Clustered Genes Encoding 2-Keto-L-Gulonate Reductase and L-Idonate 5-Dehydrogenase in the Novel Fungal d-Glucuronic Acid Pathway

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D-Glucuronic acid is a biomass component that occurs in plant cell wall polysaccharides and is catabolized by saprotrophic microorganisms including fungi. A pathway for D-glucuronic acid catabolism in fungal microorganisms is only partly known. In the filamentous fungus Aspergillus niger, the enzymes that are known to be part of the pathway are the NADPH requiring D-glucuronic acid reductase forming L-gulonate and the NADH requiring 2-keto-L-gulonate reductase that forms L-idonate. With the aid of RNA sequencing we identified two more enzymes of the pathway. The first is a NADPH requiring 2-keto-L-gulonate reductase that forms L-idonate, GluD. The second is a NAD+ requiring L-idonate 5-dehydrogenase forming 5-keto-D-gluconate, GluE. The genes coding for these two enzymes are clustered and share the same bidirectional promoter. The GluD is an enzyme with a strict requirement for NADP+/NADPH as cofactors. The kcat for 2-keto-L-gulonate and L-idonate is 21.4 and 1.1 s⁻¹, and the Km 25.3 and 12.6 mM, respectively, when using the purified protein. In contrast, the GluE has a strict requirement for NAD+/NADPH. The kcat for L-idonate and 5-keto-D-gluconate is 5.5 and 7.2 s⁻¹, and the Km 30.9 and 8.4 mM, respectively. These values also refer to the purified protein. The gluD deletion resulted in accumulation of 2-keto-L-gulonate in the liquid cultivation while the gluE deletion resulted in reduced growth and cessation of the D-glucuronic acid catabolism.

Keywords: fungi, Aspergillus, metabolism, D-glucuronate, D-glucuronic acid, L-idonate, 2-keto-L-gulonate

INTRODUCTION

The genus Aspergillus is a large group of filamentous fungi containing species that are known to be versatile decomposers of biomass polymers (de Vries and Visser, 2001). Aspergillus niger – a member of the group of black aspergilli – is widely used in industrial biotechnology due to its useful characteristics such as capacity to produce organic acids and biomass hydrolysing enzymes in high yields. Several different sugars and sugar acids resulting from the extracellular biomass hydrolysis by a mixture of secreted enzymes are catabolized by the organism through metabolic pathways. Many of these pathways are known and characterized; however, some remain still unknown and
may contain enzymes and biochemical reactions that are not described earlier. These reactions may serve as source of enzymes for biotechnological applications such as production of fuels and chemicals from biomass.

One such a biomass component with limited knowledge on its catabolism is D-glucuronic acid (D-glcUA). It occurs in the cell wall polysaccharides such as glucuronoxylan (Reis et al., 1994) in plants and ulvan (Lahaye and Robic, 2007) in algae. In nature, D-glcUA resulting from biomass hydrolysis is catabolized by saprotrophic microorganisms through different metabolic pathways. In bacteria, two different catabolic pathways for D-glcUA are known: an isomerase pathway (Ashwell, 1962) and an oxidative pathway (Dagley and Trudgill, 1965; Chang and Feingold, 1970). D-GlcUA and its close structural isomer D-galacturonic acid (D-galUA), a pectin constituent, are catabolized analogously via these pathways in bacteria. Some of the enzymes in these pathways have dual functions and are used for the catabolism of both compounds. In addition to the bacterial pathways, a different catabolic D-glcUA pathway is known in animal cells (Hankes et al., 1969). The animal pathway, also known as glucuronate-xylulose-pentose phosphate pathway or uronate cycle, contains two oxidation, two oxidation and one decarboxylation reactions resulting in formation of D-xylulose, which, after phosphorylation to D-xylulose 5-phosphate, is a metabolite of pentose phosphate pathway (Figure 1A). In fungi, the catabolic pathway for D-galUA is well known including reduction, dehydration, and an aldolase reaction and second reduction (Figure 1B) (Kuorelahti et al., 2005, 2006; Liepins et al., 2006; Hilditch et al., 2007). However, a fungal pathway for D-glcUA catabolism is only partly known.

The first enzyme for D-galUA catabolism in the filamentous fungus A. niger has most likely a dual function and is also the first enzyme in D-glcUA catabolism. The gaaA, encoding a hexuronate reductase is reducing D-galUA to L-galactonate and D-glcUA to L-gluconate (Martens-Uzunova and Schaap, 2008; Kuivanen et al., 2016). Transcription of gaaA was induced on both of these carbon sources and deletion of the gene reduced the catabolism of both carbon sources, however, did not block it completely (Kuivanen et al., 2016). In the following steps the pathways for D-galUA and D-glcUA differ, the L-galactonate dehydratase showed no activity with L-gulonate and an L-gulonate dehydratase activity was not found in A. niger (Motter et al., 2014). An enzyme that is essential for D-glcUA catabolism was identified to be a NADH dependent 2-keto-L-gulonate reductase, GluC (Kuivanen et al., 2016). Deletion of gluC gene resulted in reduced growth on D-glucUA plates and blocked the D-glucUA consumption in liquid cultivations. The L-gulonate is converted to 2-keto-L-gulonate by an unknown enzyme. For the further conversion of L-idonate, two enzyme activities have been described in the literature: the NAD+ and NADP+ dependent L-idonate 5-dehydrogenases (EC 1.1.1.366 and EC 1.1.1.264). The NAD+ dependent activity has been described in plants (Wen et al., 2010) in the pathway for L-ascorbic acid catabolism (DeBolt et al., 2006) and in bacteria as part of L-idonate catabolism (Bausch et al., 1998). The NADP+ dependent L-idonate 5-dehydrogenase activity was described for the first time already long time ago in the filamentous fungus Fusarium sp. (Takagi, 1962). However, there is no report on a fungal L-idonate 5-dehydrogenase gene or the biological function of such a gene.

In the present study, we identify a gene cluster encoding NADPH dependent, L-idonate forming, 2-keto-L-gulonate reductase and NAD+ dependent L-idonate 5-dehydrogenase which forms 5-keto-D-gluconate (Figure 1C). These genes are involved in the fungal D-glcUA catabolism and the reaction catalyzed by the latter enzyme is a direct continuation for the previously identified reaction by the action of GluC.

MATERIALS AND METHODS

Strains
The A. niger strain ATCC 1015 (CBS 113.46) was used as a wild type. The A. niger mutant strain ΔpyrG (deleted orotidine-5'-phosphate decarboxylase) was described earlier (Mojzita et al., 2010). All the plasmids were produced in Escherichia coli TOP10 cells. The Saccharomyces cerevisiae strains ATCC 90845 and a modified CEN.PK2 (MATa, len2-3/112, ura3-52, trp1-289, his3-Δ1, MAL2-8Δ, SUC2) were used in the homologous recombination for the plasmid construction and for the production of the purified GluD and GluE enzymes, respectively.

Media and Cultural Conditions
Luria Broth culture medium supplemented with 100 µg ml⁻¹ of ampicillin and cultural conditions of 37°C and 250 rpm were used with E. coli. YPD medium (10 g yeast extract l⁻¹, 20 g peptone l⁻¹, and 20 g D-glucose l⁻¹) was used for yeast pre-cultures. After the transformation of an expression plasmid in yeast, SCD-URA (uracil deficient synthetic complete media supplemented with 20 g D-glucose l⁻¹) plates were used for uracil auxotrophic selection. SCD-URA medium was used in protein production. All the yeast cultivations were carried out at 30°C and the liquid cultivations at 250 rpm. A. niger spores were generated on potato-dextrose plates and ∼10⁸ spores were inoculated to 50 ml of YP medium (10 g yeast extract l⁻¹, 20 g peptone l⁻¹) containing 30 g gelatin l⁻¹ for pre-cultures. Mycelia were pre-grown in 250-ml Erlenmeyer flasks by incubating overnight at 28°C, 200 rpm and harvested by vacuum filtration, rinsed with sterile water and weighted. In A. niger transformations, SCD-URA plates supplemented with 1.2 M D-sorbitol and 20 g agar l⁻¹ (pH 6.5) were used. A. nidulans defined minimal medium (Barratt et al., 1965) was used in the A. niger cultivations. The minimal medium used in the phenotypic characterization in liquid cultivations contained 20 g D-glucUA l⁻¹ and the pH was adjusted to 3. These cultivations were inoculated with 20 g l⁻¹ (wet) of pre-grown mycelia. The strain ΔgluA was cultivated in 24-well plates in 4 ml final volume and ΔgluE in shake flasks in 25 ml final volume. Agar plates used for phenotypic characterization contained uracil deficient SC-medium (synthetic complete), 15 g agar l⁻¹ and 10 g D-glucUA l⁻¹. Plates were inoculated with 1.5×10⁶ spores and incubated at 28°C for 3 days.
Transcriptional Analysis
The RNA sequencing for transcriptional analysis was carried out as described previously (Kuivanen et al., 2016). The protein ID numbers refer the numbers from the Join Genome Institute (JGI), MycoCosm, A. niger ATCC 1015 v.4.0 database, available at: http://genome.jgi.doe.gov/Aspni7/Aspni7.home.html.

Protein Production and Purification
The gene gluD was amplified by PCR (KAPA HiFi DNA polymerase, Kapa Biosystems, primers in Table 1) from A. niger cDNA extracted and generated from D-glucUA cultivated wild type strain. The resulting DNA fragment was digested with BamHI and NheI (both NEB) and ligated into a modified pYX212 plasmid (Verho et al., 2004) containing TPI1 promoter and URA3 selectable marker. The gluE gene was custom synthesized as a yeast codon optimized gene (GenScript, USA), released with EcoRI and BamHI (both NEB) and ligated into the modified pYX212 plasmid. For the histidine-tagged protein, gluE was amplified by PCR (primers in Table 1) and ligated in a similar manner to the modified pYX212 plasmid. A yeast strain was then transformed with the resulting plasmids using the lithium acetate method (Gietz and Schiestl, 2007). The procedure for protein production and purification was described previously (Kuivanen et al., 2016).

Enzymatic Assays
The oxidoreductase activity of purified GluD and GluE proteins was assayed using Konelab 20XT Clinical Chemistry Analyzer (Thermo Scientific). The reaction mixture contained 50 mM Tris buffer, 400 µM NAD+ or NADH, a substrate in different concentrations and purified proteins in a final concentration of 3.6 mg l⁻¹. The pH 8 was used with NAD+ and L-idonate and pH 7 with NADH and 2-keto-D-gulonate and 5-keto-D-gluconate. The reaction was started by addition of the purified protein and the formation/consumption of NADH was followed at 340 nm. The kinetic parameters were determined using the IC50 tool kit. L-Idonate and 2-keto-D-gulonate were ordered as custom synthesized by Omicron Biochemicals Inc, USA while 5-keto-D-gluconate was ordered from Sigma-Aldrich.

Gene Deletions in A. niger
The deletion cassette for gluD contained homologous 5’ (~450 bp) and 3’ flanks (~650 bp) for targeted integration and the selectable marker pyrG. The 5’ and 3’ flanks were amplified by PCR with the primers as described in Table 1. The resulting PCR amplified fragments contained 40 bp compatible ends for homologous recombination with the A. niger pyrG and EcoRI and BamHI digested pRS426. The deletion cassette for gluE was constructed in a similar manner but contained homologous 5’ and 3’ flanks of 1.5 kb (primers in Table 1). All the fragments were joined using yeast homologous recombination as described earlier (Kuivanen et al., 2015). The resulting deletion cassette for gluD was produced by PCR amplification (primers in Table 1) from the resulting plasmid and the cassette for

\[\text{IC50.tk} \]
TABLE 1 | Oligonucleotides used in the study.

| Name | Sequence | Description |
|------|----------|-------------|
| P1   | ATATACATATGCGGCGGTTCGCTATGAAATGCTATGGGAGGTG | 5′ flank of gluD, FOR |
| P2   | TCAATCACCCGATGCGGATGCTGTAAG | 5′ flank of gluD, REV |
| P3   | GCGTGATAACATGATGTGCGCATCAG | 3′ flank of gluD, FOR |
| P4   | CCGGATCAAGCTCAGTTGCTACGATCTGTTGACCCAGGCTTGACCAGGCTTGACTTGAC | 3′ flank of gluD, REV |
| P5   | ACCGATCCATGCGGATGCTGTAAG | 5′ flank of gluE, FOR |
| P6   | CTGGATATGGCATGCGGATGCTGTAAG | 5′ flank of gluE, REV |
| P7   | GCGTGATAACATGATGTGCGCATCAG | 3′ flank of gluE, FOR |
| P8   | AGCTCCACGCGGCGGCGGCGCTCTAGAACTAGTGGATCGCGGCCGCCTTTGGTAC | 3′ flank of gluE, REV |

gluE deletion was produced by linearization of the plasmid with NotI (NEB). The gluD deletion cassette was transformed to A. niger ΔpyrG strain together with the CRISPR plasmid pFC-332 (Nodvig et al., 2015) and the in vitro synthesized sgRNA (CTCCCTCATCCTGACCCCTG) (GeneArt™ Precision Synthesis Kit). The gluE deletion cassette was transformed to A. niger ΔpyrG strain without the CRISPR plasmid. Mutants with successful integration of the cassette were selected for growth in the absence of uracil and, in the case of gluD deletion, in the presence of hygromycin (for pFC-332) and in the absence of uracil (for the deletion cassette containing pyrG). Resulting transformants were screened for the correct integration of the deletion cassette and for the deletion of gluD or gluE open reading frame using diagnostic PCR (Phire direct PCR kit, Thermo Scientific, primers in Table 1).

Chemical Analyses
Samples were removed from liquid cultivations at intervals and mycelium was separated from the supernatant by centrifugation or filtration. The concentration of D-glucuronic acid was determined by HPLC using a Fast Acid Analysis Column (100 mm × 7.8 mm, Bio-Rad Laboratories, Hercules, CA, USA) linked to an Aminex HPX-87H organic acid analysis column (300 mm × 7.8 mm, Bio-Rad Laboratories) with 5.0 mM H2SO4 as eluent and a flow rate of 0.5 ml min⁻¹. The column was maintained at 55°C. Peaks were detected using a Waters 2487 dual wavelength UV (210 nm) detector. The retention times of the peaks resulting from the supernatant were compared with the retention times of standards.

RESULTS
Clustered Genes Are Induced by D-Glucuronic Acid
RNA sequencing of the A. niger wild type strain ATCC 1015 cultivated in D-glucUA as sole carbon source revealed several putative genes with induced transcription (Figure 2). Figure 2 presents the induction of transcript levels between 0 and 4 hours (Y-axis) and the absolute transcript levels at 4 h (X-axis). We selected genes that were induced on D-glcUA (Figure 2, values on Y-axis clearly above 1), had absolute transcript levels around similar or higher than that of actin at 4 h (Figure 2, X-axis) and are predicted to code for a metabolic enzyme, such as oxidoreductases. The D-galUA/D-glucUA reductase gaaA and the 2-keto-l-gulonate reductase glcC were among the most induced genes as reported earlier (Kuivanen et al., 2016). In addition, two genes, with the protein identifiers 1114837 and 1099233 (JGI, MycoCosm, A. niger ATCC 1015 v.4.0 database), putatively encoding a D-isomer specific 2-hydroxy acid dehydrogenase and an alcohol dehydrogenase, respectively, were induced. These genes are clustered in the genome in opposite directions relative to each other and share a common promoter region of 455 bp
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FIGURE 2 | RNA sequencing of A. niger wild type strain – fold change in transcript abundancies 4 h after the shift to d-glucuronic acid (y-axis) and transcript level (x-axis) 4 h after the shift to d-glucuronic acid. The genes gaaA, gluC, actin and the genes with the protein IDs 1114837 (gluD) and 1099233 (gluE) are highlighted. Transcript levels are presented as fragments per kilobase of exon per million fragments mapped (FPKM). The protein ID numbers refer the numbers from the JGI MycoCosm A. niger ATCC 1015 v.4.0 database.

FIGURE 3 | (A) The gene cluster of gluD (as predicted in JGI MycoCosm A. niger ATCC 1015 database v3.0 = ID 43297; v4.0 = ID 1114837 and sequenced from cDNA = gluD) and gluE (ID 1099233) and (B) the differences in protein sequences of GluD as predicted in the v3.0 (43297), v4.0 (1114837) and determined from cDNA.

(Figure 3A). The fold change in transcript levels after the shift to d-glcUA was exactly the same for these genes while 1114837 had slightly higher transcript abundancy (Figure 2).

The Clustered Genes gluD and gluE Code for 2-Keto-L-Gulonate Reductase and L-Idonate 5-Dehydrogenase

The open reading frames of the two genes were cloned in multicopy yeast expression vectors, expressed in yeast and the crude cell extracts were tested for activity with a small library of sugars and sugar acids. Both enzymes showed activity towards L-idonate. The 1114837 had activity with NADP⁺ as a cofactor whereas the 1099233 had activity when the cofactor was NAD⁺. In the case of 1114837 we noticed that the open reading frame that was custom synthetized according to the open reading frame as predicted in the DOE JGI A. niger ATCC 1015 v3.0 database (the protein ID in v3.0 is 43297), did not result in an active protein, however, when the gene was amplified from A. niger cDNA the resulting protein was active. In the current DOE JGI A. niger ATCC 1015 v4.0 database the exon prediction has been changed, however, both of the predictions (v3.0 and 4.0) are wrong. The sequence of the 1114837 amplified from cDNA differs from the predicted sequences: In the prediction v3.0, 21 nucleotides were predicted to be an intron and are missing in the open reading frame whereas in the prediction v4.0 the
exons 3, 4 and the intron between them are combined. This is shown in the Figure 3A (v3.0 = 43297 and v4.0 = 1114837) and the differences in the resulting protein sequences are shown in Figure 3B. The correct gene sequence was deposited at GenBank with the accession number KX443112.

The enzyme 1114837 showed, besides the activity with NADP$^+$ and L-Idonate, also activity with 2-keto-L-gulonate and NADPH as cofactor. This suggests that the enzyme is NADPH-dependent 2-keto-L-gulonate reductase. We named the gene gluD. The gene 1099233 had activity with NAD$^+$ and L-Idonate but did not show activity with 2-keto-L-gulonate and NADH. It showed, however, activity with 5-keto-D-glucurate and NADH. We conclude that the enzyme is a NAD$^+$-dependent L-Idonate 5-dehydrogenase. We named the gene gluE.

For the more detailed characterization, histidine tagged GluD and GluE proteins were produced in yeast and the kinetic parameters of the purified proteins were investigated. Purified GluD showed NADPH/NADP$^+$-dependent oxidoreductase activity toward 2-keto-L-gulonate and L-Idonate with the $k_{cat}$ values of 21.4 and 1.1 s$^{-1}$, respectively. The $K_m$ values for the substrates were 25.3 and 12.6 mM, respectively. Purified GluE protein had strictly NAD$^+$/NADH-dependent oxidoreductase activity toward L-Idonate and 5-keto-D-glucurate with the $k_{cat}$ values of 5.5 and 7.2 s$^{-1}$, respectively. The $K_m$ values for the substrates were 30.9 and 8.4 mM, respectively. Kinetic parameters of GluD and GluE are presented in Table 2 and Supplementary Figure 1.

**Deletion of gluD or gluE has an Effect on D-Glucuronic Acid Catabolism**

We also deleted the genes gluD and gluE from A. niger and tested the resulting phenotypes. For the gluD gene deletion CRISPR technology was used to remove the native gene. This was implemented using the AMA-plasmid expressing Cas9 (Nodvig et al., 2015), an in vitro synthesized sgRNA and the deletion cassette with the selectable marker pyrG. GluE gene was deleted without CRISPR using only the deletion cassette containing pyrG marker. Both of the gene deletions were confirmed with diagnostic PCR and the mutant strains were tested for growth and ability to catabolize D-glucUA.

The mutant strain ΔgluD did not show reduced growth when cultivated on agar plate with D-glucUA as sole carbon source (Figure 4). However, in the liquid cultivation on D-glucUA, a phenotype was observed for ΔgluD; 2-keto-L-gulonate accumulated in the medium after D-glucUA was consumed (Figures 5A,C). This was not observed with the wild type strain (Figure 5B). In the case of the mutant strain ΔgluE, growth on D-glucUA plate was reduced (Figure 4). In addition, the consumption of D-glucUA in liquid cultivation was almost completely disrupted in ΔgluE (Table 3).

**DISCUSSION**

D-GlucUA is a biomass component that is catabolised by many microorganisms including fungi. However, the catabolic pathway in fungi is only partly known. Recently, we identified the gene gluC that is essential for D-glucUA catabolism in the filamentous fungus A. niger (Kuivanen et al., 2016). The gene encoded an enzyme reducing 2-keto-L-gulonate to L-Idonate using NAD$^+$ as cofactor. We also showed that the gaaA gene encoding a D-galUA and D-glucUA reductase is induced on both substrates, D-galUA and D-glucUA. All this indicates that D-glucUA is first reduced to L-gulonate, then converted to 2-keto-L-gulonate by an unknown mechanism, and then reduced to L-Idonate by the GluC. In the present study, we identified a gene cluster that is involved in D-glucUA catabolism in A. niger consisting of the genes gluD and gluE. In this cluster, gluD encodes a NADP$^+$-dependent enzyme that, similar to GluC, catalyzes the reaction between 2-keto-L-gulonate and L-Idonate. The other gene in the cluster, gluE, encodes a NADH-dependent enzyme that catalyzes the reaction between L-Idonate and 5-keto-D-glucurate. The latter reaction catalyzed by GluE seems to be the next step after the formation of L-Idonate in the catabolic D-glucUA pathway in A. niger. This, still incomplete pathway is summarized in Figure 1C.

The D-glucUA pathway genes gluD and gluE are clustered in a similar manner as the D-galUA catabolic pathway genes gaaA and gaaC (Martens-Uzunova and Schaap, 2008) in the A. niger genome. An ortholog of gluD-gluE gene cluster is present in most of the sequenced aspergilli (AspGD)². In fungi, genes of the same metabolic pathway are sometimes co-localized on chromosomes, i.e., they form chromosomal clusters (Wisecaver et al., 2014). What drives the formation of these clusters is debated. The need to ensure removal of toxic intermediates (McGary et al., 2013) has been proposed as the ultimate reason, but mere transcriptional co-regulation (Gordon et al., 2015) might have other benefits too. In this case, gluD and gluE share a common promoter and transcription of the genes is induced with a similar pattern on D-glucUA. Thus, transcriptional co-regulation is a possible explanation for the formation of gluD-gluE cluster. It is also suggested that soil-dwelling fungi may have obtained genes from bacteria for catabolism of unusual carbon sources through horizontal gene transfer (Wisecaver et al., 2014; Wisecaver and Rokas, 2015). In fact, it was suggested that fungal β-glucuronidases genes are derived from bacteria allowing fungi to hydrolyse glucuronides resulting in access to released monomeric D-glucUA (Wenzl et al., 2005). In bacteria, metabolic genes are often present in clusters such as in the case of catabolic L-Idonate pathway in E. coli (Bausch et al., 2004). If such metabolic genes are acquired from bacteria via horizontal gene transfer, it may eventually lead to the formation of metabolic gene clusters in fungi as well.

In the previous study, deletion of gluC in A. niger disrupted the D-glucUA catabolism nearly completely (Kuivanen et al., 2016). Even though, GluC and GluD catalyze the same reaction and both genes are induced on D-glucUA, it seems that GluD cannot compensate the loss of GluC activity in the

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²http://aspgd.broadinstitute.org/cgi-bin/aspg2_v3/shared/show_protein_cluster.cgi?site=asp2_v9&id=2050676
TABLE 2 | Kinetic parameters of the purified GluD and GluE proteins.

| Protein | Substrate          | $V_{\text{max}}$ (µmol min$^{-1}$ mg$^{-1}$) | $K_{m}$ (mM) | $k_{\text{cat}}$ (s$^{-1}$) | $k_{\text{cat}}/K_{m}$ (M$^{-1}$ s$^{-1}$) |
|---------|--------------------|---------------------------------|-------------|-----------------|--------------------------------|
| GluD    | 2-keto-L-gulonate  | 34.9 ± 0.5                      | 25.3 ± 1.2  | 21.4 ± 0.3      | 8.46 $\times 10^2$            |
|         | L-idonate          | 1.7 ± 0.1                       | 12.6 ± 0.8  | 1.1 ± 0.0       | 8.73 $\times 10^2$            |
| GluE    | L-idonate          | 7.6 ± 0.1                       | 30.9 ± 1.5  | 5.5 ± 0.5       | 1.78 $\times 10^2$            |
|         | 5-keto-D-gluconate | 10.0 ± 0.0                      | 8.4 ± 0.1   | 7.2 ± 0.0       | 8.57 $\times 10^2$            |

Data represent means ± standard deviation from three technical repeats.

D-glucuronate  D-glucose

wild type

ΔgluD
(Δ1114837)

Δgld
(Δ1099233)

FIGURE 4 | Growth of the A. niger strains wt, ΔgluD, and Δgld on agar plates with D-glucuronic acid or D-glucose as sole carbon source.

fungal D-glcUA pathway (deletion of gluC disrupted growth on D-glcUA; Kuivanen et al., 2016). This might be due to cofactor requirements: GluC requires NADH and GluD NADPH. In fact, it is surprising and unusual that two enzymes, in this case GluC and GluD, are present for the same reaction, but have different cofactor requirements. Since both reactions are reversible a possible interpretation is that the enzyme couple may act as a NAD(P)$^+$ transhydrogenase adjusting the ratio of NAD$^+/NADH$ and NADP$^+/NADPH$. Deletion of gluD did not result in reduced or no growth on D-glcUA as sole carbon source. However, it resulted in a phenotype of accumulating 2-keto-L-gulonate when cultivating on D-glcUA. This observation further supports the hypothesis that the fungal catabolic D-glcUA pathway proceeds through the oxidation of
L-gulonate to 2-keto-L-gulonate. The oxidation of L-gulonate to 2-keto-L-gulonate is a biochemical reaction that is not described in the literature and the responsible enzyme in A. niger still remains unclear. In the case of 2-keto-L-gulonate reductase activity, an unspecific bacterial D-gulonate 2-dehydrogenase (EC 1.1.1.215) had been described that showed also activity for the reaction between L-idade and 2-keto-L-gulonate (Yum et al., 1998). This bacterial enzyme used NADP\(^+\)/NADPH as a cofactor similar to the GluD described in this study. However, we conclude that GluD is the first specific NADPH dependent 2-keto-L-gulonate reductase reported to date.

The protein product of the gene gluE, described in this study, catalyzed the reversible reaction from L-idade to 5-keto-D-gluconate using NAD\(^+\)/NADH as cofactor. A similar enzyme activity has been described in the filamentous fungus Fusarium sp. already in Takagi (1962), however, this enzyme activity was strictly NADP\(^+\)/NADPH dependent. In plants, an NAD\(^+\)/NADH enzyme (EC 1.1.1.366) oxidizing L-idade to 5-keto-D-gluconate functions in the pathway converting L-ascorbic acid to L-tartaric acid (DeBolt et al., 2006). In addition, E. coli has an L-idade 5-dehydrogenase, IldD (EC 1.1.1.264), producing 5-keto-D-gluconate from L-idade with NAD\(^+\) as cofactor in the catabolic L-idade pathway (Bausch et al., 1998). GluE has only low sequence homology toward the other characterized L-idade 5-dehydrogenases and it is the first reported fungal NAD\(^+\) dependent L-idade 5-dehydrogenase. The gluE deletion in A. niger had also a phenotype – growth was reduced and D-glucUA consumption was ceased. This is a strong indication that the gene is part of the fungal catabolic D-glucUA pathway and the pathway passes through the oxidation of L-idade to 5-keto-D-gluconate.

It is unclear how the fungal D-glucUA pathway continues after formation of 5-keto-D-gluconate. In plants, a NAD\(^+\) dependent L-idade 5-dehydrogenase forming 5-keto-D-gluconate was described (Wen et al., 2010). This was suggested to be part of the pathway for L-ascorbic acid degradation (DeBolt et al., 2006). In this pathway, the resulting 5-keto-D-gluconate is split by an aldolase to L-threo-tetruronate and glycolaldehyde. The L-threo-tetruronate is then oxidized to L-tartaric acid. In this pathway, only the L-idade 5-dehydrogenase gene had been identified. Another possibility would be a route similar to the L-idade catabolism in bacteria. In E. coli, a NAD\(^+\) specific L-idade 5-dehydrogenase is reducing the L-idade to 5-keto-D-gluconate and the 5-keto-D-gluconate is subsequently reduced to D-gluconate (Bausch et al., 2004). D-Gluconate is then phosphorylated and the resulting 6-phosphogluconate enters the Entner–Doudoroff pathway. If 5-keto-D-gluconate is reduced to D-gluconate in the fungal D-glucUA pathway in A. niger, it would connect D-glucUA catabolism with the catabolism of D-glucose. A. niger oxidizes extracellular D-glucose to D-gluconate which is then taken up and catabolized further through the phosphorylation to D-gluconate-6-phosphate and subsequently via pentose phosphate pathway (Muller, 1985). It is also suggested that some strains of A. niger catabolize D-gluconate through the non-phosphorylative Entner–Doudoroff pathway including dehydratation of D-gluconate to 2-keto-3-deoxy-gluconate which is the split to D-glyceraldehyde and pyruvate by the action of an aldolase (Elzainy et al., 1973; Allam et al., 1975). However, the

![HPLC analysis of the A. niger growth medium](A) at 0 h, (B) at 72 h with the A. niger wild type strain, and (C) with ΔgldD.

**TABLE 3 | Concentration of d-glucuronic acid (d-glucUA) in submerged cultivations by the A. niger wild type strain (wt) and ΔgldE.**

| Strain       | 0 h    | 20 h   | 50 h    |
|--------------|--------|--------|---------|
| wt           | 20.4 ± 0.0 | 12.0 ± 0.4 | 0.0 ± 0.0 |
| ΔgldE        | 20.4 ± 0.0 | 19.1 ± 0.1 | 17.8 ± 0.2 |

Data represent means ± standard deviation from three biological repeats.
fate of 5-keto-D-glucuronic acid in the fungal D-glucuronic acid pathway still remains to be unraveled.

**AUTHOR CONTRIBUTIONS**

JK and PR designed and carried out all the experimental work and analyzed the data. MA processed and analyzed the RNAseq data. JK and PR drafted the manuscript. PR designed the fundamental concept and participated in the coordination of the study. All the authors read and approved the final manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00225/full#supplementary-material

**FIGURE S1 | Oxidoreductase activity of purified GluD towards**

(A) 2-keto-L-gulonate and (B) L-idonate with NADPH and NADP⁺, respectively and oxidoreductase activity of purified GluE toward (C) L-idonate and (D) 5-keto-D-glucuronic acid with NAD + and NADH, respectively. Data represent means ± standard deviation from three biological repeats. If error bars not visible are smaller than the symbol.
Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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