.4 Hypotheses and objectives of the work

D-Galacturonate is the major component of pectin and a potential carbon source for microorganisms living on decaying plant material. Bacterial catabolic D-galacturonate pathways are known but similar pathways have not been reported in eukaryotes. However, many species of yeast and mould fungi are able to utilise and grow on D-galacturonate. This indicates that in eukaryotic microorganisms a catabolic D-galacturonate pathway exists which is different from the bacterial pathways. The aim of this work was to determine how eukaryotes catabolise D-galacturonate and what enzymes and intermediate metabolites form the pathway. The filamentous fungus *T. reesei* was chosen as the model organism.

Some plant materials such as the peel of citrus fruits and sugar beet pulp are especially rich in pectin. Large amounts of these pectin-rich residues are produced as side products of the food industry. They are mainly used as animal feed after drying and pelletising. However, these residues have relatively low feed value and drying consumes energy. Therefore it would be desirable to convert this cheap and renewable raw material to products of higher value.

2. Materials and methods

The filamentous fungus *Trichoderma reesei* was able to grow on D-galacturonate. Its genome sequence was known and was publicly available at the Joint Genome Institute website (http://genome.jgi-psf.org/Trire2/Trire2.home.html). Furthermore, *T. reesei* has been extensively studied in our laboratory and many genetic engineering techniques have been developed for working with this mould fungus. For example, a good cDNA library of the strain Rut C-30 had already been made, which facilitated cloning of the genes. *T. reesei* was therefore chosen as the model organism in this study. The strains that were used to study the different enzymes of the D-galacturonate pathway are listed in Table 2.

Table 2. The *T. reesei* strains used in this work.

Enzyme	Enzyme activity observed in strain	Gene cloned from strain	Gene deleted from strain
GAR1	Rut C-30	Rut C-30	QM6a (Mojzita *et al.,* 2010)
LGD1	Rut C-30	Rut C-30	Rut C-30
LGA1	VTT-D-80133	Rut C-30	QM6a
GLD1	Rut C-30 and QM6a	Rut C-30	
GLD2	Rut C-30 and QM6a	QM6a	

T. reesei was grown in liquid culture that contained 20 g/l of the main carbon source, 2 g/l (in GAR1 and GLD1 experiments) or 0.5 g/l (in LGD1 and LGA1 experiments) of proteose peptone and also 15 g/l KH_2PO_4, 5 g/l $(NH_4)_2SO_4$, 0.6 g/l $MgSO_4*7H_2O$, 0.6 g/l $CaCl_2*2H_2O$, and trace elements (Mandels and Weber, 1969) at 28ºC. Before harvesting *T. reesei* was typically grown on D-galacturonate for five days. Growth on this substrate was slower than on all the

other used carbon sources. Difference in the growth rate on D-galacturonate was not observed between the used *T. reesei* host strains.

The work proceeded stepwise along the pathway starting by finding the pathway's first enzyme exhibiting activity with D-galacturonate. When the reaction product of the previous enzyme was identified it was then used as the reaction substrate for the next enzyme. Some of the intermediate substrates had to be prepared chemically or enzymatically, since they were not commercially available. The enzyme activities were measured using crude mycelial extracts of *T. reesei* grown aerobically on D-galacturonate, and for comparison on other carbon sources. Enzyme activities were measured as U/mg, *i.e.* micromoles of substrate per minute per milligram of protein. The gene coding for the enzyme was identified either by purifying and sequencing the enzyme or by directly cloning a gene from the *T. reesei* cDNA library based on sequence homology to other similar enzymes. The cloned genes were expressed in *S. cerevisiae*, the enzyme activities were verified and the enzymes were further characterised. Some of the pathway's genes were deleted from *T. reesei* by replacing them with a hygromycin B resistance gene by homologous recombination. The effect of the gene deletion was then studied by growing the deletion strains on D-galacturonate. The materials and methods are described in more detail in the original articles. The used methods are listed in Table 3.

Table 3. Methods used in the original articles (I–IV).

Method	**Used in**
Chemical or enzymatic preparation of reaction substrates	III, IV
Cloning a fungal gene based on homology	II, III
Constructing a fungal deletion strain	II, IV
Dry mass measurements	II, IV
Enzyme activity measurements	I–IV
Expressing fungal genes in *S. cerevisiae*	I–IV
Metabolite analysis, HPLC or NMR	II, IV
Protein purification and partial sequencing	I, IV
RNA extraction and Northern hybridisation	I, II

3. Results

A novel catabolic D-galacturonate pathway was identified in the filamentous fungus *Trichoderma reesei*. It consisted of four enzymes which were described in four separate publications. The enzymes were NADPH-dependent D-galacturonate reductase (GAR1), L-galactonate dehydratase (LGD1), L-threo-3-deoxy-hexulosonate aldolase (LGA1) and NADPH-dependent glyceraldehyde reductase (GLD1). This fungal pathway converted D-galacturonate to pyruvate and glycerol as presented in Figure 8.

3.1 D-Galacturonate reductase (I)

T. reesei grown on D-galacturonate had an NADPH-dependent D-galacturonate reductase activity in the crude mycelial extract. When the mould fungus was grown on D-glucose, D-fructose, D-galactose, D-xylose, or glycerol this activity was absent.

3.1.1 Cloning the D-galacturonate reductase

In order to identify the gene sequence of the *T. reesei* D-galacturonate reductase, the enzyme was purified from mycelial extract after growth on D-galacturonate. The purified enzyme with an estimated molecular mass of 40 kDa was digested with trypsin and the amino acid sequences of two fragments were obtained. These peptide sequences corresponded to a *T. reesei* cDNA sequence in public databases where only the 3' end of the open reading frame was reported. The genomic sequence of *T. reesei* upstream from this partial open reading frame was compared to previously reported aldo-keto reductase sequences and six possible start codons were detected. *T. reesei* cDNA library and six different sense primers were used with an antisense primer to identify the correct start codon. In this way the open reading frame was identified and cloned. It coded

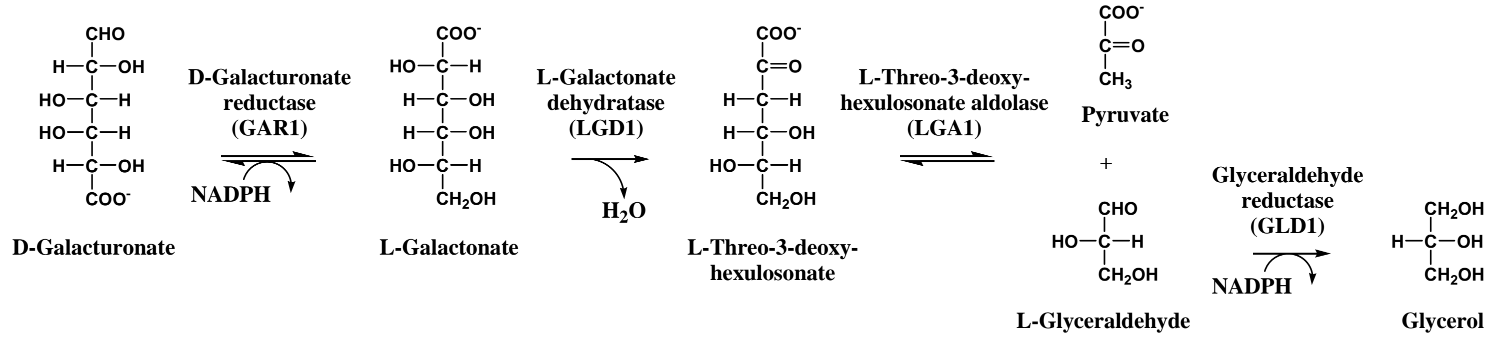


Figure 8. The D-galacturonate catabolic pathway in *T. reesei*.

for a 309 amino acid protein with a molecular mass of 33 940 Da. The corresponding genomic DNA sequence had five introns. The open reading frame was expressed in *S. cerevisiae* in a multicopy plasmid under a constitutive promoter which resulted in the production of an active enzyme in this heterologous host. The gene was called *gar1* and the sequence was deposited in NCBI GenBank with accession number AY862503. In the *T. reesei* genome database v2.0 it has a protein ID 22004.

3.1.2 Characterisation of the D-galacturonate reductase

The partially purified *T. reesei* D-galacturonate reductase used NADPH as a cofactor. The reverse reaction was observed with L-galactonate and $NADP^+$. No activity was observed with L-galactono-1,4-lactone as a substrate.

To facilitate purification the D-galacturonate reductase was produced in *S. cerevisiae* with a C-terminal polyhistidine tag. The tagged and the nontagged protein had the same activity in the crude yeast extract and the purified tagged protein was used for kinetic analysis. The D-galacturonate reductase exhibited activity in the forward direction with D-galacturonate, D-glucuronate and DL-glyceraldehyde. The Michaelis-Menten constants were K_m = 6 mM and V_{max} = 40 U/mg, K_m = 11 mM and V_{max} = 25 U/mg, and K_m = 6 mM and V_{max} = 7 U/mg, respectively. The cofactor was always NADPH, for which the Michaelis-Menten constants were K_m = 30 µM and V_{max} = 40 U/mg when D-galacturonate was used as a substrate. No activity was observed when D-glucose, D-fructose, D-xylose, D-galactose, L-arabinose, or D-mannose were tested as substrates. In the reverse direction L-galactonate and $NADP^+$ served as substrates with Michaelis-Menten constants K_m = 4 mM and K_m = 1 µM, respectively, and V_{max} = 2 U/mg. No backward reaction was observed with L-galactono-1,4-lactone, L-gulonate, glycerol, D-arabitol, L-arabitol, xylitol, galactitol or ribitol.

Expression of the D-galacturonate reductase gene was studied with Northern blot analysis on different carbon sources. When *T. reesei* was grown on D-fructose, D-xylose, lactose, D-galactose, glycerol and D-mannose a basic expression level was observed, but on D-galacturonate the expression level was 8 times and on D-glucose 3 times greater.

The D-galacturonate reductase gene in *T. reesei* was deleted by replacing it with a hygromycin B resistance gene by homologous recombination. When grown in liquid cultures with D-galacturonate and peptone for five days the host strain produced about 3 g/l of biomass, whereas the deletion strain produced

only about 0.5 g/l of biomass. Both strains produced about 0.2 g/l of biomass when only the peptone was included in the medium. Deletion of the D-galacturonate reductase gene also resulted in an impaired sporulation ability on PD (potato dextrose) agar plates. The spores could be obtained by growing the deletion strain on plates containing 10% carrot juice (Mojzita *et al.*, 2010).

3.2 L-Galactonate dehydratase (II)

T. reesei was grown on D-galacturonate and the mycelial extract was tested for different enzyme activities. When L-galactonate was added to the mycelial extract we observed the formation of a reducing sugar. The enzyme activity which formed a reducing sugar from L-galactonate was present in *T. reesei* mycelia grown on D-galacturonate but absent when grown on D-galactose, D-glucose, D-xylose, D-fructose, lactose, or glycerol. In addition a reaction mixture of L-galactonate and crude mycelial extract of *T. reesei* grown on D-galacturonate was analysed using electrospray ionisation mass spectrometry (ESI-MS). It was observed that the reaction product was a molecule with a mass of one water molecule less than the mass of L-galactonate, and had a different MS-MS spectrum from the lactone form of L-galactonate. Some pyruvate was also formed, which provided a clue about the subsequent reactions. Since no redox cofactor was involved and a water molecule was split off, the second enzyme in the pathway was concluded to be a dehydratase.

3.2.1 Cloning the L-galactonate dehydratase

Different approaches were tested in order to purify the L-galactonate dehydratase. However, in all the trials the L-galactonate dehydratase lost its activity in the purification process and we were not able to obtain pure protein that could have been sequenced. In order to identify the gene coding for an L-galactonate dehydratase the amino acid sequences of *E. coli* and *Brucella melitensis* D-galactonate dehydratases (EC 4.2.1.6) were compared to the *T. reesei* genome sequence. Six open reading frames of potential dehydratases were then cloned using *T. reesei* cDNA or RNA as a template. They all were expressed in *S. cerevisiae* under a constitutive promoter in a multicopy plasmid. The cell extracts of these yeast strains were then assayed for reducing sugar formation from L-galactonate. *S. cerevisiae* does not have L-galactonate dehydratase activity but one of the expressed open reading frames resulted in a

significant activity with L-galactonate as a substrate. This gene was called *lgd1* and the sequence was deposited in NCBI GenBank with accession number DQ181420. It coded for a protein of 450 amino acids and had one intron in the corresponding genomic DNA sequence. Since such an enzyme activity was not described previously it does not have an EC (Enzyme Commission) number. In the Brenda database (www.brenda-enzymes.info) it was listed with the preliminary number EC 4.2.1.B1. In the *T. reesei* genome database v2.0 it has a protein ID 104599.

3.2.2 Characterisation of the L-galactonate dehydratase

The L-galactonate dehydratase gene was expressed in *S. cerevisiae*, resulting in an active enzyme that produced a reducing sugar from L-galactonate. The reaction mixture was analysed by NMR spectroscopy to identify the reaction product. The results showed that the product was L-threo-3-deoxy-hexulosonate (2-keto-3-deoxy-L-galactonate) and that it predominantly existed as a pyranose ring. Over 85% of the product existed as one anomer of the pyranose ring but it could not be determined which one of the two anomers it was. The enzyme also exhibited stereospecificity at the C3 position, since it attached a hydrogen atom only to the axial position during the reaction.

The L-galactonate dehydratase activity in the crude mycelial extract of *T. reesei* grown on D-galacturonate was 0.018 U/mg protein (0.3 nkat/mg) and in the cell extract of *S. cerevisiae* expressing the *lgd1* it was 0.009 U/mg protein (0.15 nkat/mg). Here the L-galactonate concentration was 7.5 mM. The product L-threo-3-deoxy-hexulosonate was measured with a thiobarbituric acid (TBA) assay (Buchanan *et al.*, 1999).

The L-galactonate dehydratase was polyhistidine tagged either at the N- or C-terminus and produced in *S. cerevisiae*. The tag at the C-terminus resulted in an inactive protein and the N-terminally tagged protein had only about one fifth of the activity of untagged protein in yeast extract. Since the tag apparently interfered with the activity, the crude cell extract of the yeast strain expressing untagged *lgd1* was used to characterise the enzyme kinetics. Different sugar acids were tested for the substrate specificity of L-galactonate dehydratase. It produced a reducing sugar from L-galactonate and D-arabinonate. Activity with D-arabinonate was about half of that with L-galactonate. No activity was observed when D-galactonate, D-gluconate, D-xylonate, or L-gulonate were used as substrates. The K_m for L-galactonate was estimated to be 5 mM. L-

Galactonate at a concentration of 20 mM was completely converted to L-threo-3-deoxy-hexulosonate in 24 hours. This suggested that the energetic equilibrium is strongly on the side of the reaction product. The enzyme required bivalent cations (Mg^{2+}) for activity and no activity was observed in the presence of 5 mM EDTA. The TBA assay that was used to determine enzyme kinetics was disturbed by high substrate concentrations. When the same reactions were measured with 3,5-dinitrosalicylate (DNS) assay (Bernfeld, 1955) the high substrate concentration did not interfere with the assay.

Northern blot analysis was used to study expression of the L-galactonate dehydratase gene on different carbon sources. The expression level was the same on D-galacturonate, glycerol and lactose and three times higher on D-glucose and D-xylose.

The L-galactonate dehydratase gene in *T. reesei* was deleted by replacing it with a hygromycin B resistance gene by homologous recombination. When grown in liquid cultures with D-galacturonate and peptone for five days the host strain produced about 2.4 g/l of biomass, whereas the deletion strain produced only 0.1 g/l of biomass. Both strains also produced 0.1 g/l of biomass when only the peptone was included in the medium. The deletion strain also accumulated L-galactonate in the culture; in five days it produced 1.3 g/l of L-galactonate, whereas 0.4 g/l was produced by the host strain (unpublished results). The growth on D-galacturonate was restored when the *lgd1* was retransformed to the deletion strain.

3.3 L-Threo-3-deoxy-hexulosonate aldolase (IV)

The first indication of an aldolase reaction in the D-galacturonate pathway was when pyruvate was detected after incubating L-galactonate with crude mycelial extract of *T. reesei* grown on D-galacturonate. The formed pyruvate was identified by ESI-MS as well as by assaying with lactate dehydrogenase. After the L-galactonate dehydratase had been characterised and shown to produce L-threo-3-deoxy-hexulosonate, this compound was prepared and used as the reaction substrate in the search for the next enzyme on the pathway. The crude mycelial extract mixed with L-threo-3-deoxy-hexulosonate produced pyruvate. The reverse reaction was also observed, since a compound giving a signal in the TBA assay was formed when pyruvate and L-glyceraldehyde were incubated with the crude mycelial extract. The L-threo-3-deoxy-hexulosonate aldolase activity was induced in *T. reesei* during growth on D-galacturonate but not on D-

glucose, D-xylose, lactose, or glycerol. Such an enzyme activity had not been described previously.

3.3.1 Cloning the L-threo-3-deoxy-hexulosonate aldolase

The L-threo-3-deoxy-hexulosonate aldolase was partially purified from *T. reesei* mycelial extract after growth on D-galacturonate. The obtained 37 kDa protein was digested with trypsin and four peptide sequences were obtained for identifying the coding sequence from the *T. reesei* genome. The open reading frame was cloned from cDNA, and comparing it to the genomic DNA showed that there were no introns. The gene was named *lga1* and the sequence was deposited in NCBI GenBank with the accession number EF203091. It coded for a 315 amino acid protein. In the *T. reesei* genome database v2.0 it has a protein ID 60067. The gene was expressed in *S. cerevisiae* under a constitutive promoter in a multicopy plasmid and this heterologous host produced an active protein.

3.3.2 Characterisation of the L-threo-3-deoxy-hexulosonate aldolase

The L-threo-3-deoxy-hexulosonate aldolase activity in the crude mycelial extract of *T. reesei* grown on D-galacturonate was 0.16 U/mg of protein in the forward direction with L-threo-3-deoxy-hexulosonate as a substrate. L-Glyceraldehyde and pyruvate were substrates in the reverse direction and the enzyme activity was 0.20 U/mg of protein. When L-glyceraldehyde was replaced with D-glyceraldehyde the activity was 0.01 U/mg of protein. This was close to the detection limit, which was about 3% of the activity with L-glyceraldehyde. In fact D-glyceraldehyde appeared to be a reaction inhibitor, although the actual mechanism is not known. From the enzyme activity it was seen that when DL-glyceraldehyde was used as a substrate instead of L-galactonate the reaction was only about 10% (0.02 U/mg of protein) of that with L-glyceraldehyde, rather than the anticipated 50%. The L-threo-3-deoxy-hexulosonate aldolase activity in the cell extract of *S. cerevisiae* expressing the *lga1* was 0.05 U/mg of protein.

A polyhistidine tag was added to the N- or C-terminus of the L-threo-3-deoxy-hexulosonate aldolase and the protein was produced in *S. cerevisiae*. The C-terminally tagged enzyme had only about one third of the activity of untagged enzyme, but the N-terminal tag did not interfere with the activity. The purified N-terminally tagged protein was used in further enzyme characterisations.

Substrate specificity in the forward direction was tested with L-threo-3-deoxy-hexulosonate (2-keto-3-deoxy-L-galactonate), D-glycero-3-deoxy-pentulosonate (2-keto-3-deoxy-D-arabinonate), and D-threo-3-deoxy-hexulosonate (2-keto-3-deoxy-D-galactonate). Pyruvate was produced when L-threo-3-deoxy-hexulosonate or D-glycero-3-deoxy-pentulosonate was a reaction substrate. The Michaelis-Menten constants for L-threo-3-deoxy-hexulosonate were K_m = 3.5 mM and V_{max} = 20.3 U/mg and for D-glycero-3-deoxy-pentulosonate K_m = 3.8 mM and V_{max} = 12.5 U/mg. Substrate specificity in the reverse direction was tested with pyruvate and different aldehydes and ketones. Activity was observed with L-glyceraldehyde, glycolaldehyde, and methylglyoxal. The Michaelis-Menten constant K_m for pyruvate was 0.5 mM and for L-glyceraldehyde 1.2 mM, and the V_{max} was about 6.5 U/mg. For glycolaldehyde the constants were K_m = 6.5 mM and V_{max} = 0.9 U/mg.

The L-threo-3-deoxy-hexulosonate aldolase gene in *T. reesei* was deleted by replacing it with a hygromycin B resistance gene by homologous recombination. When grown in liquid cultures with D-galacturonate and peptone for six days the host strain produced about 4.7 g/l of biomass, whereas the deletion strain produced only 0.2 g/l. Both strains produced 0.1 g/l of biomass when only the peptone was included in the medium. The deletion strain also accumulated L-threo-3-deoxy-hexulosonate in the culture; in six days it accumulated 3.6 g/l of L-threo-3-deoxy-hexulosonate whereas only 0.3 g/l accumulated with the host strain. Retransforming the *lga1* to the deletion strain restored the growth on D-galacturonate.

3.4 Glyceraldehyde reductase and DHA reductase (III)

On the basis of previous knowledge from other filamentous fungi it was suspected that *T. reesei* has at least one NADPH-dependent reductase producing glycerol. It was assumed that a constitutive dihydroxyacetone (DHA) reductase and possibly also a glyceraldehyde reductase would be found. *T. reesei* was grown on D-galacturonate, glycerol, lactose, D-glucose and D-xylitol and the crude mycelial extracts were tested for reductase activity with L- or D-glyceraldehyde and NADPH. Similar activities were observed on all carbon sources, demonstrating that glyceraldehyde reductase activity was not specifically induced on D-galacturonate.

3.4.1 Cloning the glyceraldehyde reductase and DHA reductase

In order to find glycerol dehydrogenase genes in the *T. reesei* genome, the partial amino acid sequences of *A. niger* $NADP^+$-dependent glycerol dehydrogenase (Norbeck and Blomberg, 1997) were compared to the translated *T. reesei* genome sequence. Two potential open reading frames were identified. The start and the stop codons were also predicted by comparing other glycerol dehydrogenase sequences to the *T. reesei* genomic DNA. One open reading frame was cloned from *T. reesei* cDNA and called *gld1* and the other from genomic DNA, since it did not appear to have any introns, and was called *gld2*. When the cloned *gld1* sequence was compared to the corresponding genomic DNA, three introns were detected. The open reading frame that codes for a 331 amino acid protein was deposited in NCBI GenBank with the accession number DQ422037. In the *T. reesei* genome database v2.0 it has a protein ID 120911. The open reading frame of *gld2* that codes for a 318 amino acid protein was deposited in the NCBI GenBank with accession number DQ422038. In the *T. reesei* genome database v2.0 it has a protein ID 122778. Both cloned genes were expressed in *S. cerevisiae* under a constitutive promoter in a multicopy plasmid. Activities were measured in the crude cell extracts. The *gld1*-expressing strain showed activity when NADPH and DL-glyceraldehyde were used as substrates. No activity was detected in the other direction with $NADP^+$ and glycerol. The *gld2*-expressing strain did show activity with $NADP^+$ and glycerol or with NADPH and DHA, but very low activity with D- and L-glyceraldehyde.

3.4.2 Characterisation of the glyceraldehyde reductase and DHA reductase

An N-terminal polyhistidine tag was added to the GLD1 and GLD2 and the enzymes were produced in *S. cerevisiae*. The tags did not interfere with the activities and therefore the purified tagged enzymes were used in kinetic characterisations. Both enzymes were specific for NADPH as a cofactor. The GLD1 catalysed reduction of D-glyceraldehyde and L-glyceraldehyde to glycerol. The Michaelis-Menten constant K_m for both was about 0.9 mM and for NADPH about 40 µM. The enzyme also had significant activity with glyoxal, methylglyoxal and diacetyl but no activity in the other direction with glycerol and $NADP^+$. The purified GLD2 reduced DHA and oxidised glycerol. The Michaelis-Menten constant K_m for DHA was 1 mM and for NADPH 50 µM. In the oxidative direction the K_m for glycerol was 350 mM and for $NADP^+$ 110 µM.

4. Discussion

The four new enzymes of the previously unknown D-galacturonate pathway are discussed below separately and also together as a pathway. Some possible biotechnological applications of this newly discovered pathway are also presented.

4.1 The fungal D-galacturonate pathway

It is common that in a pathway one or two of the reactions are irreversible (Nielsen, 1997). An irreversible reaction creates a force which pushes metabolites only in one direction along the pathway and also enables an organism to control the flux. The second and the fourth enzyme in the fungal D-galacturonate pathway, the L-galactonate dehydratase and the glyceraldehyde reductase, catalysed an irreversible reaction. The other two reactions, catalysed by the D-galacturonate reductase and by the L-threo-3-deoxy-hexulosonate aldolase, were reversible.

While we were studying the fungal catabolism of D-galacturonate in *Trichoderma reesei*, another research group was studying this fungal pathway in *A. niger*. Their approach was a transcriptome analysis of *A. niger* to identify genes that were induced specifically on the carbon sources D-galacturonate, polygalacturonic acid or sugar beet pectin. They grew *A. niger* first on D-fructose, transferred the mycelia to media containing D-galacturonate, collected samples after 2, 4, 8 and 24 hours and used the RNA isolated from these samples in the transcriptome analysis. In addition to several genes involved in pectin degradation and potentially in D-galacturonate transport they found four hypothetical genes that formed the D-galacturonate catabolic pathway in *A. niger*. These four genes were predicted to code for D-galacturonate reductase

(GAAA), L-galactonate dehydratase (GAAB), 2-keto-3-deoxy-L-galactonate aldolase (GAAC) and glyceraldehyde reductase (GAAD) as we had reported earlier for *T. reesei*. They also noticed that a similar D-galacturonate pathway is strictly conserved in genomes of pectin-degrading filamentous fungi belonging to the subphylum *Pezizomycotina.* The fungal D-galacturonate pathway gene homologues were not found in any of the sequenced yeast fungi (Martens-Uzunova and Schaap, 2008).

On the whole the D-galacturonate pathways found in *T. reesei* and *A. niger* are very similar, but there are also some significant differences. In *A. niger* transcriptome analysis all the four pathway genes were strongly induced by D-galacturonate (Martens-Uzunova and Schaap, 2008). We observed the enzyme activities of the three first enzymes of the pathway (GAR1, LGD1 and LGA1) in *T. reesei* only when grown on D-galacturonate and not on other carbon sources, but the activity of the fourth enzyme (GLD1) was not dependent on D-galacturonate. We also studied the gene induction but used Northern hybridization, and of the pathway's three first genes that were tested only the *gar1* showed induction on D-galacturonate. The RNA that we used in the Northern blot was isolated from *T. reesei* after growing it on D-galacturonate for four days, when the residual D-galacturonate concentration in the culture was typically 6–9 g/l. The enzyme activities were still strong at that time point but it is not clear why the gene induction was not seen at the mRNA level. In the *A. niger* transcriptome analysis the pathway's genes were strongly induced after 8 hours from the transfer to D-galacturonate medium, when the residual D-galacturonate concentration was 6.2 g/l (Martens-Uzunova and Schaap, 2008). It should be noted that in the case of the *T. reesei* gene induction study that the enzyme activity measurements, the D-galacturonate concentration measurements and the Northern blot samples were not prepared from the same cultures. In addition, Northern blot analysis is not the most sensitive method for studying gene transcription; it provides mainly qualitative information of mRNA levels. Some other method, such as real-time qPCR or cDNA microarray analysis, would have been more suitable for studying quantitative transcription of genes of the *T. reesei* D-galacturonate pathway.

When comparing amino acid sequences of the *A. niger* GAAB, GAAC and GAAD to *T. reesei* LGD1, LGA1 and GLD1 it can be seen that the identities are 76%, 60% and 57%, respectively (Table 4). For these three sequences there are no other sequences with higher identities in the two organisms. The high degree of identity indicates that these enzymes probably have the same functions in

both species. By comparison, the enzymes of the glycolytic pathway in *T. reesei* and *A. niger* have 60–86% identical amino acid sequences. On the other hand the amino acid sequences of the *T. reesei* GAR1 and the *A. niger* GAAA were only 13% identical, even though both have been produced in a heterologous host and have been shown to code for an active D-galacturonate reductase enzyme. However, although the amino acid sequences were not related, the structures and functions of these two enzymes could be similar. The GAR1 was NADPH-dependent. GAAA preferred NADPH as a cofactor, but it could also use NADH. It is possible that two non-homologous genes code for the first enzyme in the *T. reesei* and *Aspergillus* pathways. It is conceivable that differing cofactor requirements in these mould fungi might have created an evolutionary pressure to use different D-galacturonate reductases. At the same time, *T. reesei* has an orthologous gene to *A. niger gaaA* in its genome, designated *gar2*, and *A. niger* has an orthologue to *T. reesei gar1*. In amino acid sequences the identity between the hypothetical GAR2 and GAAA is 70% and between GAR1 and the hypothetical *A. niger* counterpart 61% (Table 4). It is not known whether the *gar2* in *T. reesei* or the *gar1* orthologue in *A. niger* is expressed, or whether they code for active enzymes.

Table 4. The identities between amino acid sequences of the *T. reesei* and the *A. niger* D-galacturonate pathway enzymes.

	T. reesei	***A. niger***	**Identity**
D-Galacturonate reductase	**GAR1**	GAR1 ortholog	61%
D-Galacturonate reductase	GAR2	**GAAA**	70%
	GAR1	GAAA	13%
L-Galactonate dehydratase	**LGD1**	**GAAB**	76%
L-Threo-3-deoxy-hexulosonate aldolase	**LGA1**	**GAAC**	60%
Glyceraldehyde reductase	**GLD1**	**GAAD**	57%

We have shown that deleting *gar1* in *T. reesei* almost abolishes growth on D-galacturonate, and therefore the *gar2* cannot replace the deleted *gar1* (Mojzita *et al.*, 2010). Conversely, it was shown in *A. nidulans* and *A. niger* that if the activity of GAAA was lost the mould fungus did not grow on D-galacturonate as the *gar1* orthologue could not compensate for the missing activity (Uitzetter *et al.*, 1986; Mojzita *et al.*, 2010). In *A. niger* only the *gaaA* was strongly induced on D-galacturonate, whereas the *gar1* orthologue was not. Nevertheless, both

genes are conserved not only in *T. reesei* and in *A. niger* but also in the other *Pezizomycotina*. This suggests that both these genes are important for pectin-degrading fungi and could therefore indicate that both are in some way involved in D-galacturonate catabolism. Possibly the capacity to use either one of the two different D-galacturonate reductases gives these filamentous fungi an advantage to adapt to changing growth conditions more efficiently.

In the *A. niger* genome the *gaaA* and *gaaC* share the same promoter region. The arrangement with the bidirectional promoter region between these two genes is a conserved feature in fungi having the D-galacturonate pathway gene homologues. This means that in *T. reesei* the hypothetical *gar2* gene also shares the same promoter region with the *lga1*. This in turn could indicate that *gar2* is also induced on D-galacturonate in *T. reesei*.

Deleting any of the genes *gar1, lgd1* or *lga1* in *T. reesei* abolished almost all growth on D-galacturonate, indicating that there is no alternative D-galacturonate pathway. Although the *lgd1*-deletion strain did not use D-galacturonate for growth, it removed some of the D-galacturonate from the medium. At the same rate as D-galacturonate disappeared, approximately an equal amount of L-galactonate accumulated in the culture. This was not observed with the wild-type strain. In a similar way, all the D-galacturonate that was removed from the medium by the *lga1*-deletion strain appeared to be converted to L-threo-3-deoxy-hexulosonate. The concentrations of L-galactonate and L-threo-3-deoxy-hexulosonate were measured only from the growth medium; intracellular concentrations were not measured. However, it is assumed that D-galacturonate is taken up by the fungus and after that the catabolism proceeds inside the mycelia. Probably in the *lgd1*- and *lga1*-deletion strains the intracellular concentrations of the accumulated compounds became so high that they leaked out into the growth medium. Surprisingly, the *gar1*-deletion strain also removed some of the D-galacturonate from the medium, although it did not use it for growth. It was not clear to what compound D-galacturonate was converted (Mojzita *et al.*, 2010). Possibly a double deletion with *gar2* would have completely prevented the disappearance of D-galacturonate from the growth medium. The *gar1*-deletion strain was not tested for D-galacturonate reductase activity. When purifying GAR1 from the wild type *T. reesei* we observed two fractions that had activity with D-galacturonate and NADPH, but since the activity of one fraction was only about 10% compared to the GAR1 it was not further investigated. It would also be interesting to know whether the *gar2*-deletion alone would result in an altered phenotype when grown on D-

galacturonate, or whether the expression of *gar2* in a heterologous host would result in an active enzyme.

4.2 The D-galacturonate reductase

The purified GAR1 had a molecular mass of 40 kDa as estimated by SDS-PAGE, whether it originated from *T. reesei* or had been produced as a tagged enzyme in *S. cerevisiae*. The calculated molecular mass was only 33 940 Da. Glycosylation could explain the difference in mass, although this was not further studied.

The *T. reesei* GAR1 and the recently cloned and characterised *A. niger* D-galacturonate reductase GAAA (Martens-Uzunova and Schaap, 2008) had several differences. These D-galacturonate reductases had non-homologous amino acid sequences and different substrate specificities. The GAR1 was NADPH-dependent, whereas the GAAA was shown to use both NADH and NADPH as cofactors. Of the cofactors GAAA had clearly higher affinity for NADPH, as its K_m was 0.036 mM whereas that for NADH was 0.326 mM. With the polyhistidine-tagged GAR1 the K_m for NADPH was 0.030 mM, which is similar to that of GAAA. Other differences in the kinetic properties were that the GAAA has a much lower K_m value for D-galacturonate than GAR1 (0.175 mM and 6 mM, respectively) and also lower V_{max} (9 U/mg and 40 U/mg, respectively). Both GAR1 and GAAA had strongest activity with D-galacturonate as a substrate but also reacted with D-glucuronate. DL-Glyceraldehyde was not a substrate for GAAA as it was for GAR1.

There have also been earlier reports of D-galacturonate reductase activity in some other eukaryotes. However, this activity was always connected to L-ascorbic acid synthesis. Some animal enzymes, for example an NADPH-dependent glucuronolactone reductase (EC 1.1.1.20) in rat kidney and an NADPH-dependent aldehyde reductase in chicken kidney, were shown to have activity with D-galacturonate but the corresponding genes were not identified (Hara *et al.*, 1983; Hayashi *et al.*, 1984). In addition to several other substrates they could also react with D-glucuronate and DL-glyceraldehyde, as could the *T. reesei* GAR1. An NADPH-dependent D-galacturonate reductase was also purified from ripe strawberry fruit (*Fragaria* x *ananassa*) and the corresponding gene was identified (Agius *et al.*, 2003). The enzyme was 319 amino acids in size and had 30% identity with GAR1. It catalysed the reaction from D-galacturonate to L-galactonate. Compared to this main reaction it had only about

1% of activity with other substrates, D-glucuronate or L-galactose. Even more recently a D-galacturonate reductase was reported in a unicellular alga *Euglena gracilis* (Ishikawa *et al.*, 2006). This enzyme had a broad substrate specificity, reacting also with D-galacturonate, D-glucuronate and DL-glyceraldehyde. It was able to use both NADPH and NADH as an electron donor but had about fivefold higher activity with NADPH compared to NADH. On the basis of a short N-terminal sequence published it was seen that this enzyme and *T. reesei* GAR1 are not closely related.

4.3 The L-galactonate dehydratase

When the *T. reesei* L-galactonate dehydratase (LGD1) was identified no sequence was available for any enzyme with L-galactonate dehydratase activity. Of previously identified enzymes the LGD1 had highest homology with the D-galactonate dehydratase (EC 4.2.1.6) of *E. coli* and the mandelate racemase (EC 5.1.2.2) of *Pseudomonas putida* (Ransom and Gerlt, 1988). Both had 20% identity in amino acid sequences with the LGD1. A D-altronate dehydratase (EC 4.2.1.7.) that is part of the bacterial D-galacturonate pathway in *E. coli* had only 13% identity in the amino acid sequence compared to LGD1.

An L-fuconate dehydratase (EC 4.2.1.68) was partially purified from the fungus *Pullularia (Aureobasidium) pullulans* and had high specificity for the substrates L-fuconate and L-galactonate (Veiga and Guimarães, 1991). The K_m values were 1.5 mM and 3.3 mM, respectively. An excess of substrate was reported to inhibit the reaction. The enzyme was not active with D-arabinonate as a substrate. The sequence of this L-fuconate dehydratase was not available. Only recently, within the past year, have several bacterial amino acid sequences of L-fuconate dehydratases been published. For example the *Xanthomonas campestris* L-fuconate dehydratase enzyme (FucD) has 50% identity in amino acids with LGD1. This enzyme was polyhistidine tagged, purified and characterised (Yew *et al.*, 2006). It was a homodimeric enzyme and exhibited activity with L-fuconate, D-arabinonate and L-galactonate as substrates, with K_m values of 0.33 mM, 2.1 mM and 6.2 mM, respectively. This was also the order of highest activity, indicating that L-fuconate is probably the physiological substrate. Weaker reactions were observed with L-talonate, D-ribonate, and D-altronate as substrates. The structures of these substrates are presented in Figure 9. After measuring enzyme activity with a considerable number of sugar acid substrates it was concluded that the L-fuconate dehydratase is specific for sugar

acids with a common structure. These sugar acids have the hydroxyl group of the C2 and C5 on the left and those of the C3 and C4 on the right in the Fischer projection. L-Fuconate and L-galactonate both have this structure. D-Arabinonate can also adapt this structure as the hydroxyl group of the C5 is free to rotate about the C4-C5 bond. By contrast, in L-talonate and D-ribonate the hydroxyl group of the C2 and in D-altronate the hydroxyl group of the C5 is on the right and the reaction with these substrates was considerably weaker compared to L-fuconate, D-arabinonate and L-galactonate.

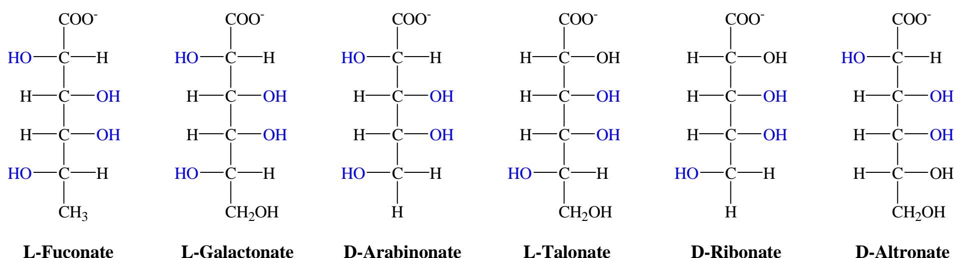


Figure 9. Substrates of the *X. campestris* FucD.

It is possible that the *T. reesei* L-galactonate dehydratase has the same stereochemistry requirement for its substrates as the L-fuconate dehydratase. We showed that the LGD1 was able to use L-galactonate and D-arabinonate but could not use D-galactonate, D-gluconate, D-xylonate or L-gulonate as reaction substrates. From this we concluded that the LGD1 catalyses the reaction with sugar acids that have the hydroxyl group of the C2 on the left and of the C3 on the right in the Fischer projection. We did not test other sugar acids with this structure as reaction substrates because they were not commercially available. Our conclusion of the stereospecificity of L-galactonate dehydratase is in agreement with what was shown for L-fuconate dehydratase.

Recently an L-galactonate dehydratase gene was cloned from another filamentous fungus, *A. niger* and expressed in *E. coli* (Martens-Uzunova and Schaap, 2008). The corresponding enzyme GAAB and *T. reesei* LGD1 have 76% identity in amino acid sequence. The purified GAAB had activity with L-galactonate as a substrate, but testing with other sugar acids was not reported. For purification purposes the GAAB was polyhistidine tagged at the N-terminus. We observed that both N- and C-terminal polyhistidine tags severely interfered with the activity of LGD1 and therefore we did not want to use the tagged

enzyme in kinetic measurements. Instead we used the cell extract of *S. cerevisiae* that was expressing the *T. reesei lgd1*.

In the determination of Michaelis-Menten constants for both the LGD1 and the GAAB (Martens-Uzunova and Schaap, 2008), there were uncertainties in the obtained activity values. In the activity measurements with LGD1 we did not add bivalent cations to the reaction as we only noticed at a later stage of the study that the enzyme had such a requirement. On the other hand the yeast cell extract itself might have contained the required amount of bivalent cations for maximum enzyme activity, since later we observed that adding $MgCl_2$ to the reaction did not increase the enzyme activity. In fact with 30 mM of $MgCl_2$ in the reaction there was only 49% of the activity left and with 5 mM of $MgCl_2$ 86% as compared to the reaction without added $MgCl_2$. We concluded that the loss of activity was caused by the high concentration of chloride ions. For measuring reactions with purified polyhistidine tagged GAAB an addition of 5 mM $MgCl_2$ was justified as there was no cell extract involved. The TBA assay (Buchanan *et al.*, 1999) was used to determine the reaction product of both fungal L-galactonate dehydratases, LGD1 and GAAB (Martens-Uzunova and Schaap, 2008). We noticed that at high substrate concentrations no reaction product was detected, which suggested that the high substrate concentration completely inhibited the enzyme activity. However, product formation was also observed at high substrate concentrations when measured using the DNS assay (Bernfeld, 1955), and it was evident that it was actually the TBA assay that was disturbed by the high substrate concentration. Comparison of the results obtained with these two assaying methods is shown in Figure 10. Because high substrate concentration interfered with the TBA assay, we determined the K_m value for the LGD1 based on the DNS assay results. The problem with this non-specific assay was that each individual reducing sugar quantified by this method has its own constant for converting absorbance values to concentrations. We did not perform a calibration for the L-threo-3-deoxy-hexulosonate, the reducing sugar that was measured here, and therefore we could not determine the V_{max} for this enzyme. Only later did we realise that the TBA assay could have been used to determine the Michaelis-Menten constants K_m and V_{max} if the reaction mixtures had simply been diluted before measuring. The group studying the *A. niger* GAAB reported that the enzyme activity was inhibited by an L-galactonate concentration of 10 mM. They measured that the K_m for L-galactonate was 3.4 mM (Martens-Uzunova and Schaap, 2008).

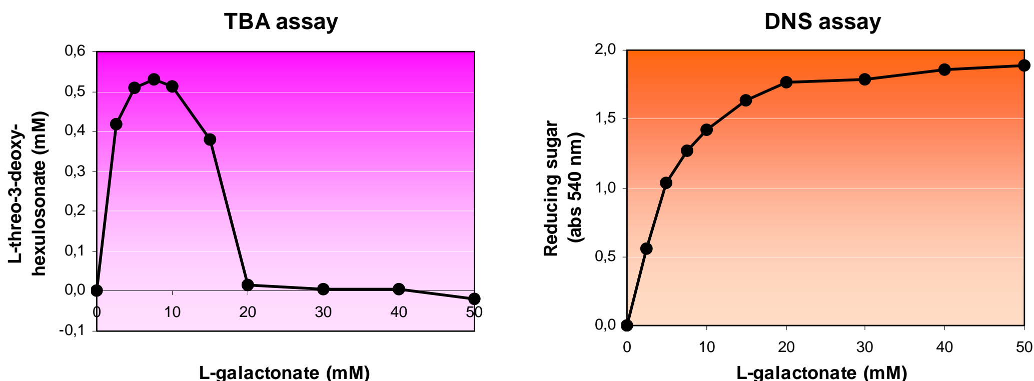


Figure 10. Comparison of the TBA and the DNS assays. The same LGD1 reaction mixtures were measured in parallel with both methods.

The L-galactonate dehydratase belongs to an enolase superfamily. These enzymes are related by their ability to catalyse thermodynamically difficult abstractions of the α-proton of a carboxylate anion substrate to form an enolic intermediate. Although the initial step is common, the substrate and the overall reaction can be very different. For example racemisation, β-elimination of water, β-elimination of ammonia or cycloisomerisation may occur (Babbitt *et al.*, 1996). The enzymes of the enolase superfamily have a structure of two conserved domains, the N-terminal α+β capping domain and the C-terminal $(\beta/\alpha)_7\beta$-barrel domain. The N-terminal domain mainly determines the substrate specificity, whereas the $(\beta/\alpha)_7\beta$-barrel domain contains the catalytic groups and the active site is at the interface between these two domains (Gerlt and Raushel, 2003; Rakus *et al.*, 2007). Unique to enolases is that a pair of bound divalent metal ions is present at the active site. These metal ions are believed to be important in stabilising the transition state of the proton transfer reaction (Richard and Amyes, 2001). The enzymes belonging to the mandelate racemase (MR) subfamily of the enolase superfamily have specific conserved residues at the $(\beta/\alpha)_7\beta$-barrel domain that are involved in metal ion binding and activity (Babbitt *et al.*, 1996). They are the Lys-x-Lys motif at the end of the second β-strand; Asp, Glu, and Glu residues that bind Mg^{2+} at the end of the third, fourth, and fifth β-strands; a His-Asp dyad at the end of the seventh and sixth β-strands; and a Glu at the end of the eighth β-strand (Yew *et al.*, 2006). All the members of the MR subfamily have these residues in common even though some catalyse the MR reaction (~100 sequences) and others a dehydration of acid sugars (~385

sequences) (Rakus *et al.*, 2007). For example these residues are present in the sequences of both L-galactonate dehydratases, *T. reesei* LGD1 and *A. niger* GAAB, as well as in the *X. campestris* L-fuconate dehydratase (Figure 11). The dehydration reaction is proposed to be irreversible. This is because the enol form of the product converts to its keto tautomer, which is more stable and greatly predominates at equilibrium relative to the enol tautomer (Figure 12) (Babbitt *et al.*, 1996).

The LGD1 had half of its maximum activity at an L-galactonate concentration of about 5 mM. When measured with the crude mycelial extract of D-galacturonate-grown *T. reesei* and a substrate concentration of 7.5 mM, the activity was 0.018 U/mg of protein. Therefore in the crude mycelial extract the maximum activity could be around 0.03 U/mg of protein. This activity is low for example when compared to the GAR1 activity. The GAR1 activity was about 0.25 U/mg of protein with the crude extract of D-galacturonate-grown mycelia and a D-galacturonate concentration of 94 mM and NADPH at 250 μM. At these concentrations the GAR1 already had the maximum activity, as the K_m for D-galacturonate was 6 mM and for NADPH 30 μM. Most probably the reaction catalysed by the LGD1 is irreversible. An enzyme with an irreversible reaction allows metabolites to run only in one direction through the pathway. Therefore the low activity of LGD1 might be beneficial. A high LGD1 activity would lead to high flux through the whole catabolic pathway. This could lead for example to a depletion of NADPH, which would probably be detrimental for the organism.

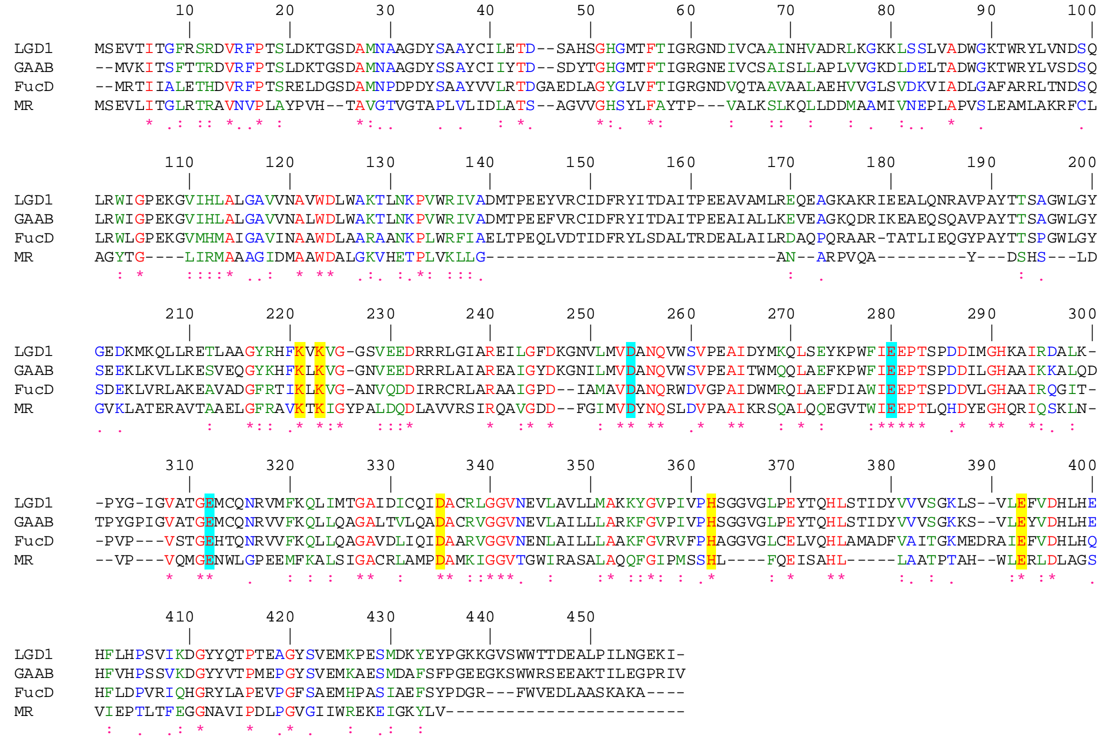


Figure 11. Active site residues and metal ion binding sites in *T. reesei* L-galactonate dehydratase (LGD1), *A. niger* L-galactonate dehydratase (GAAB), *X. campestris* L-fuconate dehydratase (FucD) and *P. putida* mandelate racemase (MR). The important active site residues are boxed in yellow and the metal binding residues in blue.

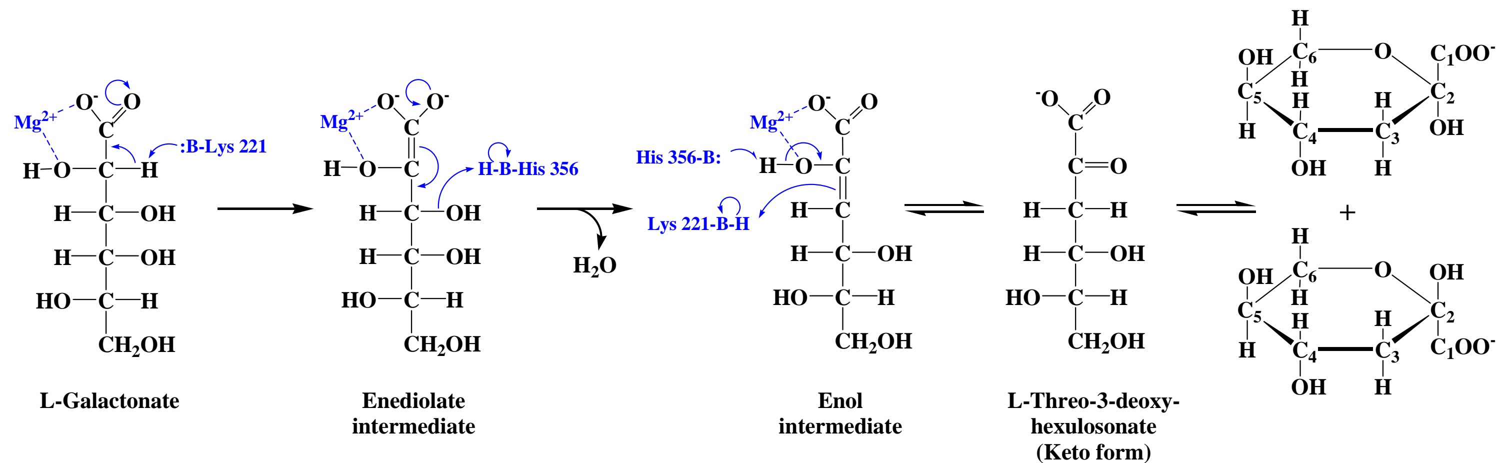


Figure 12. The proposed mechanism of the LGD1-catalysed reaction. Modified for the LGD1 from the FucD-catalysed reaction proposed by Yew *et al.*, 2006.

4.4 The L-threo-3-deoxy-hexulosonate aldolase

The L-threo-3-deoxy-hexulosonate aldolase (LGA1) is a putative member of the dihydrodipicolinate synthase (DHDPS) family (also called N-acetylneuraminate lyase family) that comprises several pyruvate-dependent class I aldolases. The enzymes of the class I aldolase family share a common TIM $(\beta/\alpha)_8$ barrel structure but catalyse diverse reactions in different pathways. They all have a conserved lysine residue at the active site that stabilises the reaction intermediate via Schiff-base formation (Lys174 in LGA1). In addition several other highly conserved residues have been found in the enzymes of the DHDPS family (Barbosa *et al.*, 2000). Such residues are also present in the LGA1 sequence, including the GxxGE motif (from Gly49 toGlu53) that is associated with binding the α-keto acid carboxylate moiety of the reaction substrate and the salt bridge forming Arg/Glu residues (Arg287 and Glu61) that link the C-terminal three-helical cluster with the $(\beta/\alpha)_8$ barrel. The DHDPS family enzymes have a common structure in which the N-terminal TIM $(\beta/\alpha)_8$ barrel is followed by a C-terminal domain of three α-helices. This structure is also predicted for the LGA1.

The LGA1 showed stereospecificity, as there was activity with L-threo-3-deoxy-hexulosonate and D-glycero-3-deoxy-pentulosonate but not with D-threo-3-deoxy-hexulosonate. This suggested that the reaction substrate must have the hydroxyl group of the C4 on the right in the Fischer projection. In addition the hydroxyl group of the C5 probably has to be on the left, as it is in L-threo-3-deoxy-hexulosonate. The D-glycero-3-deoxy-pentulosonate can also adopt this structure because the single bond at C4-C5 is free to rotate about the bond axis. The D-threo-3-deoxy-hexulosonate that was not a substrate has the opposite configuration at the C4 and C5 compared to the L-form. The structures of these compounds are compared in Figure 13. The LGA1 was also stereospecific in the reverse direction. With pyruvate only those aldehydes reacted that had a hydroxyl group at the C2 and on the left in the Fischer projection. L-Glyceraldehyde was a substrate, whereas D-glyceraldehyde was not. The other accepted substrates glycolaldehyde and methylglyoxal can rotate the hydroxyl group of the C2 on either side. There was no activity with acetaldehyde, which does not have a C2 hydroxyl group.

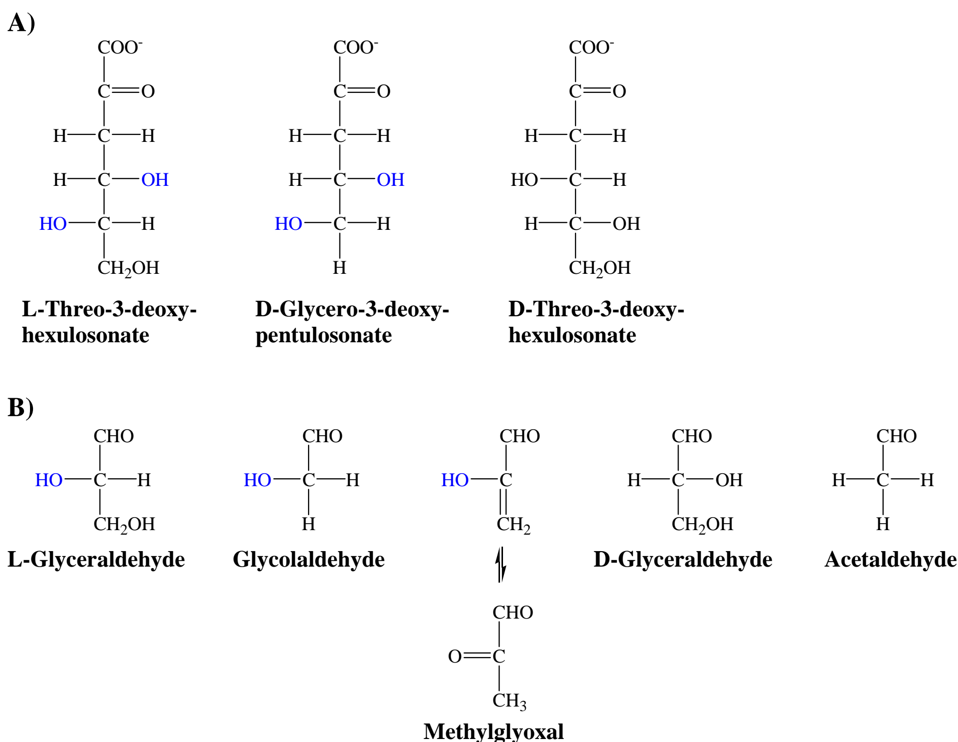


Figure 13. A) L-Threo-3-deoxy-hexulosonate and D-glycero-3-deoxy-pentulosonate were substrates in the forward reaction of the LGA1. D-Threo-3-deoxy-hexulosonate was not a reaction substrate. B) L-Glyceraldehyde, glycolaldehyde and methylglyoxal were substrates in the reverse reaction with pyruvate. D-glyceraldehyde and acetaldehyde were not reaction substrates.

A similar enzyme to the *T. reesei* LGA1, a 2-keto-3-deoxy-L-rhamnonate (L-erythro-3,6-dideoxy-hexulosonate) aldolase of *P. stipitis*, cleaves 2-keto-3-deoxy-L-rhamnonate to pyruvate and L-lactaldehyde (Twerdochlib *et al.*, 1994). In equilibrium the reaction mixture consisted of about 90% of the substrate 2-keto-3-deoxy-L-rhamnonate and 10% of the products pyruvate and L-lactaldehyde. It could be assumed that the LGA1 also has its equilibrium on the side of the substrate. We observed that the L-threo-3-deoxy-hexulosonate accumulated to a concentration of about 1.5 mM (0.3 g/l) in the culture when *T. reesei* was grown from four to six days on D-galacturonate. The K_m for L-threo-3-deoxy-hexulosonate was 3.5 mM. In the D-galacturonate pathway there is an irreversible reaction both before and after the aldolase reaction. The L-galactonate dehydratase produces L-threo-3-deoxy-hexulosonate, the substrate for the aldolase, while the glyceraldehyde reductase removes L-glyceraldehyde,

the reaction product of the aldolase reaction. It appears that the LGA1 can operate close to the equilibrium but the constant production of its substrate and the removal of its product drive the metabolism in the forward direction.

The *T. reesei* wild type strain and the *lga1*-deletion strain were both cultivated in liquid medium containing D-galacturonate. The dry masses of the produced mycelia were compared. We also measured the amount of L-threo-3-deoxy-hexulosonate in the media. Both results were reported separately as grams per litre. However, it was not taken into account that there was a lot more of the wild type mycelia in the culture compared to the *lga1*-deletion strain that hardly grew on D-galacturonate. Even so, the *lga1*-deletion strain produced significantly more L-threo-3-deoxy-hexulosonate into the growth medium than the wild type strain. The amounts of L-threo-3-deoxy-hexulosonate produced with respect to the dry masses are presented in Table 5.

Table 5. Production of L-threo-3-deoxy-hexulosonate (grams per gram of mycelial dry mass).

Strain	**2 days**	**4 days**	**6 days**
Wild type	0.09	0.08	0.06
lga1-deletion	3.9	11	19

The concentration of L-threo-3-deoxy-hexulosonate in the culture of the wild type strain did not increase along with the amount of mycelium. It appears that the flow of metabolites through the D-galacturonate pathway increased when more L-threo-3-deoxy-hexulosonate was produced. Therefore the L-threo-3-deoxy-hexulosonate concentration in the culture was relatively constant throughout the six-day experiment. The opposite was true for the *lga1*-deletion strain. A small amount of mycelium was produced in the beginning of the experiment from the peptone in the medium. The D-galacturonate could not be used for growth because the produced L-threo-3-deoxy-hexulosonate was not metabolised further. There was no increase in the mycelial dry mass after two days, but the concentration of L-threo-3-deoxy-hexulosonate increased at an almost constant rate. The mycelium corresponding to one gram of dry mass produced about 3.7 grams (18.5 mmol) of L-threo-3-deoxy-hexulosonate per day between days two and six in the experiment. L-Threo-3-deoxy-hexulosonate is produced in the irreversible reaction catalysed by L-galactonate dehydratase. The accumulation of L-threo-3-deoxy-hexulosonate did not appear to inhibit the

preceding reactions, because the production rate did not slow down. The L-threo-3-deoxy-hexulosonate concentration did not increase when all the enzymes of the pathway were present (wild type), which indicates that it is the L-galactonate dehydratase reaction that sets the limit for the flow rate. From this it could be concluded that *in vivo* in the conditions of this experiment the flow of metabolites through the D-galacturonate pathway was about 18.5 mmol/day/gram of mycelial dry mass.

4.5 The glyceraldehyde reductase and DHA reductase

There are two kinds of $NADP^+$-dependent glycerol dehydrogenases. Those classified as EC 1.1.1.72 catalyse conversion of glycerol and $NADP^+$ to glyceraldehyde, NADPH and H^+. The others are classified as EC 1.1.1.156 and catalyse the reaction of glycerol and $NADP^+$ to DHA, NADPH and H^+. In many cases a high pH was required to detect reactions *in vitro* in this direction (Viswanath-Reddy *et al.*, 1978; Schuurink *et al.*, 1990; Witteveen *et al.*, 1990). The reason for this is that hydrogen ions are produced in the dehydrogenase reaction. At high pH they are neutralised to water and do not accumulate in the reaction mixture, which would hinder the reaction (Asnis and Brodie, 1953). Under physiological pH conditions the reaction equilibrium was strongly on the side of glycerol production. Therefore the enzymes are discussed here as glyceraldehyde reductases and DHA reductases, respectively. The enzymes are rather unspecific and in general both types can catalyse a reaction with glyceraldehyde and DHA. There is, however, a preference for one of these substrates over the other.

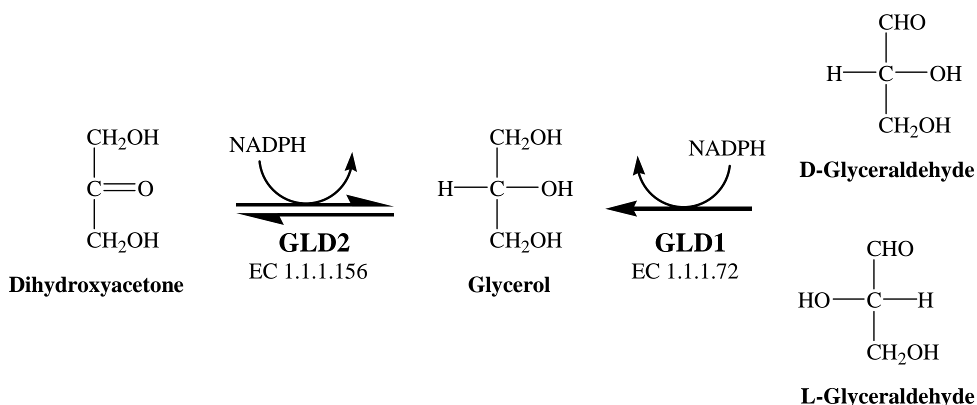


Figure 14. The reactions of NADPH-dependent DHA reductase and NADPH-dependent glyceraldehyde reductase.

Both the NADPH-dependent enzymes, DHA reductase and glyceraldehyde reductase, were found in *T. reesei*. They were called GLD2 and GLD1, respectively (Figure 14). The corresponding genes were cloned and expressed in *S. cerevisiae* and the purified enzymes were used in activity measurements. The GLD2 had the highest activity with DHA and only low activity with D- and L-glyceraldehyde in the forward direction. The reverse reaction was detected with glycerol. The purified GLD1 preferred D- and L-glyceraldehyde over DHA as a substrate. The reverse reaction with glycerol was not detected even at high pH.

NADPH-dependent reductases that catalyse production of glycerol have been found in several other fungal species. A glyceraldehyde reductase purified from *Neurospora crassa* favoured D-glyceraldehyde as a substrate over DHA and L-glyceraldehyde. The reverse reaction was measured with glycerol (Viswanath-Reddy *et al.*, 1978). The enzyme from *Rhodotorula* species used both D- and L-glyceraldehyde as substrates equally well, whereas the activity with DHA was low. The reverse reaction was not detected (Watson *et al.*, 1969). A purified DHA reductase of *Mucor javanicus* had very low activity with glyceraldehyde, but preferred DHA as a substrate. There was activity with glycerol in the reverse direction (Hochuli *et al.*, 1977).

Two NADPH-dependent glycerol-producing reductases were found in *A. niger* (Schuurink *et al.*, 1990; Witteveen *et al.*, 1990) and *A. nidulans* (Schuurink *et al.*, 1990; Sealy-Lewis and Fairhurst, 1992). In these species one enzyme was constitutively expressed, whereas the other was induced on D-galacturonate. The constitutive enzyme of both species resembled a DHA reductase. The preferred

substrate was DHA, whereas the activity with D-glyceraldehyde was about 10% of that with DHA, and there was no reaction with L-glyceraldehyde. The enzymes had activity with glycerol in the reverse direction (Schuurink *et al.*, 1990). The D-galacturonate-induced enzyme was a glyceraldehyde reductase, as was shown with the *A. niger* enzyme. It had high activity with D-glyceraldehyde (Witteveen *et al.*, 1990). The corresponding enzyme of *A. nidulans* was also proposed to be involved in D-glyceraldehyde catabolism, but activity was shown only in the reverse direction with glycerol (Sealy-Lewis and Fairhurst, 1992). The difference between these *Aspergillus* species was that when *A. nidulans* was grown on D-galacturonate it produced an additional unspecific NAD^+-dependent alcohol dehydrogenase that reduced D-glyceraldehyde to glycerol (Hondmann *et al.*, 1991).

Based on its substrate preference the *T. reesei* GLD2 belonged to the group of NADPH-dependent DHA reductases. The same conclusion was made from its amino acid sequence. There are few sequences available from similar enzymes in other filamentous fungi. The *T. reesei* GLD2 amino acid sequence was 86% identical with the *Trichoderma atroviride* Gld1 (Seidl *et al.*, 2004) and 71% identical with the *A. nidulans* GldB (de Vries *et al.*, 2003). In *T. reesei* and *T. atroviride* there were no introns in the corresponding gene, whereas the *A. nidulans* gene contained one intron. The NADPH-dependent alkyl 4-halo-3-oxobutyrate reductase (KER) of the filamentous fungus *Penicillium citrinum* did not show activity with glycerol and $NADP^+$ (Asako *et al.*, 2005). However, the amino acid sequence of KER was 67% identical with GLD2.

One role of the NADPH-dependent DHA reductase in filamentous fungi is in glycerol production. In *A. nidulans* two pathways have been shown to operate simultaneously at high osmolarity. In the main route dihydroxyacetone phosphate (DHAP) is dephosphorylated to DHA that is then reduced to glycerol (Redkar *et al.*, 1995). In the other pathway DHAP is reduced to glycerol-3-phosphate that is then dephosphorylated to glycerol (Fillinger *et al.*, 2001). Another proposed role for DHA reductase was that it rapidly converts the toxic DHA into glycerol. The enzyme apparently has high physiological importance, as it was present at high concentrations under different growth conditions (Schuurink *et al.*, 1990).

Sequences of NADPH-dependent glyceraldehyde reductases of filamentous fungi were not known before the *T. reesei* GLD1. However, *S. cerevisiae* has NADPH-dependent reductases Ypr1p and Gcy1p that preferred DL-glyceraldehyde over DHA as a substrate (Petrash *et al.*, 2001; Ford and Ellis,

2002). Their amino acid sequences have been published (Norbeck and Blomberg, 1997) and both are 37% identical with the *T. reesei* GLD1 (40% and 39% with GLD2, respectively). By comparison, the identity of GLD1 with the above-mentioned *A. nidulans* GldB, *P. citrinum* KER and *T. atroviride* Gld1 is 41%, 40% and 37%, respectively. It was observed in the transcriptome analysis of *A. niger* that *gaaD* that was the closest homologue in this species to the *T. reesei gld1* gene that was specifically induced on D-galacturonate (Martens-Uzunova and Schaap, 2008). The corresponding enzymes are 57% identical. However, the enzyme activity of GAAD has not been reported.

The main role of the NADPH-dependent glyceraldehyde reductase is in the catabolism of glyceraldehyde. D-Glyceraldehyde is produced in the non-phosphorylated pathway for D-gluconate (Elzainy *et al.*, 1973) and D-galactonate catabolism (Elshafei and Abdel-Fatah, 2001). L-Glyceraldehyde is generated in the D-galacturonate pathway. All these three pathways involve a dehydratase and an aldolase that sequentially convert the sugar acid to the corresponding 2-keto-3-deoxy sugar acid and then to pyruvate and glyceraldehyde.

It could be assumed that the *T. reesei* GLD2 is constitutively expressed and that the GLD1 is induced when grown on D-galacturonate. However, in contrast to the glyceraldehyde reductases of *A. niger* (Witteveen *et al.*, 1990) and *A. nidulans* (Sealy-Lewis and Fairhurst, 1992), the GLD1 activity was not specifically induced on D-galacturonate. The GLD1 activity was tested after growth on D-galacturonate, glycerol, lactose, D-glucose and D-xylitol and similar activity was observed with L- and D-glyceraldehyde in all the tested crude mycelial extracts. This could indicate that the GLD1 has a more general role in *T. reesei* than to reduce L-glyceraldehyde produced in the D-galacturonate pathway.

T. reesei converts D-galacturonate to pyruvate and glycerol. Pyruvate is an intermediate in several central metabolic pathways. For example, pyruvate can be further converted into carbohydrates, fatty acids, the amino acid alanine or energy. T. reesei is also able to utilise glycerol. Most probably the main route for glycerol catabolism in T. reesei proceeds via glycerol kinase and a mitochondrial FAD-dependent glycerol-3-phosphate dehydrogenase, as in A. nidulans and in most other eukaryotic organisms when growing on glycerol (Hondmann et al., 1991). The product DHAP is an intermediate of the glycolytic pathway, as can be seen in Figure 15. In another possible pathway glycerol is first converted to DHA by a reverse reaction of an NADPH-dependent DHA reductase and subsequently to DHAP by a DHA kinase. In A. oryzae this is the preferred catabolic glycerol pathway (Salazar *et al.*, 2009). This route produces NADPH

at the expense of NADH and ATP (Norbeck and Blomberg, 1997). Using this route might also be beneficial for *T. reesei* growing on D-galacturonate, since the D-galacturonate pathway consumes two molecules of NADPH per one molecule of D-galacturonate.

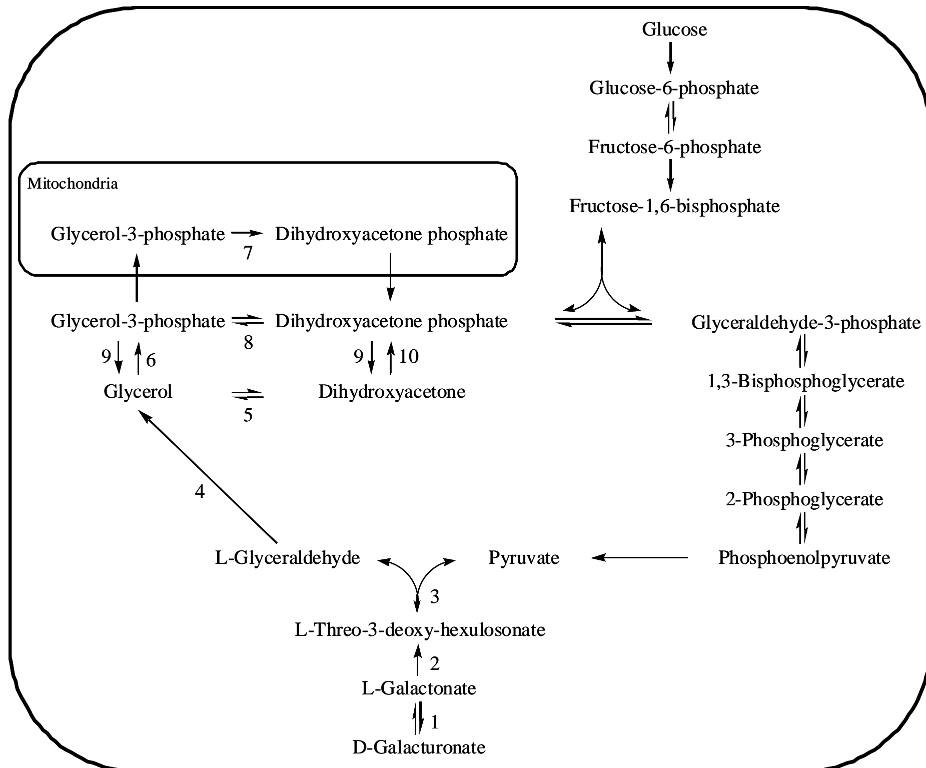


Figure 15. D-galacturonate and presumed glycerol metabolic pathways of *T. reesei* and their relationship to glycolysis. *1* NADPH-dependent D-galacturonate reductase; *2* L-galactonate dehydratase; *3* L-threo-3-deoxy-hexulosonate aldolase; *4* NADPH-dependent glyceraldehyde reductase; *5* NADPH-dependent DHA reductase; *6* glycerol kinase; *7* FAD-dependent glycerol-3-phosphate dehydrogenase; *8* NAD^+-dependent glycerol-3-phosphate dehydrogenase; *9* glycerol-3-phosphate phosphatase; *10* DHA kinase.

4.6 Biotechnological applications of the D-galacturonate pathway

The production of citrus, apple or sugar beet juice generates processing residues that contain large amounts of pectin and other carbohydrate polysaccharides. Several million dry tons of these residues are produced globally every year, which causes disposal problems. Currently, these carbohydrate-rich residues are mainly used as cattle feed, but their feed value is low because of the low protein content (Grohmann and Bothast, 1994). Furthermore, for transporting and to prevent spoilage the residues must be dried and pelletised. In the case of sugar beet this can require up to 30–40% of the total energy of the processing (Doran *et al.*, 2000). This kind of a carbohydrate-rich by-product could be a potential raw material for microbial conversion to more valuable products.

In many plant materials complexes of lignin and hemicellulose protect cellulose fibres from enzymatic hydrolysis. In citrus, apple and sugar beet processing residues the lignin content is low and the monomeric sugars can easily be released by treatment with cellulolytic and pectinolytic enzymes (Grohmann and Bothast, 1994). After extraction of sucrose from sugar beet, 70–75% of the dry mass of the remaining pulp is still carbohydrates, mainly D-glucose (21.1%), D-galacturonate (21.1%), L-arabinose (20.9%) and five other sugars (10% altogether) (Micard *et al.*, 1996).

Two fungi, *T. reesei* and *A. niger*, have been metabolically engineered to convert D-galacturonate to galactarate. In both fungi the gene coding for the D-galacturonate reductase, the *gar1* in *T. reesei* and the *gaaA* in *A. niger*, was first deleted in order to prevent them from catabolising D-galacturonate. Then a gene from *A. tumefaciens* that codes for an NAD^+-dependent D-galacturonate dehydrogenase (EC 1.1.1.203) was introduced to both strains. The enzyme catalyses the conversion of D-galacturonate to galactarate. The *T. reesei* strain produced galactarate at a high yield. The *A. niger* strain was apparently able to metabolise galactarate, which resulted in a lower yield. Galactarate has applications for example in the food, cosmetics and pharmaceutical industries. It might also be useful in synthesising polymers (Mojzita *et al.*, 2010).

The *T. reesei* strain in which the *lgd1*, the gene coding for the L-galactonate dehydratase, was deleted was unable to grow on D-galacturonate but converted it to L-galactonate. L-Galactonate could be useful as an acidifying agent in the food industry and could also have applications in the cosmetics industry. The L-galactonate dehydratase enzyme (LGD1) could be used to produce L-threo-3-

deoxy-hexulosonate from L-galactonate or D-glycero-3-deoxy-pentulosonate from D-arabinonate. The reaction catalysed by the LGD1 is irreversible and the substrate would be completely converted to the dehydrated product. L-Threo-3-deoxy-hexulosonate could also be produced from D-galacturonate with a *T. reesei* strain in which *lga1*, the gene coding for L-threo-3-deoxy-hexulosonate aldolase, was deleted. This strain did not grow on D-galacturonate but converted it to L-threo-3-deoxy-hexulosonate.

5. Conclusions and future prospects

Previously it was not known how eukaryotes catabolise D-galacturonate, although there was evidence that such a pathway exists and that it is different from the prokaryotic D-galacturonate pathways. In this study the first eukaryotic catabolic D-galacturonate pathway was identified in the filamentous fungus *Trichoderma reesei*. This novel pathway consisted of four enzymes. These four new fungal enzymes were NADPH-dependent D-galacturonate reductase, L-galactonate dehydratase, L-threo-3-deoxy-hexulosonate aldolase and NADPH-dependent glyceraldehyde reductase. In the first step D-galacturonate reductase used NADPH as a cofactor to convert D-galacturonate to L-galactonate. This was a reversible reaction. In the second step L-galactonate dehydratase removed a water molecule from L-galactonate and this irreversible reaction produced L-threo-3-deoxy-hexulosonate. In the third step L-threo-3-deoxy-hexulosonate aldolase cut the carbon chain of L-threo-3-deoxy-hexulosonate in the middle, which produced pyruvate and L-glyceraldehyde. This enzyme reaction also operated in the reverse direction. In the fourth step L-glyceraldehyde was converted to glycerol by an NADPH-dependent glyceraldehyde reductase. The backward reaction was not detected with this enzyme.

The first three enzyme activities were present in the crude mycelial extract only when *T. reesei* was grown on D-galacturonate, which indicated that these enzyme activities were involved in D-galacturonate utilisation. When the corresponding gene of any of these three enzymes was deleted in *T. reesei* the resulting strain was no longer able to grow with D-galacturonate as the only carbon source. This showed that these enzymes were essential for D-galacturonate catabolism and that there were no alternative pathways in *T. reesei*. The same set of enzymes also operated in *Aspergillus niger* in D-galacturonate catabolism, and there is genetic evidence that the same pathway is

also conserved in other plant cell wall-degrading fungi in the subphylum *Pezizomycotina* (Martens-Uzunova and Schaap, 2008).

For a long time there has been a strong interest worldwide in producing ethanol efficiently from plant biomass for use as a renewable transport fuel. Many different approaches have been developed and tested to find the best ways for ethanol production. The catabolic D-galacturonate pathways have also been seen to have some potential in this respect. It has been suggested that D-galacturonate could be converted to ethanol by *S. cerevisiae* after equipping it with a heterologous D-galacturonate pathway along with a D-galacturonate transporter (van Maris *et al.*, 2006). However, if the fungal D-galacturonate pathway were to be used, one of the main problems with this hypothetical yeast strain might be its increased need for NADPH, because two enzymes of the fungal pathway were NADPH-dependent reductases.

Converting the various components of different biomaterials to more valuable products has become currently one of the main goals in biotechnology. Filamentous fungi are important tools in biotechnology. Basic research on their physiology and enzymology, as performed in this thesis work, creates a basis from which the various biotechnological applications arise. It has already been shown that the different deletion strains constructed during this work can be used to convert D-galacturonate to L-galactonate or to L-threo-3-deoxy-hexulosonate and with additional metabolic engineering to galactarate (Mojzita *et al.*, 2010). In addition, all the four new genes of the fungal D-galacturonate pathway could be cloned and expressed in *Saccharomyces cerevisiae* as functional enzymes. Discovering new enzymes always extends the possibilities to develop new biotechnological applications.

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Publication II

L-galactonate dehydratase is part of the fungal path for D-galacturonic acid catabolism

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L-galactonate dehydratase is part of the fungal path for D-galacturonic acid catabolism

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Summary

An L-galactonate dehydratase and the corresponding gene were identified from the mould *Hypocrea jecorina* (*Trichoderma reesei*). This novel enzyme converts L-galactonate to L-threo-3-deoxy-hexulosonate (2-keto-3-deoxy-L-galactonate). The enzyme is part of the fungal pathway for D-galacturonic acid catabolism, a pathway which is only partly known. It is the second enzyme of this pathway after the D-galacturonic acid reductase. L-galactonate dehydratase activity is present in *H. jecorina* cells grown on D-galacturonic acid but absent when other carbon sources are used for growth. A deletion of the L-galactonate dehydratase gene in *H. jecorina* results in a strain with no growth on D-galacturonic acid. The active enzyme was produced in the heterologous host *Saccharomyces cerevisiae* and characterized. It exhibited activity with L-galactonate and D-arabonate where the hydroxyl group of the C2 is in L- and the hydroxyl group of the C3 is in D-configuration in the Fischer projection. However, it did not exhibit activity with D-galactonate, D-gluconate, L-gulonate or D-xylonate where the hydroxyl groups of the C2 and C3 are in different configuration.

Introduction

D-galacturonic acid is the major component of pectin and consequently an important carbon source for microorganisms living on decaying plant material. A bacterial catabolic pathway has been described while a eukaryotic pathway has remained unknown. The bacterial pathway consists of five enzymes converting D-galacturonic acid (D-galacturonate) to pyruvate and D-glyceraldehyde-3-phosphate. The intermediate metabolites are D-tagaturonate, D-altronate, D-erythro-3-deoxy-hexulosonate and D-erythro-3-deoxy-hexulosonate-6-phosphate. The enzymes are uronate isomerase, D-tagaturonate reductase, altronate dehydratase, 2-dehydro-3-deoxy-D-gluconate kinase and 2-dehydro-3-deoxy-D-gluconate-6-phosphate aldolase respectively (Ashwell *et al*., 1960; Cynkin and Ashwell, 1960; Hickman and Ashwell, 1960; Smiley and Ashwell, 1960; Meloche and Wood, 1964). There are no reports about genes which are similar to the genes of the bacterial D-galacturonic acid pathway in the sequenced genomes of eukaryotic microorganisms. There are also no reports describing these enzyme activities present in bacteria in yeasts or moulds. However, a similar pathway must exist in eukaryotic microorganisms as many species of yeast and mould can utilize and grow on D-galacturonic acid. This suggests that there is a eukaryotic pathway for the catabolism of D-galacturonic acid which is different from the bacterial pathway.

We have previously presented that the first step in the fungal pathway is an NADPH-specific D-galacturonic acid reductase generating L-galactonate. This enzyme activity was induced in the filamentous fungus *Hypocrea jecorina* (*Trichoderma reesei*) when grown on D-galacturonic acid and the activity was absent when grown on other carbon sources (Kuorelahti *et al*., 2005). Information about how L-galactonate is further catabolized is lacking.

There are only a few other studies on D-galacturonic acid catabolism in fungal microorganisms. Uitzetter *et al*. (1986) mutagenized the filamentous fungus *Aspergillus nidulans* and found that mutants lacking pyruvate dehydrogenase or pyruvate carboxylase activity were unable to grow on D-galacturonic acid, whereas a pyruvate kinase mutant was able to grow on this carbon source. This was interpreted that D-galacturonic acid is converted to pyruvate but not through phosphoenolpyruvate. It was suggested that in *A. nidulans* D-galacturonic acid is catabolized via a non-phosphorylating pathway through glyceraldehyde and pyruvate (Visser *et al*., 1988; Hondmann *et al*., 1991), and that D-glyceraldehyde is an intermediate (Uitzetter *et al*., 1986). The non-phosphorylating path was supported by a microarray analysis of genes transcribed in *Aspergillus niger* grown on D-galacturonic acid. Here genes similar to aldoketo reductase, racemase and aldolase were identified as coexpressed genes (Martens-Uzunova *et al*., 2005). It was further suggested that D-galacturonic acid is metabolized through glycerol because a glycerol kinase mutant had reduced growth on

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D-galacturonic acid (Witteveen *et al*., 1990). It was also suggested that an NADP-dependent glycerol dehydrogenase is involved in the pathway because such an enzyme was induced on D-galacturonic acid (Sealy Lewis and Fairhurst, 1992).

In this study we demonstrate that the second enzyme in the fungal pathway for D-galacturonic acid catabolism is an L-galactonate dehydratase, an enzyme which has not been described previously. We postulate a fungal pathway for the D-galacturonic acid catabolism which results in pyruvate and glycerol and has L-glyceraldehyde as an intermediate.

Results

The filamentous fungus *H. jecorina* was grown on D-galacturonic acid as a carbon source and the mycelia were disintegrated. The resulting mycelia extract was then tested for different enzyme activities with L-galactonate as a substrate. We tested for NAD(P)H linked reductases, NAD(P) linked dehydrogenase or ATP consuming kinase activity; however, we did not observe such activities. Nevertheless we noticed that the incubation of L-galactonate with the extract resulted in the formation of a reducing sugar. This was followed using the 3,5-dinitrosalicylate (DNS) assay for reducing sugars (Bernfeld, 1955). The formation of a reducing sugar without involvement of a redox cofactor suggested the presence of a dehydratase active with L-galactonate. This activity was observed only when the fungus was grown on D-galacturonic acid. It was absent when the fungus was grown on a different carbon source such as D-galactose, D-glucose, D-xylose, D-fructose, lactose or glycerol. We tested the *H. jecorina* strains Rut C-30 or QM6a.

In order to clone the corresponding gene for the dehydratase we searched the *H. jecorina* genome for sequences with homology to other dehydratases. We identified five potential open reading frames with some amino acid similarities. We amplified these open reading frames by PCR using *H. jecorina* cDNA as a template.

Table 1. ^{1}H and ^{13}C NMR chemical shifts of the product L-threo-3-deoxy-hexulosonate.

	δ (ppm)[a]		δ (ppm)[b]
H3	1.789	C1	177.53
H3′	2.162	C2	97.84
H4	3.859	C3	40.22
H5	3.604	C4	70.13
H6	3.606	C5	71.92
H6′	3.801	C6	64.18

a. Referenced to internal TSP (0 ppm).
b. Referenced to external acetone (31.5 ppm).

The PCR products were then ligated to a yeast expression vector and transformed to *Saccharomyces cerevisiae*. The *S. cerevisiae* cell extract was then analysed for the activity of forming a reducing sugar from L-galactonate. *S. cerevisiae* does not have an L-galactonate dehydratase activity but one open reading frame showed this activity when expressed in *S. cerevisiae*. We called the gene *lgd1* for L-galactonate dehydratase. The cDNA sequence of the open reading frame was deposited in GenBank and has the accession number DQ181420. The open reading frame coded for a protein with 450 amino acids and a calculated molecular mass of 50.049 Da. Comparing the cDNA with the genomic DNA revealed one intron in the genome sequence. The intron was between the nucleotides 156 and 157 of the open reading frame and contained 61 nucleotides.

The yeast extract of the strain expressing the *lgd1* was used to convert L-galactonate to the reaction product which was then identified by NMR spectroscopy. The ^{1}H and ^{13}C chemical shifts of the product are given in Table 1. From one dimensional ^{1}H spectrum of the reaction mixture (Fig. 1) the product signals were readily visible, and from two dimensional DQFCOSY and (^{1}H, ^{13}C) HSQC experiments (not shown) it was evident, that the product has a proton spin-system CH_2-CH-CH-CH_2, in which one of the CH_2 functions has typical chemical shifts of a hydroxymethyl group and the second one has quite

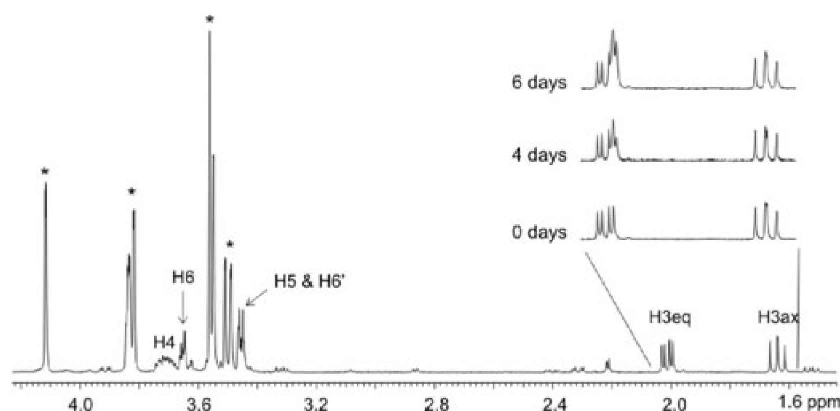

Fig. 1. 500 MHz ^{1}H NMR spectrum of the reaction mixture. The substrate signals are indicated by asterisks. For numbering of the product hydrogens, see Fig. 3. The insert shows the signals of the two protons at position 3 at different time points of incubation of the reaction mixture in D_2O. The signal from the axial proton remained unchanged, while a novel equatorial signal without the geminal coupling appeared on top of the original equatorial signal. This indicates that the enzyme retained activity in the NMR tube and that the hydrogen atom attached to the substrate in the reaction is attached to an axial position.

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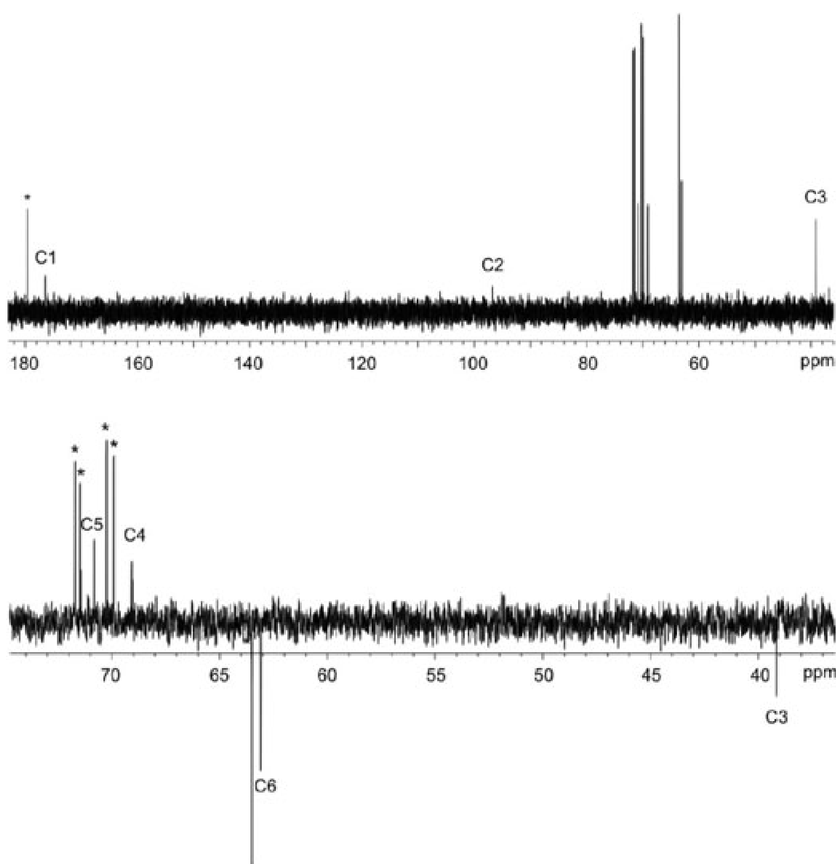

Fig. 2. ^{13}C (upper) and DEPT135 (lower) NMR spectra of the reaction mixture. The substrate signals are marked with an asterisk and the product signals are numbered according to Fig. 3. The DEPT spectrum indicates that the product has two CH_2 groups and the position of the signal of C2 in the upper spectrum is characteristic for a hemiketal structure indicating that the molecule exists predominately in ring form.

unique proton chemical shifts typical to a CH_2 group close to a keto group or a hemiketal. The DEPT spectrum (Fig. 2) further confirmed that the molecule has two CH_2 and two CH type carbon atoms. In addition to these four carbons, the ^{13}C spectrum (Fig. 2) of the product revealed two additional carbon signals. One is on the carboxyl area close to the signal of the carboxyl carbon of the substrate, L-galactonate, and the other one (97.84 ppm) is typical for a quaternary carbon in a hemiketal structure, like C2 signals in sialic acids. Thus NMR results show that the reaction product is L-threo-3-deoxy-hexulonate and that it exists predominantly as a pyranose ring (Fig. 3). The signals of one anomer dominate the spectrum of the product (over 85%), however, it was not possible to determine, which one of two anomers it is.

The enzyme retained activity still in the NMR tube and more product was formed when the sample was incubated in room temperature over several days (Fig. 1). As the reaction took place in deuterium environment (D_2O), the hydrogen attached to the molecule was a deuterium atom. We observed that the axial H3 signal did not increase with the product formation, while the equatorial H3 signal from the newly formed product had lost its geminal coupling and experienced a small upfield isotope shift of 0.02 ppm. This indicates that the equatorial hydrogen at carbon 3 must be the one that was bound to the carbon originally and the axial hydrogen at carbon 3 originates from the D_2O.

The reducing sugar was then quantified with the thiobarbituric acid assay as described by Buchanan *et al.* (1999). The activity in the extract of *S. cerevisiae* expressing the *lgd1* was 0.15 nkat mg^{-1} at an L-galactonate concentration of 7.5 mM. This was similar to the activity in the *H. jecorina* extract when grown on D-galacturonic acid. There the activity was 0.3 nkat mg^{-1} using the same L-galactonate concentration.

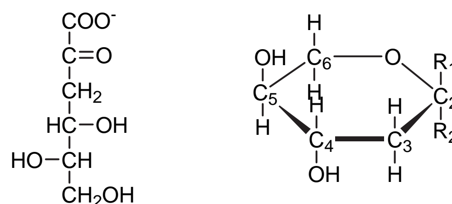

Fig. 3. L-threo-3-deoxy-hexulosonate in Fischer-projection (left). The NMR analysis revealed that it predominantly exists in the pyranose form, as shown in Haworth-projection (right). For the pyranose form two anomers are possible with R_1 as carboxyl group and R_2 the hydroxyl group or vice versa. One anomer was predominant, however, we could not identify which of the two it was.

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The LGD1 protein was tagged with a histidine tag and produced in *S. cerevisiae*. In one construct the C-terminus of the protein was tagged, in another the N-terminal end of the protein. The C-terminal tagging resulted in an inactive protein while the N-terminal tagging resulted in a protein with reduced activity. The activity found in the crude extract of the N-terminally tagged protein was about five times lower than the non-tagged protein.

As the histidine tagged LGD1 protein was either inactive or had reduced activity we used the crude cell extract of the yeast strain expressing the *lgd1* to analyse the kinetics of the L-galactonate dehydratase. To study the specificity of the enzyme we used different sugar acids and tested the activity of the enzyme to convert the sugar acid to a reducing sugar using the DNS assay. Activity was observed with the sugar acids L-galactonate and D-arabonate. The activity with D-arabonate was about 50% of the activity with L-galactonate under the conditions as specified in the *Experimental procedures*. Activity was only observed in the extract of the strain expressing the *lgd1*. No activity was observed in the control strain. When D-galactonate, D-gluconate, D-xylonate or L-gulonate was used as substrates no activity was observed in the strain expressing the *lgd1* or in the control strain.

For the substrate L-galactonate we estimated a K_m value of about 5 mM. This was measured at pH 7.0 with yeast extract containing the LGD1 enzyme at a final concentration of 1 g l^{-1} of extracted protein and following the production of L-threo-3-deoxy-hexulosonate using the thiobarbituric acid assay as described by Buchanan *et al.* (1999) or measuring the production of a reducing sugar using the DNS assay. The control strain with the empty plasmid showed no activity. The same K_m for L-galactonate was found when a *H. jecorina* extract was analysed. Using the yeast extract, an incubation of 24 h led to a complete conversion when the initial L-galactonate was 20 mM or lower, i.e. the L-threo-3-deoxy-hexulosonate concentration was equal to the initial L-galactonate concentration (data not shown). At L-galactonate concentrations above 20 mM the thiobarbituric acid assay gave values for the product formation which were lower that with the DNS assay. This was interpreted as an interference of the L-galactonic acid with the thiobarbituric acid assay.

The enzyme has an essential requirement for bivalent cations. In the presence of 5 mM EDTA we observed a complete loss of activity.

The transcription of the *lgd1* gene on different carbon sources was examined by a Northern blot analysis. The carbon sources D-galacturonate, D-glucose, glycerol, lactose and D-xylose were tested. The gene was transcribed on all these carbon sources. The transcription levels of the *lgd1* gene were normalized with the transcription levels of actin. The ratio of these two transcription levels, L-galactonic acid dehydratase over actin was the same on D-galacturonic acid, glycerol and lactose. It was three times higher on D-glucose and D-xylose.

To check if the *lgd1* gene is essential for the D-galacturonic acid metabolism we deleted the gene in a Rut C-30 strain of *H. jecorina*. For that purpose we constructed a deletion cassette which contained the hygromycin B phosphotransferase gene (*hph*), which was transformed and selected for hygromycin B resistance. A PCR of these strains showed that only 10% had the deletion cassette in the intended locus. These strains were further analysed by Southern hybridization to verify that only one copy of the deletion cassette had integrated into the correct position. In the Southern hybridizations the *lgd1* and the *hph* were probed. The *lgd1* gene was only seen in the control strain while the *hph* gene which replaced the *lgd1* gene was only in the deletion strain (not shown). The deletion strain (VTT D-05369) had the same growth rate on lactose as the host strain Rut C-30 on plate or in liquid culture but only residual growth on D-galacturonic acid while the host strain grew normal. When the *lgd1* gene was transformed back to the deletion strain the growth on D-galacturonic acid was restored. The growth rate of all the strains including the wild-type strain QM6a was compared in liquid medium containing 20 g l^{-1} D-galacturonic acid and 0.5 g l^{-1} proteose peptone or only 0.5 g l^{-1} proteose peptone as a carbon source. The residual growth of the deletion strain is due to growth on proteose peptone because all the strains produced the same amount of biomass also on proteose peptone only (Table 2).

Discussion

In this manuscript we describe the identification of a dehydratase that is active on the sugar acids L-galactonate and D-arabonate. These two sugar acids have in common that the hydroxyl groups of the C2 and the C3 in the Fischer projection are in L- and D-configuration respectively. Other sugar acids with such a configuration were not tested because they were not commercially available. The dehydratase was neither active with sugar acids where the hydroxyl groups of C2 were in D- and C3 in L-configuration as in D-galactonate, D-gluconate and D-xylonate, nor with a sugar acid with the hydroxyl groups of C2 and C3 in D-configuration as in L-gulonate.

Dehydratases and their corresponding genes, active on sugar acids with the hydroxyl groups C2 in D- and C3 in L-configuration have been described previously. A dehydratase active with D-xylonate and L-arabonate was described by Niu *et al.* (2003) and a dehydratase active with D-gluconate in the non-phosphorylated Entner–Doudoroff pathway was described in *Sulfolobus solfataricus* by Buchanan *et al.* (1999).

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Table 2. Growth of the lgd1 deletion strain and control in liquid medium.

H. jecorina strain	Lactose + peptone dry mass (g l^{-1})	D-galacturonic acid + peptone dry mass (g l^{-1})	Peptone dry mass (g l^{-1})
QM6a	0.7 ± 0.1	3.4 ± 1.3	0.1 ± 0.0
RutC30	4.2 ± 0.1	2.4 ± 0.7	0.1 ± 0.0
RutC30-*Δlgd1* A	3.3 ± 1.0	0.1 ± 0.0	0.1 ± 0.0
RutC30-*Δlgd1* B	3.9 ± 0.8	0.1 ± 0.0	0.1 ± 0.0
RutC30-*Δlgd1-lgd1* AA	4.2 ± 0.1	2.5 ± 0.6	0.1 ± 0.0
RutC30-*Δlgd1-lgd1* AB	4.1 ± 0.7	3.2 ± 0.5	0.1 ± 0.0

Dry mass (g l^{-1}) after 5 days of cultivation on 0.05% peptone and 2% of another carbon source if indicated. Values are averages of triplicates. Two parallel deletion strains were studied (A and B) and also two parallel retransformed strains (AA and AB) that were constructed by transforming *lgd1* into the deletion strain A.

A dehydratase which is similar to the enzyme described in this manuscript is the D-altronate dehydratase E.C. 4.2.1.7 (Smiley and Ashwell, 1960). D-altronate has the hydroxyl groups of C2 and C3 in L- and D-configuration like L-galactonate. The enzyme is part of the bacterial D-galacturonic acid pathway and the purified D-altronate dehydratase from *Escherichia coli* has been characterized (Smiley and Ashwell, 1960; Dreyer, 1987). A gene coding for the D-altronate dehydratase had been inferred by electronic annotation (GenBank accession number AAC76126). A CLUSTALW alignment of the L-galactonate dehydratase with D-altronate dehydratase revealed a 13% identity in the amino acid sequences. The low degree of homology suggests that L-galactonate dehydratase and D-altronate dehydratase belong to different protein families.

The homology of L-galactonate dehydratase with annotated enzymes was highest with the D-galactonate dehydratase (E.C. 4.2.1.6) of *E. coli* and the mandelate racemase (E.C. 5.1.2.2) of *Pseudomonas putida* (Ransom *et al*., 1988). Their Swiss-Protein accession codes are Q6BF17 and P11444 respectively. Both these enzymes have only 20% identity in amino acid sequences with L-galactonate dehydratase in a CLUSTALW alignment. Nevertheless, the conserved residues such as the active site and the metal binding residues of mandelate racemase subgroup of enolase superfamily suggested by Babbitt *et al*. (1996) are present in the sequence of L-galactonate dehydratase. These conserved residues were not found in the D-altronate dehydratase of *E. coli*. The reaction of each enzyme in the enolase superfamily is initiated by an abstraction of the α-proton of a carboxylic acid to form an enolic intermediate.

The reaction product of the L-galactonate dehydratase was identified as a reducing sugar. Reducing sugars are commonly measured with the DNS assay. In order to define the reaction product it was analysed by NMR. To generate a sufficient amount of reaction product L-galactonate was incubated in the yeast extract of the strain expressing the L-galactonate dehydratase gene. In this extract the reaction product did not react further, which facilitated the NMR analysis. In the *H. jecorina* mycelia extract the reaction product was degraded, making the NMR analysis more difficult. The NMR analysis showed that erythro- or threo-3-deoxy-hexulosonate was formed. Knowing the substrate of the dehydratase reaction we concluded that it was L-threo-3-deoxy-hexulosonate. The NMR analysis also revealed that it is predominantly in the pyranose form (Fig. 3). For the pyranose form two anomers are possible; the carboxyl group in R_1 and the hydroxyl group in R_2 or vice versa (Fig. 3). The NMR suggested that one anomer is predominant but it did not allow determining which of the two anomers it is. The NMR analysis revealed also that the axial hydrogen at the carbon 3 is the hydrogen that was added during the reaction. However, as there are two possible chair conformations of the pyranose ring it remains unclear which of the two protons is in axial position.

Knowing that the reaction product is L-threo-3-deoxy-hexulosonate enabled us to measure its concentration. An assay where the 2-keto-3-deoxy sugar acid gives a colour reaction with thiobarbituric acid allowed the quantification (Buchanan *et al*., 1999). The *H. jecorina* extract produced L-threo-3-deoxy-hexulosonate with a rate of 0.3 nkat mg^{-1} of extracted protein; in the extract of yeast expressing the *lgd1* the rate was 0.15 nkat mg^{-1}. The enzyme activities of the same order of magnitude indicate that the heterologous expression in yeast results in an active enzyme. The accumulation of the reaction product in the *H. jecorina* extract indicated that at least in the extract it is metabolized only slowly. In the yeast extract L-galactonate was completely converted to L-threo-3-deoxy-hexulosonate demonstrating that the energetic equilibrium is on the side of the reaction product. The irreversibility of the D-galactonate dehydratase reaction was described by Donald *et al*. (1979). The reaction results in an enolic intermediate that undergoes a spontaneous ketonization. The keto tautomer of the product is greatly favoured relative to the enol tautomer. It was also shown that the D-galactonate dehydratase is unable to enolize the product 2-keto-3-deoxy-D-galactonate.

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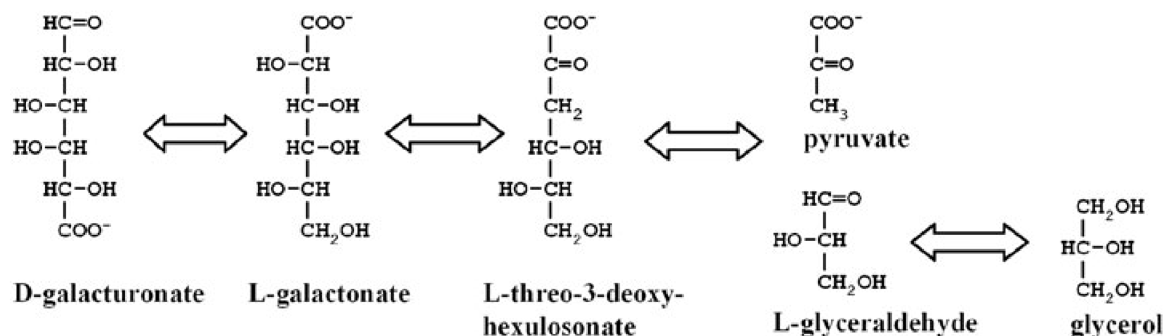

Fig. 4. Hypothetical pathway for the catabolism of D-galacturonic acid in eukaryotic microorganisms. The metabolites are represented in Fischer-projection. In this projection the C1 of the D-galacturonic acid is the reduced C6 of the L-galactonate. The first two enzymes of this pathway, the D-galacturonic acid reductase and the L-galactonate dehydratase have been identified. The L-galactonate dehydratase is the subject of this communication. The remaining two enzymes, the L-threo-3-deoxy-hexulosonate aldolase and L-glyceraldehyde reductase are hypothetical.

The L-galactonate dehydratase is part of a eukaryotic pathway for the catabolism of D-galacturonic acid. The first enzyme in this pathway is the D-galacturonic acid reductase converting D-galacturonic acid to L-galactonate (Kuorelahti *et al.*, 2005), and the L-galactonate dehydratase described in this manuscript the second. There are several observations supporting this assumption. First of all the LGD1 protein was active with L-galactonate. Second, the activity was observed in *H. jecorina* extract only when mycelia were grown on D-galacturonic acid. The activity was not observed when mycelia were grown on other carbon sources. This is already an indication that the L-galactonate dehydratase is related to the D-galacturonic acid catabolic pathway. Another indication is that we did not find any other enzyme activities with L-galactonate such as reductase, dehydrogenase or kinase activities; however, this is not a proof that these activities do not exist. A deletion of the *lgd1* gene in *H. jecorina* gives evidence that the L-galactonate dehydratase is part of the D-galacturonic acid pathway because in the resulting strain growth on D-galacturonic acid is abolished (Table 2).

In the eukaryotic pathway for D-galacturonic acid catabolism the product of this second enzyme, L-threo-3-deoxy-hexulosonate, has to be further catabolized. This part of the pathway is still unknown. A hypothetical pathway is shown in Fig. 4. In this hypothetical pathway L-threo-3-deoxy-hexulosonate is converted to pyruvate and L-glyceraldehyde by the action of an aldolase. L-glyceraldehyde is, unlike pyruvate, a metabolite which is not part of any known metabolic pathway. We hypothesize that L-glyceraldehyde is converted to glycerol. An NADP-dependent glycerol dehydrogenase is induced in filamentous fungi grown on D-galacturonate (Sealy Lewis and Fairhurst, 1992). Such an enzyme might convert L-glyceraldehyde to glycerol. Glycerol is then metabolized through glycerol-3-phosphate and dihydroxyacetone phosphate as described for the mould *A. nidulans* (Hondmann *et al.*, 1991).

The catabolic path for D-galacturonic acid in fungi is different from the corresponding pathway in bacteria. For *E. coli* a pathway was described consisting of five enzymes converting D-galacturonic acid to pyruvic acid and D-glyceraldehyde-3-phosphate (Ashwell *et al.*, 1960; Cynkin and Ashwell, 1960; Hickman and Ashwell, 1960; Smiley and Ashwell, 1960; Meloche and Wood, 1964). The enzymes of this pathway are uronate isomerase, NADH-utilizing D-tagaturonate reductase, altronate dehydratase, D-erythro-3-deoxy-D-hexulosonate kinase and D-erythro-3-deoxy-D-hexulosonate-6-phosphate aldolase. We suggest that in the fungal path only four enzymes are required, NADPH-utilizing D-galacturonate reductase, L-galactonate dehydratase, L-threo-3-deoxy-hexulosonate aldolase and a glycerol dehydrogenase that can convert L-glyceraldehyde in the reverse reaction (Fig. 4). The differences between bacteria and fungi are that in fungi NADPH is the required cofactor, no phosphorylated intermediates are involved and no isomerases are used.

That bacteria and fungal microorganisms use different catabolic pathways is not unique for D-galacturonic acid. Also other abundant carbon sources such as D-xylose and L-arabinose are catabolized differently. In bacteria D-xylose is isomerized to D-xylulose before it is phosphorylated to D-xylulose 5-phosphate (Lawlis *et al.*, 1984), while in fungi the conversion from D-xylose to D-xylulose is a two-step process involving two redox reactions (Wang *et al.*, 1980). Also the L-arabinose catabolic pathway is distinctly different in bacteria and fungi (Lee *et al.*, 1986; Richard *et al.*, 2001; 2002). Common in these examples is that the bacterial pathways have isomerase activities which is absent in their fungal counterparts.

When comparing the sequence of the open reading frame with other sequences deposited in GenBank in a

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BLAST search, we identified hypothetical proteins with high homology in other filamentous fungi such as *Neurospora crassa* and *A. nidulans*. This suggests that L-galactonate dehydratase activity is not a unique feature of *H. jecorina*, but common in filamentous fungi. We also found hypothetical proteins with high homology (more than 50% identities in the amino acid sequences) from bacteria and higher eukaryotes indicating that the L-galactonate dehydratase might also be present in organisms other than filamentous fungi.

Experimental procedures

Strains, growth conditions and protein extracts

The *E. coli* strain DH5α was used in all the cloning procedures except the deletion cassette construction where TOP10 Electrocomp Cells (Invitrogen) were used. Bacteria were grown in Luria–Bertani medium with ampicillin at 37°C. The *S. cerevisiae* strain CEN.PK2-1D (VW-1B) was the host for the heterologous expression. It was grown in synthetic medium lacking uracil when required for selection at 30°C. We used the *H. jecorina* (*T. reesei*) strains Rut C-30 or QM6a. The strains were grown in a liquid medium containing 20 g l^{-1}D-galacturonic acid/sodium D-galacturonate (pH 7.0) or another carbon source when specified, 0.5 g l^{-1} proteose peptone, 15 g l^{-1} KH_2PO_4, 5 g l^{-1} $(NH4)_2SO_4$, 0.6 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.6 g l^{-1} $CaCl_2 \cdot 2H_2O$ and trace elements (Mandels and Weber, 1969) at 28°C. The agar plates for the mould contained also 1 ml l^{-1} Triton X-100 and 20 g l^{-1} granulated Difco Agar. To make protein extracts of *H. jecorina* or *S. cerevisiae* about 100 μg of fresh mycelia or cells were mixed with 300 μl of glass beads (diameter 0.4 mm) and 400 μl of buffer [5 mM sodium phosphate pH 7.0 and Complete, EDTA-free protease inhibitor (Roche)] and disintegrated in a Mini-Bead Beater (Biospec Products) for three times for 30 s. The mixture was then centrifuged in an Eppendorf microcentrifuge at full speed for 25 min at 4°C and the supernatant used for the analysis. For fungal protein extract supernatant a second centrifugation of 10 min was needed. The protein content of the extract was estimated using the Bio-Rad protein assay and BSA as a standard. To assay the L-galactonate dehydratase activity L-galactonate was mixed with the protein extract and formation of reducing sugars detected using the DNS assay for reducing sugars following a standard protocol (Bernfeld, 1955). L-galactonate was derived from L-galactonic acid-γ-lactone as described previously (Kuorelahti *et al*., 2005).

Cloning of the L-galactonate dehydratase and Northern blot analysis

The *H. jecorina* genome at JGI database (http://gsphere.lanl.gov/trire1/trire1.home.html) was screened for genes with homology to D-galactonate dehydratases (E.C. 4.2.1.6). This enzyme catalyses reaction of D-galactonate to 2-dehydro-3-deoxy-D-galactonate and water and has a role in D-galactonate catabolism in bacteria (De Ley and Doudoroff, 1957). PCR primers containing BamHI restriction sites were designed to amplify the open reading frames and PCR was run using a cDNA library as a template (Margolles-Clark *et al*., 1996). The PCR product was ligated to a pCR2.1-TOPO vector (Invitrogen). From the resulting vector the BamHI fragment was released and ligated to the yeast expression vector p2159. This expression vector was a multicopy yeast expression vector with the constitutive *TPI1* promoter. It was derived from the pYX212 plasmid (R and D Systems) by digesting it with EcoRI and XhoI to remove the ATG and HA-tag from the multiple cloning site and introducing a BamHI restriction site to the cloning site by inserting a EcoRI and SalI cut fragment from the pUC19 plasmid (Norrander *et al*., 1983). The resulting vectors were then transformed to the *S. cerevisiae* strain CEN.PK2-1D (VW-1B). The resulting *S. cerevisiae* strains were then disintegrated by vortexing with glass beads and the yeast extract analysed for L-galactonate dehydratase activity by monitoring the production of a reducing sugar. Using the primer 5′-GGATCCACCATGTCTGAAGTCACCAT-3′ in sense and the primer 5′-GGATCCTCAGATCTTCTCTCCGTTCA-3′ in antisense direction resulted in an active L-galactonate dehydratase after expression in *S. cerevisiae*. The gene was called *lgd1*. To generate a histidine-tagged L-galactonate dehydratase with six histidines at either the N-terminal or the C-terminal end of the protein, primers containing the coding sequences for the histidines were used. For the N-terminal tag an ATG followed by the histidine coding sequence was introduced before the starting ATG-codon of *lgd1* and for the C-terminal tag the histidine coding sequence was introduced before the stop codon.

For the Northern blot analysis the *H. jecorina* strain QM6a was grown on different carbon sources. 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); 15 μg of RNA was glyoxylated, separated in a 1% agarose gel and transferred to Hybond N filter (Amersham). The Northern blot was performed using standard procedures. As a probe we used a 750 bp PCR fragment form the 5′ end of the open reading frame of the *lgd1*. To normalize the signals a PCR fragment of the gene for actin (*act1*) was also used as a probe. The intensities of the signals were measured using the Typhoon 8600 instrument (Amersham).

Quantification of the reaction product and testing the specificity of the L-galactonate dehydratase

For the quantification of the reaction product and the K_m measurement different concentrations of L-galactonate (pH 7.0) were mixed with the yeast extract of the strain expressing the *lgd1* and incubated at 28°C. The reaction mixture contained 10 mM sodium phosphate buffer pH 7.0 or 10 mM Tris-HCl, pH 7.0. The buffer had no effect on the activity. The pH did not change after the addition of L-galactonate. As a control the extract of the yeast strain with the empty p2159 plasmid was treated in a similar way. The reaction product was identified as a 2-keto-3-deoxy sugar acid in a chemical assay using thiobarbituric acid and quantified as described by Buchanan *et al*. (1999). In this colorimetric assay the absorbance was read at 549 nm and an absorbance coefficient of 67.8×10^3 M^{-1} cm^{-1} was used.

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To test the specificity of the enzyme, the yeast extract prepared as described before was mixed at a final concentration of 1 g l^{-1} of extracted protein with 10 mM sugar acids D-gluconate, D-arabonate, D-xylonate, L-gulonate, D-galactonate and L-galactonate in 10 mM sodium phosphate buffer pH 7.0. After incubating 4 h at 28°C the formation of a reducing sugar was verified with the DNS assay as described above.

NMR analysis of the reaction product

L-galactonate at a concentration of 110 mM was incubated with the yeast extract of the strain expressing the *lgd1* as described above. The reaction mixture was then analysed by NMR after different time intervals. The reaction product was identified by comparing with the NMR spectrum of pure L-galactonate.

The NMR experiments were carried out at 23°C on a Varian Inova spectrometer operating on a proton frequency of 500 MHz. The spectral widths of the 1D ^{1}H and ^{13}C spectra were 5000 Hz and 30 675 Hz respectively. In DQFCOSY and TOCSY experiments, the spectral width was 3400 Hz and matrices of 1024×128 complex data points were acquired. The spinlock time in the TOCSY was 80 ms. In HSQC the spectral widths in ^{1}H and ^{13}C dimensions were 1654 Hz and 10 000 Hz respectively, and a matrix of 1024×256 complex data points was acquired. All 2D data matrices were zero-filled once in F1 and a cosine bell weighting function was applied in both dimensions prior to the Fourier transformation.

Deletion of the L-galactonate dehydratase in H. jecorina

For the deletion of the *lgd1* gene in *H. jecorina* a deletion cassette was constructed. For the deletion cassette 1.5 kb of the genomic DNA sequence from both sides of the L-galactonate dehydratase gene were cloned and ligated to the pBluekan7-1.NotI plasmid (obtained from P. J. Punt, TNO Nutrition and Food Research, the Netherlands). The plasmid contains an expression cassette for hygromycin resistance consisting of the *A. nidulans gpdA* promoter and *trpC* terminator and the *E. coli hph*. The part upstream the *lgd1* was cloned using the primers 5′-GAGCTCAAGCTTCCACGC AGTTGCTACTTCTA-3′ and 5′-GAGCTCTGGTTATTTGGCA GAGCGAC-3′ introducing SacI and HindIII restriction sites. The SacI fragment was ligated to the SacI cloning site of the pBluekan7-1.NotI. The part downstream of the *lgd1* was cloned with the primers 5′-ACTAGTGGGGCAAAGTTGGA CATGAT-3′ and 5′-ACTAGTAAGCTTGCAATACCTGGACC AAGCTA-3′ introducing SpeI and HindIII restriction sites. The SpeI fragment was ligated to the SpeI site of the pBluekan7-1.NotI. In the resulting vector it was checked that the orientation of the two DNA fragments relative to each other was not changed, the gene for hygromycin B resistance was placed in between them and HindIII digestion could release the deletion cassette. The deletion cassette released by HindIII digestion was transformed to the *H. jecorina* Rut C-30 strain as described previously (Penttilä *et al*., 1987) and selected for hygromycin B resistance (Mach *et al*., 1994). Strains where the deletion cassette had integrated into the *lgd1* locus were identified by PCR. Primers used were from the *gpdA* promoter sequence of the deletion cassette and from the genomic DNA sequence 1.6 kb downstream from *lgd1* gene. Southern analysis was then used to verify that these transformants contained only one copy of the *hph* gene and that this copy replaced the coding sequence of the *lgd1* gene. The Southern hybridization was performed using standard procedures (Sambrook *et al*., 1989). DNA was isolated from the Rut C-30 and the strain with the *lgd1* deletion and the DNA was digested with the restriction enzymes EcoRI and HindIII. The Hybond N filter was then probed with a 800 bp fragment of the *hph* gene and a 750 bp fragment of the *lgd1*. The resulting deletion strain was called VTT D-05369.

The *lgd1* gene was transformed back into the *H. jecorina lgd1* deletion strain to verify that there had not been any other changes in the genome in addition to the *lgd1* deletion. An expression plasmid pAN52-1NotI (obtained from P.J. Punt, TNO Nutrition and Food Research, the Netherlands) was modified by PCR so that NcoI restriction site was removed and restriction sites for BamHI, SacI, SpeI, EcoRV, ClaI and ApaI were included in between the *A. nidulans gpdA* promoter and *trpC* terminator. The *H. jecorina lgd1* gene was cut from TOPO-vector with BamHI and ligated into the BamHI site of the modified pAN52-1NotI plasmid. The resulting plasmid was cotransformed into the *lgd1* deletion strain with a selection plasmid pTOC202 that has an acetamidase enzyme encoding *A. nidulans amdS* gene as a marker. Transformants were selected for acetamide resistance. The retransformation of the *lgd1* gene was verified by PCR using oligos from the *lgd1* gene and from the *gpdA* promoter and was also confirmed by growing the strains on plate with D-galacturonic acid and 10 mM acetamide.

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Publication III

Enzymes for the NADPH-dependent reduction of dihydroxyacetone and D-glyceraldehyde and L-glyceraldehyde in the mould *Hypocrea jecorina*

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Enzymes for the NADPH-dependent reduction of dihydroxyacetone and D-glyceraldehyde and L-glyceraldehyde in the mould *Hypocrea jecorina*

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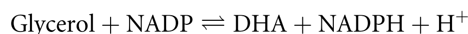
The mould *Hypocrea jecorina* (*Trichoderma reesei*) has two genes coding for enzymes with high similarity to the NADP-dependent glycerol dehydrogenase. These genes, called *gld1* and *gld2*, were cloned and expressed in a heterologous host. The encoded proteins were purified and their kinetic properties characterized. GLD1 catalyses the conversion of D-glyceraldehyde and L-glyceraldehyde to glycerol, whereas GLD2 catalyses the conversion of dihydroxyacetone to glycerol. Both enzymes are specific for NADPH as a cofactor. The properties of GLD2 are similar to those of the previously described NADP-dependent glycerol-2-dehydrogenases (EC 1.1.1.156) purified from different mould species. It is a reversible enzyme active with dihydroxyacetone or glycerol as substrates. GLD1 resembles EC 1.1.1.72. It is also specific for NADPH as a cofactor but has otherwise completely different properties. GLD1 reduces D-glyceraldehyde and L-glyceraldehyde with similar affinities for the two substrates and similar maximal rates. The activity in the oxidizing reaction with glycerol as substrate was under our detection limit. Although the role of GLD2 is to facilitate glycerol formation under osmotic stress conditions, we hypothesize that GLD1 is active in pathways for sugar acid catabolism such as D-galacturonate catabolism.

Dihydroxyacetone (DHA), D-glyceraldehyde and L-glyceraldehyde can be reduced using NADPH as a cofactor to form glycerol and NADP. Enzymes catalysing this reaction are generally called NADP:glycerol dehydrogenases. NADP:glycerol dehydrogenase activity is common in moulds and filamentous fungi. Enzymes from different species of filamentous fungi have been purified and characterized. The enzymes purified from *Aspergillus niger* [1] and *Aspergillus nidulans* [2] catalyse the reversible reaction from glycerol and NADP to DHA and NADPH. For the *A. niger* enzyme, an equilibrium constant of $3.1–4.6 \times 10^{-12}$ M was estimated for the reaction:



$$\text{Glycerol} + \text{NADP} \rightleftharpoons \text{DHA} + \text{NADPH} + \text{H}^+$$

A glycerol dehydrogenase with slightly different properties was described in *Neurospora crassa*, where D-glyceraldehyde was the preferred substrate over DHA in the reductive reaction. This enzyme was also reversible, i.e. it showed activity with glycerol and NADP [3]. The purified glycerol dehydrogenases from *A. nidulans* and *A. niger* also showed low activity with D-glyceraldehyde; however, DHA was the preferred substrate [2]. The *A. niger* enzyme was commercially available as a partly purified preparation, and partial amino acid sequences were available [4].

Abbreviations
DHA, dihydroxyacetone; DHAP, dihydroxyacetone phosphate.

Glycerol dehydrogenases have different functions in filamentous fungi. One role is to form part of the biosynthetic pathway for glycerol production. In this pathway, dihydroxyacetone phosphate (DHAP) is dephosphorylated to DHA and then reduced to glycerol by an NADP-dependent glycerol dehydrogenase [5]. This is different from the situation in yeast. Yeast lacks the enzyme activity to dephosphorylate DHAP [6]. Instead, DHAP is first reduced to glycerol 3-phosphate, which is then dephosphorylated to form glycerol. Glycerol dehydrogenase activities, however, have been reported in different yeast species [6]. In filamentous fungi, the NADP-dependent glycerol dehydrogenase was also suggested to be functional in the catabolism of DHA [2].

Another function of a glycerol dehydrogenase is to reduce glyceraldehyde. D-Glyceraldehyde is generated in the nonphosphorylated pathway for D-gluconate [7] or D-galactonate catabolism [8]. L-Glyceraldehyde was suggested to be generated in the catabolic pathway for D-galacturonate (Kuorelahti *et al.*, unpublished results). In these pathways, the sugar acids D-gluconate, D-galactonate and L-galactonate (in the D-galacturonate pathway) are converted by a dehydratase to the corresponding 2-keto-3-deoxy sugar acid, which is then split by an aldolase to form pyruvate and D-glyceraldehyde or pyruvate and L-glyceraldehyde.

A glycerol dehydrogenase is probably not part of the path for glycerol catabolism. A glycerol dehydrogenase mutant of *A. nidulans* was not affected in growth on glycerol [9]. Glycerol is catabolized in filamentous fungi through glycerol kinase and a mitochondrial glycerol 3-phosphate dehydrogenase, as in yeast [10]. *Aspergillus nidulans* probably has more than one glycerol dehydrogenase; one constitutive and one inducible on D-galacturonate [11].

A gene for a glycerol dehydrogenase, *gldB*, was identified in *A. nidulans*. This gene was shown to be effective for osmotolerance; a *gldB* disruptant did not produce glycerol, and the mutant had lost osmotolerance and showed no glycerol dehydrogenase activity [9]. A homologue of *gldB*, *gld1*, was identified in *Trichoderma atroviride*. Here, the glycerol dehydrogenase activity of the mycelial extract correlated with the transcription level of *gld1* [12].

In this study, we identified two open reading frames with high homology to previously described glycerol dehydrogenases in the genome of the filamentous fungus *Hypocrea jecorina* (*Trichoderma reesei*). These open reading frames were expressed in the yeast *Saccharomyces cerevisiae*, and the enzymes were purified and characterized. We show that one enzyme catalyses the reduction of D-glyceraldehyde and L-glyceraldehyde to glycerol, whereas the other reduces DHA. This is the first report on heterologous expression combined with kinetic characterizations of NADP-dependent glycerol dehydrogenases from mould.

Results

Partial amino acid sequences of an NADP-dependent glycerol dehydrogenase from *A. niger* had been described previously [4]. We used these sequences to find homologies in the translated *H. jecorina* genome sequence. We identified two potential genes in the genome sequence that had, after translation, homologies to the partial amino acid sequences of the *A. niger* enzyme. Comparing the nucleotide sequence with sequences of other dehydrogenases enabled us to predict the start and the stop codons and to design primers to amplify the open reading frames using PCR. For the first potential glycerol dehydrogenase gene, we predicted introns in the genomic DNA. For that reason, we amplified the open reading frame from cDNA. For the second of the two potential glycerol dehydrogenases, we predicted no introns and therefore amplified the open reading frame from the genomic DNA. We called the genes *gld1* and *gld2*, respectively.

Comparison of the cDNA of *gld1* with the genome sequence revealed that the genomic DNA indeed contained three introns. The intron sequences started after nucleotides 327, 510 and 916 of the open reading frame and contained 70, 69 and 62 nucleotides, respectively. The sequence of the open reading frame codes for a protein with 331 amino acids and a calculated molecular mass of 36 232 Da. The sequence is deposited at GenBank and has the accession number DQ422037.

The open reading frame of *gld2* coded for a protein with 318 amino acids and a calculated molecular mass of 35 663 Da. The open reading frame for *gld2* is deposited at GenBank and has the accession number DQ422038.

The *gld1* and *gld2* genes were expressed in a heterologous host, the yeast *S. cerevisiae*, under a strong and constitutive promoter. The control strain contained the empty expression vector. The cells were then disintegrated and the crude extract was analysed. *S. cerevisiae* is a suitable expression system because it does not have endogenous NADP:glycerol dehydrogenase activity.

gld1

The expression of *gld1* in *S. cerevisiae* did not result in glycerol dehydrogenase activity; that is, in the assay with glycerol and NADP as substrates, no activity was

detected. Even at an alkaline pH of 9.5, the activity was below our detection limit, which was about 0.1 nkat·mg^{-1}. Also, the control strain did not show such activity. However, in the reverse or reductive direction, we observed activity with NADPH and DL-glyceraldehyde. The reductive activity in the crude extract was estimated as 2 nkat per mg of extracted protein. In the control strain carrying the empty plasmid, this activity was below 0.1 nkat·mg^{-1}. The activity with NADPH and DL-glyceraldehyde in an extract of *H. jecorina* was about 3 nkat·mg^{-1}. The GLD1 protein was tagged with a histidine tag at the N-terminal end by adding the coding sequence for six histidines to the end of the open reading frame, and then expressed in *S. cerevisiae*. The tagged protein had a similar activity in the crude extract as the nontagged protein, indicating that the tag did not affect the protein activity. The tagged protein was then purified and further analysed.

The purified GLD1 showed activity with DL-glyceraldehyde and NADPH as a cofactor. It had a very much reduced activity with DHA (Table 1). No activity was observed with NADH as a cofactor. Other aldehydes were tested with NADPH and the results are summarized in Table 1. We found activity with glyoxal (ethane-1,2-dione), methylglyoxal (pyruvaldehyde) and diacetyl (2,3-butanedione), but no activity with C5 or C6 sugars. We tested D-glyceraldehyde and L-glyceraldehyde individually and observed similar activities; the activity with L-glyceraldehyde was only slightly lower. For D-glyceraldehyde and L-glyceraldehyde, we also observed similar Michaelis–Menten constants of about 0.9 mM (Table 1); the Michaelis–Menten constant for NADPH was about 40 μM. As with the crude extract, we did not observe oxidative activity with glycerol and NADP. Also, with other C4 and C5 polyols no activity with NADP as a cofactor was observed. We tested erythritol, ribitol, xylitol and DL-arabinitol at a concentration of 50 mM.

gld2

The expression of *gld2* in *S. cerevisiae* resulted in glycerol dehydrogenase activity. In the assay with glycerol and NADP as substrates, we found an activity of 0.5 nkat·mg^{-1} in the crude extract. Activity was also observed in the reverse direction. With DHA and NADPH, the activity was 15 nkat·mg^{-1}. GLD2 was tagged with a histidine tag at the N-terminus, in the same way as GLD1, to facilitate enzyme purification. The tagged protein had a similar activity in crude yeast extract as the untagged protein, indicating that the tag was not interfering with the protein activity. The tagged protein was purified and then used for further analysis.

In the reductive reaction, the Michaelis–Menten constant K_m for DHA was 1 mM, and the K_m for NADPH was 50 μM. The V_{max} was estimated at 2400 nkat per mg of purified protein. In the oxidative reaction, the K_m for glycerol was 350 mM and the K_m for NADP was 110 μM. The V_{max} was about 1200 nkat·mg^{-1}. In the reductive reaction, very low activity was observed with D-glyceraldehyde and L-glyceraldehyde (Table 1). Lower activities were also observed with methylglyoxal and diacetyl. In the oxidative reaction, the enzyme was active with glycerol and to a lower

Table 1. The specificities and kinetic properties of the histidine-tagged and purified GLD1 and GLD2. The reductive assay conditions were 10 mM sodium phosphate (pH 7.0) and 0.4 mM NADPH. The oxidative assay conditions were 200 mM Tris/HCl (pH 9.5) and 1 mM NADP. The activities are given in nkat per mg of protein and in kcat (in parentheses). The enzyme efficacy, V_{max}/K_m, is given in s^{-1}·M^{-1}. ND, no activity detected.

	V_{max} (nkat·mg^{-1}·s^{-1})		K_m (mM)		V_{max}/K_m (s^{-1}·M^{-1})	
	GLD1	GLD2	GLD1	GLD2	GLD1	GLD2
Dihydroxyacetone	30 (1.4)	2400 (86)	5.8	1	240	0.086
L-Glyceraldehyde	140 (5.0)	500 (18)	0.9	8	5500	2250
D-Glyceraldehyde	150 (5.5)	210 (7.5)	0.9	96	6100	78
Diacetyl	330 (12)	2500 (88)	0.9	13	13	6800
Glyoxal	375 (14)	260 (9.2)	2.4	30	5800	310
Methylglyoxal	410 (15)	3300 (120)	0.4	37 500	3600	
Acetoin	300 (11)	480 (21)	122	113	90	185
D-Ribose	160 (5.8)	ND	122	ND	48	
D-Xylose	450 (16)	ND	334	ND	48	
D-Glucose	190 (6.8)	ND	470	ND	14	
Glycerol	ND	1200 (56)	ND	350		160

extent with erythritol. Low activities were also observed with C5 and C6 sugar alcohols (Table 1). The enzyme was, like GLD1, specific for the cofactor couple NADP/NAPDH.

Discussion

There have been several reports about NADP-dependent glycerol dehydrogenases in mould. The previously purified enzymes showed activity with glycerol and NADP in the oxidizing direction and activities with DHA or D-glyceraldehyde and NADPH in the reducing direction. According to the International Union of Biochemistry and Molecular Biology (IUBMB), there are two kinds of NADP-dependent glycerol dehydrogenase. One is an enzyme with the systematic name glycerol:NADP$^+$ oxidoreductase (EC 1.1.1.72) that facilitates the reaction of glycerol and NADP to form D-glyceraldehyde and NADPH; the other is a glycerol:NADP$^+$ 2-oxidoreductase (EC 1.1.1.156) that facilitates the reaction of glycerol and NADP to form DHA and NADPH. The enzymes purified from *A. niger* and *A. nidulans* fall into the category EC 1.1.1.156, because they are mainly active with DHA, as shown by 90% smaller activity with D-glyceraldehyde and no activity with L-glyceraldehyde [2]. The glycerol dehydrogenase purified from *N. crassa* [3] is in the category EC 1.1.1.72, because this enzyme has the highest activity with D-glyceraldehyde.

There are also indications that mould can contain more than one NADP:glycerol dehydrogenase. In *A. nidulans*, it was shown that upon induction by D-galacturonic acid, a second NADP:glycerol dehydrogenase was induced [11]. Also in *A. niger*, the production of a D-glyceraldehyde-specific enzyme was induced by D-galacturonic acid [13].

All these observations harmonize with our finding that the *H. jecorina* genome has two genes coding for enzymes that are similar to NADP:glycerol dehydrogenases. Accordingly, we cloned these two open reading frames, expressed them in *S. cerevisiae* and confirmed that active enzymes were expressed. The histidine-tagged proteins were then purified and used for kinetic analysis (Fig. 1).

The *gld2* gene had the highest homology to *gldB* of *A. nidulans* and *gld1* of *T. atroviride* [12]. GLD2 had the highest activity with DHA and only low activity with D-glyceraldehyde and L-glyceraldehyde. It is consequently a glycerol:NADP$^+$ 2-oxidoreductase with the number EC 1.1.1.156. The properties of GLD2 are similar to those of the enzymes purified from *A. niger* [1] and *A. nidulans* [2]; that is, the enzyme catalyses the reversible reduction of DHA to glycerol using NADPH as a cofactor and has only low activity with D-glyceraldehyde or L-glyceraldehyde. The function of *gld2* is probably in glycerol synthesis, similar to *gldB* in *A. nidulans*.

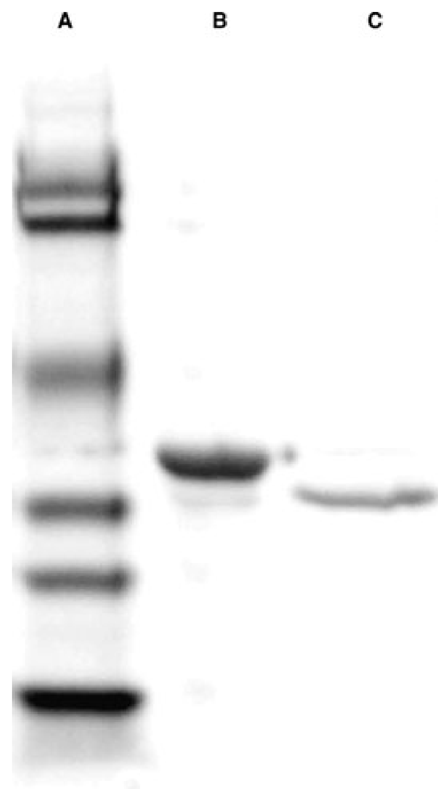


Fig. 1. SDS/PAGE of the histidine-tagged and purified GLD1 and GLD2 proteins. GLD1 is in lane B and GLD2 in lane C. Lane A contains the molecular mass markers with masses 107, 81, 48.7, 33.8, 27 and 20.7 kDa (from top to bottom).

The *gld1* gene showed highest homology to an aldo-ketoreductase from *Penicillium citrinum* [14] in a BLAST search, not considering hypothetical proteins. The kinetic properties of GLD1 were also distinctly different from those of GLD2. GLD1 had the highest activity with D-glyceraldehyde and only low activity with DHA. Thus the enzyme should be called glycerol:NADP$^+$ oxidoreductase, with the number EC 1.1.1.72. The kinetic properties of GLD1 showed some similarity to those of the glycerol dehydrogenase purified from *N. crassa* [3]. The *N. crassa* enzyme also had the highest activity with D-glyceraldehyde and lower activity with DHA. However, GLD1 had several properties that were different from those of the *N. crassa* enzyme. GLD1 had a lower activity with DHA and higher activity with L-glyceraldehyde. Another significant difference is that the *N. crassa* enzyme is reversible, i.e. shows activity with glycerol

and NADP; however, it is not clear whether glyceraldehyde or DHA is formed. With GLD1, the activity with glycerol and NADP was below our detection limits.

A possible interpretation of this difference in the reversibility of the two enzymes is that the *N. crassa* enzyme is converting glycerol to DHA, whereas GLD1 is converting glycerol to glyceraldehyde. The formation of glyceraldehyde is energetically less favourable than the formation of DHA, and is not observed for this reason. Another possible explanation is that the oxidation of glycerol by GLD1 is allosterically inhibited.

We have made a CLUSTALW alignment of GLD1 and GLD2 of *H. jecorina* together with some homologous proteins for which the protein sequences have been published and some of the kinetic properties have been described. GLD1 showed highest homology to the *P. citrinum* KER [14], the *S. cerevisiae YPR1* [15] and the *S. cerevisiae GCY1* [16]. From their kinetic properties, all these proteins can be categorized as EC 1.1.1.72. Another group of proteins that showed a high degree of homology were the *H. jecorina* GLD2, the *A. nidulans* GLDB [9] and the *T. atroviride* GLD1 [12]. These three proteins can be categorized as EC 1.1.1.156 according to their kinetic properties. The high degree of homology within these two groups of proteins might be used to predict the enzyme class of yet uncharacterized proteins.

Because GLD1 had the highest activity with D-glyceraldehyde and similar activity with L-glyceraldehyde, we would assume that the role of this enzyme is to convert D-glyceraldehyde and L-glyceraldehyde to glycerol. D-Glyceraldehyde is an intermediate in the catabolic path for D-gluconate [7] and D-galactonate [8]. L-Glyceraldehyde is an intermediate in the catabolic path for D-galacturonate [17,18].

A glycerol dehydrogenase has been described previously to be induced by D-galacturonate in the mould *A. nidulans* [11]. It would be reasonable to assume that this induced enzyme also has a role in D-galacturonate catabolism. This additional glycerol dehydrogenase in *A. nidulans* was observed when the mycelial extract was separated by native polyacrylamide gel electrophoresis, and enzyme activities with NADP and glycerol as substrates were visualized by Zymogram staining; that is, only enzymes that had activity with glycerol and NADP were visualized. As GLD1 is not active with glycerol and NADP, it must be different from the enzyme induced by D-galacturonate.

As GLD1 or any enzyme reducing L-glyceraldehyde has a clear function in D-galacturonate catabolism, we tested whether such an enzyme activity is induced. For that purpose, we grew mycelia on different carbon sources including D-galacturonate, and tested the crude mycelial extracts for activity with L-glyceraldehyde or D-glyceraldehyde and NADPH. We observed similar activities on all carbon sources, suggesting that GLD1 is not induced by D-galacturonic acid (data not shown).

NADP-dependent glycerol dehydrogenase activity has also been reported in yeast. From the fission yeast *Schizosaccharomyces pombe*, a glycerol:NADP 2-oxidoreductase was purified. This enzyme was reversible and had a 100-fold higher activity with DHA than with DL-glyceraldehyde. The active enzyme complex consisted of two different subunits with masses of 25 and 30 kDa [19]. The corresponding genes have not been identified. In this context, it is interesting to note that *S. pombe* also has an NAD-dependent glycerol dehydrogenase, a glycerol:NAD 2-oxidoreductase [20], an enzyme that has not been reported in mould.

In *S. cerevisiae*, NADP:glycerol dehydrogenase activies have not been described to the best of our knowledge. However, it was suggested that the *GCY1* of *S. cerevisiae* codes for such an enzyme, because the amino acid sequence had homologies to the purified enzyme from *A. niger* [4]. The Ypr1p of *S. cerevisiae* had a high degree of homology to Gcy1p but less to the purified enzyme from *A. niger*. *YPR1* was expressed in *E. coli* and the enzyme catalytic properties were studied. The enzyme used NADPH to reduce DL-glyceraldehyde and had about 100-fold lower activity with DHA. Ypr1p also showed activity in the oxidative direction with glycerol and NADP. However, this activity was about 4000 times lower than in the reducing direction with DL-glyceraldehyde and NADPH [15].

In this article we have shown that the same mould species can contain two distinctly different glycerol dehydrogenases, one for DHA (EC 1.1.1.156) and one for D-glyceraldehyde and L-glyceraldehyde (EC 1.1.1.72). This seems to be a common feature in different moulds, as other mould species such as *N. crassa* and *A. nidulans* contain genes with high homology to both *gld1* and *gld2*. Although the two genes have a high degree of homology, the differences in sequence are sufficient to predict the specificity.

Experimental procedures

Cloning and expression of the open reading frames for *gld1* and *gld2*

The *gld1* gene was cloned from a cDNA library of the *H. jecorina* strain Rut C-30 [21] by PCR. The following primers, introducing an *Eco*RI restriction site, were used: 5′-gaattcaacatgtcttccggaaggac-3′ and 5′-gaattcttacagcttgatga cagcag-3′. The PCR product was cloned in a TOPO vector

(Invitrogen, Carlsbad, CA, USA), and an *Eco*RI fragment of about 1 kb isolated. This fragment was then ligated to the *Eco*RI site of the p2159 vector, a vector with *TPI1* promoter and *URA3* selection marker derived from the pYX212 [17], and the orientation of the open reading frame in the expression vector was checked. The *S. cerevisiae* strain CEN.PK2-1B was then transformed with the expression vector and grown on selective medium. As a control, the same strain was transformed with the empty vector p2159.

The *gld2* gene was cloned by PCR using genomic DNA derived from the *H. jecorina* strain QM6a as a template. The following primers were used: 5′-gaattcagaatg gcctccaagacgta-3′ and 5′-gaattcttattcctcctctggccaaa-3′. The PCR product was cloned, similar to *gld1*, first in a TOPO vector and then in the expression vector p2159. The *S. cerevisiae* strain CEN.PK2-1B was then transformed with the expression vector.

The *gld1* and *gld2* genes were also expressed with N-terminal or C-terminal histidine tags. For that purpose, a coding sequence for six histidines was introduced by PCR either at the N-terminus, after the ATG, or at the C-terminus before the stop codon. The expression of these histidine-tagged proteins was done as described above.

Strains, growth conditions and cell extracts

The *E. coli* strain DH5α was used in the cloning procedures. It was grown in LB medium with ampicillin at 37 °C. The *S. cerevisiae* strain CEN.PK2-1D (VW-1B) was the host for the heterologous expression. It was grown in synthetic medium lacking uracil when required for selection at 30 °C. The *H. jecorina* (*T. reesei*) strain was Rut C-30 or QM6a. *Hypocrea jecorina* was grown in liquid medium containing 2 $g \cdot L^{-1}$ proteose peptone, 15 $g \cdot L^{-1}$ KH_2PO_4, 5 $g \cdot L^{-1}$ $(NH_4)_2SO_4$, 0.6 $g \cdot L^{-1}$ $MgSO_4.7H_2O$, 0.6 $g \cdot L^{-1}$ $CaCl_2.2H_2O$, trace elements [22], and 20 $g \cdot L^{-1}$ of the main carbon source, as specified, at 28 °C. To make mycelial or cell extracts of *H. jecorina* or *S. cerevisiae*, about 100 μg of fresh mycelia or cells were mixed with 300 μL of glass beads (diameter 0.4 mm) and 400 μL of buffer [5 mM sodium phosphate, pH 7.0, and complete, EDTA-free protease inhibitor (Roche, Basel, Switzerland)] and disintegrated in a Mini-Bead Beater (Biospec Products, Bartlesville, OK, USA) three times for 30 s. The mixture was then centrifuged in an Eppendorf microcentrifuge at full speed for 25 min, and the supernatant used for the analysis. The protein content of the extract was estimated using the Bio-Rad protein assay, and γ-globulin was used as a standard.

Enzyme purification and assays

To purify the histidine-tagged proteins, the *S. cerevisiae* cells expressing the tagged constructs were grown and a cell extract was obtained as described before. The histidine-tagged protein was purified with a nickel/nitrilotriacetic acid column (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The glycerol dehydrogenase activity was measured in a buffer containing 200 mM Tris/HCl (pH 9.5), 1 mM NADP and purified enzyme. The reaction was started by adding glycerol. When analysing cell extracts, we used a final glycerol concentration of 10 mM and a pH of 8.0. The reductase activity was measured in a buffer containing 10 mM sodium phosphate (pH 7.0) and 400 μM NADPH, which was supplemented with the cell extract or the purified enzyme. The reaction was started by adding DHA, D-glyceraldehyde, L-glyceraldehyde or any of the other substrates, and the reaction followed spectrophotometrically by monitoring the NADPH at 340 nm. When the Michaelis–Menten constants were measured, all substrates were first mixed and the reaction was then started by adding the purified enzyme. All assays were performed at 30 °C in a Cobas Mira automated analyser (Roche). L-Glyceraldehyde was synthesized from L-gulono-1,4-lactone as described previously [23,24].

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Publication IV

The missing link in the fungal D-galacturonate pathway: Identification of of the L-threo-3-deoxy-hexulosonate aldolase

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The Missing Link in the Fungal D-Galacturonate Pathway

IDENTIFICATION OF THE L-THREO-3-DEOXY-HEXULOSONATE ALDOLASE[*]

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The fungal path for the catabolism of D-galacturonate is only partially known. It is however distinctly different to the well-known bacterial path. The known elements of the fungal path are D-galacturonate reductase converting D-galacturonate to L-galactonate and L-galactonate dehydratase converting L-galactonate to L-threo-3-deoxy-hexulosonate (2-keto-3-deoxy-L-galactonate). Here we describe the missing link in this pathway, an aldolase converting L-threo-3-deoxy-hexulosonate to pyruvate and L-glyceraldehyde. Fungal enzymes converting L-glyceraldehyde to glycerol have been described previously. The L-threo-3-deoxy-hexulosonate aldolase activity was induced in the mold *Hypocrea jecorina* (*Trichoderma reesei*) during growth on D-galacturonate. The enzyme was purified from this mold and a partial amino acid sequence obtained. This sequence was then used to identify the corresponding gene from the *H. jecorina* genome. The deletion of the gene resulted in a strain unable to grow on D-galacturonate and accumulating L-threo-3-deoxy-hexulosonate. The open reading frame was cloned from cDNA and functionally expressed in the yeast *Saccharomyces cerevisiae*. A histidine-tagged protein was expressed, purified, and characterized. The enzyme catalyzed reaction was reversible. With L-threo-3-deoxy-hexulosonate as substrate the K_m was 3.5 mM and with pyruvate and L-glyceraldehyde the K_m were 0.5 and 1.2 mM, respectively.

D-Galacturonate is an important carbon source for microorganisms living on decaying plant material because D-galacturonate is the principal component of pectin. It is also of relevance in biotechnology when cheap raw materials such as pectin-rich materials like citrus peel or sugar beet pulp are to be exploited. However, knowledge about the microbial pathways for D-galacturonate catabolism is rather limited. A bacterial catabolic pathway has been described previously, whereas a fungal path is only partly known. The bacterial pathway consists of five enzymes converting D-galacturonate to pyruvate and D-glyceraldehyde-3-phosphate. The intermediate metabolites are D-tagaturonate, D-altronate, D-erythro-3-deoxy-hexulosonate, and D-erythro-3-deoxy-hexulosonate-6-phosphate. The enzymes in this path are uronate isomerase (EC 5.3.1.12) (1), NADH-utilizing D-tagaturonate reductase (EC 1.1.1.5) (2), altronate dehydratase (EC 4.2.1.7) (3), 2-dehydro-3-deoxy-D-gluconate kinase (EC 2.7.1.45) (4), and 2-dehydro-3-deoxy-D-gluconate-6-phosphate aldolase (EC 4.1.2.14) (5). The fungal path is distinctly different. The first two steps of this pathway have been described. In the first step D-galacturonate is reduced to L-galactonate by an NADPH-coupled D-galacturonate reductase (6). In *Hypocrea jecorina* (*Trichoderma reesei*) this enzyme activity was induced when grown on D-galacturonate but the activity was absent during growth on other carbon sources. The D-galacturonate reductase gene *gar1* is identified and the purified protein characterized (6). In the second step L-galactonate is converted to L-threo-3-deoxy-hexulosonate (2-keto-3-deoxy-L-galactonate) by a dehydratase (7). Also this activity was induced in *H. jecorina* when D-galacturonate was the carbon source for growth. The L-galactonate dehydratase gene *lgd1* was identified and the corresponding enzyme characterized. A deletion in the *lgd1* gene resulted in a *H. jecorina* strain unable to grow on D-galacturonate (7). It remained unclear how the L-threo-3-deoxy-hexulosonate was further metabolized, whether it was phosphorylated by a kinase as in the bacterial path and then *e.g.* split by an aldolase to result in pyruvate and L-glyceraldehyde-3-phosphate, or whether an aldolase was active on the non-phosphorylated 2-keto-3-deoxy sugar acid, or whether still another enzyme was involved. In a previous work we hypothesized that the L-threo-3-deoxy-hexulosonate is split into pyruvate and L-glyceraldehyde by aldolase (7), although such an activity has never been shown. In a subsequent reaction the L-glyceraldehyde could then be converted by a reductase to glycerol. Such a reductase that would use NADPH as a cofactor has been described previously for *H. jecorina* (8). The missing link in this pathway is an L-threo-3-deoxy-hexulosonate aldolase that would convert L-threo-3-deoxy-hexulosonate to pyruvate and L-glyceraldehyde. It was earlier suggested that D-galacturonate is metabolized through a non-phosphorylating pathway in the mold *Aspergillus nidulans* (9, 10). However it was speculated that D-glyceraldehyde is an intermediate (11). Further support for such a non-phosphorylating pathway comes from a microarray analysis of genes transcribed in *Aspergillus niger* grown on D-galacturonate where, among others, genes with homologies to aldoketo reductase, racemase, and aldolase are identified, although the gene sequences are not available (12).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) EF203091.

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EXPERIMENTAL PROCEDURES

Enzyme Activity Measurements—The aldolase activity in the forward direction was measured in a coupled assay with lactate dehydrogenase. The buffer contained 10 mM sodium phosphate, pH 7.0, 0.2 mM NADH, and lactate dehydrogenase (4 units/ml; Roche). With the fungal and yeast crude extracts the final protein concentration in the reaction was 0.5 mg/ml and with the purified histidine-tagged protein 1.1 μg/ml. The reaction was started by adding L-threo-3-deoxy-hexulosonate to the final concentration of 10 mM. When the Michaelis-Menten constant was measured this concentrations was varied between 20 μM and 40 mM. The reaction was monitored by following the NADH absorbance at 340 nm. The measurements were performed in a Cobas Mira automated analyzer (Roche) at a temperature of 30 °C. L-Threo-3-deoxy-hexulosonate was synthesized from L-galactonate as described earlier (7).

In the reverse direction the enzyme activity was assessed by measuring the production of L-threo-3-deoxy-hexulosonate using the thiobarbituric acid method as described earlier (13). The buffer contained 10 mM sodium phosphate, pH 7.0, 40 mM pyruvate, and 16 mM glyceraldehyde, and the reaction was started by adding crude protein extract to the final concentration of 0.5 mg/ml. When the affinities for the aldehyde were measured the pyruvate concentration was 60 mM, and when the affinity for pyruvate was measured the L-glyceraldehyde concentration was 24 mM, and the purified histidine-tagged protein was used at concentration 1.1 μg/ml. The reaction mixture was incubated for 1 and 1.5 h at room temperature and formation of the 2-keto-3-deoxy acid quantified as described (13). L-Glyceraldehyde was synthesized from L-gulono-1,4-lactone as described previously (14, 15). The activity is given in units, which is the activity that forms 1 μmol of product per minute.

Enzyme Purification, Identification of the Gene, and Cloning of the Open Reading Frame—The mold *H. jecorina* (*T. reesei*) strain VTT-D-80133 was grown in a medium containing D-galacturonic acid/sodium D-galacturonate 20 g/liter (pH 7.0), 0.5 g/liter proteose peptone, 15 g/liter KH_2PO_4, 5 g/liter $(NH_4)_2SO_4$, 0.6 g/liter $MgSO_4 \cdot 7H_2O$, 0.6 g/liter $CaCl_2 \cdot 2H_2O$, and trace elements (16). The mycelia were lysed by vortexing with glass beads, and the resulting protein extract was desalted by gel filtration using a PD10 column (GE Healthcare) as described (6). The protein extract in a buffer containing 5 mM sodium phosphate, pH 7.0, was then bound to a DEAE column (GE Healthcare). 33 mg of protein was bound to 10 ml of DEAE. The protein was eluted in 2.5-ml fractions with a salt gradient from 0 to 200 mM NaCl in the same buffer. The fractions were tested for L-threo-3-deoxy-hexulosonate aldolase activity in the forward direction as described above. The active fractions were identified and separated on an SDS-PAGE. A 37-kDa protein band was identified as the aldolase. The protein band was then cut out from the gel and the protein alkylated in-gel with iodoacetamide and digested with trypsin. The peptides resulting from trypsin cleavage were extracted, and the quality of the generated peptide mass fingerprint was first determined by matrix-assisted laser desorption ionization time-of-flight (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 that procedure have been described elsewhere (17).

The obtained peptide sequences were then used to identify the corresponding gene in the *H. jecorina* genome. The open reading frame was amplified by PCR using *H. jecorina* cDNA as a template, using the DyNAzyme™ EXT DNA Polymerase (Finnzymes) and introducing two BamHI restriction sites. The following primers were used: 5′-GGATCCACCATGGCCCCCCCATCCCTA-3′ and 5′-GGATCCCTACAAAGTCTTCTCAATCTC-3′. The PCR product was then cloned in a TOPO vector (Invitrogen) and sequenced.

Expression of the Aldolase Gene—From the TOPO vector described above the 1-kb BamHI fragment was released and ligated to a yeast expression vector. The expression vector was a multicopy expression vector for *Saccharomyces cerevisiae* containing *URA3* for selection and the constitutive *TPI* promoter. The plasmid was derived from the pYX212 plasmid (R&D Systems); it was modified to remove the ATG and the hemagglutinin tag from the multiple cloning sites and to introduce a BamHI restriction site in the multiple cloning sites. After the correct orientation was verified the vector was transformed to the *S. cerevisiae* strain CEN.PK2–1B and grown in selective medium. The L-threo-3-deoxy-hexulosonate aldolase with a histidine tag at the C- or N-terminal end was produced in a similar manner as described above except that that the histidine tag was introduced by PCR. To introduce the C-terminal histidine tag the primers 5′-GGATCCACCATGGCCCCCCCATCCCTA-3′ for the sense direction and 5′-GGATCCCTAGTGGTGATGATGGTGATGCAAAGTCTTCTCAATCTCC-3′ for the antisense direction were used. To introduce the N-terminal histidine tag the primers 5′-GGATCCACCATGCATCACCATCATCACCACATGGCCCCCCCATCCCTAC-3′ for the sense and 5′-GGATCCCTACAAAGTCTTCTCAATCTC-3′ for the antisense direction were used.

Characterization of the Enzyme Activity—The *S. cerevisiae* strains expressing the aldolase with or without histidine tag were disintegrated by vortexing with glass beads and the yeast extract analyzed for L-threo-3-deoxy-hexulosonate aldolase activity in the forward direction as described above. In a control experiment a similar strain but with an empty plasmid was used. The N-terminally histidine-tagged L-threo-3-deoxy-hexulosonate aldolase was purified using a nickel-nitrilotriacetic acid-agarose column (Qiagene) according to the instructions of the manufacturer. The purified enzyme was used to estimate the Michaelis-Menten constants.

Deletion of the Aldolase Gene in H. jecorina—For the deletion of the aldolase gene a deletion cassette was constructed. For the deletion cassette 1.5 kb areas from both sides of the L-threo-3-deoxy-hexulosonate aldolase gene were cloned and ligated to the pBluekan7-1.NotI plasmid (obtained from P.J. Punt, TNO Nutrition and Food Research, The Netherlands). The part upstream of the *lga1* was cloned using the oligos 5′-GAGCTCGATATCAGGAATTGAGGGGCCATTTG-3′ and 5′-GAGCTCGGCTGTTGTGATGGAAGGAT-3′ introducing SacI and EcoRV restriction sites. The SacI fragment was ligated to the SacI cloning site of the pBluekan7-1.NotI. The part downstream of the *lga1* was cloned with the oligos 5′-ACTAGTGA-

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TATCAGGCACTGGATCGTGTCATT-3′ and 5′-ACTAGT-GTAGGCATTAGGTACGTAG-3′ introducing SpeI and EcoRV restriction sites. The SpeI fragment was ligated to the SpeI site of the pBluekan7-1.NotI. The two DNA fragments were ligated to the pBluekan7-1.NotI vector in such a way that the orientation of the two DNA fragments relative to each other was not changed, and EcoRV could be used to release the deletion cassette. In this construct a gene for hygromycin B resistance was placed between the two fragments. The deletion cassette comprising of the two DNA fragments and the hygromycin B resistance gene was then released by EcoRV digestion, transformed to the *H. jecorina* VTT-D-80133 strain and selected for hygromycin B resistance using standard protocols (18). The resulting strain was called VTT-D-80133 Δ*lga1*.

Retransformation of the Aldolase Gene to H. jecorina—The aldolase gene was transformed back into the *H. jecorina* strain with the deletion in the aldolase gene to show that no other changes in the genome other than the aldolase gene deletion caused the observed phenotypes. An expression plasmid pAN52-1.Not1 (obtained from P.J. Punt, TNO Nutrition and Food Research, The Netherlands) was modified so that the NcoI restriction site was removed and restriction sites for BamHI, SacI, SpeI, EcoRV, ClaI, and ApaI were included between the *A. nidulans gpdA* promoter and *trpC* terminator. The aldolase open reading frame was released from the TOPO vector described above and ligated to the BamHI site of the modified pAN52–1.NotI plasmid. After the correct orientation of the open reading frame was confirmed the plasmid was cotransformed with the selection plasmid pTOC202, which contained the acetamidase-encoding *amdS* gene from *A. nidulans* as a selection marker. Transformants were selected for acetamide resistance. The retransformation of the aldolase gene was confirmed by PCR using oligos from the aldolase gene and from the *gpdA* promoter. The resulting strain was the VTT-D-80133 Δ*lga1 lga1*.

Growth on D-Galacturonate—The *H. jecorina* strains VTT-D-80133, VTT-D-80133 Δ*lga1*, and VTT-D-80133 Δ*lga1 lga1* were grown in a D-galacturonic acid/D-galacturonate-containing medium as described above. 250-ml shake flasks with 50 ml of medium were inoculated with 1×10^7 spores and incubated on a shaker at 28 °C, and the contents of two parallel flasks were filtered after 2, 4, and 6 days. The biomass was dried overnight at 110 °C for dry mass. The supernatants were analyzed by high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) as described earlier (19).

RESULTS

L-Threo-3-deoxy-hexulosonate Aldolase Activity—The mold *H. jecorina* was grown with D-galacturonate, D-glucose, D-xylose, lactose, or glycerol as the carbon source. The mycelia were then disintegrated by vortexing with glass beads and centrifuged to obtain a crude cytosolic cell extract. This crude extract was then tested for enzyme activities. In the forward direction with L-threo-3-deoxy-hexulosonate as a substrate we observed pyruvate production (0.16 units/mg) when the mycelia were grown on D-galacturonate, but significantly less (0.03 units/mg) when grown on other carbon sources. The same was seen in the reverse direction with L-glyceraldehyde and pyruvate as substrates. The reverse reaction was significantly stronger with L-glyceraldehyde (0.20 units/mg) as a substrate than with D-glyceraldehyde (0.01 units/mg). The reverse reaction was also inhibited by D-glyceraldehyde because D,L-glyceraldehyde was nearly as poor a substrate as D-glyceraldehyde (0.02 units/mg).

Partial Purification of the L-Threo-3-deoxy-hexulosonate Aldolase and Cloning of the Open Reading Frame—For purification of the L-threo-3-deoxy-hexulosonate aldolase the forward reaction with L-threo-3-deoxy-hexulosonate as a substrate was used to detect the enzyme. The desalted crude extract containing 33 mg of protein and 0.2-units/mg protein activity was bound to a DEAE column and eluted with a salt gradient. The protein with L-threo-3-deoxy-hexulosonate aldolase activity was eluted at a NaCl concentration between 60 and 90 mM as a single activity peak. The fraction with highest activity contained 0.1 mg/ml protein and had an activity of 4.5 units/mg of protein. The active fractions were analyzed by SDS-PAGE, and a 37-kDa protein was observed to coincide with the activity. This protein band was then cut out from the gel and processed to obtain partial amino acid sequences. Because the genome sequence of *H. jecorina* is known, these partial amino acid sequences could then be used to identify the corresponding gene sequence. The following four peptide sequences were obtained: GLVVMGSDGEAVHCTR, GFQSVPVLFGAT-EGS, AAGAAAALVLPPSYYR, and QTYYGYGGYPR. These sequences were encoded by a single gene of unknown function. We called this gene *lga1* for 2-keto-3-deoxy-L-galactonate aldolase. We then cloned the open reading frame by PCR using cDNA as a template. Comparing the cDNA clone with the genomic DNA showed that there were no introns. The open reading frame consists of 945 nucleotides, and the sequence has been deposited at GenBank™ with the accession number EF203091. The open reading frame coded for a protein with 315 amino acids and a calculated molecular mass of 33,351 g/mol.

Expression in S. cerevisiae—The open reading frame was then transferred to a yeast expression vector, which was a multicopy vector with a strong constitutive promoter. When transformed to a yeast strain this vector caused the expression of an active L-threo-3-deoxy-hexulosonate aldolase. In a crude yeast extract the activity was 0.05 units/mg of protein as measured in the forward direction, whereas in the control strain no activity could be detected.

To facilitate the purification we introduced a histidine tag to the N terminus or to the C terminus of the protein. The histidine tags were introduced by adding additional nucleotide sequence by PCR to the 3′-end, just before the stop codon or to the start of the open reading frame, just after the start codon. Both constructs were then expressed with the same vector in the same yeast strain. When testing the two modified proteins in the crude extract of *S. cerevisiae* we observed that the C-terminal tag had an activity, which was about 70% reduced when compared with the non-tagged protein. The protein with the N-terminal histidine tag however had the same activity as the non-tagged. The N-terminally tagged protein was then purified and used for more detailed enzymatic characterization.

Characterization of the Purified Protein—The histidine-tagged L-threo-3-deoxy-hexulosonate aldolase was purified

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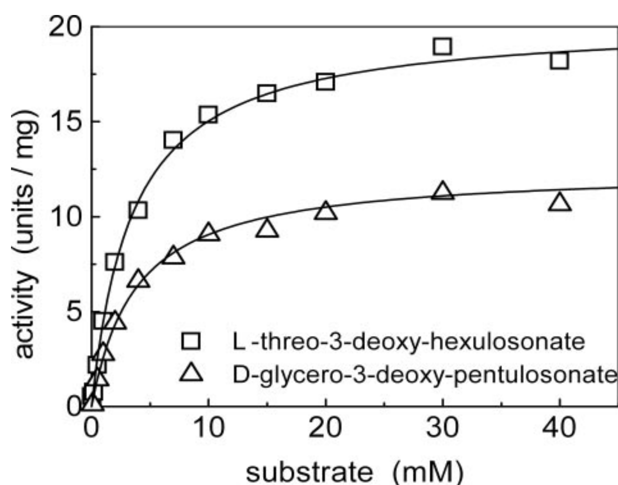

FIGURE 1. **Kinetics of the purified histidine-tagged protein.** The activity was assessed by measuring pyruvate in a coupled assay with lactated dehydrogenase. As substrates L-threo-3-deoxy-hexulosonate (*open squares*) and D-glycero-3-deoxy-pentulosonate (*open triangles*) were tested. The curves are calculated for Michaelis-Menten kinetics using the constants K_m 3.5 mM and V_{max} 20.3 units/mg for L-threo-3-deoxy-hexulosonate and K_m 3.8 mM and V_{max} 12.5 units/mg for D-glycero-3-deoxy-pentulosonate.

from the crude yeast extract using a nickel-nitrilotriacetic acid column. This resulted in a protein preparation, which gave a single band in an SDS-PAGE.

To test the specificity of the enzyme we tried as substrates D-glycero-3-deoxy-pentulosonate (2-keto-3-deoxy-D-arabonate), D-threo-3-deoxy-hexulosonate (2-keto-3-deoxy-D-galactonate), and L-threo-3-deoxy-hexulosonate (2-keto-3-deoxy-L-galactonate). The production of pyruvate was measured with a coupled enzymatic assay. Activity was observed with D-glycero-3-deoxy-pentulosonate and L-threo-3-deoxy-hexulosonate, and the kinetic constants were then estimated for each of the compounds. For the L-threo-3-deoxy-hexulosonate we found a V_{max} of 20.3 units/mg of protein corresponding to a turnover number of 11.3 s^{-1} and a K_m of 3.5 mM, for the D-glycero-3-deoxy-pentulosonate the V_{max} and K_m were 12.5 units/mg of protein (6.4 s^{-1}) and 3.8 mM, respectively (Fig. 1).

In the reverse direction we tested the activity with 50 mM pyruvate and various aldehydes and ketones at 16 mM. To analyze the product formation we used the thiobarbituric acid method (13). We observed activity with L-glyceraldehyde, glycolaldehyde, and methylglyoxal. With the other substrates tested the activity was close to the detection limit, which was about 3% of the activity with L-glyceraldehyde. We tested the aldehydes formaldehyde, acetaldehyde, D-glyceraldehyde and glyoxal, and the ketones dihydroxyacetone, diacetyl, and acetone (Table 1).

The Michaelis-Menten constants for pyruvate and L-glyceraldehyde were estimated. The K_m values were 0.5 mM for pyruvate and 1.2 mM for L-glyceraldehyde, the V_{max} was about 6.5 units/mg of purified protein which corresponds to a turnover number of 3.6 s^{-1} (Fig. 2, *A* and *B*). For glycolaldehyde a K_m of 6.5 mM and V_{max} of 0.9 units/mg were estimated (not shown).

To test whether the *lga1* was essential for the growth of D-galacturonate we deleted the gene in *H. jecorina*. A control strain where the *lga1* was expressed in the *lga1*-deletion strain was also generated. In this control strain the *lga1* was not under the natural promoter but under the *gpdA* from *A. nidulans*. These three strains were tested for growth on D-galacturonate, glycerol, and in medium without additional carbon source. The result is summarized in Fig. 3. On glycerol all three strains grew to about the same biomass. On D-galacturonate the strain with

TABLE 1
Activity with pyruvate and various aldehydes and ketones
The pyruvate concentration was 50 mM, the concentration of the cosubstrate 16 mM. The purified aldolase was incubated with the two substrates, and the product formation was tested using the thiobarbituric acid assay. (b.d. stands for below detection limit.)

Substrate	Activity
	units/mg
L-Glyceraldehyde	9.8
D-Glyceraldehyde	b.d.
Glycolaldehyde	0.5
Methylglyoxal	0.6
Formaldehyde	b.d.
Acetaldehyde	b.d.
Glyoxal	b.d.
Acetone	b.d.
Diacetyl	b.d.
Dihydroxyacetone	b.d.

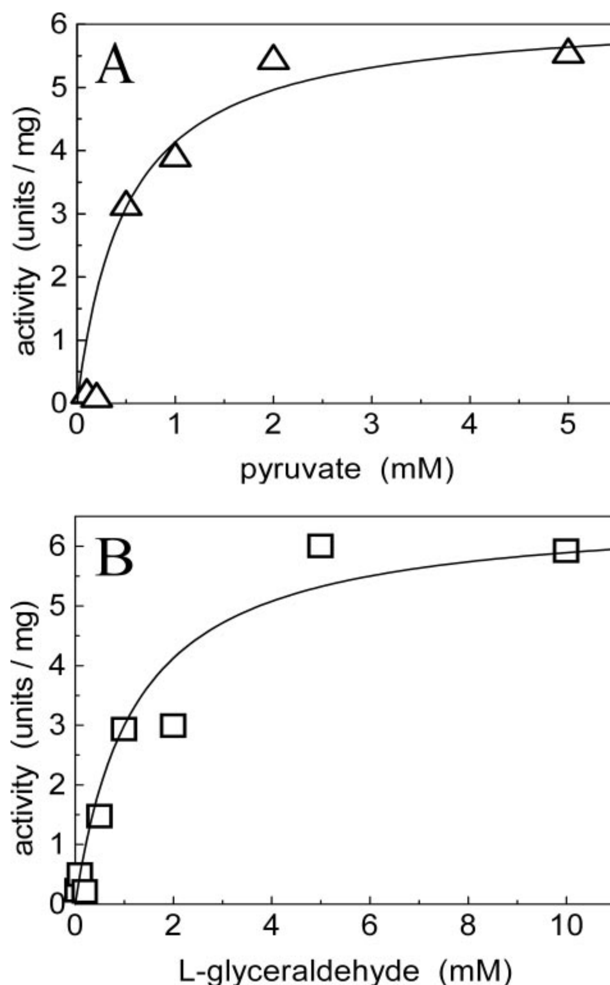

FIGURE 2. **Kinetics of the purified histidine-tagged protein in reverse direction.** The activity was assessed by measuring L-threo-3-deoxy-hexulosonate and D-glycero-3-deoxy-pentulosonate with the thiobarbituric acid assay. *A*, the L-glyceraldehyde concentration is 24 mM. *B*, the pyruvate concentration is 60 mM. The *curves* are calculated for Michaelis-Menten kinetics using the constants K_m 0.5 mM and V_{max} 6.2 units/mg for pyruvate and K_m 1.2 mM and V_{max} 6.6 units/mg for L-glyceraldehyde.

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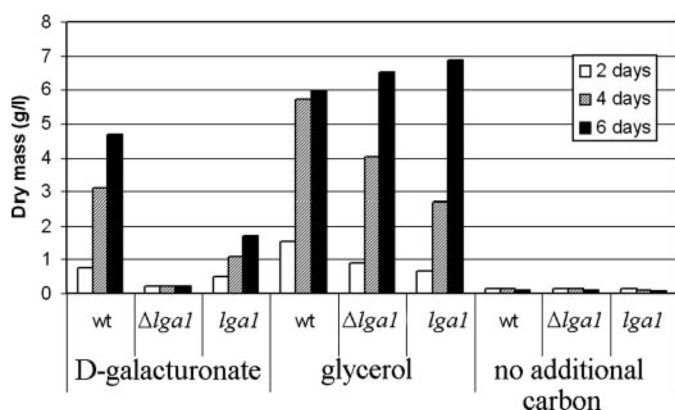

FIGURE 3. **Growth of the *lga1*-deletion strain and controls in liquid medium.** The dry mass of three strains, parent strain (*wt*), *lga1*-deletion strain (Δ*lga1*), and the control strain where the lga1 was retransformed to the deletion strain (*lga1*), were grown for 2, 4, and 6 days. The medium was supplemented with D-galacturonate or glycerol or was not supplemented with an additional carbon source.

TABLE 2

Accumulation of L-galactonate and L-threo-3-deoxy-hexulosonate in the growth medium for the parent strain (wt) and for the *lga1*-deletion strain (Δ*lga1*)

The concentrations are given in g/l.

	0 days	2 days	4 days	6 days
L-Galactonate (wt)	0.0	0.1	0.4	0.3
L-Galactonate (Δ*lga1*)	0.0	0.1	0.2	0.3
L-Threo-3-deoxy-hexulosonate (wt)	0.0	0.1	0.3	0.3
L-Threo-3-deoxy-hexulosonate (Δ*lga1*)	0.0	0.8	2.3	3.6

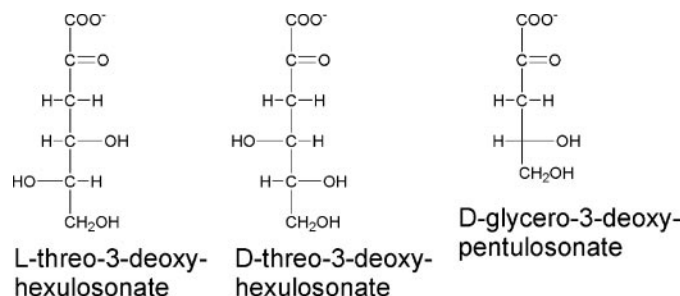

FIGURE 4. **Fischer projection of the substrates tested for activity with the LGA1 protein.** The L-threo-3-deoxy-hexulosonate (2-keto-3-deoxy-L-galactonate) and D-glycero-3-deoxy-pentulosonate (2-keto-3-deoxy-D-arabonate) were substrates.

lga1-deletion (Δ*lga1*) did not grow, whereas the control strain (*lga1*) regained the ability to grow on D-galacturonate. The growth rate was slightly reduced, which might be because of the heterologous promoter that was used. There was also some growth when a medium with no additional carbon source was used. This residual growth is because of the proteose peptone, which is always present in the medium.

In the *lga1*-deletion strain (Δ*lga1*) L-threo-3-deoxy-hexulosonate was accumulating. The growth media was analyzed by HPLC for D-galacturonate, L-galactonate and L-threo-3-deoxy-hexulosonate. During the 6 days of growth the D-galacturonate was consumed completely by the parent strain, whereas only a small fraction was consumed by the *lga1*-deletion strain. L-Galactonate accumulated with both strains to about 0.3 g/liter during the same period. L-Threo-3-deoxy-hexulosonate accumulated mainly in the deletion strain. It accumulated with a nearly constant rate to 3.6 g/liter after 6 days, whereas with the parent strain 0.3 g/liter were not exceeded.

DISCUSSION

Here we describe the identification of the missing link in the fungal D-galacturonate pathway. In previous communications we described the enzymes and the corresponding genes of D-galacturonate reductase and L-galactonate dehydratase converting D-galacturonate to L-galactonate and L-galactonate to L-threo-3-deoxy-hexulosonate (2-keto-3-deoxy-L-galactonate), respectively (6, 7). We also identified an enzyme and corresponding gene for an L-glyceraldehyde reductase (8). The missing link is an L-threo-3-deoxy-hexulosonate aldolase. Such an enzyme has not been described previously.

The L-threo-3-deoxy-hexulosonate aldolase activity is induced when D-galacturonate is the carbon source for growth but is absent on other carbon sources. This is already an indication that the L-threo-3-deoxy-hexulosonate aldolase is related to the D-galacturonate pathway. Upon deletion of the aldolase gene the growth on D-galacturonate is prevented providing evidence that the enzyme is part of the pathway. It also shows that it is part of the main catabolic path, *i.e.* there are no alternative routes. This is further supported by the observation that L-threo-3-deoxy-hexulosonate accumulated in the presence of D-galacturonate in the *lga1*-deletion strain (see Table 2).

The protein has similarities with the protein family of dihydrodipicolinate synthases. These are so called class I aldolases with a lysine in the active site, which is also involved in the catalytic reaction. In the LGA1 protein the corresponding lysine is in position 174. The protein also showed homologies to the protein family of 2-keto-3-deoxy-gluconate aldolases. These proteins are found in archae and are part of the non-phosphorylated Entner-Doudoroff pathway facilitating the aldol cleavage of 2-keto-3-deoxy-gluconate to pyruvate and D-glyceraldehyde. The protein does, however, not show significant sequence similarities to microbial 2-keto-3-deoxy-phosphogluconate aldolases. In a BLAST search the LGA1 protein had highest homology with hypothetical proteins from filamentous fungi such as those with the protein identifiers XP385304, XP957838, AAZ79448, XP754430, BAE57893, and XP660463. These proteins have between 60 and 75% identities in the amino acid sequence. They also have in common the following sequence around the catalytic lysine: HXNIXGTK*FTCXXTG-KLTRVA. The catalytic lysine is marked with an asterisk. It is likely that these proteins catalyze the same reaction as the LGA1 protein.

After the *lga1* gene coding for L-threo-3-deoxy-hexulosonate aldolase was identified. We expressed it in the heterologous host *S. cerevisiae*. This resulted in an active enzyme. The tagging of the N terminus, unlike the tagging of the C terminus, had no detrimental effect on the enzyme activity so that we chose this construct to analyze the kinetics.

To test the specificity in the forward direction we used L- and D-threo-3-deoxy-hexulosonate. We found activity with L- but not with the D-form. The two compounds differ in their stereo specificity in C5 and C6. We also tested D-glycero-3-deoxy-pentulosonate. This compound is similar to the L-threo-3-deoxy-hexulosonate except that it lacks C6 (Fig. 4). The D-glycero-

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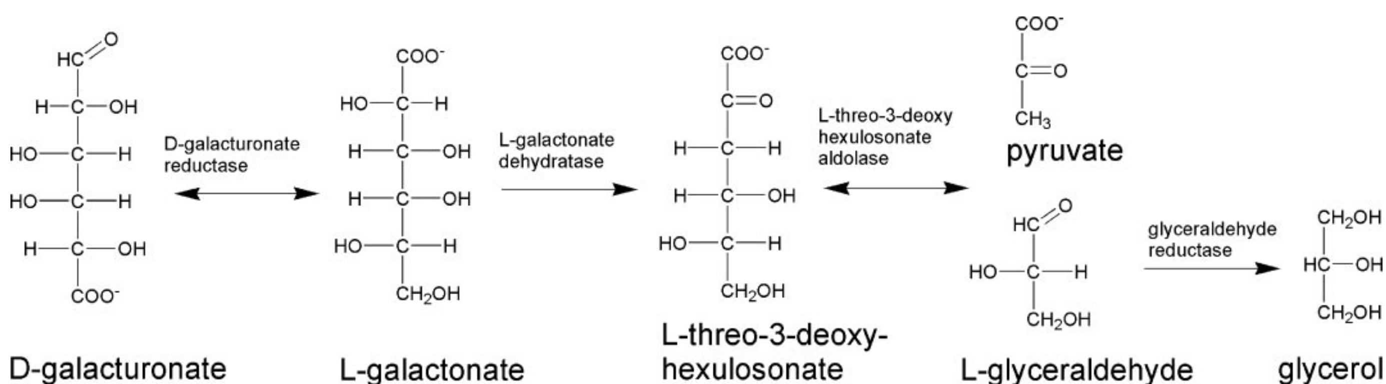

FIGURE 5. **Fungal pathway for D-galacturonate catabolism.** The metabolites are represented in Fischer projection. In this projection the C6 of D-galacturonate becomes the C1 of L-galactonate and the following metabolites. The D-galacturonate reductase and the glyceraldehyde reductase use NADPH as a cofactor. The D-galacturonate reductase and the L-threo-3-deoxy-hexulosonate aldolase catalyzed reactions were reversible, whereas for the L-galactonate dehydratase and the glyceraldehyde reductase the reverse reaction was not detected.

3-deoxy-pentulosonate showed activity, however, with a lower maximal velocity. This suggests that the aldolase recognizes the stereo specificity of the substrate only until the C4. This is supported by the activities found in reverse direction. Only the aldehydes L-glyceraldehyde, glycolaldehyde, and methylglyoxal showed activity together with pyruvate. The lack of activity with acetaldehyde suggests that the hydroxyl group on C5 is recognized and essential for the activity.

The enzyme catalyzed reaction is reversible. In the forward direction with L-threo-3-deoxy-hexulosonate as a substrate we estimated a V_{max} of 20 units/mg or 11.3 s^{-1}. In the reverse direction with pyruvate and L-glyceraldehyde as substrates the V_{max} was 6.5 units/mg or 3.6 s^{-1}. We did not estimate the equilibrium concentrations. However, for a similar enzyme, the L-erythro-3,6-dideoxy-hexulosonate (2-keto-3-deoxy-L-rhamnonate) aldolase, the equilibrium was on the substrate site. In equilibrium the substrate had about 10-fold higher concentration than pyruvate and the aldehyde (20). The K_m in forward direction was 3.5 mM. This together with the assumption that for the LGA1 reaction the equilibrium is also on the site of the substrate would suggest that L-threo-3-deoxy-hexulosonate might accumulate. We indeed found an accumulation of up to 0.3 g/liter corresponding to 1.5 mM in the growth medium for the strain without modifications. Other reactions in the pathway, such as the reactions before and after the aldolase, were not reversible. The L-galactonate dehydratase was shown to convert L-galactonate quantitatively (7), and also the glyceraldehyde reductase showed activity only with aldehyde and not with glycerol as substrate. It seems that the aldolase operates close to equilibrium, and that the catabolism through this pathway is driven by the enzymes before and after the aldolase (Fig. 5).

The catabolic path for D-galacturonate metabolism has, except for the first step, similarities to the "non-phosphorylated Entner-Doudoroff pathway". In this pathway, D-gluconate, derived from D-glucose, is converted to D-erythro-3-deoxy-hexulosonate (2-keto-3-deoxy-gluconate) which is subsequently split to pyruvate and D-glyceraldehyde. This pathway is described in a hyperthermophilic archeon (13). Similar bacterial pathways have been described for D-xylose (21) and L-arabinose (22). Common is that the 2-keto-3-deoxy-pentulosonate is split by an aldolase to pyruvate and an aldehyde.

There are also a few examples for such pathways in eukaryotes. In *Aspergilli* similar pathways for D-gluconate (23) and D-galactonate (24) were described in which an aldolase splits the 2-keto-3-deoxy-gluconate or -galactonate to pyruvate and D-glyceraldehyde. Also a yeast catabolic path for L-rhamnose is described where the 2-keto-3-deoxy-L-rhamnonate is split to pyruvate and L-lactaldehyde by an aldolase (20). In the eukaryotic pathways these aldolase activities were described but not the corresponding genes. The identification of the L-threo-3-deoxy-hexulosonate aldolase gene might help to identify the other aldolases.

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Title
Identification of the fungal catabolic D-galacturonate pathway

Abstract
Pectin is a natural polymer consisting mainly of D-galacturonic acid monomers. Microorganisms living on decaying plant material can use D-galacturonic acid for growth. Although bacterial pathways for D-galacturonate catabolism had been described previously, no eukaryotic pathway for D-galacturonate catabolism was known at the beginning of this work. The aim of this work was to identify such a pathway.

In this thesis the pathway for D-galacturonate catabolism was identified in the filamentous fungus *Trichoderma reesei*. The pathway consisted of four enzymes: NADPH-dependent D-galacturonate reductase (GAR1), L-galactonate dehydratase (LGD1), L-threo-3-deoxy-hexulosonate aldolase (LGA1) and NADPH-dependent glyceraldehyde reductase (GLD1). In this pathway D-galacturonate was converted to pyruvate and glycerol via L-galactonate, L-threo-3-deoxy-hexulosonate and L-glyceraldehyde.

The enzyme activities of GAR1, LGD1 and LGA1 were present in crude mycelial extract only when *T. reesei* was grown on D-galacturonate. The activity of GLD1 was equally present on all the tested carbon sources. The corresponding genes were identified either by purifying and sequencing the enzyme or by expressing genes with homology to other similar enzymes in a heterologous host and testing the activities. The new genes that were identified were expressed in *Saccharomyces cerevisiae* and resulted in active enzymes. The GAR1, LGA1 and GLD1 were also produced in *S. cerevisiae* as active enzymes with a polyhistidine-tag, and purified and characterised. GAR1 and LGA1 catalysed reversible reactions, whereas only the forward reactions were observed for LGD1 and GLD1. When *gar1*, *lgd1* or *lga1* was deleted in *T. reesei* the deletion strain was unable to grow with D-galacturonate as the only carbon source, demonstrating that all the corresponding enzymes were essential for D-galacturonate catabolism and that no alternative D-galacturonate pathway exists in *T. reesei.*

A challenge for biotechnology is to convert cheap raw materials to useful and more valuable products. Filamentous fungi are especially useful for the conversion of pectin, since they are efficient producers of pectinases. Identification of the fungal D-galacturonate pathway is of fundamental importance for the utilisation of pectin and its conversion to useful products.

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Many microorganisms that live on decaying plant material can use D-galacturonate for growth. Eukaryotic catabolic D-galacturonate pathway was not known before. In this thesis work the pathway for D-galacturonate catabolism was identified in the filamentous fungus *Trichoderma reesei.* The pathway consisted of four enzymes: NADPH-dependent D-galacturonate reductase (GAR1), L-galactonate dehydratase (LGD1), L-threo-3-deoxy-hexulosonate aldolase (LGA1) and NADPH-dependent glyceraldehyde reductase (GLD1). In this pathway D-galacturonate was converted to pyruvate and glycerol via L-galactonate, L-threo-3-deoxy-hexulosonate and L-glyceraldehyde. The enzyme activities of GAR1, LGD1 and LGA1 were present in crude mycelial extract only when *T. reesei* was grown on D-galacturonate. The corresponding genes were identified and cloned. They were functionally expressed in *Saccharomyces cerevisiae*, and the enzymes were characterised. GAR1 and LGA1 catalysed reversible reactions, whereas only the forward reactions were observed for LGD1 and GLD1. When *gar1*, *lgd1* or *lga1* was deleted in *T. reesei* the deletion strain was unable to grow with D-galacturonate as the only carbon source, demonstrating that all the corresponding enzymes were essential for D-galacturonate catabolism and that no alternative D-galacturonate pathway exists in *T. reesei.*

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