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DISSERTATION

Molecular Physiology of Reductive Monosaccharide Catabolism by *Hypocrea Jecorina*

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Preface

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I really enjoyed working with you and thank you very much for your support, discussion and critical reading of my publications and of course - of my thesis. Thank you for being such a good colleague.

Sincere thanks are given to all the people of the division Molecular Biotechnology for helpfulness and argument every time I needed it and of course for sharing a very good time over the last years.

I will miss you...

Special thanks go to my family and friends.

Thank you for being yourself and being here. I love you!

La chance ne sourit qu'aux esprits bien préparés
(Chance favors the prepared mind)

Louis Pasteur

Summary

Despite the comprehensive knowledge on the enzyme systems degrading hemicelluloses to their monomers, little is however known about the further metabolism of the hemicellulose monomers in filamentous fungi, and how this metabolism regulates the synthesis of the respective hemicellulases.

This work was concerned with several enzymes of *Hypocrea jecorina* (anamorph *Trichoderma reesei*) involved in L-arabinose and D-xylose catabolism. The initial reductive step of these two pentose pathways was shown to be catalyzed by D-xylose reductase. The enzyme does not account for all pentose reductase activity indicating the presence of additional genes encoding for enzymes with similar function. This suggestion was confirmed by the finding of four putative genes encoding aldose or D-xylose related reductases in the genome of this fungus. Characteristics of the recombinant protein and of cell free extracts showed significant activity of this enzyme with D-galactose, which was presumed to initialize an additional catabolism for this aldose, which is essential for full induction of the main cellulases of *H. jecorina* by lactose, a feasible industrial application.

For catalysis of the following step of pentose catabolism two related enzymes were identified, which evolved as separate branches from the evolutionary line leading to mammalian D-sorbitol dehydrogenases. Both enzymes display largely the same substrate specificity, but their differences in substrate affinity are physiologically relevant. Interestingly these enzymes are also involved in a novel reductive D-galactose pathway, one catalyzing the second step, the conversion of galactitol to L-xylo-3-hexulose and the other one mainly converting D-sorbitol to D-fructose. Investigation of the metabolic role of these two dehydrogenases suggested a common phenomenon in fungi - the partially compensation of functional loss of one enzyme by the other one.

Finally the *in vivo* performance of the linkage enzyme, which connects the L-arabinose and D-xylose metabolism by converting L-xylulose to xylitol demonstrated also the presence of further enzyme(s) with similar characteristics. Besides this key bioconversion, the enzyme catalyzes the reduction L-sorbose to D-sorbitol *in vivo* thereby demonstrating its role in the reductive D-galactose pathway.

Kurzfassung

Im Gegensatz zu den umfassenden Kenntnissen über die komplexen Enzymsyssteme, die zum hydrolytischen Abbau der Hemizellulosen zu deren monomeren Bausteinen führen, weiß man heute noch immer wenig über den weiteren Katabolismus jener Monomere und inwiefern diese die jeweilige Hemizellulaseproduktion regulieren.

Diese Arbeit befasst sich mit einigen im Pentosekatabolismus involvierten Enzymen eines industriellen Zellulase- und Hemizellulaseproduzenten, *Hypocrea jecorina*, ein filamentöser Pilz, der seit geraumer Zeit von industrieller Bedeutung ist. Die D-Xylosereduktase katalysiert den Anfangsschritt sowohl im D-Xylose- als auch im L-Arabinose-Abbauweg, vier weitere Gene, die für ebensolche Enzyme kodieren, konnten in der durchsequenzierten Gendatenbank von *H. jecorina* identifiziert werden. Die Eigenschaften des rekombinanten Proteins sowie zellfreie Extrakte des Pilzes demonstrierten neben der enzymatischen Aktivität mit D-Xylose und L-Arabinose auch eine signifikante Reaktion mit D-Galaktose. Diese reduktive Umwandlung stellt nicht nur den Anfangsschritt eines parallel zum Leloir-Abbauweg operierenden katabolen Weges dar, sondern ist auch essentiell zur maximalen Induktion der Zellulaseproduktion durch Laktose.

Die darauffolgende Umwandlung im Pentosekatabolismus wird durch zwei verwandte Enzyme (sog. Alkoholdehydrogenasen) katalysiert, die sich während der Evolution als zwei getrennte Gruppen zu unterschiedlichen Zeitpunkten aus den Sorbitoldehydrogenasen entwickelten. Beide Enzyme zeigen fast das gleiche Substratspektrum, wobei unterschiedliche Substrataffinitäten von physiologischer Relevanz sind. Interessanterweise spielen diese Enzyme auch im neuartigen reduktiven D-Galaktose-Abbauweg wichtige Rollen. Eines der beiden als Katalysator der Umwandlung von Galactitol zu L-Xylo-3-hexulose und das andere oxidiert D-Sorbitol zu D-Fruktose. Diese beiden genau untersuchten Dehydrogenasen lassen auf ein, den Pilzen eigenen Phänomen schließen: Ein Enzym kann das Fehlen des Anderen zumindest zum Teil kompensieren indem es dessen Umwandlungsreaktion katalysiert, jedoch meist mit geringerer Aktivität.

Zuletzt ergab die Untersuchung eines „Verbindungsenzyms", das durch die Umwandlung von L-Xylulose zu Xylitol, die beiden Pentose-Abbauwege, nämlich den der L-Arabinose und der D-Xylose miteinander verknüpft. Auch für diesen katalytischen Schritt gibt es mehrer Enzyme und eine weitere Biokonversion, die Reduktion von L-Sorbose zu D-Sorbitol wird von diesem Enzym katalysiert, was seine Rolle im reduzierenden D-Galactose-Abbauweg beweist.

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Introduction

Biomass represents a useful and valuable resource to man. After World War II petrochemicals began to dominate the synthetics market and today, 65 % of our clothing is made from oil, as are virtually our inks, paints, dyes, pharmaceuticals, plastics and hundreds of intermediate chemicals. To date, all transportation fuels and many chemicals are produced from crude oil or natural gas. At a certain moment in the future, an alternative carbon source is required due to the decreasing reserves of these fossil materials and/or to meet environmentally based policy goals. Because it takes millions of years to convert biomass into coal, fossil fuels are not renewable in the time frame over which we use them. Plant biomass as a renewable carbon source is the only foreseeable sustainable source of oganic fuels, chemicals and materials (Aristidou and Penttila 2000).

While biomass has served as a substrate in microbial processes for the production of alcoholic beverages for thousands of years, it has only been recently that broader applications of this material have been envisaged. Thus it is that biotechnologists are now developing efficient systems for the production of liquid fuels, pharmaceuticals and chemicals from "waste" organic materials (Magee and Kosaric 1985). Various agricultural residues, such as corn fiber, corn stover, wheat straw, rice straw, and sugarcane bagasse, contain about 20 - 40 % hemicellulose. Industrial processes using this latter material have traditionally made use of only the hexose component of the holocellulose. Therefore the pentose sugars, which may comprise as much as 40 % of the plant material, have in most cases been wasted.

For economic reasons, the substrate cost for a commercial process must be reduced to an absolute minimum. Due to the high cost of the large amounts of cellulase enzymes needed, successful utilization of cellulosic material as a renewable carbon source largely depends on the development of economically feasible process technologies for the production of the cellulase enzyme complex. As the enzyme production cost varies greatly with the carbon source used, utilization of cheap alternative substrates is seen as an attractive way to improve process economics. With regard to their availability at no extra cost, lignocellulosic wastes have gained considerable interest to be used as carbon sources in cellulase enzyme fermentation processes. From table 1, it is evident that large quantities of certain agricultural residues are generated annually on a global basis. In many of these materials, a significant proportion of the total carbohydrate content exists in pentosans. Thus it can be seen that biomass resources are available in sufficient quantity to merit evaluation of their use in the manufacture of valuable products. The significant proportion of these materials that are represented by hemicellulosic carbohydrates in turn makes examination of pentosan conversion necessary.

Residue	Global availability (10^6 t a^{-1})	COMPOSITION (weight %)				
		D-glucose	D-xylose	L-arabinose	Lignin	Ash
Rice straw	180	39	13.9	4.3	9.9	12.4
Barley straw	53	37.5	15	3.96	13.8	10.8
Corn stover	150	37	13.9	3	15.1	4.3
Wheat straw	550	34.7	18	2.2	14.5	9.6
Wood residues	25		pentosans			
Hardwood		60	23		28	-
Soft wood		53	7		23	-

Table 1 composition and availability of agricultural residues (Magee and Kosaric 1985)

The three major organic components of land-plant biomass are cellulose, lignin and **hemicellulose**. Whereas cellulose is a linear homopolymer of D-glucose units, the hemicelluloses are heteropolysaccharides composed of two or more monosaccharides such as D-xylose, L-arabinose, D-mannose, D-glucose, D-galactose and 4-O-methyl-D-glucuronic acid. Some of them are partially esterified with acetic, ferulic and p-coumaric acids. The primary structure of hemicelluloses depends on the source and can vary even between different tissues of a single plant. Often two or three different hemicelluloses occur in the same plant species, but in different proportions. A major hemicellulose component of hardwood is O-acetyl-4-O-methyl-D-glucurono-D-xylan and a minor component is glucomannan (Timell 1967; Wilkie 1983), whereas a major softwood hemicellulose is O-acetyl-D-galacto-D-gluco-D-mannan. The next most abundant softwood hemicellulose is L-arabino-D-glucurono-D-xylan which, in contrast to hardwood xylan, is not acetylated. Minor hemicelluloses found in wood are various types of galactans (e.g., in compression wood) and arabinogalactans (in larchwood). The most abundant hemicelluloses of cereals and grasses are arabinoxylans (v d Veen *et al.* 1991,Wilkie, 1979). A hypothetical fragment of a plant xylan which shows the major structural features found in this group of hemicelluloses is depicted in Fig. 1. Every xylan backbone is built of ß-1,4-linked D-xylopyranosyl residues. The xylopyranosyl residues are randomly substituted with α-1,2-linked 4-O-methyl-D-glucuronic acid and α-1,3-linked L-arabinofuranose. In cereal xylans, one xylopyranosyl residue may carry two α-L-arabinofuranosyl substituents at positions 2 and 3. Hardwood glucuronoxylans are partially acetylated. In cereals, arabinoglucuronoxylans composed of up to 10 % of L-arabinofuranosyl residues are esterified at position 5 by ferulic acid or p-coumaric acid (Mueller-Harvey *et al.* 1986).

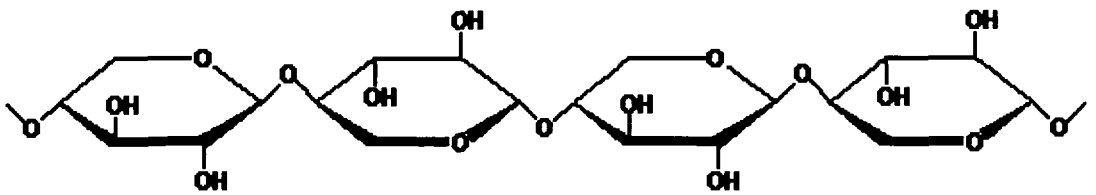


Fig. 1 schematic picture of xylan backbone

Due to the heterogeneity and complex nature of hemicelluloses and especially its main component xylan, its complete breakdown requires the action of a complex of several hydrolytic enzymes with diverse specificity and modes of action. Thus it is not surprising for xylan-degrading organisms to produce an arsenal of polymer-degrading proteins. The **xylanolytic enzyme system** carrying out the xylan hydrolysis is usually composed of a repertoire of hydrolytic enzymes: β-1,4-endoxylanase, β-xylosidase, α-L-arabinofuranosidase, α-glucuronidase, acetyl xylan esterase, and phenolic acid esterase (Kulkarni *et al.* 1999). An illustration of their actions is shown Fig. 2.

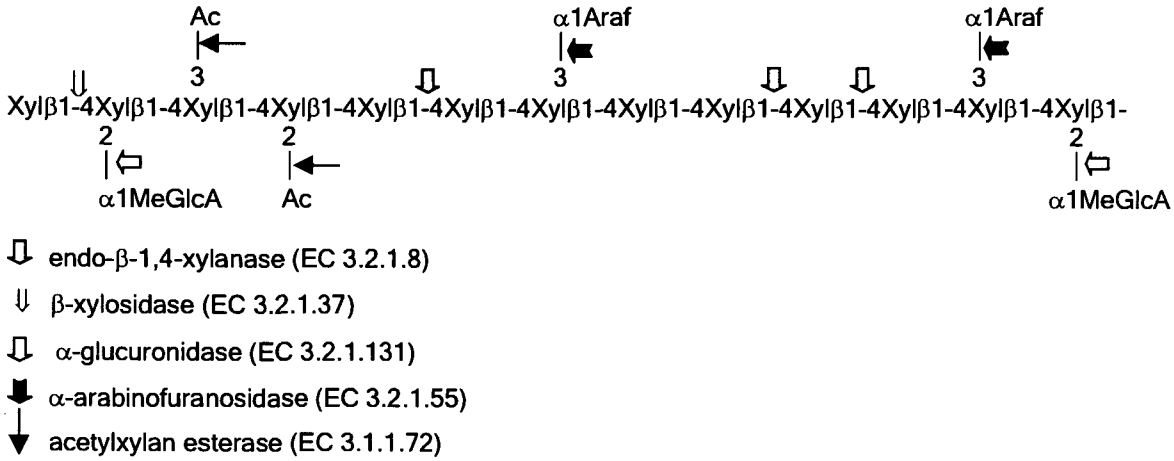


Fig. 2 Hypothetical plant xylan and the enzymes required for its complete hydrolysis

All these enzymes act cooperatively to convert xylan into its constituent sugars. The principal xylan depolymerizing enzyme is endo-β-1,4-xylanase. This enzyme cleaves mainly in unsubstituted parts of the main chain. Other enzymes work with this xylanase synergistically. They attack either the polymeric xylan, creating new sites for endo-β-1,4-xylanase, or they attack the linear and substituted oligosaccharides liberated by endo-β-1,4-xylanase. The presence of such a multifunctional xylanolytic enzyme system is quite widespread among fungi (Belancic *et al.* 1995) and bacteria (Dey *et al.* 1992). Filamentous fungi are particularly interesting producers of xylanases since they excrete the enzymes into the medium and their enzyme levels are much higher than those of yeast and bacteria. Numerous fungal enzymes have been sufficiently purified and characterized, and their genes isolated and sequenced. Some of these xylanases have also been crystallized and their tertiary structures established (Krengel 1996; Krengel and Dijkstra 1996; Rose *et al.* 1987; Torronen *et al.* 1994).

However, fungal xylanases are generally associated with cellulases (Steiner *et al.* 1987). Selective production of xylanase is possible in the case of *Trichoderma* and *Aspergillus* species.

The filamentous fungus *Trichoderma reesei* (*Hypocrea jecorina*) is one of the most efficient producers of xlyanases and cellulases. It produces at least four β-1,4-xylanases (XYNI, XYNII, XYNIII, XYNIV), two cellobiohydrolases (CBHI, CBHII), five endo-β-1,4-glucanases (EGI, EGII, EGIII, EGIV, EGV) and two β-D-glucosidases (BGLI, BGLII) (Clarkson *et al.* 2001; Nogawa *et al.* 2001; Xu *et al.* 1998).

Extensive literature available on the main **endo-β-1,4-xylanases** of *Trichoderma* species has been reviewed by Wong and Saddler (1992). The low molecular mass endo-β-1,4-xylanases like XYNI and XYNII belong to the glycanase family 11. These xylanases from *T. reesei* (Torronen *et al.* 1992) have been crystallized and their tertiary structure determined (Fig.3). These enzymes appear as small, well-packed molecules formed mainly of β-sheets, whose twisted structure forms a cleft where catalytic groups are located. Two glutamic acid residues in the cleft function as catalytic groups. The loop hanging over the cleft undergoes a conformational change on substrate binding (Havukainen *et al.* 1996; Torronen *et al.* 1994).

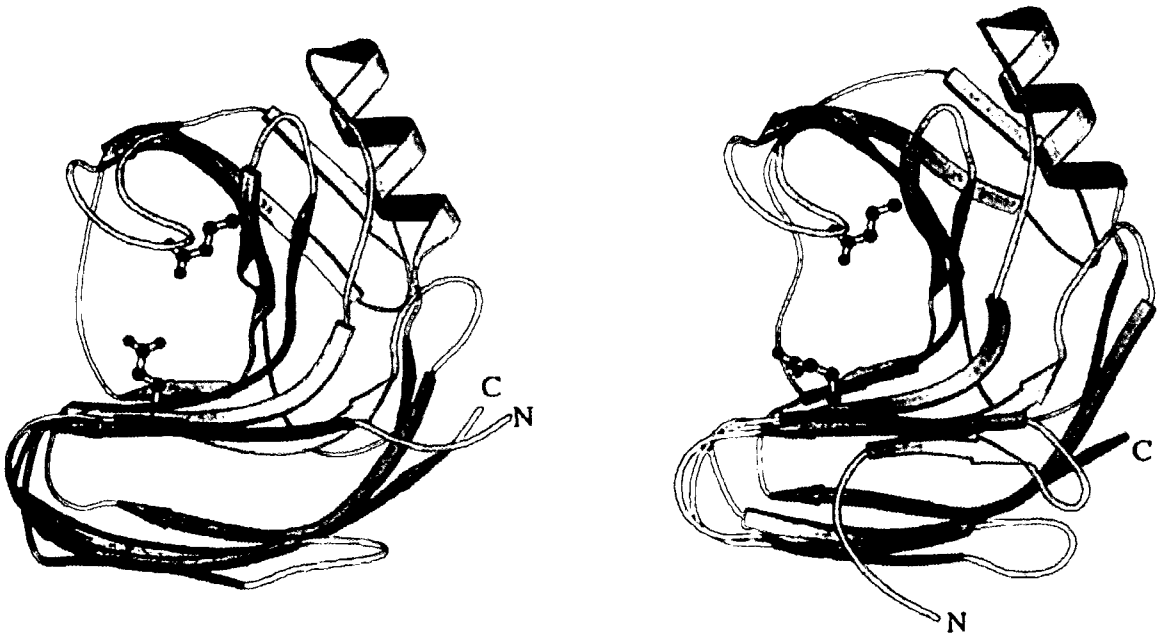


Fig. 3 Ribbon presentation of the main fold of the two specific xylanases of *Trichoderma reesei*. The carboxyl groups in the clefts are the carboxyl groups of two catalytic glutamic acid residues and they are indicated by balls and sticks. Dispite the great similarity in tertiary structure, the enzymes show differences in catalytic properties, pointing to differences in the substrate binding sites.

Much less information is available on **β-xylosidase**, an important saccharifying hydrolase. It hydrolyzes β-1,4-xylooligosaccharides with a degree of polymerization 2 to 7, at a fixed concentration, with the rate rising with increasing chain length. The enzyme also attacks to a certain extent a debranched beechwood xylan and a glucuronoxylan, forming D-xylose as the only product (Herrmann *et al.* 1997; Margolles-Clark *et al.* 1996).

The role of **α-glucuronidase**, **α-arabinofuranosidase**, and **acetyl xylan esterase** is to liberate side groups from different xylans or from substituted oligosaccharides formed from the polysaccharide by xylanase. The removal of side groups makes the xylopyranosyl residues of either the xylan main chain or xylooligosaccharides more accessible to degradation by xylanase or β-xylosidase.

For a review on the molecular structure and hydrolytic pattern of xylanases see (Subramaniyan and Prema 2002).

The principal hemicellulose in softwoods is **galactoglucomannan**. The main structural features of this hemicellulose and the enzymes required for its complete hydrolysis are shown in Fig. 4

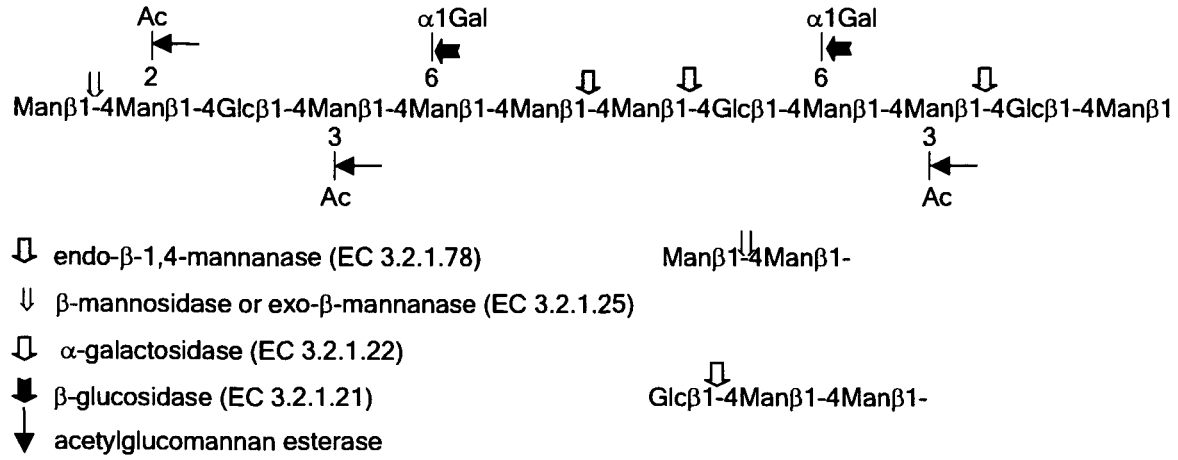


Fig. 4 Hypothetical plant galactoglucomannan and the enzymes required for its complete hydrolysis.

Endo-β-1,4-mannanases are enzymes that hydrolyze the β-1,4-glycosidic linkages in mannans, galactomannans, glucomannans and galactoglucomannans. As glucomannans and galactoglucomannans contain, in addition to mannose units, glucose units in the backkbone of the polymers, they might also be hydrolyzed by specific endoglycanases (endo-β-glucomannanases) which do not act on polymers with a mannan backbone (Ademark *et al.* 1998; Stalbrand *et al.* 1995; Stålbrand *et al.* 1993; Torrie *et al.* 1990). Mannanases from a variety of different organisms have been studied, including bacteria, fungi, higher plants and animals (reviewed by Dekker and Richards (1976)). The degradiation of galactomannan and galactoglucomannan by β-mannanase is greatly affected by the extent and pattern of substitution of the mannan backbone. The interference of D-galactosyl side groups with hydrolysis has been carefully analyzed using β-mannanase s from *A. niger* (McCleary and Matheson 1983) and *T. reesei* (Tenkanen *et al.* 1997). A multiplicity of extracellular β-mannanases appears to be common among fungi and has been noticed, for example, in *T. reesei* (Stalbrand *et al.* 1995; Stålbrand *et al.* 1993) and *T. harzianum* (Torrie *et al.* 1990). Although the purification of different β-mannanases of *A. niger* have been reported by (Eriksson and Winell 1968; McCleary 1979; McCleary 1988; Tsujisaka *et al.* 1972), it has not been clear if more than one β-mannanase is secreted. Th one extracellular β-mannanase was characterized by (Ademark *et al.* 1998) and showed a very similar activities as the two major β-mannanases from *T. reesei* (Harjunpaa *et al.* 1995).

The complete conversion of glactomannan into D-galactose and D-mannose requires the presence of two additional enzymes, **α-galactosidase** and **β-mannosidase**. A report on the cloning and characterization of these genes from *Aspergillus niger* has been reported by Ademark *et al.* (2001). An α-galactosidase of *T. reesei* is already characterized (Savel'ev *et al.* 1997) and its crystal structure has been investigated (Golubev *et al.* 2004). A β-mannosidase of *T. reesei* has also been purified, crystallized and its structure was analyzed (Aparicio *et al.* 2002).

Despite the comprehensive knowledge on the enzyme systems degrading hemicelluloses to their monomers, little is however known about the further metabolism of the hemicellulose monomers in filamentous fungi, and how this metabolism regulates the synthesis of the respective hemicellulases. With respect to hemicellulose hexoses (D-galactose, D-mannose), excessive information is available from work with *S. cerevisiae* (Fukasawa and Nogi 1989). The D-galactose metabolism known as **Leloir pathway** has been characterized (Frey 1996) and also studied in *T. reesei* in the EC-project Eurofung 2; QLK3-1999-00729 (Seiboth *et al.* 2004; Seiboth *et al.* 2002; Seiboth *et al.* 2002). This pathway requires the three enzymes galactokinase, galactose-1-P uridyltransferase, and UDP-galactose 4-epimerase. The transformation of D-galactose into D-glucose-1-phosphate by these enzymes is illustrated in Fig. 5. The process takes place through the initial phosphorylation of D-galactose to D-galactose-1-phosphate by the action of galactokinase. D-galactose-1-phosphate is transformed into D-glucose-1-phosphate in the presence of galactose-1-uridyltransferase and UDP-glucose, which is cycled through UDP-galactose and thereby acts as a cofactor for the transformation of D-galactose-1-phosphate into D-glucose-1-phosphate.

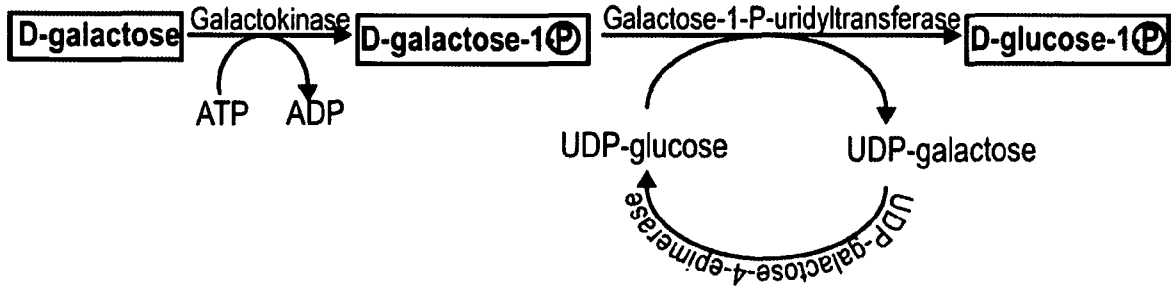


Fig.5 Leloir pathway

Interestingly **pentose metabolism** generally has received little attention in filamentous fungi. The bioconversion of D-xylose by yeasts in relation to the production of ethanol under anaerobic conditions has been extensively reviewed by Jeffries (Jeffries 1983). In this catabolism the D-xylose is first converted to D-xylulose and then phosphorylated (Fig. 6). A basic difference seems to exist between prokaryotes and eukaryotes in the initial metabolism: Bacteria generally employ an isomerase to convert D-xylose to D-xylulose (Lawlis *et al.* 1984), whereas yeasts and fungi carry out the same conversion through a two-step reduction and oxidation (Chiang and Knight 1960b).

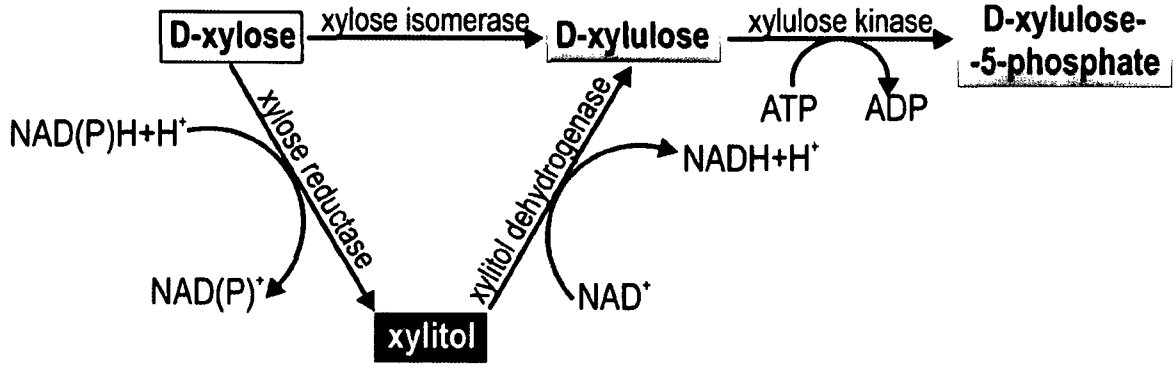


Fig. 6 D-xylose conversion in bacteria (white) and fungi (grey)

Chiang and coworkers (Chiang and Knight 1959; Chiang and Knight 1960a; Chiang and Knight 1960b; Chiang and Knight 1961; Chiang *et al.* 1958) established that the pathways of L-arabinose and D-xylose degradation are combined, L-arabinose metabolism proceeding that of D-xylose (Fig. 7). More recent studies with *Aspergillus niger* and *Pichia stipitis* mutants have provided evidence for the connection between L-arabinose and D-xylose breakdown in fungi (de Vries *et al.* 1994; Shi *et al.* 2000; Witteveen *et al.* 1989). A mutant of *Aspergillus niger* unable to grow on D-xylose and L-arabinose has been isolated. Enzymatic analysis revealed a deficiency in D-xylulose kinase activity. After transfer of growing mycelium to a medium containing either D-xylose or L-arabinose, the mutant accumulates large amounts of L-arabinitol and xylitol, as shown by ^{13}C NMR spectroscopy. These data and an analysis of enzyme activities induced by D-xylose and L-arabinose in the wild type strain led to the following catabolic pathway for D-xylose and L-arabinose (Fig. 7):

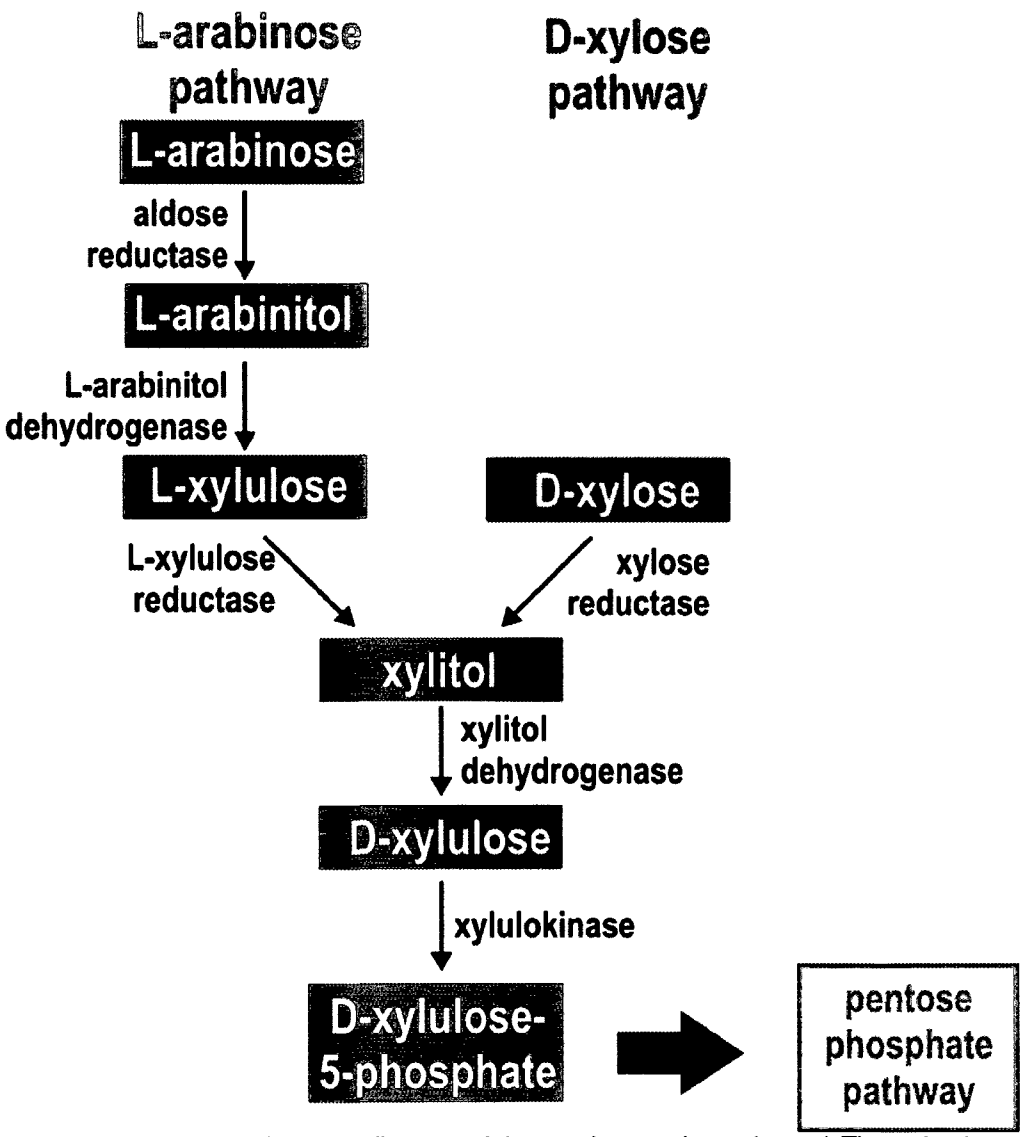


Fig. 7 Fungal L-arabinose pathway (red) proceed the D-xylose pathway (green). The reduction steps of the sugars to the corresponding polyols are all NADPH dependent. The oxidation steps of the polyols to the sugars are all NAD^+ dependent. Fractionation of cell-free extracts gave information about the specificity of the enzymes and showed that all reactions are catalyzed by different enzymes.

However, important points were still unclear. One is the nature of the enzyme(s) catalyzing the conversion of L-arabinose to L-arabinitol. The Boehringer-Mannheim metabolic chart (http://www.expasy.ch/cgi-bin/show_image?C3) lists this step as being catalyzed by the same enzyme as that from D-xylose to xylitol, i.e. aldose (aldehyde) reductase. In fact, the aldose reductase of *A. niger* and of several yeasts has a broad substrate specificity, including D-xylose and L-arabinose (Ditzelmüller *et al.* 1984; Verduyn *et al.* 1985,Witteveen, 1989). However, in *A. niger* two enzymes, differentially responding to the presence of D-xylose and L-arabinose in the medium, and with different affinity for D-xylose and L-arabinose are present (Witteveen, 1989).

The analysis of pentose metabolism is also valuable in the light of understanding the mechanisms that play a role in extracellular enzyme production. The induction of extracellular arabinases by pentose sugars and ploYols generated by the metabolic pathway of L-arabinose and D-xylose catabiolism in *A. niger* has been studied by (vd Veen *et al.* 1991). Thereby L-arabinitol was found to be a good inducer for α-L-arabinofuranosidase and endo-arabinase activities. A similar mechanism could be suggested in *T. reesei.*

The basic factors for efficient production of hemicelluloytic enzymes are the choice of an appropriate inducing substrate and an optimum medium composition. Xylan, the main inducer of several xylanases, is a high molecular mass polymer and therefore cannot penetrate the cell wall. The low molecular mass fragments of xylan play a key role in the regulation of xylanase biosynthesis. These molecules are liberated from xylan by the action of small amounts of constitutively produced enzymes. In vitro experiments have shown that production xylanases and cellulases is induced by both monosaccharides and disaccharides. Among several sugars, the disaccharide sophorose was the most powerful soluble inducer of *T. reesei* cellulases (Sternberg and Mandels 1982). Good cellulose induction has also been reported with lactose, an industrial production medium which promotes also good growth (Chaudhuri and Sahai 1993) and L-sorbose (Nogawa *et al.* 2001). Efficient inducers of xylanase production include xylan, xylobiose, D-xylose, lactose, L-sorbose, and sophorose (Chaudhuri and Sahai 1993; Xu *et al.* 1998; Zeilinger *et al.* 1996). Xiong and Coworkers studied the effect of different pentoses on the production of hydrolytic enzymes by *Trichoderma reesei* and they achieved a good production of xylanases and cellulases on L-arabinose as the main carbon source (Xiong *et al.* 2004). These facts should suggest extensive studies on pentose metabolism in filamentous fungi.

Outline

The work started with investigation of D-xylose catabolism of *Hypocrea jecorina* (anamorph *Trichoderma reesei*) thereby I concentrated on the second step catalyzed by xylitol dehydrogenase (Xdh1). Cloning and identification of the gene *xdh1* was performed as well as recombinant protein production, purification and enzyme characterization. The metabolic characterization of the *xdh1* deletion mutant exhibited a residual growth on D-xylose and xylitol. This fact initiated the search for a second enzyme with similar properties as xylitol dehydrogenase. A possible candidate was striktly found: *lad1* encoded L-arabinitol dehydrogenase (Lad1). Growth experiments with different mutant strains of *H. jecorina* deficient in each polyol dehydrogenase encoding gene and in both genes (=double deletion) clearly showed that the loss of the xylitol dehydrogenase step in D-xylose catabolism can be partially compensated by *lad1*-encoded L-arabitol-4-dehydrogenase. Moreover the occurrence of further enzymes, besides these two, with similar enzyme properties could be ruled out in *H. jecorina*.

Already working with Lad1, the protein was recombinantly expressed in *E. coli*. During purification procedure, the fused GST was removed and an extensive characterization of the enzyme was carried out. Predominantly I was interested if the Lad1 can catalyze the oxidation of galactitol because recent experiments on D-galactose metabolism indicated a second (reductive) pathway for D-galactose catabolism with galactitol as intermediate. Lad1 showed fairly high activity on galactitol, but the product of this conversion was not identifiable. Final NMR experiments identified L-xylo-3-hexulose as product. This finding initiated the screening for further unusual Lad1 conversion products. Every available hexitol was tested as potential substrate and we observed a very broad substrate specificity of this enzyme. The investigation of oxidation products exhibited a second 3-hexulose - D-arabino-3-hexulose - as D-talitol conversion product. Since this enzyme appeared very interesting with such unusual conversions, I have been analyzing if it is a specific enzyme for fungi or if it occurs also in other organisms. Detailed research in different databases showed no occurrence of Lad in any other genomes than of fungi. A similar substrate profile could be found of sorbitol dehydrogenases (Sdh). Phylogenetic analysis and search for Sdh in databases of different fungi, displayed the evolution of a fungal specific class of enzymes arosen from sorbitol dehydrogenases.

L-sorbose, a strong inducer of cellulases, was published being an intermediate in the reductive D-galactose catabolism in *Aspergillus nidulans*. I presumed the possibility that the reaction product of the second step of this D-galactose pathway of *H. jecorina* - L-xylo-3-hexulose - can be converted to L-sorbose and investigated the next steps. Since it was known from another working group that the L-xylulose reductase is able to convert L-sorbose to D-sorbitol and two possible enzymes able to catalyze the oxidation of D-sorbitol to D-fructose were available of my work, I wanted to identify this step in L-sorbose catabolism. Therefore I reinvestigated the characteristics of xylitol dehydrogenase in much more detail. This displayed a smaller substrate spectrum but better performances of Xdh1 on several carbohydrates especially with D-sorbitol. Further research on metabolic characteristics and

activity assays of the single deletion mutants and the double deletion mutant confirmed the exhibited enzyme data: Xdh1 accounts for the D-sorbitol dehydrogenase activity in *H. jecorina*. The proof of conversion of L-sorbose by L-xylulose reductase was also carried out by metabolic analysis of a Δ*lxr1* (encoding L-xylulose reductase) mutant strain of *H. jecorina.*

To complete the D-xylose catabolism of *H. jecorina* the enzyme catalyzing its first step was investigated. The gene *xyl1,* encoding D-xylose reductase, was cloned and characterized. The recombinant protein expression, purification and characterization of the enzyme was performed as well, showing similar substrate specificities as former published D-xylose reductases of other organisms. The metabolic data of the *xyl1* deletion mutant indicated also the occurrence of further enzyme(s) with similar characteristics than Xyl1. An interesting outcome of this work is the activity of this D-xylose reductase with D-galactose, which is also reduced in the Δ*xyl1* strain and therefore can catalyze the first step in the reductive D-galactose catabolism - the reduction to galactitol as well. The deletion of *xyl1* had also a significant effect on cellulase induction by lactose, which is of industrial relevance not only because *H. jecorina* is used as industrial cellulose producer but also because lactose is important as the only soluble cellulase inducer which is feasible for industrial use.

Chapter One

D-xylose metabolism by *Hypocrea jecorina*: loss of the xylitol dehydrogenase step can be partially compensated by *lad1*-encoded L-arabinitol-4-dehydrogenase

Summary

With the goal of the genetic characterization of the D-xylose pathway in *Hypocrea jecorina* (anamorph *Trichoderma reesei*), we cloned the *xdh1* gene encoding NAD-xylitol dehydrogenase, which catalyzes the second step of fungal D-xylose catabolism. This gene encodes a 363-aa protein with a mass of 38 kDa, belongs to the zinc containing alcohol dehydrogenase family, exhibits high sequence identity to the published sequences of xylitol dehydrogenases from yeast origins, but contains a second, additional binding site for Zn^{2+}. The enzyme catalyzed the NAD-dependent oxidation of xylitol and D-sorbitol, and the NADH-dependent reduction of D-xylulose and D-fructose. No activity was observed with NADP, L-arabinose, or L-arabinitol. A single 1.4-kb transcript was formed during growth on xylan, D-xylose, L-arabinose, L-arabinitol, and with lower abundance on xylitol, D-galactose, D-galactitol and lactose, but not on D-glucose and glycerol. X*dh1* deletion mutants exhibited 50 % reduced growth rates on D-xylose and xylan, whereas growth rates on xylitol remained unaltered. These mutants contained 30 % of the xylitol dehydrogenase activity of the parent strain, indicating the presence of a second xylitol dehydrogenase. This activity was shown to be due to the *lad1*-encoded L-arabinitol-4-dehydrogenase, because *H. jecorina xdh1/lad1* double-deletion strains failed to grow on xylan, D-xylose or xylitol. In contrast, *lad1* deletion strains of *H. jecorina* grew normally on these carbon sources. These results show that *H. jecorina* contains a single xylitol dehydrogenase which is encoded by *xdh1* and is involved in metabolism of xylan or D-xylose, and that the *lad1*-encoded L-arabinitol-4-dehydrogenase can compensate for it partially in mutants with a loss of *xdh1* function.

Introduction

D-xylose is a major constituent of plant hemicelluloses, where it forms the ß-1,4-xylan backbone of hardwood. ß-1,4-xylans are heteropolysaccharides that have a backbone of ß-1,4-linked xylopyranosyl residues, and contain side groups such as D-glucuronic acid, L-arabinose, p-coumaric acid and ferulic acid. They constitute 20 - 35 % of the roughly 830 gigatons of annually formed renewable plant biomass (Timell 1965).

Both prokaryotic and eukaryotic microorganisms can use xylan as a carbon source for growth. The bacterial pathway for D-xylose catabolism is well established. It consists of an isomerase and a kinase that sequentially convert D-xylose to D-xylulose, and D-xylulose 5-phosphate, which is an intermediate of the pentose phosphate pathway. This pathway is absent from fungi, where D-xylose goes through NADPH-linked reduction and NAD-linked oxidation reactions instead, before phosphorylation of D-xylulose occurs (Fig. 8). The enzymes catalyzing the first two steps (aldose reductase [EC 1.1.1.21] and xylitol dehydrogenase [EC 1.1.1.9]) have been characterized mainly from yeasts. They are nonspecific and can use other sugars in addition to D-xylose and xylitol, respectively, at approximately the same rates. The genes encoding the enzymes involved in fungal D-xylose catabolism have also been cloned from different yeasts (for a review see (Jeffries and Shi 1999)) and, in part (i.e. aldose reductase and xylulose-5-phosphate kinase) from *Aspergillus niger* (Hasper *et al.* 2000; van Peij *et al.* 1998). Further, cloning of a gene encoding a xylitol dehydrogenase from *H. jecorina* has been reported, but neither its nucleotide nor its amino acid sequence has been made available (Wang *et al.* 1998).

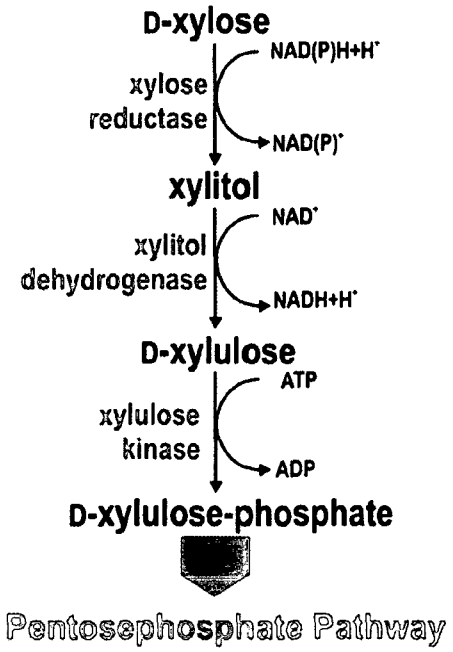


Fig. 8
Fungal D-xylose pathway

A genetic analysis of the D-xylose metabolizing pathway in yeast showed that aldose reductase and xylitol dehydrogenase are indeed essential for D-xylose degradation. In contrast, an aldose reductase knock-out mutant of *A. niger* was still able to grow on D-xylose, although at a reduced rate (Hasper *et al.* 2000), suggesting that multiple enzymes are involved in the first step of the D-xylose catabolic pathway in filamentous fungi.

Xylan breakdown by the ascomycete *Hypocrea jecorina* (anamorph: *Trichoderma reesei*) has received the strongest interest because of the application of the corresponding xylanases in the pulp and paper industry and the food industry. The extracellular addition of intermediates of the D-xylose metabolic pathway leads to differential expression of the two major xylanase genes (Mach and Zeilinger 2003), stressing the need to understand this pathway in more detail for the metabolic engineering of xylanase formation in this fungus. As a first step toward the genetic engineering of the D-xylose catabolic pathway in *H. jecorina*, we attempted to identify the genes and proteins involved.

Here we report the cloning of the *xdh1* gene, encoding the NAD-xylitol dehydrogenase of *H. jecorina*. We show that deletion of the gene partially affects the growth of *H. jecorina* on D-xylose and that L-arabinitol-4-dehydrogenase (encoded by *lad1*) partially compensates for the loss of *xdh1* function under these conditions. However, we will also show that *lad1* is not involved in D-xylose metabolism in a background in which *xdh1* is functional; this result identifies the *lad1* bypass as a rescue mechanism only.

Experimental procedures

Strains and culture conditions

The *H. jecorina* parent strains used in this study were QM9414 (ATCC 26921) and the *pyr4* negative mutant TU-6 (ATCC MYA-256, (Gruber *et al.* 1990)). All strains were maintained on malt extract agar and auxotrophe strains supplemented with uridine (10 mM). Strains were grown in 1-liter Erlenmeyer flasks on a rotary shaker (250 rpm) at 30°C with 250 ml of the medium described by (Mandels and Andreotti 1978) and containing various carbon sources at final concentration of 10 g/liter.

For the analysis of the effect of different carbon sources on gene expression, the different *H. jecorina* strains were pregrown on 1 % (wt/vol) glycerol (20 h), the mycelia were harvested by filtration and washed with tap water, equal amounts of mycelia (1 ± 0.2 g [wet weight]/liter [mean and standard deviation]) were transferred to flasks containing the different carbon source (1 % [wt/vol]), and cultivation was continued.

Escherichia coli strains ER1647 and BM25.8 (Novagen, Madison, WI) were used for genomic library screening, strains XL-1 Blue and XLOLR (Stratagene, La Jolla, CA) were used for cDNA library screening and JM109 (Promega, Madison, WI) was used for plasmid propagation.

Determination of fungal growth

To determine hyphal growth on agar plates, a small piece of agar was placed in the centre of an 11-cm plate, and the increase in colony diameter measured twice daily. To measure growth in submerged cultures, the increase in biomass dry weight was recorded. To this end, mycelia were harvested after appropriate times, washed extensively with tap and then distilled water, and dried to constant weight.

Preparation of cell extracts

To prepare cell-free extracts for enzyme activity assays, *H. jecorina* strains QM9414 and ΔXDH1 were grown for 24 h on the medium described by (Mandels and Andreotti 1978). The mycelia were then harvested by filtration through a precooled linen cloth, washed with cold tap water, ground to a fine powder under liquid nitrogen, and homogenized by sonicating a concentrated mycelial suspension (1 g [wet weight] per 2.5 ml of buffer A [0.1 M Tris-HCl {pH 7.5}, 1 mM EDTA and 5 mM β-mercaptoethanol]) ten times for 30 sec each time at 2°C. The resulting homogenate was centrifuged at 10000 × g for 20 min at 4°C. The supernatant, which had a protein content of between 8 and 15 mg/ml, was used as a cell extract.

Enzyme assay

Xylitol dehydrogenase activity was assayed by the procedure of (Chakravorty and Horecker 1966), using 0.1 M glycine buffer (pH 8.6) and 0.1 M glycylglycine (pH 7.0) for the forward and reverse reaction, respectively. Enzyme (cell extract or purified glutathione S-transferase [GST]-Xdh1 fusion) was added in amounts sufficient to produce a change in the A_{340} of between 0.02 and 0.1/minute. The reaction was started by the addition of substrate or enzyme, respectively. Activity was expressed as units, 1 U corresponding to the conversion of 1 µmol of substrate per min, and reported as specific activities (units per milligram of protein). The protein concentration was determined by using a protein assay from Bio-Rad Laboratories (Munich, Germany).

SDS-PAGE

Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed with 10 % polyacrylamide gels as described by Ausubel *et al.* (2003). Gels were stained with Coomassie Blue.

Extraction and quantification of mycelial xylitol

To measure the intracellular xylitol concentration, mycelia of *H. jecorina* were harvested, washed with cold tap water, and resuspended in 1 ml of destilled water. The suspension was then snap-frozen at -75 °C for 1 h, thawed, boiled for 10 sec, and finally homogenized in a pre-cooled Potter-Elvehjem glass homogenizer. The homogenate was centrifuged (10.000 x g, 4°C, 10 min), and the xylitol concentration in the supernatant quantified by high-pressure liquid chromatography with an H^+ exchange column (Bio-Rad Aminex HPX-H^+), with 10 mM H_2SO_4 at 55 °C as mobile phase, and with isocratic elution. Compounds were detected by a refractive index detector. The data were averaged and deviated by not more than 3 %. For calculation of the intracellular xylitol concentration, 1 g of mycelial dry weight was assumed to be equivalent to a 2.4-ml intracellular volume (Slayman and Tatum 1964) without considering intracellular compartimentation.

Cloning of the *H. jecorina xdh1* gene

An alignment of different xylitol dehydrogenases from the National Center for Biotechnology Information data bank revealed the conserved amino acid sequences TGICGSDVH and GHYVQGGM to be potentially suitable for amplifying a corresponding fragment of *H. jecorina*. Consequently, the primers xdhfor1 (5´- ACCGGCATCTGCGGCTCCGATGTCC -3´) and xdhrev2 (5´-CCGGTGATGCAGGTCCCGCCATAC-3´) were deduced directly from the respective nucleotide sequence of a hypothetical protein (NCU00891.1) in the *Neurospora crassa* genome database (http://www-genome.wi.mit.edu/annotation/fungi/neurospora/) which shows a high level of similarity to xylitol dehydrogenases. Using 100 ng of *H. jecorina* QM9414 genomic DNA as template in a total volume of 50 µl in an automated temperature cycling device (Biotron, Biometra, Göttingen, FRG); a reaction mixture containing 2.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1 % (vol/vol) Triton X-100, 0.4 µM of each primer, 0.2 mM of each dNTP and 0.5 units of *Taq*-Polymerase (Promega, Madison, WI); and an amplification program consisting of 1 min initial denaturation (94°C), 30 cycles of amplification (1 min 94°C, 1 min 54°C, 1 min 74°C), and a final extension period of 7 min at 74°C. These primers allowed the amplification of a 650-bp fragment, which was isolated and used to screen a genomic λ BlueSTAR library (Novagen, Madison, WI) of *H. jecorina* QM9414. The corresponding

xdh1 gene was located on a 2.5-kb *Sac*I fragment and ligated into pBluescript SK(+) resulting in pXDH, and sequenced by means of a LI-cor 4200 automatic sequencer (LI-cor Inc., Lincoln). To confirm the position of putative introns, a complete *xdh1* cDNA fragment, isolated from a λ HybriZAP cDNA library (Stratagene, La Jolla, CA) of *H. jecorina* QM9414 grown on D-xylose, was sequenced.

To amplify a 1.2-kb fragment of the *H. jecorina lad1* by PCR, the following primers lad1fw (5'-ACAGCTCGCCATGTCGCCTTC-3') and lad1rv (5'-GCACCTCACTCTCAATCCAGGC -3') were derived from the published *H. jecorina lad1* sequence (Corpet 1988). Standard conditions were used for amplification. The corresponding *lad1* gene was located on ≈11-kb *Not*I genomic λ clone H1 and partially sequenced.

The assembled DNA sequences were deposited in GenBank (*xdh1*: Accession No. AF428150; *lad1*: No. AY225444).

Sequence analysis

The 2.5-kb fragment was analyzed using BLAST programs (Altschul *et al.* 1990), and a multiple sequence alignment was done by using MultiAlin (Ditzelmüller *et al.* 1984). Consensus binding sequences in the *xdh1* and *lad1* 5' regions were identified manually.

Nucleic acid isolation and hybridization

Fungal mycelia were harvested by filtration, washed with tap water, frozen, and ground in liquid nitrogen. Nucleic acids (DNA, total RNA) were extracted as described previously (Seiboth *et al.* 2002). Standard methods (Sambrook *et al.* 2001) were used for electrophoresis, blotting and hybridisation of nucleic acids.

Probes used for Northern hybridizations were a 1.9-kb *Acc*65I *act1* fragment (actin encoding), the 1.2-kb *lad1* fragment (see above) and the 1.4-kb *xdh1* cDNA fragment. The relative abundances of transcripts were determined by densiometric measurements of autoradiographs derived from different exposure times (only values with linear correlation [r>0.9] were used).

Overexpression of Xdh1 in *E. coli*

To obtain purified *H. jecorina* xylitol dehydrogenase, the full length cDNA of *xdh1* was overexpressed as a GST fusion in *E. coli*. To this end, the *xdh1* coding region was PCR-amplified from the cDNA clone using primers GEX-XDHfwd (5'- CTGCTGGATCCATGGCGACTCAAACGATC -3') and GEX-XDHrev (5'- AGGGCGGCGGCCGCTTACACCTTCTCGTTG -3'). PCR amplification was performed with *Pfu* polymerase (Promega, Madison, WI) by using an initial denaturation cycle of 45 sec at 94°C, followed by 28 cycles of amplification (45 sec at 94°C, 45 sec at 50°C and 3 min at 72°C), and a final extension step of 10 min at 72°C. The amplicon was cut with *Bam*HI and *Not*I and cloned into pGEX4-2T (Amersham Biosciences, Vienna, Austria). After verification by sequencing, the GST-Xdh1 fusion protein was overexpressed in *E. coli* BL21 (Stratagene, La Jolla, CA) and purified by using glutathione-Sepharose 4B (Amersham Biosciences) according to the manufacturer's protocol. For storage at -80°C or -20°C respectively, 20 % (vol/vol) sterile glycerol was added.

Construction of *H. jecorina xdh1, lad1* and *xdh1/lad1* knock-out mutants

To construct a *xdh1* knockout vector, a genomic fragment containing further 3 kb upstream and 4 kb downstream of the *xdh1* coding region was isolated from the λ phage clone described above, and the *xdh1* coding region replaced by the hygromycin resistance conferring expression cassette from pRLMex30 (Mandels and Andreotti 1978). To this end, a 3-kb *Apa*I-*Cla*I *xdh1* upstream fragment was cloned into pBluescript SK(+), the 2.8-kb *Xho*I-*Hind*III hygromycin cassette cloned into the resulting vector, and finally a 2.8-kb *Apa*I-*Sal*I fragment of the *xdh1* downstream region was added to this vector to result in the final deletion vector pΔXDH. Transformation of *H. jecorina* QM9414 was done by the protocol of Gruber *et al.* (1990) with an 8.0-kb *Acc*65I-*Bst*XI fragment of pΔXDH.

To construct a *lad1*-knock out strain, the *lad1* coding region was replaced by the *H. jecorina pyr4* marker (Chakravorty and Horecker 1966). To this end, the 2.7-kb *Sal*I *pyr4* fragment was cloned between a 1-kb *BamH*I-*Mlu*I upstream fragment and a 2-kb *EcoRV*-*Nco*I downstream fragment of *lad1* in pBluescript SK(+), resulting in the final deletion vector pΔLAD1. For transformation of *H. jecorina* TU-6, a 5.7-kb *Apa*I-*Xba*I fragment of pΔLAD1 was used.

To obtain strains deleted in both dehydrogenases, *H. jecorina* Δ*lad1* was transformed with the 8.0-kb *Acc*65I-*Bst*XI fragment of pΔXDH.

Results

Cloning and characterization of *H. jecorina xdh1* and its encoded protein

Based on the PCR approach outlined above, a 650-bp *H. jecorina* xylitol dehydrogenase fragment was amplified and used to isolate a 2.5-kb genomic subclone which included the complete structural *xdh1* gene. The results of Southern analysis of chromosomal DNA digested with different restriction nucleases are consistent with the occurrence of a single *xdh1* in the *H. jecorina* genome (data not shown). Nucleotide sequence analysis revealed an open reading frame of 1,210 bp, interrupted by a single intron of 118-bp, thus encoding a 363-amino-acid protein with a calculated mass of 38 kDa. An analysis of the amino acid sequence with PROSITE (http://www.expasy.ch/prosite) identified the protein as a member of the zinc-containing alcohol dehydrogenase family, showing highest sequence identity to the xylitol dehydrogenase of *Candida* sp. strain HA167 (former *Galactocandida mastodermitis*). When the Xdh1 protein sequence was compared with the *Neurospora* genome sequence database, four ORFs yielded significant degrees of similarity (e-values of e^{-160}, $1e^{-60}$, $1e^{-48}$, and $1e^{-44}$, respectively). The highest similarity was found with the hypothetical protein NCU00891.1, from which the primers were developed. The second highest similarity was obtained with NCU00643.1. However, submitting NCU00643.1 to a BLASTP search revealed that it is highly similar to L-arabinitol-2-dehydrogenase of *H. jecorina* (Corpet 1988). The two even less similar proteins (NCU07022.1, NCU01905.1) could not be identified with reasonable certainty.

A parsimony analysis of the aa-sequence of *H. jecorina* Xdh1 and those of several other prokaryotic and eukaryotic proteins to which it showed highest similarity in BLAST search revealed that Xdh1 and the hypothetical protein NCU00891.1 of *N. crassa* formed a strongly supported terminal branch within a dichotomous cluster containing all other yeast xylitol and D-sorbitol dehydrogenases and for which two D-sorbitol dehydrogenases of *Schizosaccharomyces pombe* represented a basal

ancestor (Fig. 9). Seven bacterial alcohol and D-sorbitol dehydrogenases formed a sister clade to this cluster. The two *H. jecorina* and *N. crassa* L-arabinitol dehydrogenases formed a clearly different cluster. These data provide evidence that the protein encoded by *H. jecorina xdh1* clearly is a member of the fungal xylitol and D-sorbitol dehydrogenases family and, in addition, that only one such member is present in the *Neurospora* genome sequence database.

Fig. 10 shows an alignment of the amino acid sequence of *H. jecorina* xylitol dehydrogenase with those of its closest yeast neighbors: the polyol and coenzyme binding sites as well as the zinc binding sites are well conserved, but there is generally only poor conservation outside these areas. It is interesting that *H. jecorina* Xdh1 and *N. crassa* Xdh1 (NCU00891.1) - in contrast to the yeast xylitol dehydrogenases - have two predicted binding sites for Zn^{2+} instead of one: one site, consisting of C_{50}, H_{75} and E_{161}, which is typical for D-sorbitol and xylitol dehydrogenases, and a second site, comprising C_{105}, C_{108}, C_{111}, C_{119}, which is typical for alcohol dehydrogenase and which is not found in the other xylitol dehydrogenases described so far from yeast origins.

Regulation of *xdh1* gene expression

The transcriptional regulation of *xdh1* was studied by Northern analysis: during growth on D-xylose or xylan, *H. jecorina* accumulated a single, 1.4-kb *xdh1* transcript (Fig. 11). An *xdh1* transcript accumulated on L-arabinitol to an abundance similar to that on D-xylose or xylan, whereas its abundance was found to be higher on L-arabinose but significantly lower on D-galactose, galactitol, lactose, or xylitol. Generally, the *xdh1* transcript was most abundant in young cultures, and its abundance was sharply decreased during further cultivation (data not shown). No *xdh1* transcript could be detected during growth on D-glucose and glycerol.

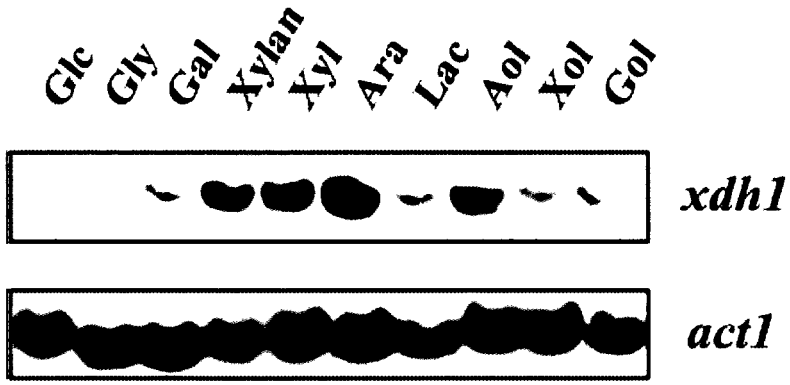


Fig. 11 Induction of xdh1 transcription in *H. jecorina* by different carbon sources

Northern analysis of *xdh1* transcript levels during growth on various carbon sources. Samples were obtained after 6 h after transfer from a glycerol culture to the respective carbon source. In the case of the carbon sources lactose and xylan samples were taken after 24 h of batch growth (conidial inoculum). Abbreviations: Glc, D-glucose; Gly, glycerol; Gal, D-galactose; Xyl, D-xylose; Ara, L-arabinose; Lac, Lactose; Aol, L-arabinitol; Xol, xylitol; Gol, galactitol.

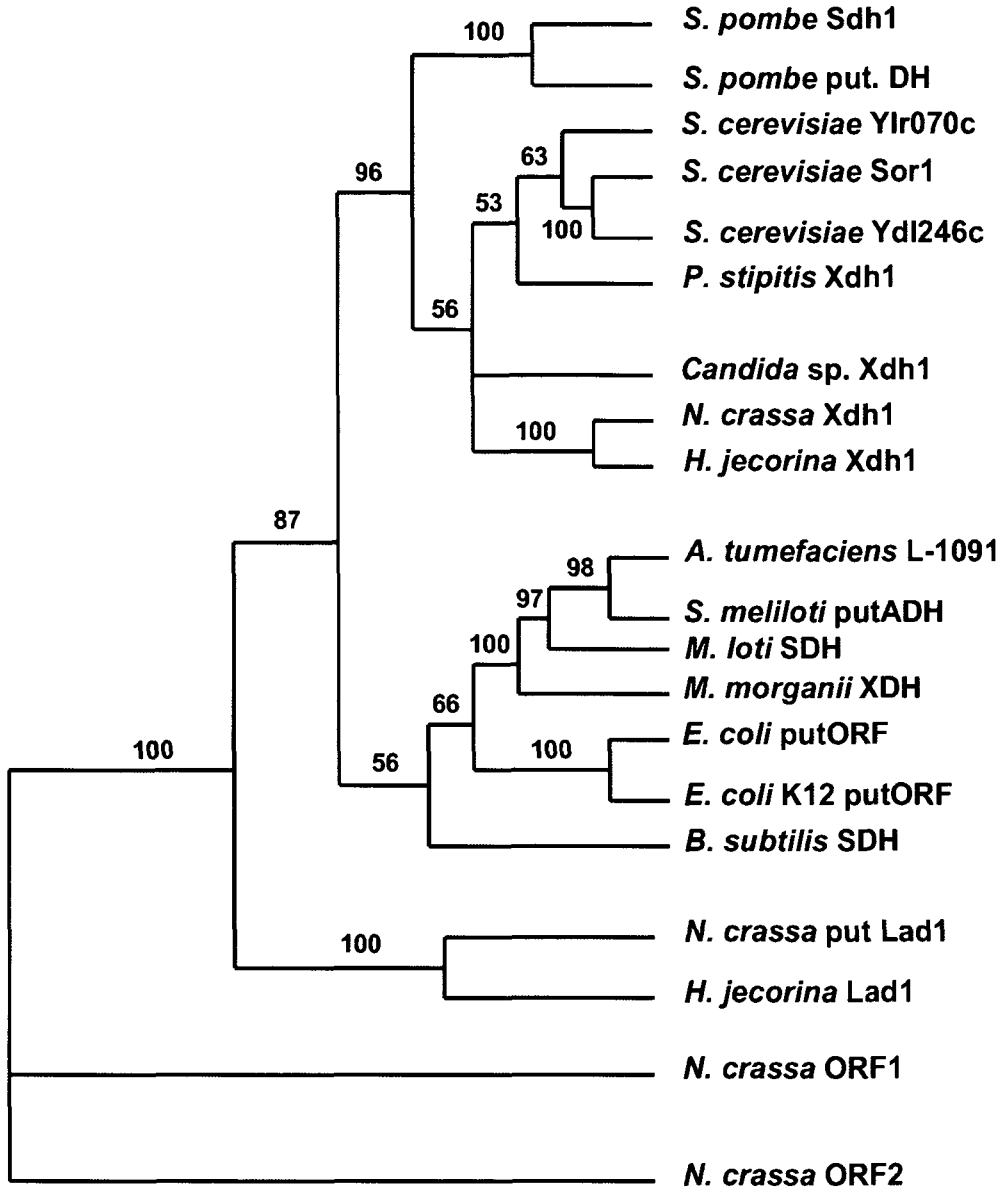


Fig. 9 Phylogenetic tree inferred by parsimony amalysis of the amino acid sequences of xylitol dehydrogenases and D-sorbitol dehydrogenases.

The tree shown is the single most parsimonous tree. The numbers given over selected branches indicate the percentage of 500 bootstrap resampled data sets supporting the clade to the right of the branch, and are given only for values >50. Proteins used in this tree are: *S. pombe* Sdh1 (P36624), *S. pombe* putative dehydrogenase (S38345), *S. cerevisiae* Ylr070c (NP_013171.1), *S. cerevisiae* Sor1 (NP_012693.1), *S. cerevisiae* Ydl246c (NP_010035.1), *P. stipitis* Xdh1 (P22144), *Candida* sp. Xdh1 (AAC24597.1), *N. crassa* Xdh1 (NCU00891.1), *H. jecorina* Xdh1 (AF428150.), *Agrobacterium tumefaciens* L-1091 (NP_356336.1), *Sinorhizobium meliloti* putative ADH (NP_386632.1), *Mesorhizobium loti* SDH (NP_105675.1), *Morganella morganii* XDH (AAA25324.1), *E. coli* putative ORF product(NP_288210.1), *E. coli* K12 putative ORF product (NP_416288.1), *Bacillus subtilis* SDH (NP_388496.1), *N. crassa* putative L-arabitol-2-dehydrogenase (NCU00643.1), *H. jecorina* L-arabitol-2-dehydrogenase (AAL08944.1), *N. crassa* ORF1 product (NCU07022.1), *N. crassa* ORF2 product (NCU01905.1)

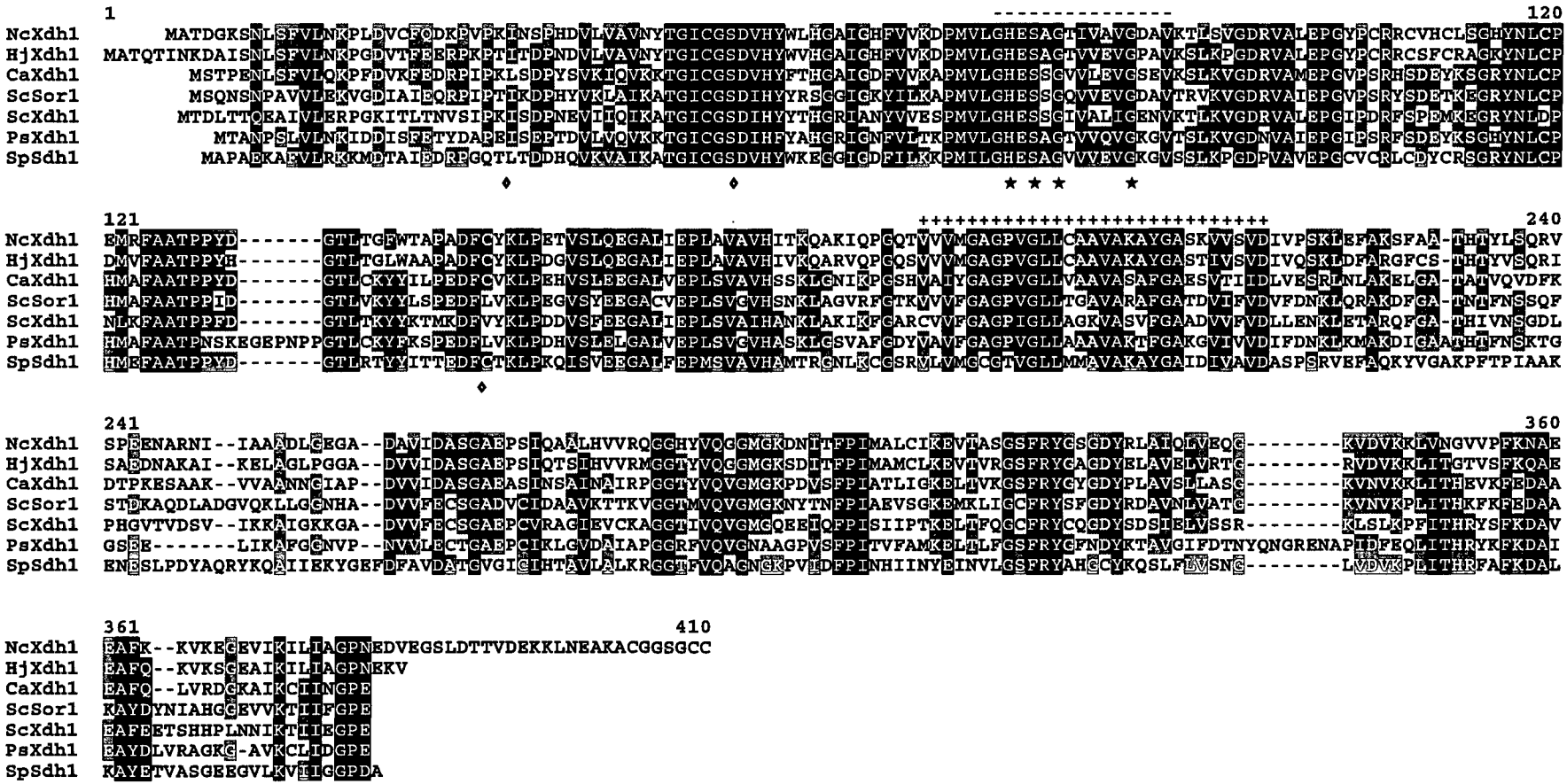


Fig. 10 Alignment of *H. jecorina* xylitol dehydrogenase with other xylitol and D-sorbitol dehydrogenases.

Dehydrogenases were as follows: HjXdh1 (*H. jecorina*), NcXdh1 (*N. crassa;* NCU00891.1), CaXdh1 (*Candida* sp. HA167, AAC24597.1), ScSdh1 (*S. cerevisiae*, NP_012693.1), ScXdh1 (*S. cerevisiae*, NP_013171.1), SpSdh1 (*S. pombe*, P36624), PsXdh1 (*P. stipitis*, P22144). Diamonds indicate the first zinc binding site (C_{50}, H_{75}, E_{161}) and stars the second zinc binding site (C_{105}, C_{108}, C_{111}, C_{119}). The area marked with "–" indicates the zinc-containing alcohol dehydrogenase signature and the area marked with "++" indicates a NAD-binding site. Residues in white on a black background are conserved in at least 90 % of the proteins, residues in white on grey in 40 %.

Substrate specificity of Xdh1

H. jecorina Xdh1 was overexpressed in *E. coli* as a fusion to GST, purified to physical homogeneity (Fig. 12), and used to investigate the substrate specificity of the enzyme.

Fig. 12 SDS-PAGE of purified GST-xylitol dehydrogenase fusion protein.
Ten micrograms of protein was loaded on the gel, which was stained with Coomassie blue.
MW, molecular weight markers

The enzyme was active with xylitol and D-sorbitol in the forward reaction and with D-xylulose and D-fructose in the reverse reaction. No activity was observed with L-arabinose or L-arabinitol as a substrate. NAD or NADH was exclusively required as a coenzyme; NADP or NADPH yielded less then 5 % activity (Table 2). We also tested whether Mg^{2+} was necessary and found full activity in the absence of Mg^{2+}. These data are largely consistent with those reported for xylitol dehydrogenase from the yeast *Candida* (Lunzer *et al.*, 1998) and suggest that the two enzymes - despite of a different number of zinc binding sites - have similar substrate specificities.

Table 2. Substrate specificity of recombinant Xdh1 from *H. jecorina*

Substrate	Cosubstrate [mM]	K_m [mM]	V_{max} [U/mg protein]
xylitol	NAD (0.3)	25 [± 7]	0.06 [± 0.02]
D-sorbitol	NAD (0.3)	24 [± 7]	0.04 [± 0.01]
galactitol	NAD (0.3)	ND	< 0.002 [± 0.001]
L-arabinitol	NAD (0.3)	ND	< 0.002 [± 0.001]
D-xylulose	NADH (0.15)	4.5 [± 0.8]	0.10 [± 0.03]
D-fructose	NADH (0.15)	400 [± 120]	0.15 [± 0.04]
D-galactose	NADH (0.15)	ND	< 0.005 [± 0.002]
NAD^+	xylitol (50)	0.025 [± 0.010]	0.06 [± 0.002]
NADH	D-xylulose (5)	0.35 [± 0.014]	0.10 [±0.03]
$NADP^+$	xylitol (50)	ND	< 0.005 [± 0.001]

ND, not determined

Xdh1 is involved in but not essential for growth on D-xylose

To study whether xylitol dehydrogenase is essential for metabolism of D-xylose in *H. jecorina*, a knockout mutant in which *xdh1* coding region was replaced by the *E. coli hph* gene (encoding hygromycin B phosphotransferase) under the control of *H. jecorina* expression signals was constructed (Mach *et al.* 1994). Several *xdh1* disruptants, verified by Southern analysis (Fig. 13), were obtained.

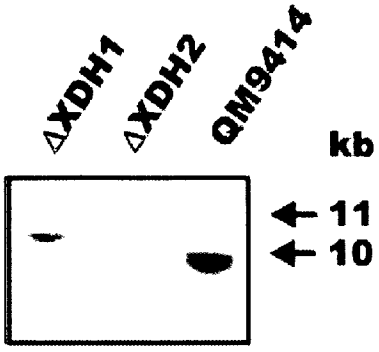


Fig. 13 Southern analysis of xdh1 deletion strains.

Genomic DNA was digested with *Eco*RV and probed with a 2.5-kb *Sac*I fragment of *xdh1*. Replacement of the *xdh1* coding region by the hygromycin resistance-conferring expression cassette in the *xdh1* deletion strain leads to an ≈900-bp increase in the size of the 10-kb hybridizing fragment in strain QM9414 to ≈11 kb in the *xdh1* deletion strain.

All of them exhibited considerably slower growth on D-xylose, but clearly were still able to grow on this carbon source. Interestingly, growth on xylitol - on which already the parent strain grew rather slowly - was unaffected in the deletion mutant (Fig. 14 and 15).

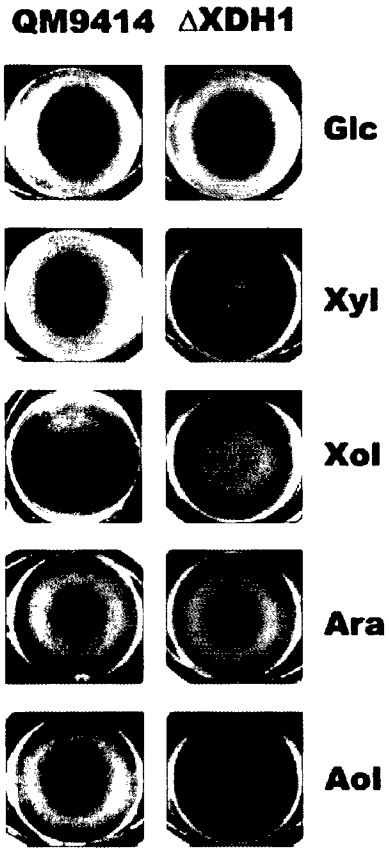


Fig. 14 (left) **Comparison of the growth behaviors of parent strain QM9414 and the *xdh1* deletion strain on plates** containing D-glucose (Glc), D-xylose (Xyl), xylitol (Xol), L-arabinose (Ara), L-arabinitol (Aol) as carbon sources.

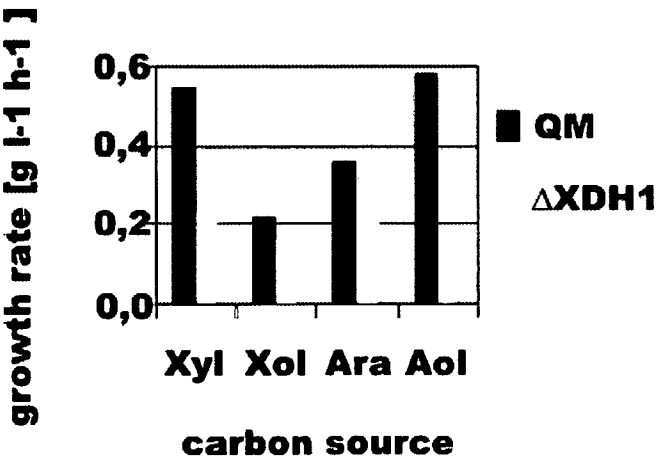


Fig. 15 Effect of the *xdh1* deletion on biomass production during growth on various carbon sources in shake flasks. QM, QM9414.

Consistent with this finding, cell extracts from the deletion mutant still contained xylitol dehydrogenase activity, albeit at a significantly reduced level (Table 3), thus indicating that the *xdh1* gene product does not account for all xylitol dehydrogenase activity of *H. jecorina* ΔXDH1. A microscopic examination of the mutant further showed that during growth on D-xylose, the hyphae of the mutant appeared swollen and contained thicker cell walls (Fig. 16). This morphology correlates with the accumulation of much higher concentrations of xylitol in the hyphae of strain ΔXDH1 than in those of the parent strain as (112 vs. 55 mM, respectively). During growth on xylitol, on the other hand, the hyphae of strain ΔXDH1 showed no changes in morphology compared with the morphology of the parent strain, consistent with the similar rates of growth of both strains on xylitol as a carbon source.

Table 3. Effect of *xdh1* gene deletion in *H. jecorina* on total polyol dehydrogenase activities

Substrate	Concentration [mM]	Source QM9414	Source ΔXDH1
xylitol	150	0.33 [± 0.05]	0.11 [±0.03]
D-sorbitol	150	0.27 [± 0.04]	0.08 [± 0.02]
L-arabinitol	150	0.08 [± 0.02]	0.06 [± 0.02]
galactitol	150	< 0.01 [± 0.005]	<0.01 [± 0.005]
D-fructose	200	0.02 [± 0.01]	<0.01 [± 0.005]
D-xylulose	10	0.08 [± 0.02]	0:01 [± 0.005]

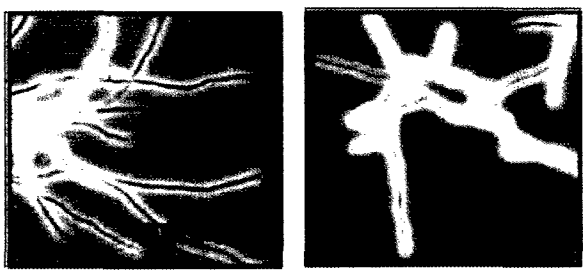


Fig. 16 Microscopic views of strains QM9414 and ΔXDH1 grown on D-xylose

Interestingly, when the *xdh1*-delta mutant was tested for growth on other carbon sources, growth on L-arabinitol but not on L-arabinose also was significantly affected: in submerged cultivation, the increase in biomass density was reduced by more than 50 %, which is in the same range as the reduction observed for D-xylose (Fig. 7 and 8), but no apparent morphology changes were observed.

L-arabinitol-4-dehydrogenase is responsible for the residual xylitol dehydrogenase activity in *H. jecorina xdh1* deletion mutant

The results described above demonstrate that at least one more enzyme with xylitol dehydrogenase activity is present in *H. jecorina* and can partially compensate for the loss of Xdh1 function. A potential candidate for such an enzyme is *lad1*-encoded L-arabinitol-4-dehydrogenase, because it also catalyzes the oxidation of xylitol to D-xylulose (Richard *et al.* 2001). To study this possibility, the *lad1* gene and its flanking regions were cloned and used to construct a *xdh1 lad1* double-deletion strain of

H. jecorina. First, *H. jecorina lad1* deletion mutants were constructed by replacing the *lad1* coding region by the *H. jecorina pyr4* gene (Fig. 17).

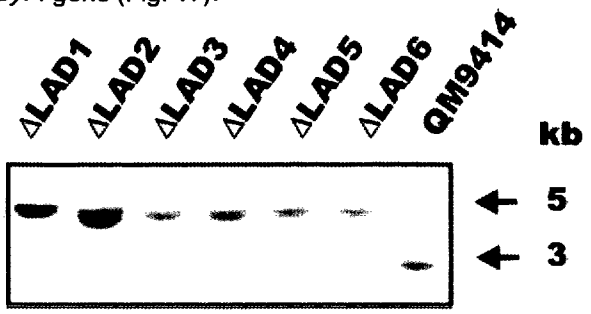


Fig. 17 Southern analysis of *lad1* deletion strains.
Genomic DNAs of strain QM9414 and *lad1* deletion strains were digested with *Bam*HI and probed with a 1-kb *Bam*HI-*Mlu*I fragment of *lad1*. in the *lad1* deletion strains, replacement of the *lad1* coding region by the *H.jecorina pyr4* marker leads to a 1.7-kb increase in the size of the hybridizing fragment. This change leads to an increase in the size of the 3-kb hybridizing fragment in strain QM9414 to a ≈4.7-kb fragment in the *lad1* deletion strains.

The corresponding mutant strains were selected for growth on minimal medium with D-glucose as carbon source and were tested for disruption of *lad1*. Ten mutants were identified by Southern analysis to have undergone a single integration event in the *lad1* locus. It is noteworthy that, in contrast to previous reports, percentage of integration to the homologous locus was found to be by about 50 % in the case of *lad1*. Second, to construct a *xdh1/lad1* double-deletion strain of *H. jecorina,* the *xdh1* coding region was replaced in a *lad1* deleted strain as described above for strain ΔXDH1. Four transformants which showed the expected integration into the *xdh1* locus (Fig. 18) and in which the *lad1* locus remained disrupted were obtained.

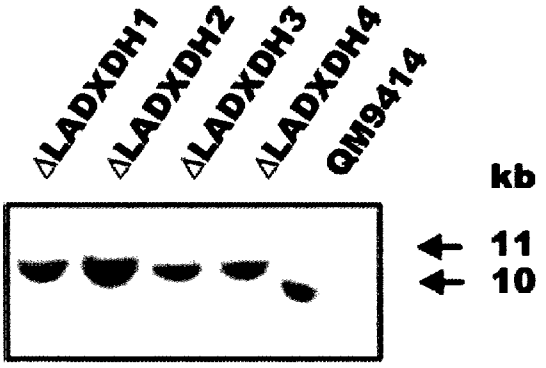


Fig. 18 Southern analysis of *xdh1lad1* deletion strains performed as described in the legend of Fig. 17.

The double-deletion mutant and the *lad1* mutant were consequently tested for growth on D-xylose and xylitol (Fig. 19). The data clearly show that the double-deletion strain lost the ability to grow on either of these carbon sources. Cell extracts from mycelia of strain ΔLADXDH1 that had been pregrown on glycerol and then transferred to D-xylose for 10 h did not contain any xylitol dehydrogenase activity, providing evidence that *lad1* is responsible for the residual xylitol

dehydrogenase activity still present in the *xdh1* deletion mutant. In contrast, the *lad1* single-deletion strain grew equally well on all of these carbon sources, demonstrating that the *lad1* gene product is not involved in D-xylose and xylitol degradation in strains of *H. jecorina* when the *xdh1* gene product is still functional.

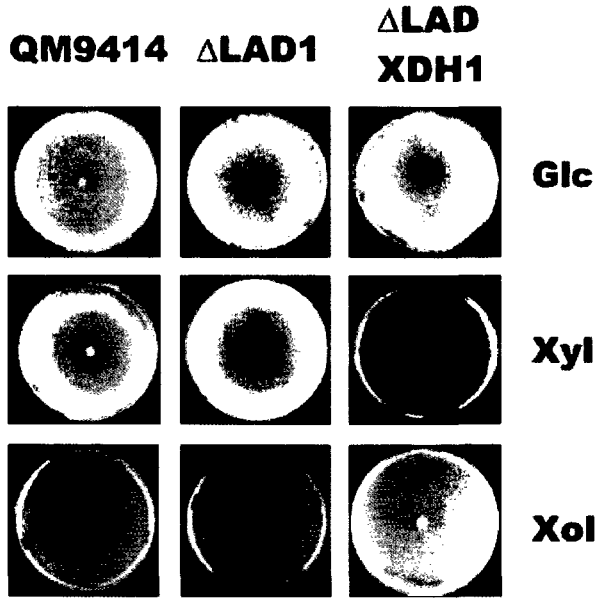


Fig. 19 Comparison of the growth behaviors of parent strain QM9414 and deletion strains on plates containing D-glucose (Glc), D-xylose (Xyl), and xylitol (Xol) as carbon sources.

The compensation of *xdh1* loss of function by the *lad1* gene product is theoretically in conflict with the data of Richard *et al.* (2001) showing that *lad1* is not expressed on D-xylose. However, their data were obtained with carbon catabolite-derepressed strain RUT C-30. Therefore, we examined *lad1* expression in the QM9414 background and in strain ΔXDH1. Consistent with the findings of (Richard *et al.* 2001), we did not find *lad1* expression in strain QM9414, but we did find it in the *xdh1* deletion strain (Fig. 20). Therefore, *lad1* is transcribed under conditions in which *xdh1* function is impaired.

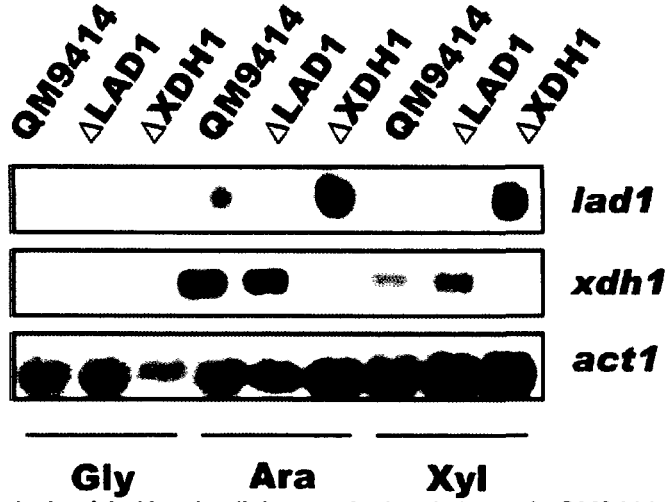


Fig. 20 Northern analysis of *lad1* and *xdh1* transcript levels in strain QM9414 and deletion strains. Samples were obtained 6 hours after transfer from a glycerol culture (Gly) to L-arabinose (Ara) and D-xylose (Xyl) cultures.

Discussion

In the present work we have characterized a xylitol dehydrogenase from the filamentous fungus *H. jecorina* at the molecular and functional level. According to its primary structure, it belongs to the family of Zn^{2+}-containing long-chain alcohol dehydrogenases, which also includes the xylitol, D-sorbitol, and L-iditol dehydrogenases from yeasts (Habenicht *et al.* 1999; Kotter *et al.* 1990; Persson *et al.* 1993; Richard *et al.* 1999). However, it differs from these enzymes in that it contains two predicted binding sites for Zn^{2+}: in addition to site one which has three ligands typical for binding an active Zn^{2+} site (C_{50}, H_{75}, and E_{161}) and which is conserved in all members of this enzyme family (Habenicht *et al.* 1999; Kotter *et al.* 1990; Persson *et al.* 1993; Richard *et al.* 1999), there is a second site (C_{105}, C_{108}, C_{111}, C_{119}) which is not present in most of the other xylitol dehydrogenases (Persson *et al.* 1993). Therefore, it was concluded that the ligands of the second Zn^{2+} atom of the long-chain human alcohol dehydrogenase (C_{97}, C_{100}, C_{103}, and C_{111}) are not conserved in the yeast xylitol dehydrogenase, and it was suggested that this structural zinc atom is missing in all xylitol dehydrogenases, and that this feature is characteristic of D-sorbitol dehydrogenases in general. However, the D-sorbitol dehydrogenase from *S. pombe* contains a similar second zinc binding site which is similar to that in *H. jecorina*, and the putative *N. crassa* xylitol dehydrogenase (NCU00891.1) also contains such a site. The phylogenetic analysis of xylitol and D-sorbitol dehydrogenases in this study showed that both, the yeast and the filamentous fungal xylitol dehydrogenases arose from the *S. pombe* ancestor, implying that the enzymes in yeast lost the second zinc binding site during evolution. That *S. pombe* is an evolutionary ancestor of *Saccharomyces* and *Candida* has also been evidenced by 28S gene sequence analysis (Kurtzman and Robnett 1998). In addition, and despite containing two zinc-binding sites, the *H. jecorina* xylitol dehydrogenase has the same substrate specificity as the yeast xylitol dehydrogenases (Gruber *et al.* 1990; Lunzer *et al.* 1998; Persson *et al.* 1993; Richard *et al.* 1999), namely, oxidation of xylitol and D-sorbitol, reduction of D-xylulose and D-fructose, and no activity on L-arabinitol or L-arabinose. Although no extensive kinetic analysis was attempted, we conclude in analogy that this enzyme is a typical L-iditol:NAD^+-2-oxidoreductase (EC 1.1.1.14).

The Expression of *xdh1* is adaptive, as no *xdh1* transcript could be detected in mycelia grown on D-glucose or glycerol, whereas it accumulates during growth on D-xylose, xylitol and also on L-arabinose and L-arabinitol. The effect of the latter two components may be direct or indirect, since the catabolism of L-arabinitol forms xylitol. The lack of *xdh1* expression on D-glucose and glycerol would be indicative of regulation by carbon catabolite repression, but since the *xdh1* transcript also does not accumulate on glycerol in the *cre1*-truncated mutant *H. jecorina* RUT C-30 (unpublished data), this is clearly not the case. Therefore, the presence of the *xdh1* transcript during growth on D-xylose is most likely due to induction, which would be consistent with the mode of regulation of the other two genes of the D-xylose catabolic pathway in *A. niger* (xylose reductase and xylulose-5-phosphate kinase; (Hasper *et al.* 2000; van Peij *et al.* 1998). In addition, the *Aspergillus* xylulose-5-phosphate kinase was shown to be induced by L-arabinose and L-arabinitol but not by xylitol. As for the transactivator protein mediating this response, the *A. niger* xylose reductase was shown to be under the control of XlnR, the *A. niger* transcriptional activator of xylanase and cellulase biosynthesis (van Peij *et al.* 1998; vanKuyk *et al.* 2001), whereas its xylulokinase was still inducible by D-xylose in an XlnR-negative mutant. Whether an XlnR homologue would also be involved in regulation of *xdh1* by D-

xylose in *H. jecorina* is unclear, however, as we did not detect any nucleotide sequences matching the consensus for binding of XlnR (GGCTAA) in the *xdh1* 5' upstream region. In *H. jecorina*, the genes encoding the two xylanases are differently expressed, and only *xyn1* but not *xyn2* is regulated by the XlnR homologue Xyr1 (Mach and Zeilinger 2003; Wurleitner *et al.* 2003). It is therefore possible that this coordinated regulation of genes for xylan and D-xylose metabolism in *A. niger* does not exist in *H. jecorina,* a possibility which may reflect the different natural habitats of the two fungi.

An intriguing finding of this study was that a loss-of-function mutant of *xdh1* was still capable of growing on xylitol and - although with lower growth rates - also on D-xylose, implying the presence of at least one more enzyme oxidizing xylitol or a less effective new pathway for D-xylose catabolism. The results obtained with the *xdh1/lad1* double-deletion mutant conclusively showed that the enzyme responsible for this residual activity is L-arabinitol-4-dehydrogenase, Lad1. The lack of both Xdh1 and Lad1 leads to a complete loss of ability to grow on D-xylose and xylitol. Lad1 and Xdh1 belong to the same family of zinc-containing alcohol dehydrogenases, and a phylogenetic analysis of Xdh1 and Lad1 from various sources showed that the clade containing Lad1 is basal to that of Xdh1; these data imply that the more specific Xdh1 protein may have evolved from the rather broadly specific Lad1 protein. Such an assumption is also supported by the finding that *H. jecorina xdh1* was not able to enable a loss-of-function mutant of *lad1* to grow on L-arabinose, consistent with the inability of Xdh1 to oxidize L-arabinitol. Compensation of loss-of-function mutants in one pentose catabolic pathway by enzymes from another pathway may also explain the results obtained with *A. niger*, i.e. that a xylose reductase mutant was still able to grow with reduced rate on D-xylose (Hasper *et al.* 2000). The pathways for D-xylose and L-arabinose catabolism are both initiated by an aldose reductase, but at present it is not known whether the same enzyme, two isoenzymes with the same substrate specificity, or two enzymes with different substrate specificities catalyze the steps in the two pathways.

Despite of the lack of L-arabinitol-oxidizing activity of Xdh1, the *xdh1* mutant showed a significantly decreased growth rate on L-arabinitol, an intriguing result in view of the lack of effect on growth on xylitol in this mutant. Although we cannot rule out the possibility that the *xdh1* gene product also has a regulatory function, we interpret the findings in terms of an imbalance in substrates, products, and/or coenzymes such that flux like that in the wild type cannot be maintained. The fact that these findings are observed only when the first (NADPH-specific) step (L-arabinose reductase) is omitted suggests that the NADPH/NADP ratio may be the critical variable: filamentous fungi possess a cytosolic pyridine nucleotide transhydrogenase (e.g. *N. crassa* genome database: NCU01140.1 (http://www-genome.wi.mit.edu/annotation/fungi/neurospora) which is responsible for maintaining a balance between NADH/NAD and NADPH/NADP ratios. The catabolism of L-arabinose to L-xylulose-5-phosphate requires two NADPH and two NAD^+ molecules, and it is likely that the transhydrogenase is at least partially involved in the regeneration of NAD^+ for the L-arabinitol-4-dehydrogenase and xylitol dehydrogenase reactions through reduction of the $NADP^+$ formed in the aldose reductase reactions. Thus, under conditions where only L-arabinitol-4-dehydrogenase accounts for pentitol reductions, the regeneration of NAD^+ on behalf of only one NADPH generated by the D-xylulose reductase reaction located downstream may not produce sufficient activity to catalyze L-arabinitol oxidation with the same velocity as in the wild type. This interpretation is also supported by the generally lower growth rate of the wild type on both L-arabinitol and xylitol. However, the proposed role of the pyridine nucleotide transhydrogenase needs to be verified by reverse genetics first.

Chapter Two

The metabolic role and evolution of L-arabinitol 4-dehydrogenase of *Hypocrea jecorina*

Summary

L-arabinitol 4-dehydrogenase (Lad1) of the cellulolytic and hemicellulolytic fungus *Hypocrea jecorina* (anamorph: *Trichoderma reesei*) has been implicated in the catabolism of L-arabinose, and genetic evidence is also available that it is involved in the catabolism of D-xylose in *xdh1* (xylitol dehydrogenase) mutants and of D-galactose in *gal1* (galactokinase) mutants of *H. jecorina*. In order to identify the substrate specificity of Lad1, we have recombinantly produced the enzyme in *E. coli* and purified it to physical homogeneity. The resulting enzyme preparation catalyzed the oxidation of pentitols (L-arabinitol, xylitol) and of hexitols (D-allitol, D-sorbitol, L-iditol, L-mannitol) to the same corresponding ketoses as mammalian sorbitol dehydrogenase (SDH), albeit with different catalytic efficacies, showing highest k_{cat}/K_m for L-arabinitol. However, it oxidized galactitol and D-talitol at C4 exclusively, yielding L-xylo-3-hexulose and D-arabino-3-hexulose, respectively. Phylogenetic analysis of Lad1 showed that it is a member of a terminal clade of putative fungal L-arabinitol dehydrogenase orthologues which separated during evolution of SDHs. Juxtapositioning of the Lad1 3D-structure over that of SDH revealed major aa-exchanges at topologies flanking the binding pocket for D-sorbitol. A *lad1* gene disruptant was almost unable to grow on L-arabinose, L-arabinitol, D-talitol, D-sorbitol and galactitol, exhibited a significantly reduced growth on D-galactose and a slightly reduced growth on D-glucose. Growth on L-arabinose, L-arabinitol, D-talitol, D-sorbitol and galactitol was completely eliminated in a mutant in which also the *xdh1* gene had been disrupted. These data show that Lad1 is indeed essential for the catabolism of L-arabinose, but that it also constitutes an essential step in the catabolism of several hexoses, and emphasized the importance of such reductive pathways of catabolism in fungi.

Introduction

D-galactose metabolism via the Leloir pathway is a ubiquitous trait in pro- and eukaryotic cells (Frey 1996). It involves the formation of D-galactose-1-phosphate by galactokinase (EC 2.7.1.6), its transfer to UDP-glucose in exchange with D-glucose-1-phosphate by galactose 1-phosphate-uridyltransferase (EC 2.7.7.12), and the epimerization of the resulting UDP-galactose to UDP-glucose by UDP-glucose 4-epimerase (EC 5.1.3.2). However, alternative pathways of D-galactose metabolism have been reported in plants (Gross and Phar 1982; Schnarrenberger *et al.* 1995) and bacteria (Bettenbrock and Alpert 1998; Chassy and Thompson 1983; De Ley and Doudoroff 1957; Shuster and Doudoroff 1967). In the fungus *Aspergillus niger*, the presence of an oxidative, non-phosphorylated pathway of galactose catabolism has been suggested (Elshafei and Abdel-Fatah 2001).

For the fungus *Hypocrea jecorina* (anamorph: *Trichoderma reesei*), the D-galactose-containing disaccharide lactose is the only soluble carbon source for industrial cellulase production or formation of heterologous proteins under the control signals of cellulase promoters (Penttilä 1998; Persson *et al.* 1991). The metabolism of D-galactose and its regulation is therefore of interest for the improvement of the biotechnological application of this fungus. Interestingly, *Hypocrea jecorina* contains - in addition to the standard Leloir pathway (Seiboth *et al.* 2002; Seiboth *et al.* 2002) - also a reductive pathway via galactitol as an intermediate (Seiboth *et al.* 2003). Molecular genetic evidence suggests that the *lad1*-encoded Lad1 catabolizes galactitol (Seiboth *et al.* 2003). However, the product of the oxidation of galactitol by this enzyme has not been identified.

Lad1 is believed to participate in a fungal-specific pathway of L-arabinose utilization involving an NADPH-linked reductase, which forms L-arabinitol. This is converted to L-xylulose by Lad1 followed by an NADPH-linked L-xylulose reductase, which forms xylitol from L-xylulose (Richard *et al.* 2001; Richard *et al.* 2002). However, genetic evidence for the involvement of either of these proteins in L-arabinose metabolism has not yet been presented. On the other hand, we have recently shown that *lad1* compensates for the loss of xylitol dehydrogenase activity in *xdh1* mutants (Seiboth *et al.* 2003).

The aim of this study therefore was (i) to identify the product of galactitol oxidation by Lad1; (ii) to verify that Lad1 is indeed involved in L-arabinose metabolism *in vivo*; and (iii) to identify whether Lad1 is also involved in other monosaccharide catabolic pathways in *H. jecorina*. In addition, we will show that Lad1 is a fungal orthologue of the yeast/mammalian SDH and we will highlight the structural differences and similarities between these two protein groups.

Experimental procedures

Strains and culture conditions

H. jecorina strains used in this study were QM9414 (ATCC 26921) and the *pyr4* negative mutant TU-6 (ATCC MYA-256, (Gruber *et al.* 1990)). All strains were maintained on malt extract agar and auxotrophic strains supplemented with uridine (10 mM). Strains were grown in 250 ml in 1-l Erlenmeyer flasks on a rotary shaker (250 rpm) at 30°C in the medium described by (Mandels and Andreotti 1978) with the respective carbon source at a final concentration of 10 g/l.

Escherichia coli strain JM109 (Promega, Madison, WI) was used for plasmid propagation.

Determination of fungal growth

To determine hyphal growth on agar plates, plates were inoculated by placing a small piece of agar into the centre of an 11-cm plate, and the increase in colony diameter measured twice daily.

Cloning of the *H. jecorina lad1* gene and construction of a *lad1* knock-out mutant

The cloning of *lad1*, and its use to obtain *lad1*-knockout strains of *H. jecorina* has been described previously (Seiboth *et al.* 2003).

Overexpression of *lad1* in *E.coli* and purification of Lad1

To obtain purified *H. jecorina* Lad1, the *lad1* was overexpressed as a glutathione-S-transferase fusion in *E. coli*. To this end, the *lad1* coding region was PCR-amplified from cDNA using primers GEX-Lad fwd (5´- GCAATTCACAGGGATCCATGTCGCCTTCC -3´) and GEX-Lad rv3 (5´- CTTGGTCGCAGCGGCCGCTCAATCCAGG -3´). PCR amplifications were performed with *Pfu* polymerase (Promega, Madison, WI), using an initial denaturation cycle of 45 sec at 94°C, followed by 30 cycles of amplification (45 sec at 94°C, 45 sec at 56°C and 3 min at 72°C). The final extension step was 10 min at 72°C. The amplicon was cut with *BamHI* and *NotI* and cloned into pGEX4-2T (Amersham Biosciences, Uppsala, Sweden) and, after verification by sequencing, the GST-Lad1 fusion protein was overexpressed in *E. coli* BL21 (Stratagene, California). Purification using Glutathione Sepharose 4B and thrombin cleavage of fused protein bound to column matrix was performed according to the manufacturer's protocol (Amersham Biosciences). Physical homogeneity of the overproduced protein was verified by SDS-PAGE in 10 % polyacrylamide gels as described by Ausubel *et al.*, 2003, using Coomassie Blue protein staining. Homogenously purified fractions were stored until use at -80°C or -20°C, respectively, in the presence of 1 % (w/v) BSA.

Enzyme assay

Lad1 activity was determined spectrophotometrically by measuring the rate of change in absorbance at 340 nm for NAD reduction or NADH oxidation at 25°C, using a Pye Unicam SP6-400 Spectrophotometer connected to an United Technologies Packard Model 641 recorder. Reactions were initiated by adding an aliquot of recombinant enzyme to a 1.0 ml-reaction mixture in a 10-mm half-micro disposable cuvette (Brand Wertheim, Germany). Measurements were made by varying the substrate concentration over the range of 10 to 100 mM with a constant coenzyme concentration of 0.25 mM for both (NAD and NADH) in either 100 mM glycine/NaOH pH 8.6 or 100 mM glycylglycine/NaOH pH 7.0. Activities are expressed as kat (nkat), 1 nkat corresponding to the conversion of 1 nmol of substrate per sec, and given as specific activities (kat/mg protein). Protein concentration was determined with the Bio-Rad Protein Assay (Bio-Rad Laboratories, München, Germany). Michaelis-Menten constant K_m and maximal velocity V_{max} were graphically determined by direct linear plotting (Cornish-Bowden and Eisenthal 1978; Eisenthal and Cornish-Bowden 1974).

Monosaccharides and polyols used were purchased from Sigma except for D-allitol from Omicron Biochemicals, Inc. (IN, USA), and L-mannitol and D-talitol from SACHEM s.r.o. (Praha, CZ).

Large scale production of hexitols and hexuloses by Lad1

Conversion of the hexitols into the corresponding ketohexoses was done in 1 or 2 ml volumes consisting of 150 mM hexitol, 1 mM NAD^+ in 100 mM glycine/NaOH pH 8.6 and 0.02 to 0.1 U of purified Lad1. To maintain a constant NAD^+ concentration, 150 mM pyruvate (Sigma, Germany) and 5 U lactate dehydrogenase (Sigma, Germany) were added. For conversion of ketohexoses into hexitols, the reactions (1 ml vol.) consisted of 150 mM ketohexose, 1 mM NADH, 150 mM L-lactat (Merck, Germany), 10 U lactate dehydrogenase in 100 mM glycylglycine/NaOH pH 7.0, and 0.001 to 0.01 U purified Lad1. The mixtures were incubated for 20 h at 37°C. Controls were prepared by boiling the assay immediately after addition of enzyme.

The reaction mixtures were deionized by passage through columns containing DOWEX 50W×8 (H^+ form) and DOWEX 1×8 ($HCOO^-$ form) and concentrated by evaporation at 40°C to a volume of 1 ml. Aliquots (0.2 ml) were subjected to HPLC on an Aminex HPX-87C column (300×7.8 mm; Bio-Rad, Germany) connected to a Bio-Rad 1755 refractive index detector, using water as the mobile phase (85°C, flow rate of 0.6 ml/min). Products were identified by their absence in the control reactions. Appropriate fractions from successive runs were pooled and concentrated to dryness by evaporation.

Chemical analyses.

For GC and GC-MS analyses, samples were dried and redissolved in pyridine. Carbohydrates and hexitols were converted into trimethylsilyl derivatives by treatment with N,O-bis-(trimethylsilyl)-trifluoroacetamide:trimethyl-chlorosilane (10:1, v/v) for 60 min at 75°C. GC was carried out on a Hewlett Packard 6890 equipped with a cool on-column injector, a DB-5ms capillary column (20 m x 0.18 mm i.d., 0.18 µm film thickness; J&W Scientific) and a flame ionization detector. The carrier gas was helium (1.5 ml/min constant flow). The temperature program was: 1 min hold at 85°C, 85-120°C at 10°C/min, 120-180°C at 3°C/min. GC-MS was carried out with a Varian 3400CX coupled to a Varian Saturn 3 ion trap mass spectrometer (operated in the EI mode). A HP-5ms column (50 m x 0.2 mm i.d., 0.33 µm film thickness) was used with helium as the carrier gas at a head pressure of 44 psi (at 130°C) and a temperature gradient of 130-320°C at 6°C/min.

NMR spectra were recorded in D_2O on a BRUKER AVANCE 400 Spectrometer at 400.13 MHz for ^1H and 100.62 MHz for ^{13}C at 298 K, using a 5 mm inverse broadband probehead. Chemical shifts were referenced to tetramethylsilane.

Phylogenetic analysis

Protein sequences were aligned first with Clustal X 1.81 (Thompson *et al.* 1997) and then visually adjusted using Genedoc 2.6.002 (Nicholas *et al.* 1997). Phylogenetic analyses were performed in PAUP* 4.0b10 using sequence of the putative SDH of *Schizosaccharomyces pombe* (NP_595120) as an outgroup. Parsimony analysis was performed using a heuristic search, with a starting tree obtained via stepwise addition, with random addition of sequences with 1000 replicates. Gaps were treated as missing characters. Stability of clades was evaluated by 500 bootstrap rearrangements.

Results

Lad1 oxidizes galactitol to L-xylo-3-hexulose

We have previously shown (Seiboth *et al.* 2003) that a Δ*lad1* strain is strongly impaired in its ability to grow on galactitol, and that a *gal1/lad1*-double mutant (which is in addition deficient in galactokinase and thus blocked in the Leloir pathway of D-galactose catabolism) is unable to grow on D-galactose (Seiboth *et al.* 2004). In order to verify that this is due to a loss of the galactitol dehydrogenase activity in the *lad1*-delta strain, we tested whether *H. jecorina* Lad1 can in fact utilize galactitol as a substrate. To this end, the protein was recombinantly produced in *E. coli* as a fusion to GST, purified by affinity chromatography, and the GST-moiety removed by cleavage with thrombin. The so obtained enzyme preparation was apparently pure (Fig. 21) and used for all further investigations.

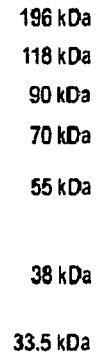


Fig. 21
SDS-PAGE of purified Lad1

Purified Lad1 oxidized galactitol with a K_m of 60 (± 10) mM and a V_{max} of 1.2 (± 0.09)*10E-11 kat (Fig. 22), thus proving that Lad1 acts on galactitol as a substrate. In order to identify the product of the oxidation of galactitol, the reaction product was purified by ion exchange chromatography and HPLC, and subjected to NMR analysis. By these means, the ketohexose formed was shown to be a 3-hexulose by a series of 2D experiments and finally identified as L-xylo-3-hexulose by comparison of the ^{13}C NMR spectrum with previously published data (Angyal *et al.* 1976); cf. Fig. 23). In order to confirm that this unusual ketohexose was indeed the product of the reaction and not an artefact, it was also used as a substrate for the backward reaction of Lad1. This experiment provided unequivocal evidence that the enzyme formed galactitol from L-xylo-3-hexulose, yielding a K_m and V_{max} of 80.7 mM and 0.20 nkat/mg protein, respectively.

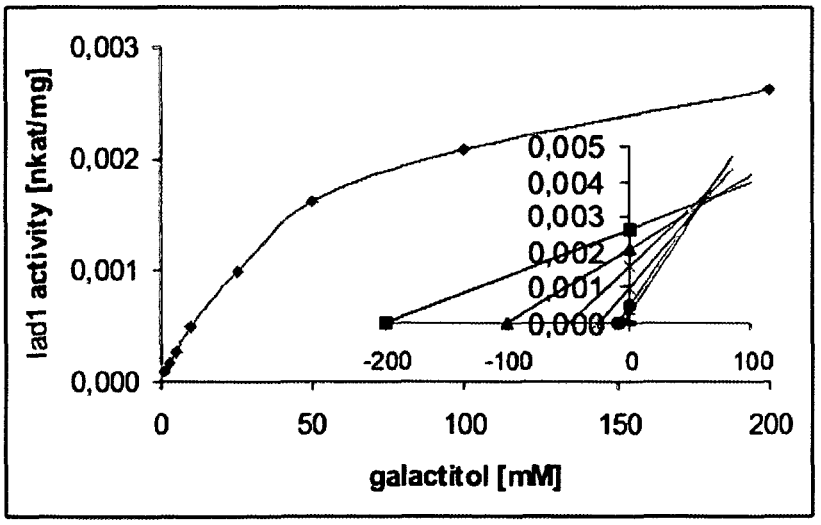


Fig. 22 Affinity of Lad1 of *H. jecorina* for galactitol.
The inset shows the Eisenthal-Cornish-Bowden direct linear plot from which K_m and V_{max} were determined.

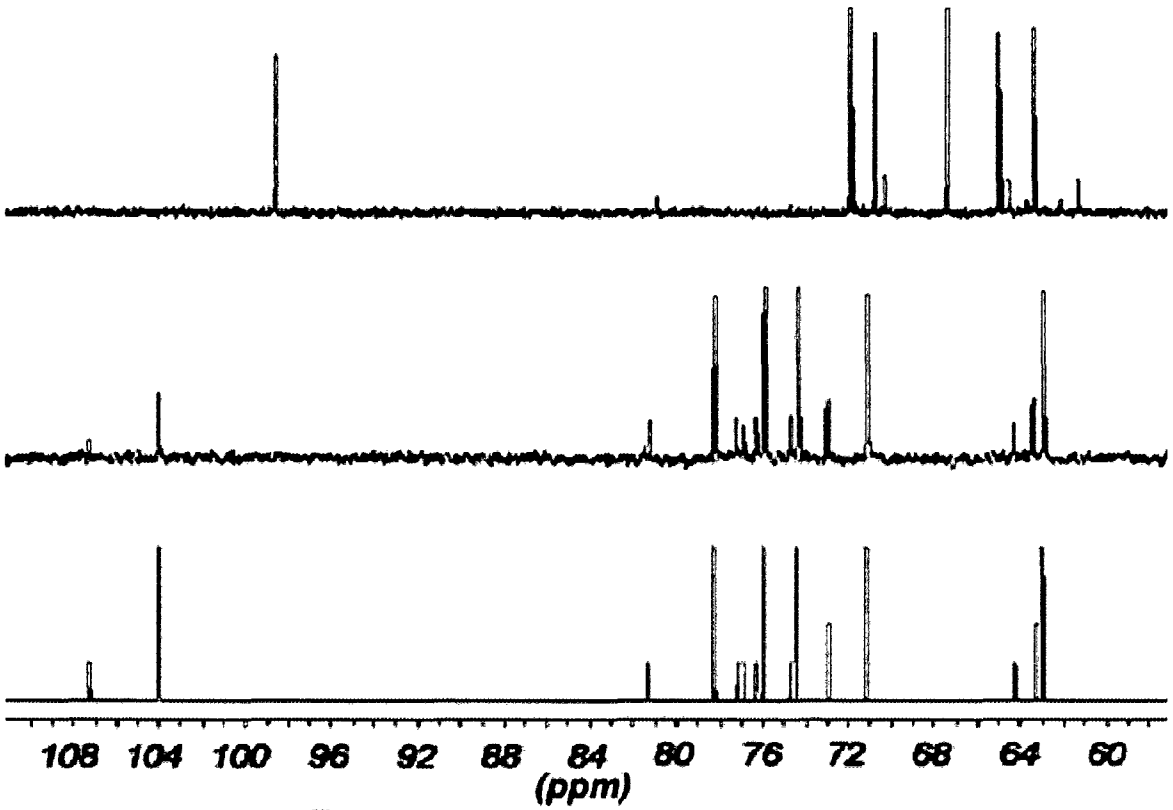


Fig. 23 ^{13}C NMR spectrum of the formed 3-hexulose (top panel) and spectrum of xylo-3-hexulose (lower panel) compiled from data reported by (Angyal *et al.* 1976)

Enzymatic conversion of hexitols and ketohexoses by Lad1

The identification of L-xylo-3-hexulose as product of the oxidation of galactitol by Lad1 raised the question whether other hexitols would be similarly oxidized at C4 by the enzyme. We have therefore investigated the substrate and product specificity of the enzyme towards various hexitols. Reaction products were identified by GC and GC-MS. Table 4 lists the results from this investigation, and the respective substrate-product relationships established are given in Fig. 24: D-sorbitol, D-allitol, L-mannitol and L-iditol were oxidized at C2, yielding D-fructose, D-psicose, L-fructose and L-sorbose, respectively. D-talitol, in contrast, behaved like galactitol as it was also oxidized at C4, yielding D-arabino-3-hexulose (Angyal *et al.* 1976). Lad1 had no activity on D-mannitol. With the exception of D-talitol, the maximum velocities (k_{cat}) for the various hexitols were significantly lower than for L-arabinitol and being lowest with D-allitol and galactitol. Comparison of the substrate specificity constants (k_{cat}/K_m) showed the same trend, but with even increased differences.

Table 4 Substrate specificity of *H. jecorina* Lad1

polyol oxidations were performed in 0.1 M glycine buffer, pH 8.6 at a constant NAD^+-concentration of 0.25 mM whereas carbonyl reduction were performed in 0.1 M glycylglycine buffer, pH 7.0 at a constant NADH-concentration of 0.2 mM and both at 25°C. Mean values [± S.D.] based on at least four separate experiments are given.

substrate	K_m [mM]	V_{max} [nkat]	k_{cat} [kat/mol]	k_{cat}/K_m [$M^{-1}s^{-1}$]
L-arabinitol	4.5 [± 1]	0.213 [± 0.009]	0.8546 [± 0.0361]	201.6407 [± 52.8330]
D-talitol	24.5 [± 2.5]	0.146 [± 0.0072]	0.5849 [± 0.0289]	24.2481 [± 3.6533]
galactitol	60 [± 10]	0.012 [± 0.0009]	0.0438 [± 0.0033]	0.7607 [± 0.1816]
D-sorbitol	46 [± 4]	0.0342 [± 0.0006]	0.1372 [± 0.0024]	3.0101 [± 0.3141]
D-allitol	11.3 [± 1]	0.00816 [± 0.00012]	0.0327 [± 0.0005]	2.9238 [± 0.3013]
L-mannitol	37 [± 2]	0.0273 [± 0.0006]	0.1095 [± 0.0024]	2.9724 [± 0.2257]
L-iditol	191.25 [± 9.5]	0.021 [± 0.0009]	0.0723 [± 0.0031]	0.3799 [± 0.0351]
D-arabino-3-hexulose	580.11 [± 31.2]	0.969 [± 0.0468]	5.0091 [± 0.2418]	8.6822 [± 0.8830]
L-xylo-3-hexulose	80.68 [± 10.3]	0.197 [± 0.0243]	0.6772 [± 0.0837]	8.6659 [± 2.1404]
D-fructose	96.2 [± 1]	0.00834 [± 0.00006]	0.0335 [± 0.0002]	0.3479 [± 0.0061]
D-psicose	80.5 [± 4]	0.011 [± 0.00024]	0.0164 [± 0.0004]	0.2042 [± 0.0146]
L-sorbose	19.4 [± 2]	0.00122 [± 0.00006]	0.0018 [± 0.0001]	0.0956 [± 0.0145]
L-tagatose	27.8 [± 1]	0.00248 [± 0.00006]	0.0037 [± 0.0001]	0.1333 [± 0.008]
D-sorbose	114.8 [± 1.5]	0.00105 [± 0.000012]	0.0016 [± 0.00004]	0.0137 [± 0.0003]

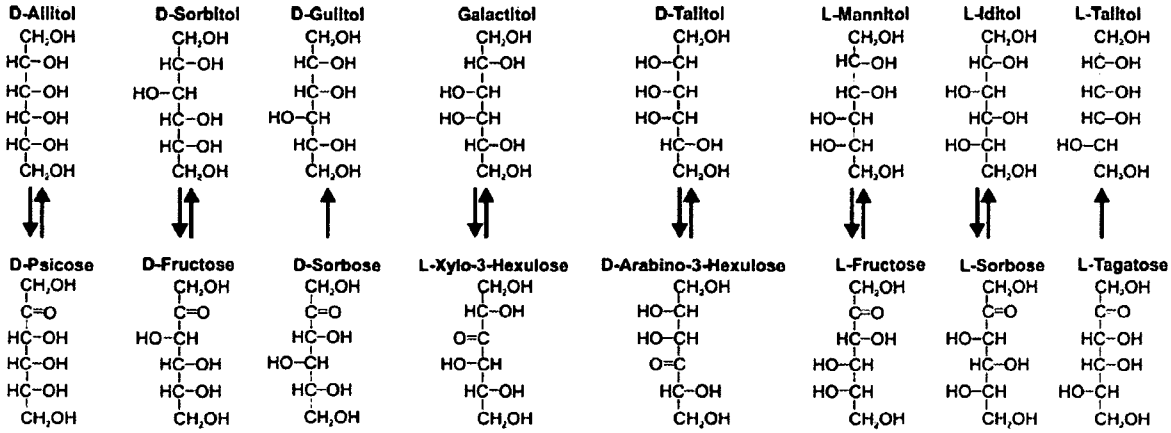


Fig. 24 Substrate-product relationships of *H. jecorina* Lad1

Reactions of Lad1 as established experimentally in this work. Oxidation of polyols was studied at 8.6, and reduction of ketoses at pH 7.0. Oxidation of D-gulitol and L-talitol was not investigated due to unavailability of the respective polyols in amounts sufficient for the analysis.

Lad1 is essential for the *in vivo* metabolism of L-arabinose and various hexitols

The above data showed that Lad1 can catalyze the oxidation of various hexitols, but with far less efficacy than L-arabinitol or other pentitols (data not shown). However, evidence is missing so far that the enzyme is indeed responsible for the metabolism of any of these polyols *in vivo*. To test this, we made use of a *H. jecorina* recombinant strain in which the *lad1* coding region had been replaced by the *H. jecorina pyr4* gene (Seiboth *et al.* 2003). The results (Fig. 25) show that this mutant was unable to grow on minimal medium with L-arabinose, grew weakly on L-arabinitol as a carbon source, and had a slightly reduced growth rate on D-glucose and D-xylose. The very weak growth on L-arabinitol was completely eliminated in a mutant in which both *lad1* and the *xdh1,* encoding xylitol dehydrogenase, (Richard *et al.* 2002) genes were disrupted (Fig. 25). However, as Xdh1 accounts only for a minor part of L-arabinose catabolism, Lad1 is clearly of major importance for the L-arabinose catabolic pathway of *H. jecorina*.

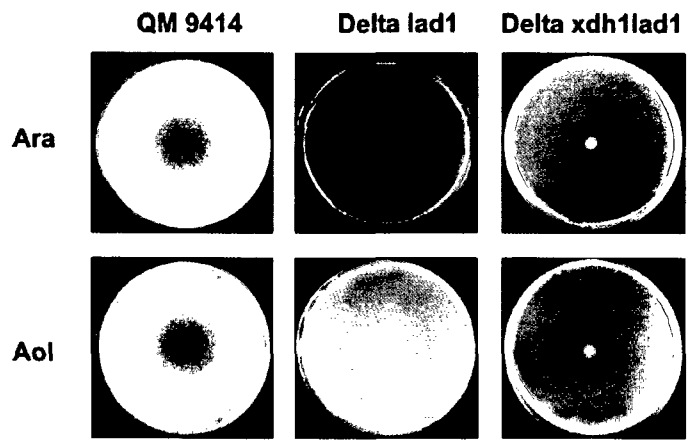


Fig. 25 Growth of *H. jecorina* QM 9414, a *lad1* knock-out mutant and a *xdh1lad1* doublemutant on L-arabinose, L-arabinitol Growth of *H. jecorina* on L-arabinose (Ara) and L-arabinitol (Aol) as carbon source on plates, incubated for three days.

Because of the poor catalytic efficacy of Lad1 on the hexitols, the *lad1*-mutant was also tested for its effect on growth on some of those hexitols which were identified as substrates of Lad1 (hexitols not investigated were unavailable in the amounts needed for these experiments). *H. jecorina* was capable of growing on galactitol, D-talitol, D-sorbitol, and L-mannitol. With the exception of L-mannitol, growth on all the other carbon sources was strongly reduced in the *lad 1* mutant (Fig. 26). *H. jecorina* failed to grow on D-allitol and L-iditol.

	QM 9414	Delta lad1
L-arabinitol	+++	-
galactitol	++	-
D-talitol	++	-
D-sorbitol	+++	-
L-mannitol	+	-
D-allitol	-	-
L-iditol	-	-

Fig. 26 Semiquantitative assessment of growth of *H. jecorina* on several hexitols:
(+++) strong growth up to (-) no growth.

Lad1 is the fungal version of higher eukaryotic SDHs

The data described above revealed that Lad1 acts largely - albeit with different affinities - on the same substrates as mammalian SDH, therefore suggesting that Lad1 may be a fungal orthologue of this enzyme. To test this, we first made a BLAST search of GenBank and the genome databases of *Neurospora crassa* (http://www-genome.wi.mit.edu/annotation/fungi/neurospora/), *Fusarium graminearum* (http://www-genome.wi.mit.edu/annotation/fungi/fusarium/index.html), *Aspergillus fumigatus* (http://www.sanger.ac.uk/Projects/A_fumigatus/) and *Aspergillus nidulans* (http://www-genome.wi.mit.edu/annotation/fungi/aspergillus/). Using the aa-sequence of Lad1 as a query, single putative proteins were obtained from *N. crassa* and *A. fumigatus*, but two proteins of high similarity were obtained for *F. graminearum* and even three for *A. nidulans*. Best hits from organisms outside of the fungal kingdom were obtained with SDHs from mammals, insects and fruits. Using the putative SDH from *Schizosaccharomyces pombe* as an outgroup, parsimony analysis of the respective aa-sequences of the matching SDHs and the putative Lads encoded by genome sequence contigs of the fungal databases (Fig. 27) clearly shows that both D-sorbitol and L-arabinitol dehydrogenases form three strongly supported clades from their common ancestor: One leading to enzymes from filamentous fungi, one leading to plant SDHs, and another one leading to mammalian SDHs. It is interesting to note that Lad1 of *H. jecorina* formed a strongly supported terminal clade with one putative protein from all other fungi investigated, suggesting that this clade represents the true Lad1 orthologue. However, the two other *A. nidulans* proteins and the second protein from *F. graminearum* formed basal branches to this terminal clade, suggesting their formation earlier in evolution. This analysis suggests that Lad1 from *H. jecorina* is a member of the most recent developed orthologous proteins in a fungal branch during evolution of the SDHs.

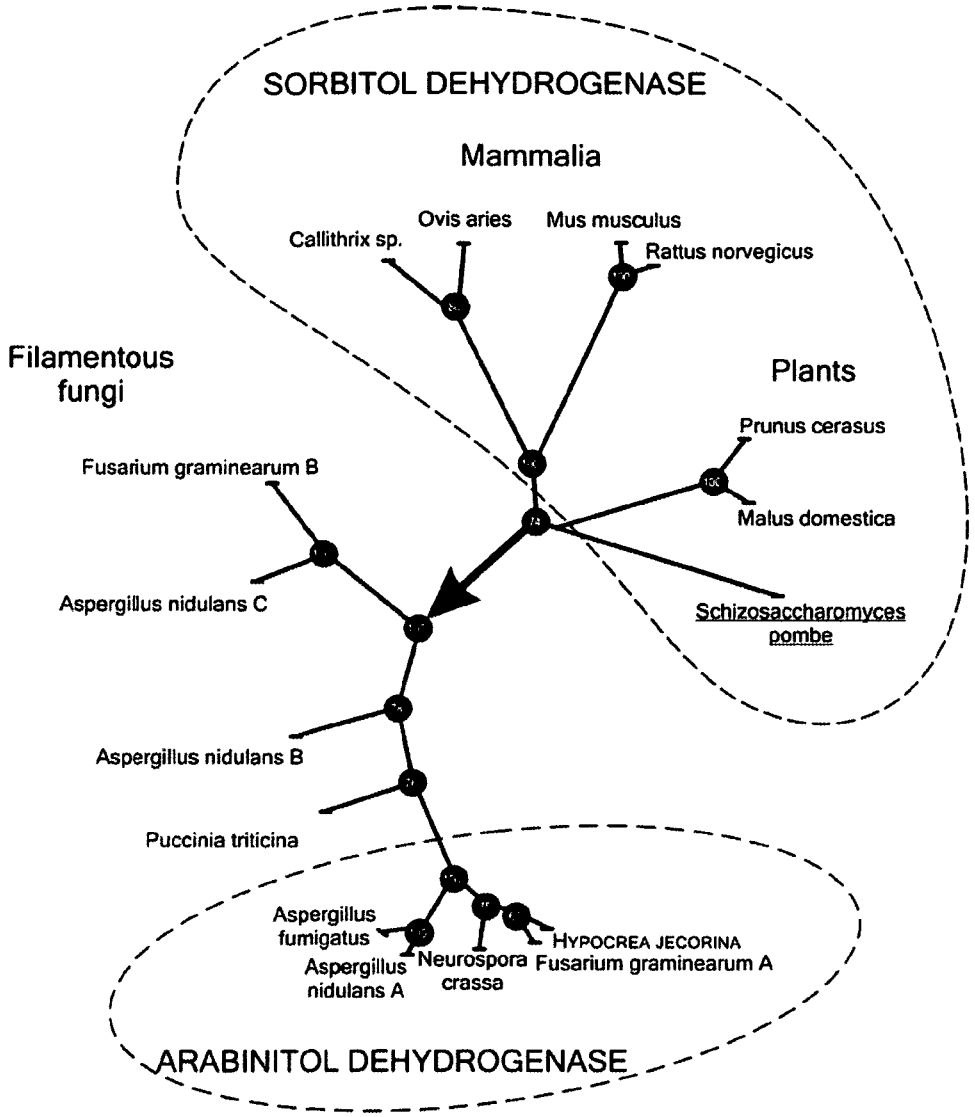


Fig. 27 Evolution of Lad and SDHs.

The radial tree shown is one out of a total of two most parsimonious trees rooted against a putative SDH from *S. pombe* (NP_595120). Numbers at the nodes give bootstrap coefficients (500 random rearrangements). The position of the filamentous fungal D-sorbitol/L-arabinitol dehydrogenases is indicated by a yellow background, and proteins orthologous to *H. jecorina* Lad1 are indicated by a dashed oval. SDHs of different mammalia and plants are indicated by a dashed oval over a white background. The aa-sequences of the respective proteins were retrieved either from GeneBank, or translated from nt-sequences present in the respetive genome databases. Accession numbers: *Callithrix* sp. (AAB69288), *Ovis aries* (S10065), *Mus musculus* (NP_666238), *R. norvegicus* (NP_058748), *Prunus cerasus* (AAK71492), *Malus domestica* (AAL23440), *S. pombe* (NP_595120), *F. graminearum* B (contig. 1.289 [18300, 19800]), *A. nidulans* C (AN8552), *A. nidulans* B (contig 1.75 [104000, 105900]), *Puccinia triticina* (AAP42830), *A. fumigatus* (contig 4846 [24742, 24047]), *A. nidulans* A (AN0942), *N. crassa* (XP_324823), *F. graminearum* A (contig 1.30 [40485, 41668]), *H. jecorina* (AY225444).

The amino acids essential for binding of D-sorbitol are conserved in Lad1

While the enzymatic characteristics of Lad1 are similar to that of SDH in many respects, the preference for pentitols instead of hexitols and the formation of 3-hexuloses from galactitol and D-talitol is a significant difference. We therefore wondered whether this difference would be reflected in a difference between the amino acids known to participate in substrate binding and catalysis by SDH (Johansson *et al.* 2001). To answer this question, we first aligned various mammalian SDHs with the various Lad1 homologues from filamentous fungi (Fig. 27), and predicted the domain structure of the proteins (Fig. 28). This demonstrated that Lad1 and SDHs are structurally strongly conserved, but it also showed that the proteins from the terminal Lad1 clade in Fig. 4 contained a number of amino acid exchanges which were absolutely conserved within this terminal clade but conferred a functional difference to SDH. We thus considered it likely that these aa-exchanges may be responsible for the altered substrate specificity of Lad1 with respect to SDH. In order to see how these aa influence SDH/Lad1 structure, we used the protein explorer on the consurf webpage (http://consurf.tau.ac.il/) to draw a 3D-picture of Lad1 based on the SDH coordinates (Fig. 28). As can be seen, all these amino acids which are involved in polyol binding in the SDH (Johansson *et al.* 2001) are absolutely conserved in Lad1 as well and thus cannot determine the binding efficacy of hexitols and pentitols. On the other hand, many of the amino acids adressed above, which are conserved among the members of the Lad1 terminal clade but which are functionally different from those present in other SDHs, are located at the facing rims of the two domains of the protein which form the substrate binding cleft. It is noteworthy that many of these changes represent exchange of hydrophobic or basic positively charged amino acids against polar or hydrophobic ones, respectively, thereby clearly creating a different environment at the active center.

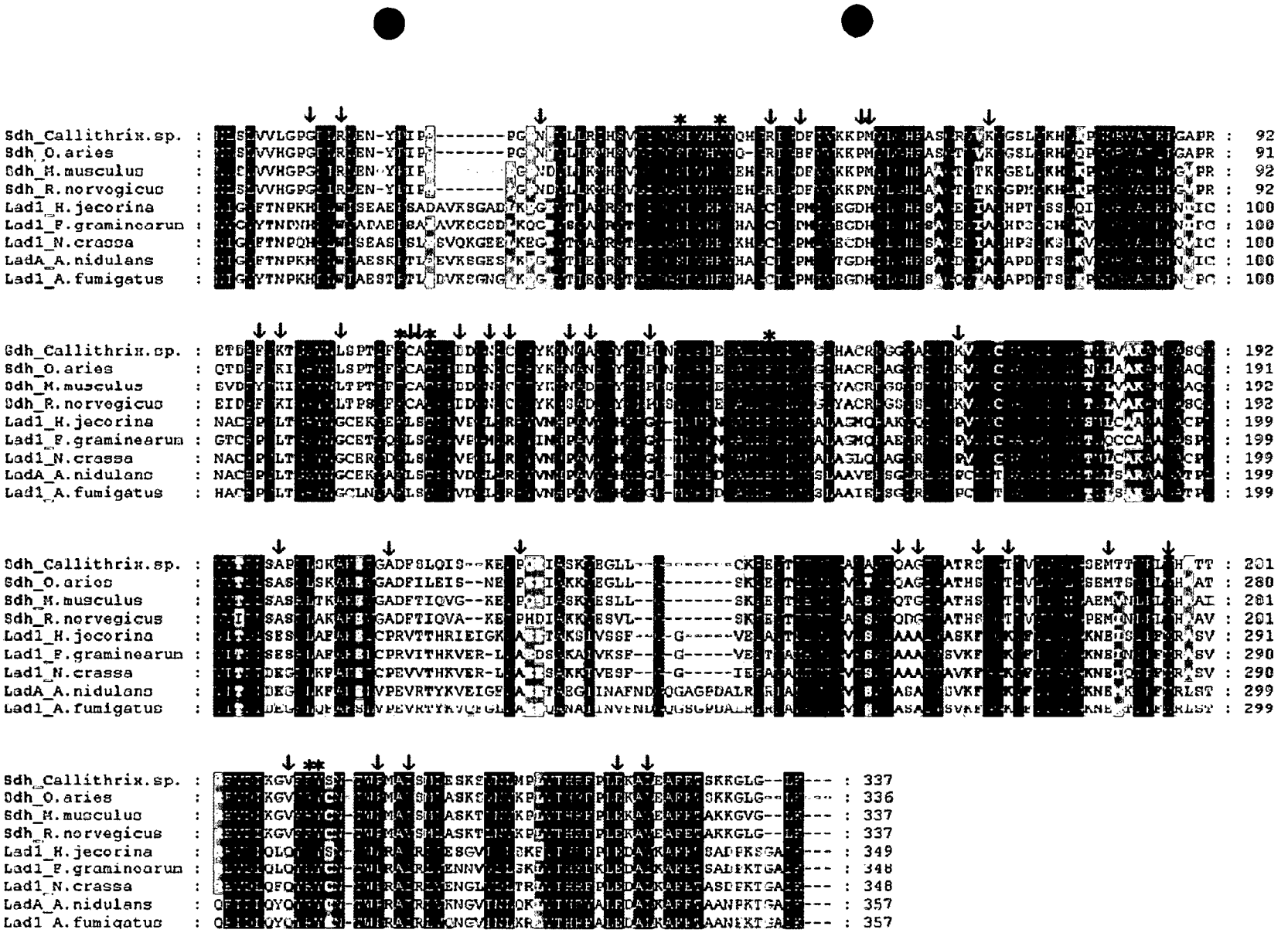
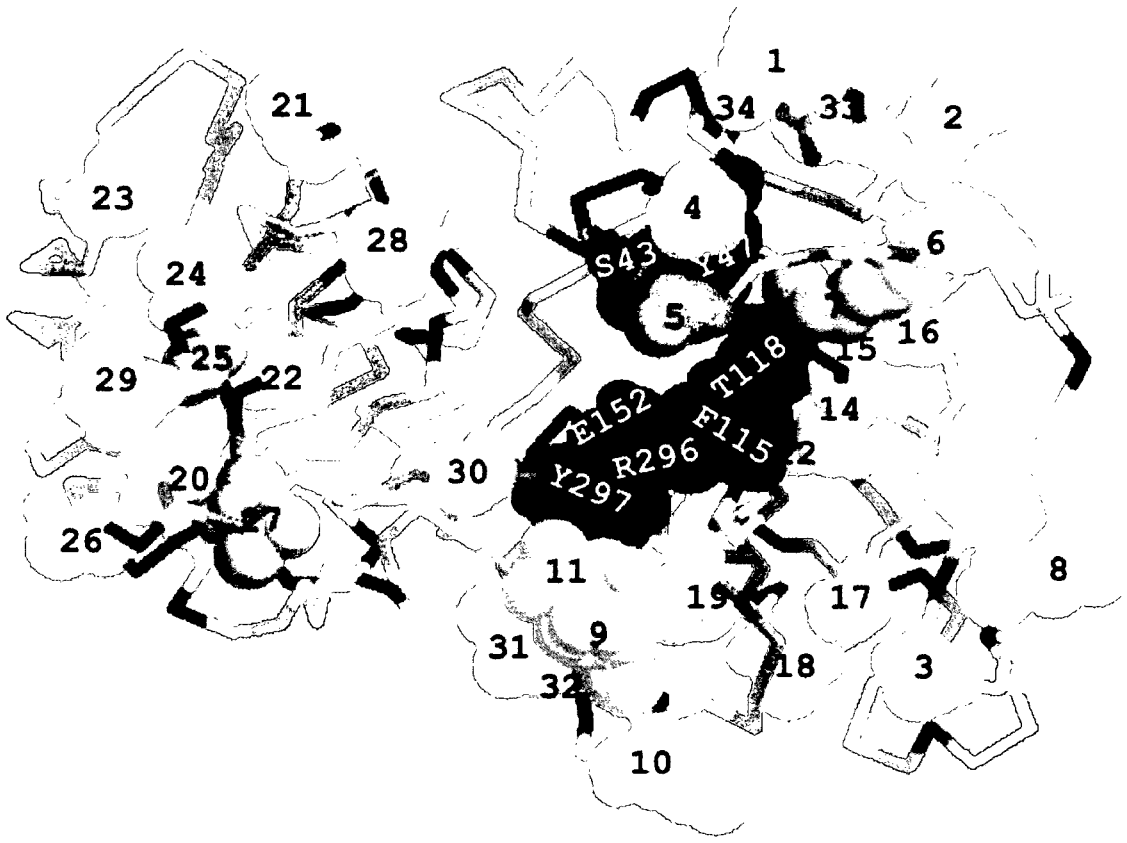


Fig. 28 Sequence alignment of mammalian and fungal sorbitol dehydrogenases

Amino acids involved in binding of D-sorbitol (Johansson *et al.* (2001) are marked by stars, amino acids conserved between Lad1 and SDH are indicated by black background, and amino acids functionally conserved among fungal Lad1 but not in SDH are given by vertical arrows.



1_N14→H	9_F101→P	18_D134→V	27_T266→K
2_R17→W	10_K103→L	19_P140→G	28_M276→E
3_D29→G	11_L109→G	20_T171→P	29_V282→M
4_R52→C	12_C116→L	21_P202→E	30_V294→Q
5_D55→P	13_A117→S	22_A213→P	31_I303→R
6_P61→D	14_D121→V	23_E226→A	32_E306→R
7_M62→H	15_N124→L,M	24_T255→A	33_Q327→D
8_K74→A	16_A126→R	25_G257→A	34_D330→K
	17_A132→P	26_T263→A	

Fig. 29 Lad1 displays significant differences in amino acids flanking the active site.

The 3D-model of Lad1 was fit according to the 2D-structure of SDH (Johannson *et al.*, 2001). Amino acids involved in binding of D-sorbitol (Johannson *et al.*, 2001), and conserved between Lad1 and SDH, are indicated by blue globes. The respective amino acids and their position are indicated by single letters and numbers. The black numbers on the lighter coloured globes indicate the amino acids which are conserved among all fungal Lad1 orthologues but different to SDHs. The aa-exchanges which are indicated by the respective black numbers are listed below the 3D-model.

Discussion

In this paper, we provide evidence that Lad1 of *H. jecorina* is a fungal orthologue of the eukaryotic SDH (L-iditol:2-oxidoreductase, EC 1.1.1.14) protein. The result from a phylogenetic analysis suggests that filamentous fungi have formed a separate branch of SDHs which are especially adapted to the reductive catabolism of hemicellulose monosaccharides available in their environment (e.g. L-arabinose, D-xylose). A comparison of the substrate specificity of Lad1 with that of mammalian SDHs shows that Lad1 has a much higher catalytic efficacy with pentitols than with hexitols. It is therefore intriguing that all the aa-residues which have been shown to be involved in the binding of D-sorbitol by SDH (i.e. S43, Y47, F115, T118, E152, R296 and Y297) are strictly conserved in Lad1 as well. Obviously, the different efficacy of substrate conversion depends on the presence of the amino acids flanking the active site cleft. As shown in the putative 3D-model, we have identified a number of aa-changes - conserved among members of the terminal arabinitol dehydrogenase cluster but significantly different to mammalian SDHs - which are located in this area of the protein. Although merely speculative at the moment, we consider it possible that these amino acids are reponsible for the differences in the activity and affinity pattern between Lad1 and SDH.

The fact that *N. crassa* or *H. jecorina* contain only a single protein (i.e. Lad1) with similarity to mammalian SDHs is consistent with our claim that arabinitol dehydrogenase is the fungal version of SDH, and is consistent with the fact that no further SDH-encoding gene present in the *N. crassa* (http://www-genome.wi.mit.edu/annotation/fungi/neurospora/) or *H. jecorina* (data not shown) genome. However, some of the fungi (*A. fumigatus, F. graminearum* and *A. nidulans*) contained one or two further genes encoding proteins with high similarity, and which arose earlier in evolution. Unfortunately, all these proteins are only known from the respective gene sequence, and thus their enzymatic properties - if they are transcribed and translated at all - are not known. The aa-changes adressed above are only partially present in these putative proteins, and thus a knowledge of their substrate specificity may provide a clue as to identify the amino acids responsible for the differences in the substrate specificity in SDH and Lad1.

Apart of the generally different pattern of activity against pentitols and hexitols, most of the substrate-product pairs of Lad1 and SDH are the same, i.e. they use the same catalytic mechanism. A strong difference in the substrate specificity between the two, however, is the oxidation of galactitol and D-talitol. Lad1 oxidizes them at C4, yielding L-xylo- and D-arabino-3-hexulose, respectively. One of the corresponding products of the SDH reaction (D-tagatose) is not reduced by Lad1 (unpublished data), and the other one (L-psicose) was unavailable for this study, but the two 3-hexuloses are converted to galactitol and D-talitol, thus proving that their identification as products of the reaction is no artefact. The occurence of these two 3-hexuloses in nature has so far not been reported, although the D-xylo-3-hexulose-6-phosphate is an intermediate in the autotrophic carbon dioxide metabolism in archaebacteria (Yaoi *et al.* 2000). Also, (Reichert 1994) reported that an L-glucitol dehydrogenase of a *Pseudomonas* sp. formed L-xylo-3-hexulose from galactitol, but the physiological relevance of this finding has not been pursued further. It is possible that the changes in the structure of the active center, which have accompanied the change in substrate preference as discussed above, may have resulted in a binding of galactitol and D-talitol in such a way that the zinc atom is coordinated to C4.

However, a more detailed interpretation of these data requires the determination of the 3D structure of Lad1 first.

The at least 10-fold higher k_{cat}/K_m values of Lad1 for the pentitols L-arabinitol and xylitol than for the various hexitols are in accordance with the postulated main role of this enzyme in pentose metabolism. In this study, we have provided evidence for such a role *in vivo*, thus proving that the enzyme indeed takes part in catabolism of L-arabinose. The high k_{cat}/K_m values of Lad1 are also consistent with the role of this enzyme in D-xylose catabolism in an *xdh1* (xylitol dehydrogenase-encoding) knock-out mutant (Seiboth *et al.* 2003). The very low k_{cat}/K_m values for galactitol are therefore in contrast to the role of Lad1 in the alternative D-galactose degrading pathway in *H. jecorina* shown in this paper, and may explain the transient accumulation of up to 400 mM galactitol during its action (Seiboth *et al.* 2004). The identification of L-xylo-3-hexulose as the product of galactitol oxidation and thus as an intermediate of this pathway raises the question, which enzymes may participate in its further metabolism. Phosphorylation of a 3-hexulose at the C6 hydroxyl group by hexokinase has not been studied yet, and there are reports claiming that the substrate specificity of hexokinase is restricted to C2 in ketohexoses (Machado de Domenech and Sols 1980). In bacteria, D-xylo-3-hexulose-6-phosphate is isomerized by the enzyme 3-hexulose-6-phosphate isomerase to fructose-6-phosphate (Martinez-Cruz *et al.* 2002) however, we were unable to find any sequences with sufficient similarity to the 3-hexulose-6-phosphate isomerase gene from *E. coli* (NP_418039) in the genome databases of *Fusarium graminearum* and *Neurospora crassa*.

(Fekete *et al.* 2004) have recently reported that galactitol is oxidized to L-sorbose in *A. nidulans* by L-arabinitol dehydrogenase from cell-free extracts. We do not know yet whether *A. nidulans* and *H. jecorina* L-arabinitol dehydrogenases differ in their reactions patterns, or whether the L-sorbose accumulating in cell-free extracts was due to more than one enzymatic step. We are currently studying the three Lad-proteins of *A. nidulans* to clarify this discrepancy.

Using the Δ*lad1* strain, we were also able to study the role of *lad1* in the catabolism of other hexitols, although we must note that these experiments are no absolute proof for an involvement for the enzymatic reaction of Lad1 and could also be due to an indirect effect of *lad1* knock-out on another gene. On the other hand, the lack of growth of the wildtype strain on D-allitol and L-iditol may either be due to a lack of uptake of these hexitols, or die to a lack of *lad1* expression by these compounds, because *H. jecorina* can grow on the corresponding products of the Lad1 reaction (D-psicose and L-sorbose, respectively). Conversely, Lad1 is clearly not involved in the metabolism of D-mannitol; it is likely that this hexitol is oxidized by L-xylulose reductase (EC 1.1.1.10), which acts as a mannitol dehydrogenase (Richard *et al.* 2002).

Chapter THREE

L-sorbose metabolism in *Hypocrea jecorina*

Summary

The catabolism of L-sorbose was investigated in the fungus *Hypocrea jecorina* (*Trichoderma reesei*), an industrial producer of cellulases, because L-sorbose is a potent inducer of cellulases and a putative intermediate in the catabolism of lactose, the major soluble carbon source for industrial cellulase production. Therefore we compared growth and enzyme expression on L-sorbose and D-sorbitol in strains deleted in the *lxr1* (L-xylulose reductase) gene, the *xdh1* (xylitol dehydrogenase) gene, the *lad1* (L-arabinitol 4-dehydrogenase) gene, and a Δ*xdh1/lad1* double deletion strain. Almost no growth on L-sorbose and D-sorbitol was found in the Δ*xdh1/lad1* and Δ*xdh1* strain while Δ*lad1* strain could grow with a reduced growth rate. Growth of the Δ*lxr1* strain was only affected on L-sorbose but not on D-sorbitol. D-sorbitol dehydrogenase activity in cell free extracts from D-sorbitol grown mycelia was lost in the Δ*xdh1/lad1* and strongly reduced in the Δ*xdh1,* whereas the reduction in the Δ*lad1* strain was considerably lower. Northern analysis showed that both dehydrogenase genes are induced by D-sorbitol. The major importance of xylitol dehydrogenase over L-arabinitol dehydrogenase in L-sorbose/D-sorbitol catabolism is also reflected in its significantly higher k_{cat}/K_m values for D-sorbitol (312 [±102] vs 3 [±0.13]). We thus conclude that L-sorbose is catabolized via L-xylulose reductase to D-sorbitol and catalyzed under the major participation of xylitol dehydrogenase and under minor participation of L-arabinitol dehydrogenase to D-fructose.

Introduction

L-sorbose (L-xylo-2-hexulose) has originally been identified in the juice of the mountain ash (*Sorbus aucuparia*), but occurs naturally also in several other plants particularly *Rosaceae* (Webb and Burley 1962). While most bacteria can assimilate L-sorbose, its catabolism in fungi is less well understood because it inhibits the β-1,3-glucan synthase involved in fungal cell-wall synthesis and is consequently toxic (Mishra and Tatum 1972). Mutants resistant to L-sorbose have been isolated from *Neurospora crassa* (Klingmüller 1967; Klingmüller *et al.* 1970) and *Aspergillus nidulans* (Elorza and Arst 1971), most of them mapping at loci involved in hexose transport, but no mutants in L-sorbose metabolism were obtained. In contrast, we have recently shown that the lack of *Aspergillus nidulans* to grow on L-sorbose is not apparent when ammonium ions are used as a nitrogen source (Fekete *et al.* 2004), suggesting that fungi are able to assimilate this monosaccharide.

Hypocrea jecorina (*Trichoderma reesei*) is a fungus which is industrially used for the production of cellulases (Penttilä 1998; Persson *et al.* 1991). We became interested in its pathway for L-sorbose catabolism, because L-sorbose is a potent inducer of cellulases in *H. jecorina* (Nogawa *et al.* 2001), and thus an understanding of L-sorbose metabolism may enable us to manipulate the pathway towards improved cellulase gene expression. In addition, a second pathway for catabolism of D-galactose besides the Leloir pathway is present in *H. jecorina* which involves the reduction of D-galactose to galactitol catalyzed by L-arabinitol 4-dehydrogenase (Seiboth *et al.* 2004). A second pathway for D-galactose is also found in *A. nidulans* involving L-sorbose as an intermediate (Fekete *et al.* 2004). An elucidation of L-sorbose catabolism may thus identify the later steps of this pathway in fungi. This is especially interesting since D-galactose is part of lactose (1,4-O-β-D-galactopyranosyl-D-glucose) a disaccharide which induces cellulases and is therefore used as carbon source for the industrial production of cellulases.

With respect to the pathway of L-sorbose catabolism in fungi, Crocken and Tatum (1968) (Crocken and Tatum 1968) suggested that in *N. crassa* L-sorbose is first reduced to D-sorbitol, and then oxidized to D-glucose. (Elorza and Arst 1971) also proposed the reduction of L-sorbose to D-sorbitol, but postulated that D-sorbitol is oxidized to D-fructose in *A. nidulans*. The enzymes involved in either pathway have not been identified yet. (Elorza and Arst 1971) made use of a putative D-sorbitol dehydrogenase mutant (*sbA3;* (Roberts 1963)). Unfortunately, strain *sbA* can not be a mutant in a D-sorbitol dehydrogenase, because the mutant phenotype vanishes in the presence of ammonium as a nitrogen source (Fekete *et al.* 2004).

Sorbitol dehydrogenase (L-iditol:NAD+-oxidoreductase, EC 1.1.1.14) is a member of the alcohol dehydrogenase family, which displays a broad specificity against various polyols. Two enzymes with activity on D-sorbitol, encoded by different genes, have been identified in *H. jecorina*: a xylitol dehydrogenase [xylitol:NAD^+ 2-oxidoreductase (D-xylulose forming), EC 1.1.1.9], and a L-arabinitol 4-dehydrogenase [L-arabinitol:NAD^+ 4-oxidoreductase (L-xylulose-forming) EC 1.1.1.12]. The latter has been shown to be evolutionary linked to mammalian D-sorbitol dehydrogenases, yet having developed distinctly different substrate binding affinities (Pail *et al.* 2004). Both enzymes catalyze the oxidation of D-sorbitol to D-fructose, but their involvement in L-sorbose catabolism has not been investigated yet.

In this paper we have therefore critically re-examined whether the reductive/oxidative pathway proposed by (Elorza and Arst 1971) is responsible for L-sorbose catabolism in *H. jecorina*, thereby making use of recombinant mutants in xylitol dehydrogenase and L-arabinitol 4-dehydrogenase encoding genes. In addition, we tested the hypothesis that the recently described L-xylulose reductase of *H. jecorina*, which has L-sorbose reductase activity (Richard *et al.* 2002), may be responsible for reduction of L-sorbose to D-sorbitol *in vivo*.

Materials and Methods

Strains and culture conditions

H. jecorina strains used in this study are listed in Table 5. All strains were maintained on malt extract agar. Strains were grown in 250 ml in 1-l Erlenmeyer flasks on a rotary shaker (250 rpm) at 30°C. For growth on L-sorbose, strains were pregrown in the medium described by Mandels and Andreotti (1978) with peptone as carbon source at a final concentration of 5 g/l for 24 h and then replaced to a medium contaning 5 mM phosphate buffer (pH 5.0) with 1.4 g/l $(NH_4)_2SO_4$, 2.0 g/l KH_2PO_4, 0.3 g/l $MgSO_4$, 0.4 g/l $CaCl_2$, 0.5 mg/l $FeSO_4$, 16 mg/l, $MnSO_4$, 14 mg/l $ZnSO_4$, 17 mg/l $CoCl_2$ and 10 g/l L-sorbose.

Escherichia coli strain JM109 (Promega, Madison, WI) was used for plasmid propagation.

Table 5 Strains of *H. jecorina* used in this study

Strain	Genotype	Reference
QM9414	wild-type	(Mandels, 1975)
ΔXDH1	*xdh1*-knock out	(Seiboth *et al.*, 2003)
ΔLAD1	*lad1*-knock out	(Seiboth *et al.*, 2003)
ΔXDH1LAD1	*xdh1-lad1*-double knock out	(Seiboth *et al.*, 2003)
ΔLXR1	*lxr1*-knock out	(Seiboth *et al.*, 2004)

Determination of fungal growth

To determine hyphal growth on agar plates, plates were inoculated with a small piece of agar in the centre of an 11-cm plate. Biomass in submerged cultivations was measured by filtering 2 x 20 ml of culture onto pre-dried and pre-weighted Whatman GF/C filters. After washing each filter with at least 20 ml of deionized water, the harvested biomass was dried to constant weight (80°C for at least 3 days).

Preparation of cell-free extracts

H. jecorina strains were pregrown for 24 h on Mandels-Andreotti medium with glycerol as carbon source and replaced for 5 h to L-sorbose and D-sorbitol as carbon source. Mycelia were then harvested by filtration, washed with cold tap water, squeezed between filter paper and ground to a fine powder in

liquid nitrogen. One gram (wet weight) of ground mycelium was mixed with 3 ml of extraction buffer (0.1M Tris-HCl pH 7.5, 1mM EDTA, 5mM β-mercaptoethanol), suspended thoroughly and homogenized by sonification (15 x 20 sec power and cycle 70 (Bandelin Sonoplus HD60, Berlin, Germany) at 2°C, with intermittent 2 min cooling periods). The resulting homogenate was centrifuged at 10000 x g for 20 min at 4°C. The supernatant (average protein concentration 8 -15 mg/ml) was used as a cell-free extract for enzyme assays and for storage on -20°C glycerol was added to a final concentration of 20 % (v/v).

Recombinant expression of Xdh1 and Lad1 in *E. coli*

H. jecorina dehydrogenases were expressed as GST fusion protein as described recently (Pail *et al.* 2004; Seiboth *et al.* 2003). Purification using Glutathione Sepharose 4B and thrombin cleavage of fused protein bound to a column matrix was performed according to the manufacturer's protocol (Amersham Biosciences, Uppsala, Sweden). Physical homogeneity of the overproduced protein was verified by SDS-PAGE in 10 % polyacrylamide gels and Coomassie Blue protein staining as described by (Ausubel *et al.* 2003). Homogenously purified fractions were either used directly or stored until use at -80°C in the presence of 1 % (w/v) BSA.

Enzyme assays

Conditions for the spectrophotometric assays for Xdh1 and Lad1 have been described previously (Pail *et al.* 2004; Seiboth *et al.* 2003). Activities are expressed as nkat, one nkat corresponding to the conversion of one nmol of substrate per sec, and given as specific activities (kat/mg protein). Protein concentration was determined with the Bio-Rad Protein Assay (Bio-Rad Laboratories). Michaelis-Menten constant K_m and maximal velocity V_{max} were graphically determined by direct linear plotting (Cornish-Bowden and Eisenthal 1978; Eisenthal and Cornish-Bowden 1974). Molecular masses of Xdh1 and Lad1 of 38,400 and 39,891 Da were used for calculation of k_{cat}.

RNA isolation and hybridization

This was performed essentially as described previously (Seiboth *et al.* 2002). Total RNA was isolated as described by Chomczynski and Sacchi (1987). Standard methods (Sambrook *et al.* 2001) were used for electrophoresis, blotting and hybridisation of nucleic acids. Probes for hybridization were: a 1.2-kb (1164 bp) cDNA *lad1* PCR fragment (Pail *et al.* 2004), a 1.2-kb cDNA *xdh1* PCR fragment (Seiboth *et al.* 2003), and a 290-bp 18S RNA fragment.

Results

Xylitol dehydrogenase is of major importance for assimilation of D-sorbitol by *H. jecorina*

We have previously shown that xylitol dehydrogenase and L-arbinitol 4-dehydrogenase catalyzes both the oxidation of D-sorbitol in vitro. Therefore, we examined the ability of the respective deletion mutant strain to grow on D-sorbitol by measuring the increase in mycelial dry weight in submerged cultures

(Fig. 29). The strain deleted in the *xdh1* gene and the double knock-out mutant were almost unable to grow on D-sorbitol whereas the deletion of *lad1* only resulted in a reduced growth rate compared to the parent strain on this carbon source. Having established the relative roles of Xdh1 and Lad1 in the assimilation of D-sorbitol, we tested the contribution of these enzymes to the catabolism of L-sorbose. Consistent with the data obtained for D-sorbitol, the double knock-out mutant failed to grow on L-sorbose, while the single mutants mirrored the effect on growth on D-sorbitol. These data show that xylitol dehydrogenase is of major importance to D-sorbitol assimilation and that a D-sorbitol dehydrogenase non uilizing mutant is not able to catabolize L-sorbose.

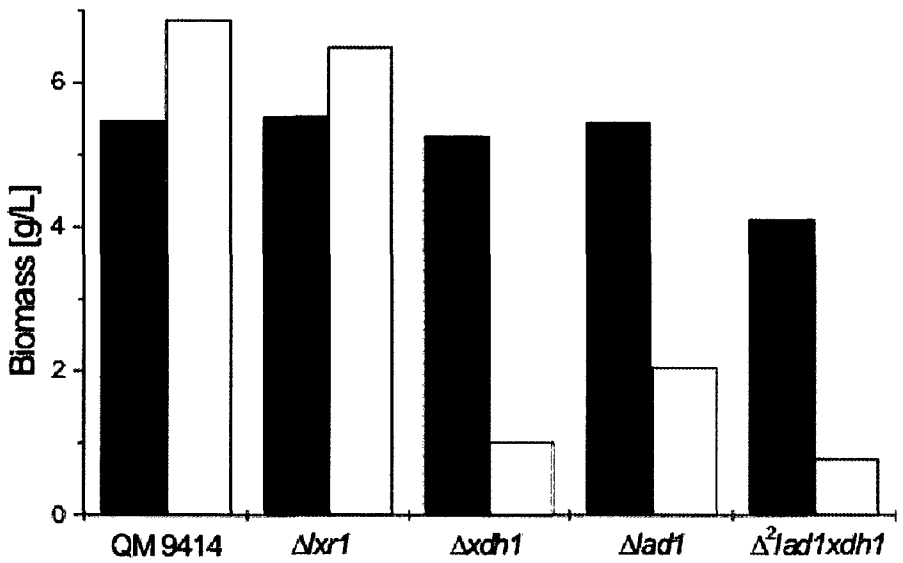


Fig 29 Biomass accumulation of various *H. jecorina* strains on D-glucose (filled bars) **and on D-sorbitol** (open bars) after 42 hours.

Xylitol dehydrogenase accounts for the major *in vitro* D-sorbitol dehydrogenase activity of *H. jecorina*

The initial hypothesis of this paper was that either Xdh1 or Lad1 or both may be responsible for the D-sorbitol dehydrogenase activity in *H. jecorina*, and therefore are involved in catabolism of L-sorbose. We grew the parent strain of *H. jecorina*, together with the three isogenic strains on glycerol, and then replaced the formed mycelia to L-sorbose and D-sorbitol. Cell-free extracts prepared from the parent strain exhibited clear D-sorbitol dehydrogenase activity, whereas the activity was reduced to 58.4 % (± 10.3) in the L-arabinitol 4-dehydrogenase mutant, and to 16.6 % (± 5.6) in the xylitol dehydrogenase mutant. D-sorbitol dehydrogenase activity was below the detection limit in the double knock-out mutant (Table 6). We can therefore conclude that xylitol dehydrogenase accounts for the major portion of the D-sorbitol dehydrogenase of *H. jecorina*, and that Xdh1 and Lad1 are the only enzymes with this activity under the given conditions in this fungus. In contrast expression levels on L-sorbose were only constitutive, which would indicate that L-sorbose does not induce D-sorbitol dehydrogenase activity.

Table 6 D-sorbitol dehydrogenase activity of the *H. jecorina* strains QM9414, ΔXDH1, ΔLAD1, and ΔXDH1LAD1 after transfer to D-sorbitol (Mycelia were pregrown on glycerol (24 hrs) and then transferred to D-sorbitol for 6 hrs).

Strain	D-sorbitol dehydrogenase specific activity [nkat/mg protein]
QM9414	0.00048 (± 0.00004)
Δ*lad1*	0.00028 (± 0.00003)
Δ*xdh1*	0.00008 (± 0.00002)
Δ*lad1xdh1*	N.D.

L-xylulose reductase catalyzes the reduction of L-sorbose to D-sorbitol *in vivo*

Data presented until here indicate that the assimilation of both D-sorbitol as well as L-sorbose require xylitol dehydrogenase or L-arabinitol dehydrogenase, thus suggesting that L-sorbose is metabolized via D-sorbitol. A candidate enzyme catalyzing the conversion of L-sorbose to D-sorbitol in vitro was identified by (Richard *et al.* 2002). They have recently reported that the *lxr1*-encoded L-xylulose reductase shows activity with L-sorbose. Consequently, we tested a Δ*lxr1* strain whether it would be able to grow on L-sorbose or D-sorbitol. The results, shown in Fig. 30, confirm that the Δ*lxr1* strain is unable to grow on L-sorbose, but can assimilate D-sorbitol just as the wild-type strain. L-xylulose reductase is therefore the enzyme converting L-sorbose to D-sorbitol.

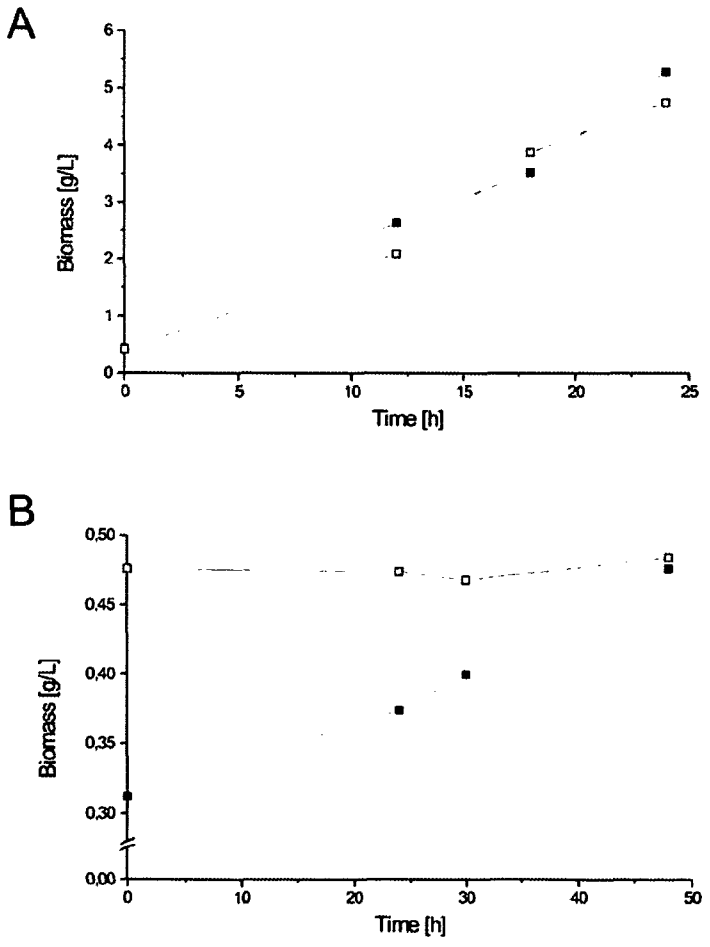


Fig. 30. Growth of *H. jecorina* QM9414 (■) and Δ*lxr1* (□) on D-glucose (A) and L-sorbose (B). Strains were pregrown on peptone and replaced on L-sorbose containing medium as described in Materials and Methods.

Xylitol dehydrogenase has a significantly better performance on D-sorbitol than L-arabinitol dehydrogenase

The superior role of Xdh1 in D-sorbitol catabolism should in theory also be reflected in its catalytic properties. We have previously reported preliminary kinetic data (K_m) of Xdh1 for D-sorbitol (24 mM) which compares favourably with that of Lad1 for D-sorbitol (46 mM). As K_m values only poorly reflect the *in vivo* activity of an enzyme, we have also determined the performance of Xdh1 for D-sorbitol. In contrast to (Seiboth *et al.* 2003) we now characterized the enzyme without the GST fused to the recombinant protein, because of the general better performance (Table 7). This shows clearly that the k_{cat}/K_m values of Xdh1 for D-sorbitol are 100-fold, and for D-fructose are about 90-fold higher compared to Lad1, therefore explaining the major role of Xdh1 in D-sorbitol metabolism. Interestingly, Xdh1 also had a high performance with D-allitol and L-iditol (210.7 [± 86.7] $M^{-1}.s^{-1}$ and 80 [± 19.4] $M^{-1}.s^{-1}$, respectively), whereas that with the presumed natural substrate xylitol was comparably small (436 [± 180.5]).

Table 7 Comparison of the **kinetic characteristics of recombinant** produced **xylitol dehydrogenase and L-arabinitol 4-dehydrogenase**

		Xylitol dehydrogenase	L-arabinitol 4-dehydrogenase[1]
k_{cat} ($kat.mol^{-1}$)	D-sorbitol	4.73 [± 0.32]	0.137 [± 0.0024]
	D-fructose	0.79 [± 0.01]	0.034 [± 0.0002]
k_{cat}/K_m ($M^{-1}.s^{-1}$)	D-sorbitol	311.7 [± 102.3]	3.01 [± 0.31]
	D-fructose	31.8 [± 1.29]	0.35 [± 0.01]

[1] values for L-arabinitol 4-dehydrogenase were taken from [Pail et al. (2004)]

xdh1 and *lad1* expression on D-sorbitol and L-sorbose

The conclusion drawn above is only correct if the the enzymes do not influence the formation of each other. In order to test this, we examined the expression of *xdh1* and *lad1* upon transfer to D-sorbitol and L-sorbose in the parent strain and in the knock-out strains (Fig. 31). As can be seen in the Northern analysis both genes are induced by D-sorbitol. On L-sorbose only in the single mutants transcripts of the respective genes were detected, while only extremely low levels of both genes could be observed after prolonged exposition (data not shown) in QM9414. This could indicate that during the very slow catabolism of L-sorbose only extremely low levels of D-sorbitol accumulate which are consequently converted by the low constitutive level of the dehydrogenases. Whereas in the single knock out strains enough D-sorbitol accumulates to induce the transcription of the respective genes.

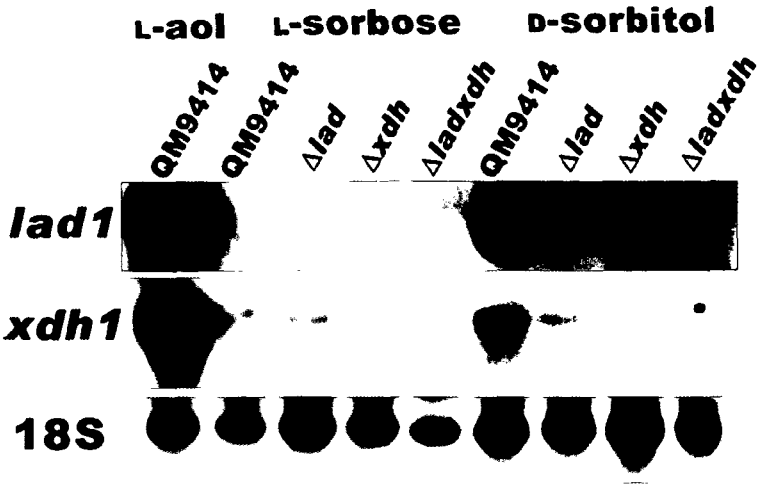


Fig. 31. Expression of *lad1* and *xdh1* upon transfer to D-sorbitol and L-sorbose as a carbon source, respectively

Discussion

Despite of the fact that L-sorbose is a strong inhibitor of ß-glucan synthase and therefore fungal growth (Mishra and Tatum 1972), *H. jecorina* is capable of assimilating L-sorbose and grow on it at a low rate. We have therefore used this fungus as a model to assess the pathway by which L-sorbose is catabolized. The results from this paper clearly demonstrate that L-sorbose is reduced to D-sorbitol by a ketoreductase, L-xylulose reductase Lxr1, and subsequently oxidized to D-fructose by either xylitol deyhdrogenase Xdh1 or L-arabinitol dehydrogenase Lad1. This pathway therefore confirms the scheme proposed for *A. nidulans* by (Elorza and Arst 1971). The subsequent metabolism of D-fructose has not been investigated in *H. jecorina*, but we assume that it will take place by a hexokinase, as in *A. nidulans* (Fekete *et al.* 2004). The involvement of a fructokinase, as in mammalian systems (Vestling *et al.* 1950), can still not be ruled out at this stage. However, screening the *H. jecorina* genome sequence database (Preliminary sequence data was obtained from The Department of Energy Joint Genome Institute (www.jgi.doe.gov) prior to publication of the genome sequence) for genes encoding an orthologue of mammalian fructokinase did not produce convincing results (unpublished findings); furthermore, the presence of fructokinase activity (i.e. formation of fructose-1-phosphate) has so far not been reported for any fungus.

Although both xylitol dehydrogenase and L-arabinitol dehydrogenase are able to oxidize D-sorbitol to D-fructose, gene knock out experiments showed that a deletion in *xdh1* has a much more drastic effect than that of *lad1* and that xylitol dehydrogenase is therefore the major enzyme participating in D-sorbitol catabolism. This conclusion is also supported by a comparison of the kinetic characteristics of Xdh1 and Lad1, as the former has a much higher K_m/K_{cat} value for D-sorbitol. (Pail *et al.* 2004) have recently demonstrated that L-arabinitol dehydrogenase evolved as a separate branch from the evolutionary line leading to mammalian sorbitol dehydrogenases, to which they exhibit the highest similarity of all fungal enzyme candidates. It is intriguing to see that this evolution towards

effective L-arabinose metabolism has been compensated by the evolution of another enzyme - Xdh1 - for D-sorbitol. This finding also shows that, despite of the fact that Xdh1 and Lad1 display largely the same substrate specificity, differences in substrate affinity may be physiologically relevant. How the fungal cell actually manages to distinguish between these two enzymes for the action on D-sorbitol is unclear at the moment. However, the rather high K_m values of the enzymes involved (Pail *et al.* 2004; Richard *et al.* 2001; Richard *et al.* 2002; Seiboth *et al.* 2003) and the structure of the pathways suggests that enzyme-enzyme interactions and metabolite channeling [Jaklitsch , 1988 #193] could take place in the reductive pathways of D-xylose, L-arabinose, D-galactose and L-sorbose catabolism. We have already shown that Lad1 can at least partially replace the role of Xdh1 in D-xylose and L-arabinose catabolism but that Xdh1 can only contribute to a very small part in L-arabinose catabolism.

(Fekete *et al.* 2004) have recently provided evidence for L-sorbose as an intermediate in the reductive pathway of D-galactose catabolism in *A. nidulans*, but did not identify the enzymes involved in its further metabolism. It is likely that the enzymes identified in this paper - L-xylulose reductase, xylitol dehydrogenase and L-arabinitol dehydrogenase - will also be responsible for D-galactose metabolism *via* the reductive pathway after formation of L-sorbose. Double deletion strains in *lxr1* or *xdh1* together with the Leloir pathway galactokinase gene *gal1* are still necessary to prove this point. If our assumption is correct, then almost the whole structure of the reductive D-galactose catabolic pathway would have been elucidated, the only piece left being the step between L-xylo-3-hexulose and L-sorbose (Pail *et al.* 2004). It is also striking that the order of reactions and the participating enzymes are identical to those assumed to take place in the L-arabinose catabolic pathway. This may explain why the enzymes of the pathway are induced by both D-galactose and L-arabinose (Seiboth *et al.* 2004; Seiboth *et al.* 2002).

The possibility of L-sorbose as an intermediate of D-galactose metabolism in *H. jecorina* would also be interesting in view of the reported ability of L-sorbose to induce cellulases in this fungus (Nogawa *et al.* 2001), as it would potentially link two inducers - lactose and L-sorbose - to a common pathway and possibly also common mechanism. We are currently testing the induction of cellulase formation by L-sorbose in different mutants of the Leloir and reductive D-galactose catabolic pathways.

Chapter FOUR

Characterization of the metabolic role of *Hypocrea jecorina* D-xylose reductase

Summary

The *Hypocrea jecorina* (anamorph: *Trichoderma reesei*) D-xylose reductase encoding gene (*xyl1*) was cloned and characterized. Transcription of *xyl1* was most abundant during growth on D-xylose, xylan, L-arabinose and L-arabinitol. Low levels were also found on lactose, D-galactose and xylitol. The encoded protein Xyl1 is a member of the aldo/ketoreductases superfamily and shows high identity (up to 60%) to its homologues in *Aspergillus niger* and *Pichia guilliermondii*. The enzyme catalyzes the reduction of D-xylose, L-arabinose, D-ribose, D-galactose and D-glucose with NADPH as cofactor. Disruption of the *xyl1* gene strongly reduces growth on D-xylose and L-arabinose but the remaining growth and residual aldose reductase activity indicate the presence of at least one other enzyme with similar substrate specificities. In addition, Δ*xyl1* strains show a slower growth on D-galactose and its D-galactose reductase activities were reduced. The reduction in growth of Δ*xyl1* is lower compared to strains, in which the Leloir pathway of D-galactose catabolism is interrupted by the deletion of the *gal1* gene encoding galactokinase. Strains deleted in *xyl1* and *gal1* are almost unable to grow on D-galactose. Furthermore the deletion of *xyl1* resulted in a strong reduction of cellulase transcript levels during growth on lactose, although the effect was not as strong as in *gal1* deleted strains. This indicates that the *H. jecorina* D-xylose reductase is involved in the reductive catabolism of D-xylose, L-arabinose and D-galactose and that both the reductive and the Leloir pathway operate in parallel during normal growth on D-galactose. The operation of both pathways is necessary for high level induction of cellulases on lactose.

Introduction

Trichoderma reesei, the anamorph of the pantropical ascomycete *Hypocrea jecorina*, occurs as a saprophytic fungus in tropical soils. It is consequently able to metabolize a wide spectrum of carbon sources including several polysaccharides and therefore produces a large variety of degrading enzymes like hydrolases, glucanases, etc. Because of the high expression levels of some cellulase genes (e.g. *cbh1*, encoding cellobiohydrolase 1, which makes up 60% of the total secreted proteins upon growth on cellulose (Gritzali and Brown 1979), *H. jecorina* is used for the industrial production of cellulolytic and hemicellulolytic enzymes and for the expression of recombinant proteins under control of the strong cellulase promoters (Nevalainen *et al.* 1994; Uusitalo *et al.* 1991).

Hemicelluloses are among the major polysaccharides used by *H. jecorina*. Two of their main constituents - D-xylose and L-arabinose - are metabolized by an interconnected pathway: L-arabinose is first reduced to L-arabinitol and then converted to L-xylulose, which in turn is reduced to xylitol (Witteveen *et al.* 1989). After further conversion to D-xylulose and phosphorylation it enters the pentose phosphate pathway. D-xylose is reduced directly to xylitol which is then metabolized by the same reactions as described before (Chiang and Knight 1960b). So far, the enzymes of the D-xylose-degradading steps have been described in yeasts (*Pachysolen tannophilus*, *Candida shehatae* and *Pichia stipitis* (Bolen *et al.* 1996; Ho *et al.* 1990; Verduyn *et al.* 1985) and in *Aspergillus niger* (Hasper *et al.* 2000). but in *H. jecorina* only the enzymes catalyzing the oxidation of L-arabinitol (L-arabinitol-4-dehydrogenase, EC 1.1.1.12) (Richard *et al.* 2001) and of xylitol (xylitol dehydrogenase, EC 1.1.1.9) (Wang *et al.* 1998) and the reduction of L-xylulose (L-xylulose reductase, EC 1.1.1.10) (Richard *et al.* 2002) have been described. In this work we cloned and characterized the enzyme D-xylose reductase (Xyl1) which, as we show, is responsible for the first step of both pathways, the reduction of D-xylose or L-arabinose, respectively.

Beside the pentoses mentioned above D-galactose is also a major constituent of plant cell walls, e.g. of galactomannan, xylan and pectin (de Vries and Visser 2001). The Leloir pathway for D-galactose metabolism in both prokaryotes and eukaryotes is already well established (Frey 1996; Holden *et al.* 2003). The first step is the phosphorylation of D-galactose by a galactokinase (EC 2.7.1.6) (de Geus *et al.* 2003), followed by the transfer of the D-galactose-1-phosphate to UDP-glucose in exchange for D-glucose-1-phosphate which is catalyzed by D-galactose-1-phosphate uridylyltransferase (EC 2.7.7.12) (Seiboth *et al.* 2002). In a side reaction the UDP-galactose is epimerized to UDP-galactose by UDP-glucose-4-epimerase (EC 5.1.3.2) (Seiboth *et al.* 2002). The D-glucose-1-phosphate is then converted to D-glucose-6-phosphate by a phosphoglucomutase and enters glycolysis. However, alternative pathways for D-galactose metabolism have been reported in plants (Gross and Phar 1982; Schnarrenberger *et al.* 1995) and bacteria (Abranches *et al.* 2004) and recently we have shown that *H. jecorina* strains defective in the galactokinase gene are still able to grow on D-galactose (Seiboth *et al.* 2004). In this work we will provide evidence for the existence of a second D-galactose-degrading pathway in *H. jecorina* and we will also show that enzymes from the L-arabinose / D-xylose metabolism are involved in it.

The D-galactose metabolism is of particular interest in regard to cellulase gene induction by lactose (Ilmen *et al.* 1997). Lactose (1,4-O-ß-D-galactopyranosyl-D-glucose) is virtually the only soluble inducer for a feasible industrial production of cellulases in *H. jecorina*. It has been shown previously

that cellulase genes in a *H. jecorina* strain disrupted in the galactokinase - encoding *gal1* gene cannot be induced by lactose while induction by sophorose - the putative cellulose derived inducer - remains unaltered (Seiboth *et al.* 2004). Consequently we investigated if a *xyl1* disruption has a similar effect.

Experimental procedures

Strains and culture conditions

H. jecorina strains used in this study were QM9414 (ATCC 26921), the *pyr4* negative mutant TU-6 (Gruber *et al.* 1990) and a galactokinase deletion mutant ΔGAL1 (Seiboth *et al.* 2004). Strains were grown in 1 l flasks on a rotary shaker (250 rpm) at 30°C, using 250 ml of the medium described by (Mandels and Andreotti 1978) with the respective carbon source at a final concentration of 1 % (w/v).

For transcript analysis, strains of *H. jecorina* were pregrown for 22 h on glycerol (1 % w/v), the mycelia harvested by filtration, washed with tap water, equal amounts of mycelia transferred to flasks containing the respective carbon source (1 % w/v), and cultivation continued for 3 h and 6 h. In case of lactose and xylan, expression was analyzed after 24 h of growth.

Escherichia coli strains ER1647 and BM25.8 (Novagen, Madison, WI) were used for library screening, and strain JM109 (Promega, Madison, WI) for plasmid propagation.

Cloning of the *H. jecorina xyl1* and sequence analysis

To amplify a *H. jecorina xyl1* fragment by PCR, the primers xylfw1 (5'-CTCTTCATCGTCTCCAAG-3') and xylrv1 (5'-AGRTANGGRTGRTGYTCDATYTG-3') corresponding to the conserved aa-sequences LFIVSK and QIEHHPYL, were derived from an alignment of the *A. niger* D-xylose reductase (AAF61912) to the putative D-xylose reductases of *Neurospora crassa* (http://www.broad.mit.edu/annotation/fungi/neurospora/). PCR amplification from genomic DNA was performed using the following conditions: 1 min initial denaturation (94°C) was followed by 30 cycles of amplification (1 min 94°C, 1 min 50°C, 1 min 72°C), and a final extension period of 7 min at 72°C. The amplified 400 bp fragment was cloned into pGEM-T (Promega), sequenced. Its translated aa sequence showed up to 68 % aa identity to other fungal D-xylose reductase in a NCBI BLAST and was therefore was used to screen a genomic λBlueSTAR library (Novagen) of *H. jecorina* QM9414. The *xyl1* gene was located on an about 5 kb *Sac*I subclone, ligated into pBluescript SK(+) (Stratagene, La Jolla, CA) to yield pXYL1 and sequenced, using a LI-COR 4200 (LI-COR Inc., Lincoln, Nebraska) automatic sequencer. DNA and protein sequences were analyzed using BLAST programs (Altschul *et al.* 1990) and Expasy (http://www.expasy.org/). The *xyl1* nucleotide sequence was deposited in the GeneBank database under Accession Number AY116507.

Construction of *H. jecorina* mutant strains

H. jecorina xyl1 deletion strains were constructed by replacing the *xyl1* coding region with the 2.7 kb *H. jecorina pyr4* fragment. 1.3 kb of a *xyl1* 5' fragment (*Sal*I/*Eco*RI) from pXYL1 was cloned together with the 2.7 kb *Sal*I *pyr4* fragment in an *Eco*RI/*Sal*I digested pBluescript SK(+). The resulting vector was digested with *Apa*I, blunted, and the insert excised with *Eco*RI and ligated into a *Sma*I/*Eco*RI digested pUC19 (GIBCO BRL Life Technologies, Maryland, USA). The 1.2 kb 3' *xyl1* fragment (*Bgl*II-*Hin*dIII) was ligated into the *Bam*HI-*Hin*dIII site of this vector to yield deletion vector pΔXYL1PYR4.

Transformation of *H. jecorina* TU-6 was performed with the 5.2 kb *Eco*RI-*Hin*dIII fragment excised from pΔXYL1PYR4 according to Gruber *et al.* (1990). Transformants were selected for growth on minimal medium with glucose (1 %, w/v) as carbon source.

The Δ*gal1*/Δ*xyl1* strain was constructed by transformation of a Δ*xyl1* strain with the 10.8 kb *gal1* deletion fragment of pΔGAL1 as described by Seiboth et al., 2004. In this vector the *H. jecorina gal1* coding region was replaced by the *A. nidulans amdS* (encoding acetamidase; (Kelly and Hynes 1985).

Retransformation of the Δ*xyl1* strains was done by cotransformation with plasmid pXYL1 together with a 2.7 kb *Xho*I-*Hin*dIII fragment of the hygromycin B-resistance conferring expression cassette from pRLMex30 (Mach *et al.* 1994). Retransformation of the Δ*gal1*/Δ*xyl1* strain was done as described (Seiboth *et al.* 2004). Retransformants were checked for single ectopic integration of the respective gene into the genome and strains carrying a single copy were used as controls in further experiments.

Nucleic acid isolation and hybridization

Fungal mycelia were harvested by filtration, washed with sterile tap water, shock frozen in liquid nitrogen and ground to a fine powder. For DNA extraction, the mycelial powder was suspended in extraction buffer (0.1 M Tris-HCl, pH 8.0; containing 1.2 M NaCl and 5 mM EDTA), incubated for 20 min at 65°C, cooled down on ice, mixed with 0.5 vol. phenol and 0.5 vol. chloroform and centrifuged (16.000x g, 15 min). Following an extraction with chloroform, the DNA was precipitated with 1 vol. of isopropanol and washed with 70 % (v/v) ethanol. Total RNA was isolated as described by (Chomczynski and Sacchi 1987). Standard methods (Ausubel *et al.* 2003) were used for electrophoresis, blotting and hybridization of nucleic acids.

Probes for hybridization were: a 1 kb cDNA *xyl1* fragment from pGEX-XYL1, a 1.2 kb *Nco*I-*Pst*I *gal1* cDNA fragment (Seiboth *et al.* 2004) and PCR amplicons for *cbh1* (1.2 kb), *cbh2* (1 kb) and 18S rRNA (300 bp).

Determination of fungal growth

To determine hyphal growth on agar plates, plates were inoculated with a small piece of agar in the centre of an 11-cm plate, and the increase in colony diameter measured daily twice. To measure growth in submerged cultures, the increase in biomass dry weight was recorded. Therefore, mycelia were harvested after appropriate times, washed extensively with distilled water and dried to constant weight in an oven at 80 °C. Data shown are the average of the three separate measurements, which deviated by not more than 15 %.

Preparation of cell-free extracts

H. jecorina strains were grown for 24 h in Mandels-Andreotti medium with either L-arabinose, D-galactose, lactose or D-xylose as carbon source. Mycelia were harvested by filtration, washed with cold tap water, squeezed between filter paper and ground to a fine powder in liquid nitrogen. One gram (wet weight) of ground mycelium was mixed with 3 ml of extraction buffer (0.1 M Tris-HCl pH 7.5; containing 1 mM EDTA and 5 mM β-mercaptoethanol), suspended thoroughly, and homogenized by sonification (15 x 20 sec; power and cycle 70 (Bandelin Sonoplus HD60, Berlin, Germany) at 2°C, with

intermittent 2 min cooling periods). The resulting homogenate was centrifuged at 15000 × g for 20 min at 4°C. The supernatant (average protein concentration 7-23 mg/ml) was stored at -80°C after addition of glycerol (20 %, final concentration) and used as a cell-free extract for enzyme assays.

Expression of *H. jecorina* Xyl1 in *E. coli*

The *H. jecorina xyl1* cDNA was expressed as a glutathione-S-transferase (GST) fusion in *E. coli*. The *xyl1* coding region was PCR-amplified from the cDNA by RT PCR using the Reverse Transcription System (Promega, Madison, WI) together with primers GEX-XYLfwd (5'-GAAAACAGGATCCATGGCGTCTCCCAC-3') and GEX-XYLrev (5'-TTTTGCGGCCGCTTAGATCTTTCTTTGTTTCTC-3') to introduce a *Bam*HI and *Not*I site. PCR amplification was performed with Pfu polymerase (Promega, Madison, WI), using an initial denaturation cycle of 45 sec at 94°C, followed by 30 cycles of amplification (45 sec at 94°C, 45 sec at 56°C and 3 min at 72°C). The final extension step was 10 min at 72°C. The amplicon was consequently cut with *Bam*HI and *Not*I and cloned into pGEX4-2T (Amersham Biosciences, Uppsala, Sweden). After verification by sequencing, the GST-Xyl1 fusion protein was transformed into *E. coli* BL21 (Stratagene, La Jolla, CA) and purified using Glutathione Sepharose 4B according to the manufacturer protocol (Amersham Biosciences, Uppsala, Sweden). 1 % (w/v) BSA was added for storage at -80°C to the recombinant protein solution.

Biochemical analysis

The protocols described by (Ausubel *et al.* 2003) were used for SDS-PAGE (using 10% polyacrylamide gels).

Enzymatic assays

Aldose reductase activity (cell free extract or purified GST-Xyl1 fusion) was determined spectrophotometrically by measuring the rate of change in absorbance at 340 nm for NADPH oxidation or NADP reduction at 30°C, using a Pye Unicam SP6-400 Spectrophotometer (Cambridge, UK) connected to an United Technologies Packard Model 641 recorder (Hartford, CT). Reactions were initiated by adding an aliquot of enzyme to a 1.0 ml-reaction mixture in a 10-mm half-micro cuvette. Measurements were made by varying the substrate concentration over the range of 5 to 100 mM with a constant coenzyme concentration of 0.15 mM for both (NADPH and NADP) in either 100 mM glycylglycine/NaOH pH 7.0 or 100 mM glycine/NaOH pH 8.6. Coenzyme activities were determined by varying their concentration (0.01 to 0.3 mM) at a constant substrate concentration (D-xylose and xylitol; 50 mM) in the buffers described above. Enzyme was added in amounts sufficient to produce a change in A_{340} between 0.02 and 0.1 per min. The reaction was started by addition of substrate or addition of recombinant enzyme, respectively. Activities are expressed as kat (nkat), 1 nkat corresponding to the conversion of 1 nmol of substrate per sec, and given as specific activities (kat/mg protein). Protein concentration was determined with the Bio-Rad Protein Assay (Bio-Rad Laboratories, München, Germany). Michaelis-Menten constant K_m and maximal velocity V_{max} were graphically determined by direct linear plotting (Cornish-Bowden and Eisenthal 1978; Eisenthal and Cornish-Bowden 1974). Molecular mass of Xyl1 used for calculation of k_{cat} was 36406 Da.

Results

Cloning of a *H. jecorina* xylose reductase

A gene encoding a putative D-xylose reductase was cloned based on conserved regions from orthologous proteins from *A. niger* and *N. crassa*. A 400 bp fragment was amplified from *H. jecorina* genomic DNA by PCR and used to isolate a genomic clone from a *H. jecorina* λBlueSTAR library. It contained an open reading frame of 1428 bp, interrupted by two introns of 356 and 97 bp, respectively, the position of which was verified by sequencing of the corresponding cDNA clone. Southern analysis of differentially digested chromosomal DNA suggests that *xyl1* occurs as a single copy in the *H. jecorina* genome but a number of additional weak hybridizing bands were also found after longer exposition times (data not shown). This is consistent with our BLAST search results of the preliminary genomic sequence data of *Hypocrea jecorina QM6a* (*Trichoderma reesei*) (www.jgi.doe.gov) which identified a number of related sequences.

The deduced polypeptide comprises 324 aa's with a calculated molecular mass of 36,406 Da. It shows high identity across the entire aa sequence to the XyrA of *Aspergillus niger* (AA61912, 63 %) and moderate identity to the D-xylose reductases of *Pichia guilliermondii* (54 %) and *Candida tenuis* (49 %). According to its aa sequence *H. jecorina* Xyl1 belongs to the superfamily of aldo/ketoreductases (AKR) which also includes a number of related monomeric NADPH-dependent oxidoreductases, such as aldehyde reductase, aldose reductase, prostaglandin F synthase, D-xylose reductase, and rho crystalline (Bohren *et al.* 1989). Xyl1 contains motifs that have been shown to be involved in substrate and coenzyme binding in yeast D-xylose/aldose reductases (Lee 1998). The aa's Ile_{271}, Pro_{272}, Lys_{273} together with Arg_{266} are highly conserved and thought to bind NADPH. It is noteworthy that the Ser residue which follows this motif in all other aldose reductases studied is replaced by a Thr in *H. jecorina*. The YXXXK sequence which is considered the active site of the enzyme appears - as in yeasts - on two positions (34-38 and 50-54). In contrast *A. niger* XyrA and human aldose reductase contain only one YXXXK. These double YXXXK motifs together with the GXXXGXG coenzyme binding motif are typical for the short chain dehydrogenases/reductases (SDR) family. Yeast D-xylose reductases are considered hybrids of aldo/ketoreductases and SDRs, but both *H. jecorina* Xyl1 and *A. niger* XyrA lack the GXXXGXG sequence. This data places Xyl1 between XyrA and yeast D-xylose reductases on the evolutionary tree.

Regulation of *xyl1* transcription

Investigation of the upstream region of *xyl1* revealed multiple binding sites for the carbon catabolite repressor protein Cre1 (Ilmen *et al.* 1996), two for the cellulase regulator Ace1 (Aro *et al.* 2003) but no binding sites for the transcriptional activator Ace2 (Aro *et al.* 2001), the Hap2/3/5 proteins (Zeilinger *et al.* 2001) or XlnR (van Peij *et al.* 1998), were detected (Fig. 32A). Northern analysis of total RNA from *H. jecorina* showed that the *xyl1* transcript was most abundant during growth on D-xylose and on xylan but it was also found on L-arabinose and L-arabinitol. A moderate *xyl1* transcript level was detected on lactose and a weak expression level on D-galactose and xylitol was observed. After prolonged exposition of the Northern blot, a low level of constitutive expression appeared on all carbon sources

tested (Fig. 32B). The expression follows the same pattern as it has been reported previously for *H. jecorina* xylitol dehydrogenase (Seiboth *et al.* 2003) indicating a common regulation.

Interestingly, *xyl1* expression requires an intact Leloir pathway, because the transcript was significantly less abundant in a *gal1* and *gal7* mutant on D-galactose (Fig. 32C). However expression on lactose as a carbon source was unaffected.

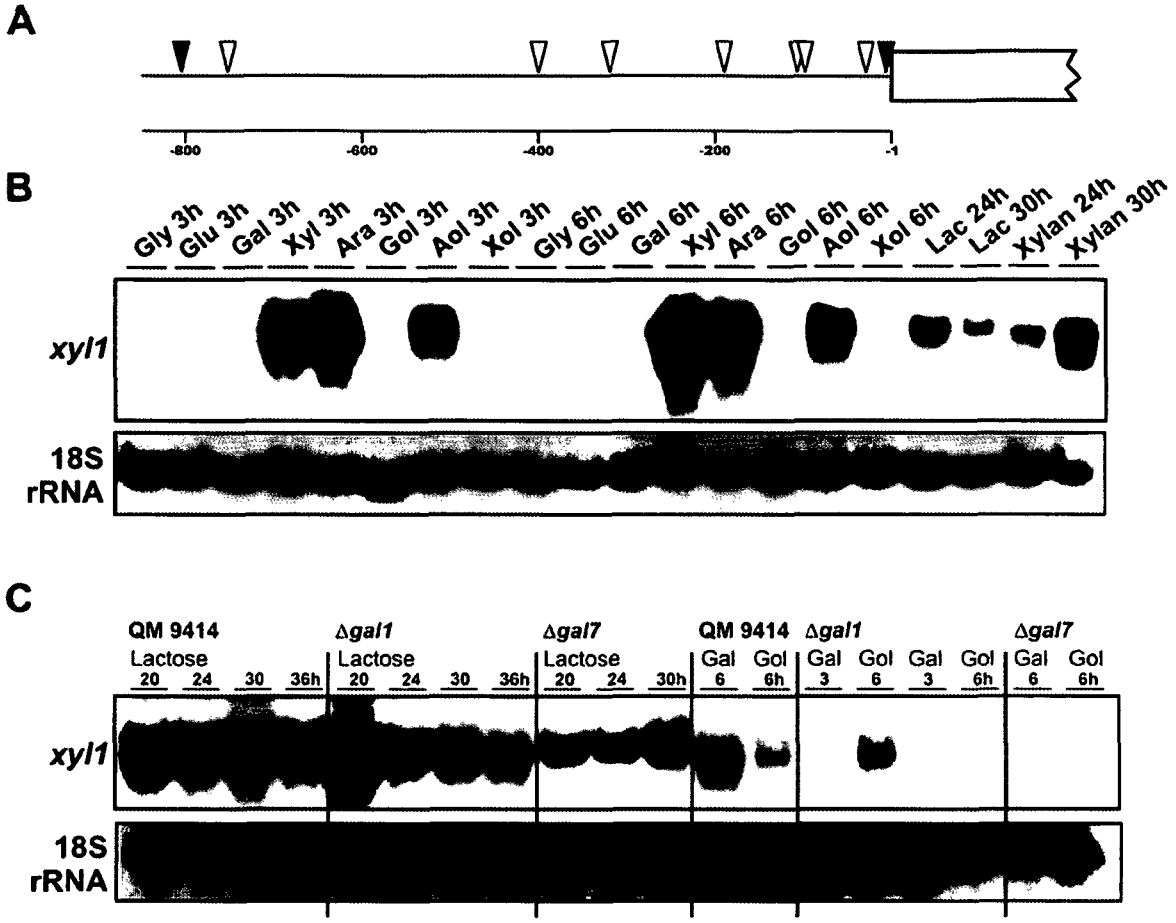
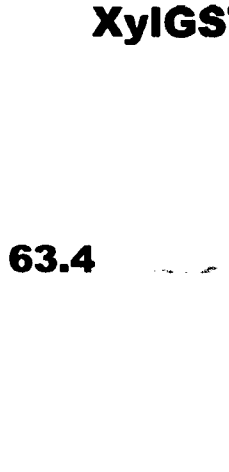


Fig. 32 Transcriptional regulation of *xyl1*

(A) Upstream region of the *xyl1* gene containing binding sites for AceI (filled triangles) and Cre1 (empty triangles). (B) Expression of *xyl1* on various carbon sources. (C) Expression of *xyl1* in strains disrupted in Leloir pathway genes. Abbreviations used are: Gly, glycerol; Glc, D-glucose; Gal, D-galactose; Xyl, D-xylose; Ara, L-arabinose; Gol, galactitol; Aol, L-arabinitol; Xol, xylitol; Lac, lactose. If not indicated otherwise, samples shown were obtained after 6 h of growth in replacement medium as described in Materials and Methods. In case of the carbon sources lactose and xylan a direct conidial inoculum was made. The 18S rRNA served as a loading control.

D-Xylose reductase catalyzes the NADPH dependant reduction of D-xylose, L-arabinose and D-galactose

The *xyl1* cDNA was expressed as a GST fusion in *E. coli* and purified by affinity chromatography (Fig. 33). Interestingly, several attempts to remove the GST residue by thrombin cleavage resulted in inactivation of the enzyme and so we decided to examine the substrate specificity of the uncleaved fusion protein. D-xylose, L-arabinose, D-ribose, D-galactose and D-glucose were converted by Xyl1



whereas no activity was detected with D-arabinose or D-mannose. The enzyme was specific for NADPH as coenzyme. NADH exhibited only a very small fraction of the performance with NADPH (458,000 vs. 1,000 $M^{-1}s^{-1}$ at 50 mM D-xylose. At 0.15 mM NADPH, the enzyme displayed highest performance with D-xylose (361.8 $M^{-1}s^{-1}$), followed by L-arabinose (30 $M^{-1}s^{-1}$), D-ribose (27.1 $M^{-1}s^{-1}$) and D-galactose (5.3 $M^{-1}s^{-1}$). The corresponding backward reactions (at 0.15 mM $NADP^+$) with xylitol, L-arabinitol, and galactitol were considerably lower with 21.8, 1.5, and 0.34 $M^{-1}s^{-1}$.

Fig. 33 SDS-PAGE of purified recombinant GST fused xylose reductase (XylGST) of *H. jecorina*

D-xylose reductase is to a significant part involved in D-xylose and L-arabinose metabolism

To investigate the role of *xyl1 in vivo* a deletion mutant was constructed by replacing the coding region of *xyl1* with the *pyr4* gene of *H. jecorina*. Transformation of *H. jecorina* yielded several transformants which were selected for growth on minimal medium. Purified transformants were tested for growth on D-xylose as sole carbon source and strains with slower growth were subjected to Southern analyses. All five strains exhibiting slower growth on D-xylose, showed integration of the deletion cassette in the *xyl1* locus (data not shown). Growth tests showed a reduced growth rate of Δ*xyl1* strains not only on D-xylose but also on L-arabinose compared to QM9414, whereas growth on D-glucose and glycerol remained unaffected (Fig. 34A-C). The fact that the Δ*xyl1* strains of *H. jecorina* could still grow on D-xylose and L-arabinose, however, suggested the presence of additional enzymes with the ability to catabolize these pentoses. Aldose reductase activity assays of cell-free extracts prepared from mycelia of QM9414 and Δ*xyl1* grown on D-xylose and L-arabinose confirmed this suggestion (Table 8). Reductase activity using NADPH as coenzyme was reduced to about 2-5 % compared to QM9414, indicating the major importance of Xyl1 in D-xylose and L-arabinose catabolism. No aldose reductase activity was found in QM9414 and Δ*xyl1* with NADH as coenzyme neither on D-xylose nor L-arabinose (Table 7).

Table 8 D-xylose reductase and D-glucose-6-phosphate dehydrogenase activities (given in [nkat·(mg cell free extract)$^{-1}$]) in cell free extracts derived from either QM9414 or the Δ*xyl1* strain grown on D-xylose and on L-arabinose respectively. D-xylose reductase measurements were performed with 50 mM D-xylose and 0.2 mM NADPH and D-glucose-6-phosphate dehydrogenase activities were measured with 3 mM D-glucose-6-phosphate and 6 mM NADP.

	D-xylose reductase		D-glucose-6-P DH	
	QM9414	Δ*xyl1*	QM9414	Δ*xyl1*
D-xylose	166.8	6.3	396.8	591.5
L-arabinose	105.9	1.9	219.9	307.5

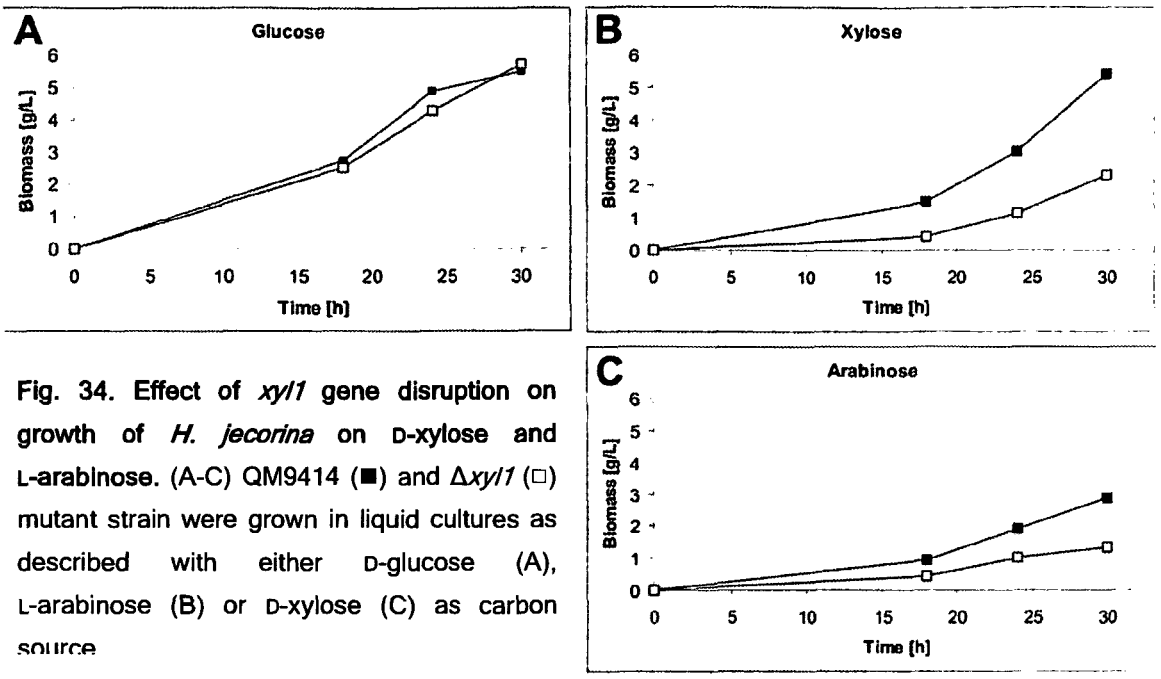


Fig. 34. Effect of *xyl1* gene disruption on growth of *H. jecorina* on D-xylose and L-arabinose. (A-C) QM9414 (■) and Δ*xyl1* (□) mutant strain were grown in liquid cultures as described with either D-glucose (A), L-arabinose (B) or D-xylose (C) as carbon source.

Xyl1 is involved in the reductive pathway for D-galactose metabolism

We have recently described the presence of a second pathway besides the Leloir pathway for D-galactose metabolism in *H. jecorina* which proceeds via galactitol (Seiboth *et al.* 2004). The enzyme catalyzing the first step in this pathway, the reduction of D-galactose to galactitol, has not been identified yet. Therefore we tested whether the deletion of *xyl1* influences the ability of *H. jecorina* to grow on D-galactose (Fig. 35). As can be seen, growth of the Δ*xyl1* strain was reduced compared to the parent strain QM9414, although not to the same extent as observed in *H. jecorina* Δ*gal1*, which is blocked in the Leloir pathway. A *H. jecorina* double deletion mutant in both *gal1* and *xyl1*, prepared by transforming strain Δ*xyl1* with the *gal1* deletion cassette, resulted in almost complete inability to grow on plates with D-galactose and growth in shake flasks was almost completely inhibited. Cell free extracts from *H. jecorina* Δ*xyl1* grown on D-galactose, exhibited a reduced D-galactose reductase activity of about 25 % compared to QM9414 (Table 9). Therefore, we conclude that Xyl1 is able to catalyze the first step in the reductive pathway of D-galactose catabolism, the reduction of D-galactose to galactitol. Our data also demonstrate that this pathway is already active when the Leloir pathway is in operation. Retransformation of the double deletion strain with *gal1* resulted in the restoration of the Δ*xyl1* phenotype (data not shown).

Table 9 D-xylose reductase activity (given in [nkat·(mg cell free extract)$^{-1}$]) with 50 mM D-xylose and 0.2 mM NADPH in cell free extracts derived from either the parent strain or the Δ*xyl1* strain grown on D-galactose.

QM9414	ΔXYL1
4.85	0.94

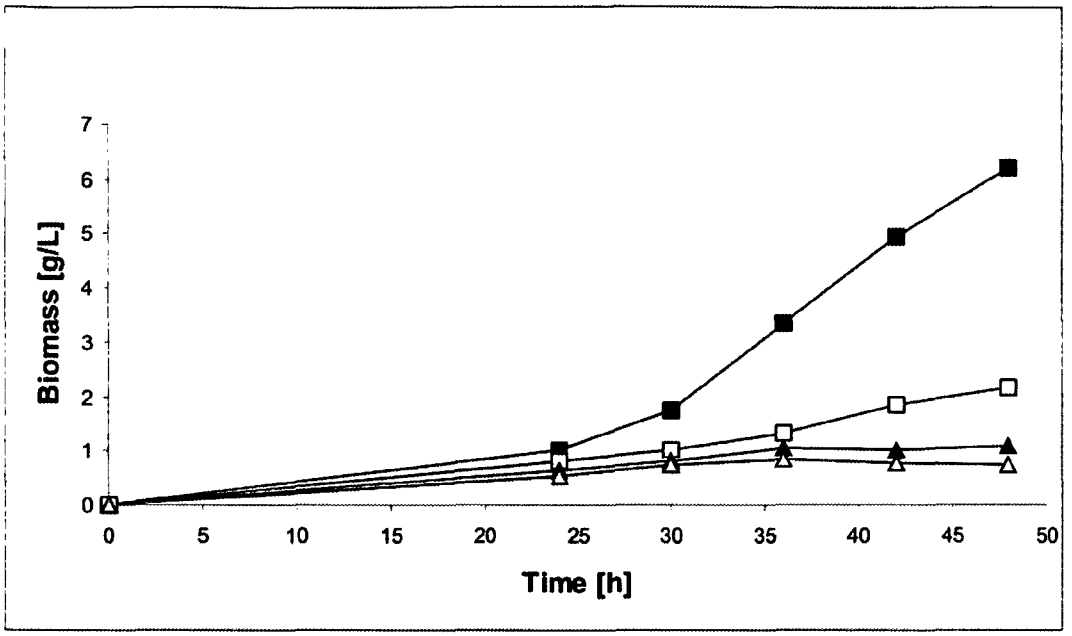


Fig. 35 Effect of a *xyl1* and *gal1xyl1* deletion on growth on D-galactose.
The parent strain QM9414 (■) and the Δ*xyl1* (□), Δ*gal1* (▲) and Δ*gal1xyl1* (△) mutant strains were grown in liquid cultures as described with D-galactose as carbon source.

xyl1 is essential for *cbh1* and *cbh2* expression on lactose

Lactose is a carbon source inducing cellobiohydrolase gene transcription. We have previously shown that blockage of the Leloir pathway by *gal1* gene deletion results in an impairment of this induction (Seiboth *et al.* 2004). Having identified Xyl1 as one of the enzymes responsible for the reductive pathway of D-galactose catabolism, we investigated the effect of the alteration of this pathway on cellulase induction. *H. jecorina* QM9414, Δ*xyl1*, Δ*gal1* and Δ*xyl1*/Δ*gal1* were grown on lactose and the accumulation of the transcripts of two major cellulase genes *cbh1* and *cbh2* compared by Northern Analysis. Fig. 36 shows that the expression of both genes is strongly reduced in the Δ*xyl1* strain, although not to the same extent as in the Δ*gal1* strain. The Δ*xyl1*/Δ*gal1* strain exhibited the same reduced level of cellulase transcripts as the Δ*gal1* mutant strain, indicating that the effect is not additive.

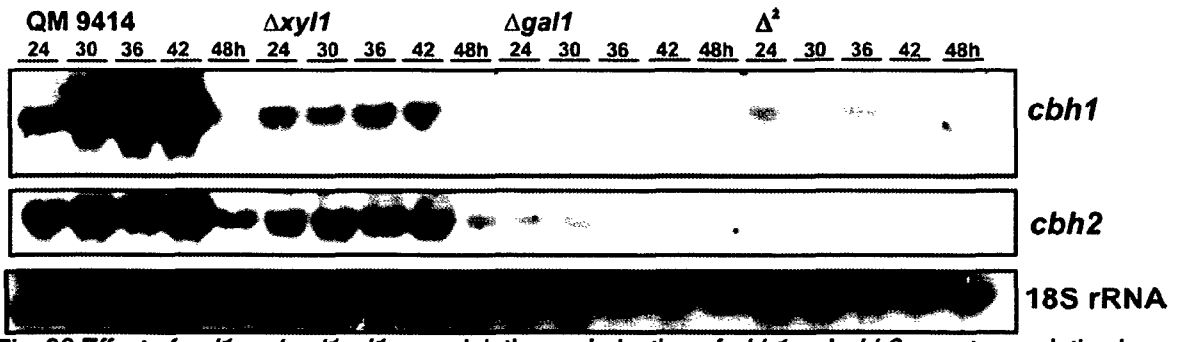


Fig. 36 Effect of *xyl1 and gal1xyl1* gene deletion on induction of *cbh1* and *cbh2* gene transcription by lactose in *H. jecorina*. Northern analysis of *cbh1* and *cbh2* transcript accumulation in *H. jecorina* QM9414 and strain Δ*xyl1*, Δ*gal1xyl1* on lactose. The 18s RNA was used as loading control.

Xyl1 deletion does not affect induction of the Leloir pathway genes by D-galactose

Downregulation of *gal1* in a Δ*xyl1* strain would be a straightforward explanation of the reduction of *cbh1* and *cbh2* gene expression, since in a Δ*gal1* strain cellulase induction is impaired. The results shown in Fig. 37, however, indicate that the Leloir gene *gal1* is expressed in the Δ*xyl1* strain at roughly the same level as in QM9414. This is in contrast to the downregulation of the *xyl1* transcript in a *gal1* or *gal7* negative background and supports the idea that an active reductive pathway is important for cellulase induction.

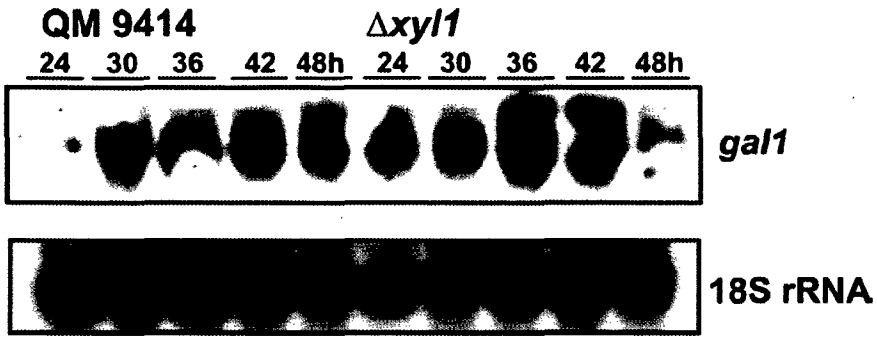


Fig. 37 Effect of *xyl1* gene deletion on induction of *gal1* and *gal7* gene transcription by lactose.
Northern analysis of *gal1* and *gal7* transcript accumulation in *H. jecorina* QM9414 and the Δ*xyl1*, Δ*gal1* and Δ*gal1*/Δ*xyl1* mutant strains upon growth on lactose for 24 to 48h.
The 18S rRNA served as a loading control.

Discussion

Here we have described the cloning of a gene, *xyl1*, encoding a D-xylose reductase from *H. jecorina* which is of major relevance for growth on D-xylose and L-arabinose, and essential for the reductive pathway of D-galactose catabolism. Performance analysis of the recombinantly produced enzyme showed that it is primarily a D-xylose reductase, and that the activities with L-arabinose and D-galactose are only due to side specifities. Nevertheless the reduction of D-xylose and L-arabinose reductase activity in cell-free extracts of the Δ*xyl1* strain was similar, indicating that the same enzyme - encoded by *xyl1* - is responsible for the reduction step in the D-xylose and L-arabinose catabolic pathway in *H. jecorina* . The major involvement of this enzyme is striking in view of the fact that the *H. jecorina* genome database contains at least four more putative aldose reductase genes (Fekete, E. and Seiboth, B., unpublished data) and that in *A. niger* two enzymes are involved (Witteveen *et al.* 1989). The function of the additional aldose reductases, if any, remains to be established. However, aldose reductases have also been implicated in other cellular processes such as detoxification of possible harmful aldehydes (Crabbe 2003), and the proteins encoded by these genes may therefore - except of accounting for the small residual aldose reductase activity in the Δ*xyl1* strain - serve different purposes in *H. jecorina* than *xyl1*.

Xyl1 showed also to be involved in the reductive catabolic pathway of D-galactose although the reduction of D-galactose reductase activity was comparably lower on D-galactose grown mycelia. These data provide one more piece of evidence that the reductive D-galactose pathway and the

D-xylose/L-arabinose catabolic pathway use the same enzymes. This fact may also explain why the enzymes catabolizing D-galactose- and L-arabinose-containing polymers (α- and ß-galactosidase, α-arabinofuranosidase) pathways are induced by both D-galactose and L-arabinose (Fekete *et al.* 2002; Kristufek *et al.* 1994).

The findings of downregulation of *xyl1* in Δ*gal1* deletion strains imply that the reductive pathway of D-galactose metabolism and the Leloir pathway are corregulated and coexist under the same physiological conditions. The reductive pathway is therefore not an alternative, salvage pathway, but part of the usual catabolism of D-galactose. The physiological reasons for this are unclear at the moment. One possible explanation would be to avoid accumulation of D-galactose-1-phosphate and/or UDP-glucose too high transient intermediary concentrations, which have been reported to be toxic in some cells (Leslie 2003). This could serve to compensate for the fact that - in contrast to e.g. yeast (Webster and Dickson 1988) - both *gal1* as well as *gal7* are only poorly regulated by D-galactose in *H jecorina*, being expressed constitutively, and induced by D-galactose only to the double level (Seiboth *et al.* 2004; Seiboth *et al.* 2002). Interestingly, the downregulation of *xyl1* in the Δ*gal1* strain was not observed on lactose as a carbon source, suggesting that it may depend on the rate of D-galactose metabolism, which is much slower than on D-galactose alone.

We have previously shown that the induction of cellulase formation by lactose requires the Gal1 galactokinase, which led us to speculate that either D-galactose or D-galactose-1-phosphate may be inducers of cellulase formation. Maybe the dependence of *xyl1* on *gal1* does not involve the same mechanism as that of expression of the cellulase genes *cbh1* and *cbh2*. The present findings that a deletion of *xyl1* also leads to a significantly reduced induction of cellulase gene transcription would suggest that Xyl1 plays a similar, additional role to Gal1 in lactose induction. However, the deletion in *xyl1* results in a reduced transcritption level of *cbh1* and *cbh2*, but significantly higher than in *gal1* deletion, whereas the deletion of both *gal1* and *xyl1* yields a transcript reduction to a similar amount as in the single deletion of *gal1*, indicating that the involvement of *xyl1* in cellulase induction by lactose is of secondary importance and not additive; the latter observation implies that both Gal1 and Xyl1 are involved in the formation of the same inducing compound. We have recently stressed that the intracellular concentration of either D-galactose or D-galactose-1-phosphate is probably critical for induction, because no cellulase gene expression is observed during incubation with D-galactose, not even in a carbon catabolite derepressed strain (Seiboth *et al.* 2004). We could also recently support this assumption by showing that it is the galactokinase activity, and not presence of the protein, which determined cellulase induction (Hartl, L. Kubicek, C.P. and Seiboth, B., manuscript in preparation). One scenario, which may therefore explain the effect of the *xyl1* deletion, and which would be in accordance with our speculations on the role of the simultaneously operating reductive pathway, would be that D-galactose catabolism only via the Leloir pathway results in intermediate levels incompatible with cellulase induction, and that the reductive bypass is required for allowing intermediate concentrations to accumulate at levels appropriate for induction. Experiments have now been initiated to quantify Leloir pathway intermediates during growth on lactose and D-galactose at defined growth rates of *H. jecorina*.

Discussion

In this work I have been characterizing some key genes and their encoded enzymes, which are responsible for the catabolism of aldoses in the filamentous fungus *Hypocrea jecorina*.

The initial step in this catabolism is a reduction of an aldose to the corresponding polyol and was shown to be catalyzed by an aldose reductase. The identified and analyzed enzyme Xyl1 belongs to the family of D-xylose aldose reductases and its performance showed that it acts primarily as D-xylose reductase. Nevertheless the reduction of D-xylose and L-arabinose reductase activity in cell-free extracts of the Δ*xyl1* mutant was similar, indicating that this enzyme is responsible for catalyzing the first step of both pentose catabolic pathways, of D-xylose and L-arabinose. In contrast to these findings the reduction of D-xylose reductase activity was much lower on D-galactose grown mycelia. Preliminary data of Fekete, E. and Seiboth, B. (unpublished data) suggest that at least one of the additional aldose reductases of *H. jecorina* can have a stronger influence on the reductive D-galactose pathway because it is strongly induced on D-galactose. Neverthelss, the Xyl1 catalyzes also the reduction of D-galactose, which has been demonstrated to be the first step of a reductive D-galactose catabolism occurring additionally to the Leloir pathway.

A further interesting result of this investigation was the downregulation of *xyl1* in Δ*gal1* deletion strains, implying that the reductive pathway of D-galactose metabolism and the Leloir pathway are corregulated and coexist under the same physiological conditions. The reductive pathway is therefore not an alternative, salvage pathway, but part of the usual catabolism of D-galactose.

It was previously shown that the induction of cellulase formation by lactose requires the Gal1 galactokinase, which suggested that either D-galactose or D-galactose-1-phosphate may be inducers of cellulase formation. The present findings that the deletion of *xyl1* lead to a significantly reduced induction of cellulase gene transcription, suggest that Xyl1 plays a similar, additional role to Gal1 in lactose induction. However, the deletion of *xyl1* results in a reduced transcription level of *cbh1* and *cbh2*, the most expressed cellulases of *H. jecorina*, but significantly higher transcript accumulation occurs than in *gal1* deletion, whereas the deletion of both *gal1* and *xyl1* yields a transcript reduction to a similar amount as in the single deletion of *gal1*, indicating that the involvement of *xyl1* in cellulase induction by lactose is of secondary importance and not additive. The latter observation implies that both Gal1 and Xyl1 are involved in the formation of the same inducing compound. We have recently stressed that the intracellular concentration of either D-galactose or D-galactose-1-phosphate is probably critical for induction. We could also recently support this assumption by showing that it is the level of galactokinase activity, and not presence of the protein, which determined cellulase induction (Hartl, L. Kubicek, C.P. and Seiboth, B., manuscript in preparation). One scenario, which may therefore explain the effect of the *xyl1* deletion, and which would be in accordance with our speculations on the role of the simultaneously operating reductive pathway, would be that D-galactose catabolism only via the Leloir pathway results in intermediate levels incompatible with cellulase induction, and that the reductive bypass is required for allowing intermediate concentrations to accumulate at levels appropriate for induction.

The following step in D-xylose catabolism was shown to be catalyzed by *xdh1* encoding xylitol dehydrogenase, which has been characterized at the molecular and functional level. This enzyme belongs to the family of Zn^{2+}-containing long-chain alcohol dehydrogenases. Structural similarities were found with the long-chain human alcohol dehydrogenase and it was suggested that this feature is characteristic of D-sorbitol dehydrogenases in general.

The remaining growth of a loss-of-function mutant of *xdh1* on xylitol and - although with lower growth rates - also on D-xylose, implied the presence of at least one more enzyme oxidizing xylitol or a less effective new pathway for D-xylose catabolism. The results obtained with the *xdh1 lad1* double-deletion mutant conclusively showed that the enzyme responsible for this residual activity is L-arabinitol-4-dehydrogenase, Lad1. The lack of both Xdh1 and Lad1 leads to a complete loss of ability to grow on D-xylose and xylitol thus demonstrating the absence of further enzymes with the same catalytic activity.

Lad1 belongs also to the family of zinc-containing alcohol dehydrogenases, and the phylogenetic analysis of Xdh1 and Lad1 from various sources showed that the clade containing Lad1 is basal to that of Xdh1 and is appropriate for fungi. These data imply that the more specific Xdh1 protein may have evolved from the rather broadly specific Lad1 protein. Besides the affirmation of this assumption with extensive studies on enzyme performances with a broad spectrum of substrates, the finding that *H. jecorina xdh1* was not able to enable a loss-of-function mutant of *lad1* to grow on L-arabinose also supported this hypothesis. Compensation of loss-of-function mutants in one catabolic pathway by enzymes from another pathway seems to be a common phenomenon in fungi. This was already observed by investigation the aldose reductase of *H. jecorina*, because a *xyl1* deletion mutant showed growth on D-xylose, although with slower rate. The same observation was reported in *A. niger* (Hasper *et al.* 2000).

A comparison of the substrate specificity of Lad1 with that of mammalian SDHs showed that Lad1 has a much higher catalytic efficacy with pentitols than with hexitols. It is therefore intriguing that all the aa-residues which have been shown to be involved in the binding of D-sorbitol by SDH (i.e. S43, Y47, F115, T118, E152, R296 and Y297) are strictly conserved in Lad1 as well. Obviously, the different efficacy of substrate conversion depends on the presence of the amino acids flanking the active site cleft. As shown in the putative 3D-model, a number of aa-changes have been identified - conserved among members of the terminal L-arabinitol dehydrogenase cluster but significantly different to mammalian SDHs - which are located in this area of the protein. Although merely speculative at the moment, it is possible that these amino acids are responsible for the differences in the activity and affinity pattern between Lad1 and SDH.

Apart of the generally different pattern of activity against pentitols and hexitols, most of the substrate-product pairs of Lad1 and SDH are the same, i.e. they use the same catalytic mechanism. A strong difference in the substrate specificity between the two, however, is the oxidation of galactitol and D-talitol. Lad1 oxidizes them at C4, yielding L-xylo- and D-arabino-3-hexulose, respectively. One of the corresponding products of the SDH reaction (D-tagatose) is not reduced by Lad1 (unpublished data), and the other one (L-psicose) was not available for this study, but the two 3-hexuloses are converted to galactitol and D-talitol, respectively, thus proving that their identification as products of the reaction is no artefact. The occurrence of these two 3-hexuloses in nature has so far not been reported, although the D-xylo-3-hexulose-6-phosphate is an intermediate in the autotrophic carbon

dioxide metabolism in archaebacteria (Yaoi *et al.* 2000). Also, (Reichert 1994) reported that an L-glucitol dehydrogenase of a *Pseudomonas* sp. formed L-xylo-3-hexulose from galactitol, but the physiological relevance of this finding has not been pursued further. It is possible that the changes in the structure of the active centre, which have accompanied the change in substrate preference as discussed above, may have resulted in a binding of galactitol and D-talitol in such a way that the zinc atom is coordinated to C4. However, a more detailed interpretation of these data requires the determination of the 3D structure of Lad1 first.

The at least 10-fold higher k_{cat}/K_m values of Lad1 for the pentitols L-arabinitol and xylitol than for the various hexitols are in accordance with the postulated main role of this enzyme in pentose metabolism. The evidence for such a role *in vivo* has been also provided, thus proving that the enzyme indeed takes part in catabolism of L-arabinose. The very low k_{cat}/K_m values for galactitol are therefore in contrast to the role of Lad1 in the alternative D-galactose degrading pathway in *H. jecorina* and may explain the transient accumulation of up to 400 mM galactitol during its action (Seiboth *et al.* 2004). The identification of L-xylo-3-hexulose as the product of galactitol oxidation and thus as an intermediate of this pathway, raises the question, which enzymes may participate in its further metabolism? Phosphorylation of a 3-hexulose at the C6 hydroxyl group by hexokinase has not been studied yet, and there are reports claiming that the substrate specificity of hexokinase is restricted to C2 in ketohexoses (Machado de Domenech and Sols, 1980). In bacteria, D-xylo-3-hexulose-6-phosphate is isomerized by the enzyme 3-hexulose-6-phosphate isomerase to fructose-6-phosphate (Martinez-Cruz *et al.* 2002); however, we were unable to find any sequences with sufficient similarity to the 3-hexulose-6-phosphate isomerase gene from *E. coli* (NP_418039) in the unpublished but restricted accessible genome database of *Hypocrea jecorina*.

(Fekete *et al.* 2004) have recently reported that galactitol is oxidized to L-sorbose in *A. nidulans* by L-arabinitol dehydrogenase from cell-free extracts. However, the recombinant LadA of *A. nidulans*, which has the best similarity to Lad1 of *H. jecorina* converted galactitol also to L-xylo-3-hexulose (unpublished data) suggesting that the L-sorbose accumulates in cell-free extracts, which is due to more than one enzymatic step.

Using the Δ*lad1* strain, I also studied the role of *lad1* in the catabolism of other hexitols, although these experiments are not absolute proof for an involvement for the enzymatic reaction of Lad1 and could also be due to an indirect effect of *lad1* knock-out on another gene. On the other hand, the lack of growth of the wildtype strain on D-allitol and L-iditol may either be due to a lack of uptake of these hexitols, or due to a lack of *lad1* expression by these compounds, because *H. jecorina* can grow on the corresponding products of the Lad1 reaction (D-psicose and L-sorbose, respectively). Conversely, Lad1 is clearly not involved in the metabolism of D-mannitol; it is likely that this hexitol is oxidized by L-xylulose reductase (EC 1.1.1.10), which acts as a mannitol dehydrogenase (Richard *et al.* 2002).

Furthermore this latter enzyme is able to convert L-sorbose, which is a possible intermediate of the reductive D-galactose catabolism, to D-sorbitol and its conversion to D-fructose was already shown to be mainly achieved by xylitol dehydrogenase. Although both xylitol dehydrogenase and L-arabinitol dehydrogenase are able to catalyze this bioconversion, gene knock out experiments showed that a deletion in *xdh1* has a much more drastic effect that of *lad1*. Also the comparison of the kinetic characteristics of Xdh1 and Lad1 confirm the major participation of Xdh1 in D-sorbitol conversion. The

next step, the phosphorylation of D-fructose can be performed by hexokinase as has been shown for *A. nidulans* (Fekete *et al.* 2004) and the resulting D-fructose-6-phosphate can enter the glycolysis.

This possible L-sorbose catabolism was proposed for *A. nidulans* by (Elorza and Arst 1971) and it is likely that the enzymes identified in this work - L-xylulose reductase, xylitol dehydrogenase, L-arabinitol dehydrogenase and D-xylose reductase - will also be responsible for D-galactose metabolism *via* the reductive pathway. Double deletion strains in *lxr1* or *xdh1* together with the Leloir pathway galactokinase gene *gal1* are still necessary to prove this point. If this assumption is correct, then almost the whole structure of the reductive D-galactose catabolic pathway would have been elucidated, the only piece being left is the step between L-xylo-3-hexulose and L-sorbose (Pail *et al.* 2004). It is also striking that the order of reactions and the participating enzymes are identical to those assumed to take place in the L-arabinose catabolic pathway. This may explain why the enzymes of the pathway are induced by both D-galactose and L-arabinose (Seiboth *et al.* 2004; Seiboth *et al.* 2002).

The possibility of L-sorbose as an intermediate of D-galactose metabolism in *H. jecorina* would also be interesting in view of the reported ability of L-sorbose to induce cellulases in this fungus (Nogawa *et al.* 2001), as it would potentially link two inducers - lactose and L-sorbose - to a common pathway and possibly also common mechanism.

Appendix

Chapter ONE was published in Eukaryotic Cell (2003), 867-875:

D-xylose metabolism by *Hypocrea jecorina*: loss of the xylitol dehydrogenase step can be partially compensated by *lad1*-encoded L-arabinitol-4-dehydrogenase

Bernhard Seiboth, Lukas Hartl*, Manuela Pail* and Christian P. Kubicek
*both authors contributed equally to this work

Chapter TWO was published in European Journal of Biochemistry (2004) 271(10):1864-1872

The metabolic role and evolution of L-arabinitol 4-dehydrogenase of *Hypocrea jecorina*

Manuela Pail, Thomas Peterbauer, Bernhard Seiboth, Christian Hametner, Irina Druzhinina and Christian P. Kubicek

Chapter Three will be submitted to Microbiology SGM

L-sorbose metabolism in *Hypocrea jecorina*

Manuela Pail, Christian Gamauf, Bernhard Seiboth, and Christian P. Kubicek

Chapter Four is already submitted to Molecular Microbiology

Metabolic characterization of the *Hypocrea jecorina* D-xylose reductase

Christian Gamauf, Manuela Pail, Bernhard Seiboth, and Christian P. Kubicek

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List of Publications

Peer-reviewed journals

Zeilinger, S., Schmoll M., Pail, M., Mach, R. L. and Kubicek, C. P. (2003) Nucleosome transactions on the *Hypocrea jecorina (Trichoderma reesei)* cellulase promoter *cbh2* associated with cellulase induction. Mol Gen Genomics (2003) 270: 46-55

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Seiboth, B., Hartl, L., Pail, M., Fekete, E., Karaffa, L., Kubicek, C. P. (2003). The galactokinase of *Hypocrea jecorina* is essential for cellulase induction by lactose but dispensable for growth on D-galactose. Mol Microbiol. (2004) 51(4):1015-25

Pail, M., Seiboth, B., Peterbauer, T., Hametner, C., Druzhinina, I., Kubicek, C. P. (2004) The metabolic role & the evolution of L-arabinitol 4-dehydrogenase (Lad1) of *Hypocrea jecorina (Trichoderma reesei)*. European Journal of Biochemistry (2004) 271(10):1864-1872.

Gamauf, C., Pail, M., Kubicek, C. P., and Seiboth, B. (2004) Metabolic characterization of the *Hypocrea jecorina* D-xylose reductase (to be submitted to Mol Microbiol)

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Pail, M., Seiboth, B., and Kubicek, C. P. (2004)
The L-arabinitol dehydrogenases of Aspergillus nidulans (in preparation)

Oral presentation

Pail, M. (2002) Characterization of the *Hypocrea jecorina (Trichoderma reesei)* xylitol dehydrogenase. Joint meeting of ÖGBM, ÖGBT, ÖGGGT and ANGT 2002, Salzburg, Austria

Pail, M. (2003) The metabolic role and evolution of L-arabinitol 4-dehydrogenase (Lad1) of *Hypocrea jecorina*. Joint meeting of ÖGBM, ÖGBT, ÖGGGT and ANGT 2003, Graz, Austria

Seiboth, B., Hartl, B., Pail, M., and Kubicek, C.P. (2003) D-xylose metabolism by *Hypocrea jecorina:* metabolic and regulatory consequences of a loss of the xylitol dehydrogenase step (2003). 14th International Congress of the Hungarian Society for Microbiology, Balatonfüred, Hungary

Seiboth, B., Pail, M., and Kubicek, C.P. (2004) The alternative D-galactose catabolic pathway in *Hypocrea jecorina*: a stowaway of D-xylose and L-arabinose catabolism. 7th European Conference on Fungal Genetics, Copenhagen, Denmark

Pail, M., Seiboth, B., and Kubicek, C. P. (2004) Novel aspects of pentose metabolism in filamentous fungi. 15th Congress of the Hungarian Society for Microbiology, Hungary

Poster presentation

Zeilinger, S., Schmoll M., Pail, M., Mach, R. L. and Kubicek, C. P. (2002) Nucleosome transactions on the *Hypocrea jecorina cbh2* promotor reveal a novel role for the carbon catabolite repressor protein Cre1 in chromatin rearrangement. 6th European Conference on Fungal Genetics, Pisa, Italy

Seiboth, B., Pail, M. and Kubicek, C. P. (2002) *Hypocrea jecorina gal1* (galactokinase) is involved in *gal7* induction on galactose and cellulase induction on lactose. 6th European Conference on Fungal Genetics, Pisa, Italy

Hartl, L., Seiboth, B., Pail, M. and Kubicek, C. P. (2002) Characterization of the *Hypocrea jecorina* xylitol dehydrogenase. 6th European Conference on Fungal Genetics, Pisa, Italy

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Best poster award for this presentation

Kubicek, C. P., Seiboth, B., Pail, M., Hartl, L., Druzhinina, I. (2003). L-Arabinitol-4-dehydrogenase of *Hypocrea jecorina* is a fungal orthologue of the eukaryotic sorbitol dehydrogenase and a key enzyme for hemicellulose degradation. 14th Congress of European Mycologists. Yalta, Crimea, Ukraine.

Pail, M., Seiboth, B., Peterbauer, T., Kubicek, C. P. (2004) L-arabinitol dehydrogenases of *Hypocrea jecorina* and *Aspergillus nidulans*. 7th European Conference on Fungal Genetics, Copenhagen, Denmark

Pail, M., Seiboth, B., Kubicek, C. P. (2004) L-arabinitol dehydrogenases of *Aspergillus nidulans*. Joint meeting of ÖGBM, ÖGBT, ÖGGGT and ANGT 2004, Innsbruck, Austria

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EDUCATION AND TRAINING

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• Name and type of organisation providing education and training	Technical University of Vienna, Institute of Chemical Engineering, Division Gene Technology and Applied Biochemistry
• Principal subject	Molecular Physiology of Reductive Monosaccharide Catabolism by *Hypocrea Jecorina*
• Title of qualification awarded	**PhD**
October 2001	**Graduation as Master of Science (Diplomingeneurin) at Technical University of Vienna**
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• Name and type of organisation providing education and training	Technical University of Vienna, Institute of chemical engineering
• Title of diploma thesis	Adaptation of LM-PCR for chromatinanalysis of the *cbh2*-promotor in *Hypocrea jecorina* and the influence of the *cbh2* activating element on nucleosome positioning
Dates (from – to)	1993 - 2001
• Name and type of organisation providing education and training	Technical University of Vienna
• Principal subjects/occupational skills covered	Studies of technical chemistry/ biochemistry, biotechnology and food chemistry
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