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Categorisation of sugar acid dehydratases in *Aspergillus niger*

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ABSTRACT

In the genome of *Aspergillus niger* five genes were identified coding for proteins with homologies to sugar acid dehydratases. The open reading frames were expressed in *Saccharomyces cerevisiae* and the activities tested with a library of sugar acids. Four genes were identified to code for proteins with activities with sugar acids: an L-galactonate dehydratase (*gaaB*), two D-galactonate dehydratases (*dgdA*, *dgdB*) and an L-rhamnonate dehydratase (*lraC*). The specificities of the proteins were characterised. The L-galactonate dehydratase had highest activity with L-fuconate, however it is unclear whether the enzyme is involved in L-fuconate catabolism. None of the proteins showed activity with galactaric acid or galactarolactone.

1. Introduction

In fungi, several sugar acid dehydratases are known that split off a water molecule from a sugar acid to generate the 2-keto-3-deoxy derivative of the sugar acid. Only in a few cases the gene sequences are known that code for the enzymes.

In filamentous fungi, an L-galactonate dehydratase was identified in *Hypocrea jecorina* (Kuorelahti et al., 2006), *Aspergillus niger* (Martens-Uzunova and Schaap, 2008) and *Botrytis cinerea* (Zhang et al., 2011). The enzymes in these species are close homologues and convert L-galactonate to 3-deoxy-L-threo-hex-2-ulosonate (2-keto-3-deoxy-L-galactonate). This enzyme is part of the eukaryotic D-galacturonic acid pathway. In the pathway, D-galacturonate is reduced to L-galactonate and 3-deoxy-L-threo-hex-2-ulosonate, the reaction product of the L-galactonate dehydratase, is split by an aldolase to pyruvate and L-glyceraldehyde. The latter is then reduced further to glycerol (Richard and Hilditch, 2009).

In the literature, also other eukaryotic pathways are described that use a sugar acid dehydratase. For example, L-rhamnose is oxidised to L-rhamnonate which is then reacting with a dehydratase. The resulting 3,6-dideoxy-L-erythro-hexulosonic acid (2-keto-3-deoxy rhamnonate) is subsequently split by and aldolase to pyruvate and L-lactaldehyde. The gene for the L-rhamnonate dehydratase was identified in *Scheffersomyces stipitis* as part of a cluster that contained all the genes coding for the enzymes for L-rhamnose catabolism (Watanabe et al., 2008; Koivistoinen et al., 2012a).

Similar to the L-rhamnose pathway is the fungal L-fucose pathway. L-Fucose is oxidised to L-fuconate which is then reacted with a dehydratase and the reaction product split by an aldolase to pyruvate and L-lactaldehyde (Guimarães and Veiga, 1990). For this pathway only the enzyme activities were described.

There are also reports that D-galactose can be catabolised by a similar pathway in filamentous fungi. Many fungal microrganisms use the Leloir pathway for D-galactose catabolism. In filamentous fungi such as *A. niger* and *H. jecorina* also an oxidoreductive pathway exist for D-galactose catabolism, which is actually the main pathway for this sugar in *A. niger* (Mojzita et al., 2012a). A third pathway for D-galactose catabolism was described in an *A. niger* strain that could be described as a non-phosphorylated De Ley–Doudoroff pathway (De Ley and Doudoroff, 1957). In this pathway D-galactose is oxidised by an NAD-utilising dehydrogenase to D-galactono-lactone which is then hydrolysed. The resulting D-galactonate is converted by a dehydratase to 3-deoxy-D-threo-hex-2-ulosonate (2-keto-3-deoxy D-galactonate) which is then split by an aldolase to pyruvate and D-glyceraldehyde (Elshafei and Abdel-Fatah, 2001). The genes coding for enzymes with such enzyme activities have not been described.

In this study we cloned all open reading frames of the homologues of sugar acid dehydrates that are present in the genome of the *Aspergillus strain* ATCC 1015 and expressed them in the yeast

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Saccharomyces cerevisiae. After the expression we analysed the dehydratase activities with a library of sugar acids.

2. Materials and methods

2.1. Cloning the open reading frames

The open reading frame for the protein with the Protein ID 191792 in the genome of the *A. niger* strain ATCC 1015 (http://genome.jgi-psf.org/) (GenBank accession number: EHA27292.1) was cloned by amplifying the 3 exons of the gene by PCR and recombining them with the yeast expression vector by homologues recombination in yeast. The exons were amplified by PCR from genomic DNA using the primers lraC_ORF_A_F/lraC_ORF_A_R, lraC_ORF_B_F/lraC_ORF_B_R and lraC_ORF_C_F/lraC_ORF_C_R (Table 1). The *S. cerevisae* strain CEN.PK2-1D was transformed with the three resulting PCR products and the EcoRI and BamHI digested plasmid p2159 according to the method of Gietz and Woods (Gietz and Woods, 2002). The plasmid p2159 was derived from the pYX212 (R&D systems) by modifying the multiple cloning site (Verho et al., 2004). The transformants were selected for uracil prototrophy. The resulting plasmid was verified by sequencing.

The gene for the Protein ID 38317 (GenBank accession number: EHA18083.1) had no intron and was amplified by PCR with the primers sodA_ORF_F and sodA_ORF_R (Table 1). The PCR product was digested with EcoRI and NheI and ligated to the corresponding sites of p2159.

The two exons of the gene for the Protein ID 49896 (GenBank accession number: EHA19069.1) were amplified by PCR using the primer pairs dgdA_ORF_A_F/dgdA_ORF_A_R and dgdA_ORF_B_F/dgdA_ORF_B_R. The yeast expression vector was then made by homologous recombination in yeast with the XmaI/NheI digested p2159.

The gene for the Protein ID 50500 (GenBank accession number: EHA20544.1) consisted of 4 exons that were amplified with the primers dgdB_ORF_A_F, dgdB_ORF_A_R, dgdB_ORF_B_F, dgdB_ORF_B_R, dgdB_ORF_C_F, dgdB_ORF_C_R, dgdB_ORF_D_F, dgdB_ORF_D_R. The four resulting fragments and the EcoRI and NheI digested pYX212 were ligated through homologous recombination in yeast.

The gene for the Protein ID 53563 (GenBank accession number: EHA22098.1) was amplified by PCR from cDNA of *A. niger* grown on D-galacturonic acid using primers gaaB_ORF_F and gaaB_ORF_R (Table 1). The PCR product was digested with XmaI and BamHI and ligated to the corresponding sites of p2159. All constructs were verified by sequencing.

The expression vectors with the different open reading frames were then used to transform the yeast strain CEN.PK2-1D (European *S. cerevisiae* Archive for Functional Analysis) and the resulting strains cultivated in selective glucose medium in the absence of uracil.

2.2. Cell extract and activity assay

Overnight cultures in 50 ml of synthetic complete medium with 2% D-glucose lacking uracil were harvested by centrifugation, washed once and resuspended in 1 ml of 10 mM phosphate buffer. To make a cell extract the resuspended cells were added to 2 ml vials containing approximately 1 ml of glass beads (0.5 mm diameter Sigma) and 80 μl of protease inhibitors (Complete, Roche). The cells in the vials were disrupted in two rounds of three cycles of 30 s each in the Precellys 24 machine (Bertin Technologies). The cell extracts were centrifuged for 20 min, at +4 °C in 14,000 rpm in a table-top centrifuge and the supernatants were used for the enzyme activity assays. The protein concentration was estimated using the Protein Assay kit (BioRad) using BSA as standard. For the dehydratase activity the cell extract was added to medium containing 10 mM of a sugar acid from our sugar acid library and 20 mM sodium phosphate buffer pH 7.0. The sugar acid library contained the following sugar acids salts: L-rhamnonate, L-galactonate, D-gulonate, D-gluconate, L-gulonate, D-arabonate, D-lyxonate, L-lyxonate, D-xylonate, meso-galactarate (muconate), L-fuconate, D-mannonate, L-mannonate, D-ribonate, L-arabonate, D-galactonate, D-galacturonate, D-glucuronate, galactarolactone and glucarolactone. Galactarolactone and glucarolactone were produced from D-galacturonic acid and D-glucuronic acid respectively using the purified uronate dehydrogenase (*udh*) from *Agrobacterium tumefaciens* and NAD as described previously (Boer et al., 2010). The reaction was stopped by mixing 45 μl of this mixture with 5 μl of 12% (w/v) trichloroacetic acid (TCA). The production of 2-keto-3-deoxy sugar was measured with the thiobarbituric acid assay as described by

Table 1
List of the primers used to amplify all the gene sequences of the characterised proteins: gaaB (GenBank: EHA22098.1), dgdA (GenBank: EHA19069.1), (dgdB GenBank: EHA20544.1), sodA (GenBank: EHA18083.1) and lraC (GenBank: EHA27292.1).

Primer	Sequence
gaaB_ORF_F	TCCCCCCGGGACCATGGTCAAGATCACAAGT
gaaB_ORF_R	TTAGGATCCTTTACCTCCCCACTATACTATC
lraC_ORF_A_F	TAAATCTATAACTACAAAAAACACATACAGGAATTAAAATGGGTTCTACCGGACAATT
lraC_ORF_A_R	ATGTTACTGTCAATCAACCAGTGACCTCCTTTGACATTATGATAATCACCTCCGG
lraC_ORF_B_F	TTGATGGTGTGGGATCCGGAGGTGATTATCATAATGTCAAAGGAGGTCACTGGTT
lraC_ORF_B_R	CATCTTCTCGAACAGGTCATTGGTATCTCTGGGGTCAGCACCAAGCAGGAAGCGC
lraC_ORF_C_F	CCATCAACACTTGAGCGCTTCCTGCTTGGTGCTGACCCCAGAGATACCAATGAC
lraC_ORF_C_R	TTCAGTTAGCTAGCTGAGCTCGACTCTAGAGGATCTTAAGTAGACTCCTGCTGGTTCT
dgdB_ORF_A_F	TAAATCTATAACTACAAAAAACACATACAGGAATTAAAATGGCTCCCATCAAGTCCAT
dgdB_ORF_A_R	GTCTCCAAATTGTCTGCCAGACATGCTCGATGTCATCAGCCTCATAACCAACAAT
dgdB_ORF_B_F	GAGATTATTGGGAGGATTGTTGGTTATGAGGCTGATGACATCGAGCATGTCTGGC
dgdB_ORF_B_R	TAACGCACTTGAGGCCCTGCGCGATACGGGCCTTGGCAGCTACCTCAACATCACT
dgdB_ORF_C_F	GGAGGTGACCGGCCAAGTGATGTTGAGGTAGCTGCCAAGGCCCGTATCGCGCAGG
dgdB_ORF_C_R	AGCAACTTGCATTGAAGCTGCAAGGGCAATTGGACCGAGGGGACAGTGCGGCGCA
dgdB_ORF_D_F	GTATGACGTGGCGATTGCGCCGCACTGTCCCCTCGGTCCAATTGCCCTTGCAGCT
dgdB_ORF_D_R	TTCAGTTAGCTAGCTGAGCTCGACTCTAGAGGATCTTACTACCACTCTCGGATTCCCC
sodA_ORF_F	TTACCTGAATTCAAAATGACCGAACTAAGAATCAC
sodA_ORF_R	TCCACCGCTAGCTCAATCATAACTAGCAACCG
dgdA_ORF_A_F	ACATACAGGAATTCGAGCTCGGTACCCGGGATCATGGTCAAGATCAAGTCTATCG
dgdA_ORF_A_R	CAGGCCATTTGCCATATGTGCTCAATGTCATCTGCTTCGTAGCCTTGAAATC
dgdA_ORF_B_F	TGACATTGAGCACATATGGC
dgdA_ORF_B_R	CGTTCATTGTTCCTTATTCAGTTAGCTAGCCTACCACTCCCGGATCCCTC

Buchanan et al. (1999). For each sugar acid a control was made with a yeast strain with the same expression vector but not expressing any additional gene. With none of the sugar acids any activity to form a 2-keto-3-deoxy sugar acid was detected. The sugar acids that were not commercially available were prepared from the corresponding sugars as described previously (Yew et al., 2006). Care was taken that in the thiobarbituric acid assay the sugar acid concentrations did not exceed 10 mM. For that reason the reaction mixture was diluted prior the thiobarbituric acid assay for the estimation of the Michaelis–Menten constants.

3. Results

Protein ID 191792: The protein from the *A. niger* strain ATCC1015 (LraC) with the Protein ID 191792 was expressed in *S. cerevisiae* and the enzyme activities tested with crude cell extracts and the sugar acids as detailed in Table 2. High activity was observed with L-rhamnonate and some with L-mannonate. L-Rhamnonate and L-mannonate have a similar chemical structure (Fig. 1); the only difference between them is at the C6 where the L-rhamnonate has no hydroxy group. The Lra3 of *S. stipitis* is a homologue enzyme. Also with this enzyme, activities with L-rhamnonate and L-mannonate were observed (Koivistoinen et al., 2012a). The Michaelis–Menten constants were estimated with L-rhamnonate as substrate (Vmax = 0.095 nkat per mg of extracted protein and Km = 2.99 mM, Fig. 2).

Protein ID 38317: The protein with the ID 38317 (SodA) was expressed in yeast *S. cerevisiae* and the activity tested with our library of sugars. With none of the sugars we tested dehydrates activity was observed with the crude cell extract (Table 2).

Protein ID 49896: The protein with the ID 49896 (DgdA) was expressed in *S. cerevisae* and its crude cell extract was tested as described above (Table 2). It showed a high activity with D-galactonate as substrate. Michaelis–Menten constants were estimated with D-galactonate as substrate. The Michaelis Menten constants are Vmax = 8.52 nkat per mg of extracted protein and Km = 8.9 mM (Fig. 3).

Protein ID 50500: The protein with the ID 50500 (DgdB) was expressed in *S. cerevisiae* and tested as described above (Table 2).

Table 2
Dehydratase activities of the five sugar acid dehydratases after expression in *S. cerevisiae* with different sugar acids. The activities are given in nkat/mg of extracted protein. The detection limit was estimated 0.01. The (–) indicates that no activity was observed or that the activity was close to the detection limit.

Substrate	gaaB	lraC	dgdA	dgdB	sodA
L-Rhamnonate	–	0.07	–	–	–
L-Galactonate	0.05	–	–	–	–
D-Gulonate	–	–	–	–	–
D-Gluconate	0.03	–	–	–	–
L-Gulonate	–	–	–	–	–
D-Arabonate	–	–	–	–	–
D-Lyxonate	–	–	–	–	–
L-Lyxonate	–	–	–	–	–
D-Xylonate	–	–	–	–	–
Mucic acid	–	–	–	–	–
L-Fuconate	0.52	–	–	–	–
D-Mannonate	0.02	–	–	–	–
L-Mannonate	0.02	0.05	–	–	–
D-Riborate	–	–	–	–	–
L-Arabonate	–	–	–	–	–
D-Galactonate	–	–	0.82	0.23	–
D-Galacturonate	–	–	–	–	–
D-Glucuronate	0.02	–	–	–	–

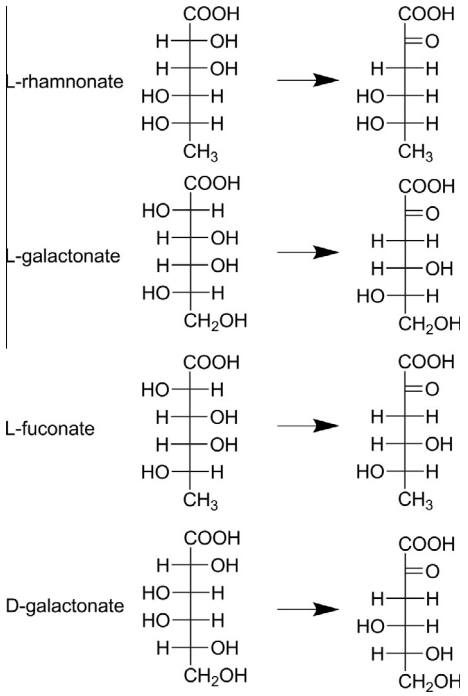

Fig. 1. Fischer projection of the sugar acids that show activity with the dehydratases present in *A. niger* and their reaction products. The LraC shows activity with L-rhamnonate and L-mannonate, the GaaB activity with L-galactonate and L-fuconate and the DgdA and DgdB have activity with D-galactonate.

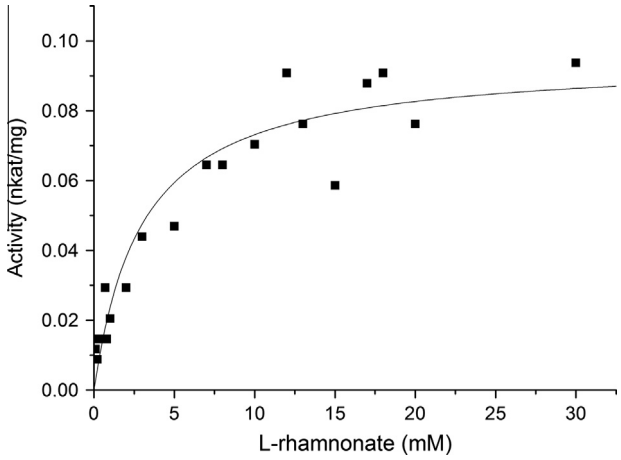

Fig. 2. Kinetic properties of the protein with the Protein ID 191792 (LraC), in a crude cell extract, using L-rhamnonate as substrate. The curve was calculated assuming Michaelis–Menten kinetics and using the constants Vmax = 0.095 nkat/mg and Km = 3 mM.

We also tested the DgdB with 6 histidines added at the N-terminal end. This his-tagged protein however did not show activity with any sugar acid. The non-tagged protein showed high activity with D-galactonate suggesting that it is a D-galactonate dehydratase. For the Michaelis–Menten constants we estimated a Vmax of 2.21 nkat per mg of extracted protein and a Km of 21.74 mM (Fig. 3).

Protein ID 53563: The protein with the ID 53563 (GaaB) was expressed in *S. cerevisiae* and the crude cell extract was tested against the sugar acids (Table 2). It showed a weak activity with several sugars: D-gluconate, D- and L-mannonate, and D-glucuronate. Considerable activity was observed with L-galactonate and an even higher activity was observed with L-fuconate. Both sugars have the

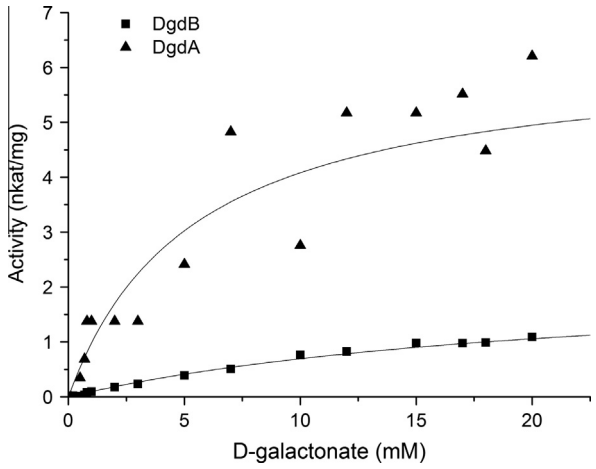

Fig. 3. Comparison of kinetic properties between the protein with the Protein ID 49896 (DgdA) and with the Protein ID 50500 (DgdB) using D-galactonate as substrate. The Michaelis–Menten curve for DgdA was calculated using Vmax = 8.52 nkat/mg and a Km = 8.9 mM. The Michaelis–Menten curve for DgdB was calculated with Vmax = 2.21 nkat/mg and a Km = 21.74 mM.

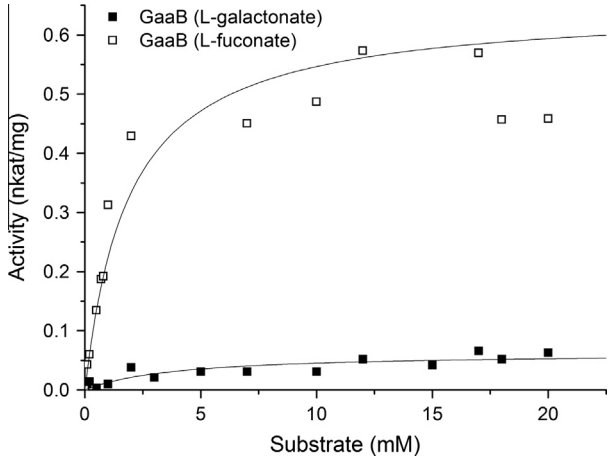

Fig. 4. Comparison of kinetic properties of the protein with the ID 53563 (GaaB) in crude cell extracts, using either L-galactonate or L-fuconate as substrate. The Michaelis–Menten curve with L-galactonate was calculated using the constants Vmax = 0.063 nkat/mg and Km = 3.92 mM as with L-fuconate, using the constants Vmax = 0.65 nkat/mg and Km = 1.89 mM.

same configuration except that L-fuconate is lacking the hydroxyl group at the C6 (Fig. 1). We tested the activities with these two sugars in order to estimate the Michaelis–Menten constants. The constants were estimated as Vmax = 0.65 nkat per mg of extracted protein and Km = 1.89 mM, for L-fuconate; for L-galactonate, Vmax = 0.063 nkat per mg of extracted protein and Km = 3.92 mM (Fig. 4). All proteins were also tested for activity with galactarolactone and glucarolactone that was produced from D-galacturonate and D-glucuronate immediately before use using an uronate dehydrogenase. None of the proteins showed activity with these lactones.

4. Discussion

The *A. niger* strain ATCC 1015 is a wild type strain that was used in the first patented citric acid process in the beginning of the last century. It has a wide array of hydrolytic and oxidative enzymes involved in the breakdown of plant lignocellulose and pectin and can utilise a large variety of carbon sources. It is also one of the *A. niger* strains where the genome sequence is available which made it suitable for this study.

The genome of the *A. niger* strain ATCC 1015 contains five genes that code for proteins with homology to sugar acid dehydratases. The open reading frames of these genes were expressed in the yeast *S. cerevisiae* from a multi copy vector with a strong promoter. The yeast cells were then lysed and the crude cell extract tested for dehydratase activities with about 20 different sugar acids. We did not purify the enzymes. For purification enzymes are often tagged with an affinity tag such a histidine tag. For the eukaryotic dehydratases this tagging had however a detrimental effect on the enzyme activities. In the case of the *S. stipitis* L-rhamnonate dehydratase the enzyme was inactivated (Koivistoinen et al., 2012a) and in the case of the *H. jecorina* L-galactonate dehydratase the enzyme activity was lost when C-terminally tagged and activity was reduced when the enzyme was tagged N-terminally (Kuorelahti et al., 2006). To have comparable results we expressed the open reading frames of the different dehydratases from the same yeast expression vector and normalised the activity to the total protein of the extract. The dehydratases are producing a 2-keto-3-deoxy sugar acid that can be detected with the thiobarbituric acid assay which is essentially detecting deoxy sugars. Without the overexpression of any dehydratase no activity with any sugar acid could be detected indicating that this expression system is suitable for testing this class of enzymes.

Of the five proteins, the protein with the ID 191792 (LraC) had the highest homology with the L-rhamnonate dehydratase Lra3 from *S. stipitis* (Watanabe et al., 2008). The corresponding gene is also part of a cluster that was previously identified as a potential cluster of genes of the L-rhamnose catabolic pathway (Koivistoinen et al., 2012a). The protein had indeed L-rhamnonate dehydratase activity after expression in *S. cerevisiae*. Similar to the *S. stipitis* protein it showed also activity with L-mannonate (Koivistoinen et al., 2012a). Also a procaryotic L-rhamnose dehydratase was described previously. This enzyme had, besides the activity with L-rhamnonate, activity with L-mannonate but also with L-lyxonate and D-gulonate (Rakus et al., 2008).

The protein with the ID53563 (GaaB) is a homologue to the L-galactonate dehydratase that was previously identified in *H. jecorina* (Lgd1) and in the *A. niger* strain CBS 120.49 (ATCC 9029) (GaaB) (Kuorelahti et al., 2006; Martens-Uzunova and Schaap, 2008). The enzyme that was studied here (*A. niger* strain ATCC1015) is also an L-galactonate dehydratase similar to the previously characterised proteins and has a similar Km value. The protein showed also activity with L-fuconate. L-Galactonate and L-fuconate have a similar chemical structure; the only difference is that L-fuconate has no hydroxyl group at the C6 (Fig. 1). The activity with L-fuconate was higher than with L-galactonate and the affinities were similar (Km L-galactonate = 3.92 mM/L-fuconate = 1.89 mM; Fig. 4). This could be an indication that this enzyme is not only functional in the D-galacturonic acid pathway

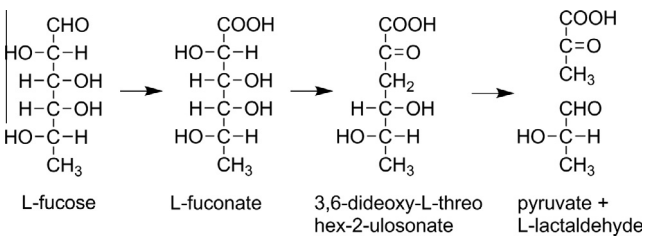

Fig. 5. The eukaryotic L-fucose pathway as suggested by Guimarães and Veiga. L-Fucose is oxidised to L-fuconate in an NAD requiring reaction with L-fuconolactone as an intermediate. L-Fuconate is then converted by the action of a dehydratase and an aldolase to pyruvate and L-lactaldehyde.

but also in the L-fucose pathway. An eukaryotic L-fucose pathway with L-fuconate as an intermediate was described at the level of enzyme activities (Fig. 5) (Guimarães and Veiga, 1990), however no genes were related to this pathway. In order to see if such an L-fucose pathway exists in this *A. niger* strain we tested for growth on L-fucose but did not observe growth in liquid medium. However L-fucose disappeared from the medium. To test whether the disappearance of L-fucose is due to the GaaB activity we tested growth and disappearance of L-fucose from the medium with a strain that had a deletion in the gaaB (*A. niger* ATCC 1015 Δ*gaaB* (Kuivanen et al., 2012)). Also this strain did not grow on L-fucose but L-fucose disappeared from the medium. Since it was not clear whether this strain is catabolising L-fucose it cannot be decided whether this enzyme is functional also in the L-fucose pathway. The next enzyme in the L-fucose pathway would be an aldolase to split the 2-keto-3-deoxy-L-fucose to pyruvate and L-lactaldehyde (Fig. 5). The aldolase from the D-galacturonic acid pathway might catalyse also this reaction. The aldolase from *H. jecorina* D-galacturonic acid pathway, Lga1, a close homologue of the *A. niger* GaaC, was unspecific except for the substrate configuration at the C4 (Hilditch et al., 2007). All this could suggest that the L-galactonate dehydratase or a close homologue might be functional in the L-fucose pathway in related species but in this strain the L-fucose pathway is not active.

The proteins with the ID 49896 and 50500 showed activity with D-galactonate. These D-galactonate dehydratases could be part of an oxidative pathway for D-galactonate catabolism that was previously decribed in an *A. niger* strain (Elshafei and Abdel-Fatah, 2001). In this pathway D-galactose is oxidised by an NAD dependent dehydrogenase to D-galactono-lactone which is either spontaneously or with the aid of a lactonase hydrolysed to the linear D-galactonate. A D-galactonate dehydratase produces then 2-keto-3-deoxy D-galactonate which is then split by an aldolase to pyruvate and D-glyceraldehyde. These enzyme activities have been reported but so far the corresponding genes were elusive. It is also not clear whether this pathway is active in all *A. niger* strains. In the *A. niger* strain ATCC 1015 D-galactose is predominantly catabolised through the oxido-reductive pathway that is similar to the L-arabinose pathway (Seiboth and Metz, 2011). Deletions of the genes of this pathway such as the *ladB* (Mojzita et al., 2012b), *xhrA* (Mojzita et al., 2012a) or *sdhA* (Koivistoinen et al., 2012b) resulted in reduced growth on D-galactose. It might be that the oxidative D-galactose pathway with D-galactonate as intermediate is only active in selected strains or that it is induced only in unusual conditions. This pathway would also require an aldolase splitting the 2-keto-3-deoxy-D-galactonate to pyruvate and D-glyceraldehyde. The aldolase from the D-galacturonic acid pathway is not suitable for this. At least the enzyme from *H. jecorina* is not active with 2-keto-3-deoxy-D-galactonate (Hilditch et al., 2007).

With the protein with the ID38317 (SodA), we did not see any activity with any of the sugar acids tested. This suggests that this enzyme is either not a sugar acid dehydratase or our library did not contain the substrate for this enzyme. It is also possible that the heterologous expression did not result in an active protein. Rakus et al. obtained a crystal structure from the L-rhamnonate dehydratase from *Salmonella typhimurium* and suggested a reaction mechanism for the dehydratation. In this mechanism the His329 which is hydrogen bonded to the Asp302 abstracts a proton from the C2 to generate the enoldiolate intermediate (Rakus et al., 2008). The corresponding amino acid sequences of the *A. niger* enzymes are in Fig. 6. While all proteins with sugar acid dehydratase activity have histidine and aspartic acid in the corresponding positions, the SodA does not. This could be interpreted that this protein is not a sugar acid dehydratase.

It was previously observed that when the gene for the D-galacturonic acid reductase *gaaA* was deleted, growth on D-galacturonic acid stopped. However when in addition to the *gaaA* deletion a

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                *                                *
DgdA      SVDILQPDIAHCGGISELRRIASMAETYDVAIAPHCPLGS
DgdB      SVDVLQPDIAHAGGISETKRIATMAETYDVAIAPHCPLGP
LraC      NLDILQPDVMWVGGMTELLKVSALASAYDLPVVPHASGPY
GaaB      ALTVLQADACRVGGVNEVLAILLLARKFGVPIVPHSGGVG
SodA      AVDGIGLKISKTGGLTRGRRVRDICLAAGYTMSVQDTSGS
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Fig. 6. Alignment of proteins sequences of the *A. niger* proteins with homologies to sugar acid dehydratases. The asterisks at D256 and H283 of the DgdA indicate the amino acids that are in the catalytic moiety functioning as the base that is abstracting the proton from the C2 during the dehydratation. The SodA is lacking the aspartic acid and the histidine in the equivalent positions.

D-galacturonic acid dehydrogenase was expressed, the resulting strain was growing again on D-galacturonic acid (Mojzita et al., 2010). This would suggest that *meso*-galactaric acid (mucic acid) or the galactaro-1,4-lactone that is formed by the dehydrogenase is catabolised. The only pathway that is described for galactaric acid catabolism involves a dehydratase or in the case of galactaro-1,4-lactone an galactarolactone cycloisomerase (Andberg et al., 2012) to produce 2-keto-3-deoxy galactarate. The cycloisomerase is from the same protein family as the sugar acid dehydratases. Since the genome of *A. niger* has no homologue of the bacterial galactarate dehydratase and galactarate has a similar structure as D- or L-galactonate, we tested the dehydratases for activity with galactaric acid galactaro-1,4-lactone, however no activity was detected with any of the enzymes. This suggests that there is either another pathway for galactarate or proteins from another protein family are active.

5. Conclusions

A. niger has one gene for a L-rhamnonate dehydratase, *lraC*, that is part of the pathway for L-rhamnose catabolism and one gene for L-galactonate dehydratase, *gaaB*, that is part of the pathway for D-galacturonic acid catabolism. The LraC hac also activity with L-mannonate. The GaaB has a higher activity with L-fuconate than with L-galactonate, however it is unclear whether this gene is involved in L-fucose catabolism. *A. niger* has also two genes for D-galactonate dehydratase, *dgdA* and *dgdB*. An oxidative pathway for D-galactose catabolism with D-galactonate dehydratase as a part of the pathway had been suggested however the remaining genes of this pathway have not been identified.

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