Reduction of quinones and phenoxy radicals by extracellular glucose dehydrogenase from *Glomerella cingulata* suggests a role in plant pathogenicity

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The plant-pathogenic fungus *Glomerella cingulata* (anamorph *Colletotrichum gloeosporioides*) secretes high levels of an FAD-dependent glucose dehydrogenase (GDH) when grown on tomato juice-supplemented media. To elucidate its molecular and catalytic properties, GDH was produced in submerged culture. The highest volumetric activity was obtained in shaking flasks after 6 days of cultivation (3400 U l\(^{-1}\), 4.2% of total extracellular protein). GDH is a monomeric protein with an isoelectric point of 5.6. The molecular masses of the glycoforms ranged from 95 to 135 kDa, but after deglycosylation, a single 68 kDa band was obtained. The absorption spectrum is typical for an FAD-containing enzyme with maxima at 370 and 458 nm and the cofactor is non-covalently bound. The preferred substrates are glucose and xylose. Suitable electron acceptors are quinones, phenoxy radicals, 2,6-dichloroindophenol, ferricyanide and ferrocenium hexafluorophosphate. In contrast, oxygen turnover is very low. The GDH-encoding gene was cloned and phylogenetic analysis of the translated protein reveals its affiliation to the GMC family of oxidoreductases. The proposed function of this quinone and phenoxy radical reducing enzyme is to neutralize the action of plant laccase, phenoloxidase or peroxidase activities, which are increased in infected plants to evade fungal attack.

**INTRODUCTION**

*Glomerella cingulata* (anamorph *Colletotrichum gloeosporioides*) is found worldwide, but predominantly in subtropical climates. It causes anthracnose which is responsible for extensive crop losses. The disease is characterized by dark, sunken, lenticular necrotic lesions containing the acervuli of the pathogen. Common hosts include fruits such as mango, papaya, avocado, strawberry, apple and citrus fruits, and non-cultivated plant species are also affected (Bailey & Jeger, 1992; Medeiros et al., 2010). Since members of the genus *Colletotrichum* cause major economic losses and are used as model organisms for fungal development, infection processes, host resistance, signal transduction and the genetic basis of plant–pathogen interactions, two *Colletotrichum* species, *C. graminicola* and *C. higginsianum*, were recently sequenced by the *Colletotrichum* Sequencing Project (http://www.broadinstitute.org/annotation/genome/colletotrichum_group/MultiHome.html, http://www.mpiz-koeln.mpg.de/english/research/pmi-dpt/Fungal_genomes/Colletotrichum/index.html).

Infection by members of the genus *Colletotrichum* is characterized by two stages. During the first stage (biotrophic development) the fungus exerts only minimal damage to host cells to avoid the arsenal of constitutive antimicrobial compounds of the host organ or triggering active resistance such as hypersensitive cell death and callose deposition (Latunde-Dada, 2001; Perfect et al., 1999). The second (necrotrophic) stage is initiated by an increase in ethylene concentration (Flashman & Kolattukudy, 1994) and the reduction of fungitoxic dienes and resorcinols (Latunde-Dada, 2001). Here, the fungus lives on nutrients obtained from invaded host cells. This second stage of development, which often occurs post-harvest in the case of *Colletotrichum* species, is linked to an increased expression of plant-cell-wall-degrading enzymes such as endo-polygalacturonases and pectinlyases (Medeiros et al., 2010; Perfect et al., 1999), cellulases, xylanases and galactosidases (Collmer & Keen, 1986; Cooper & Wood, 1975) in a concerted effort to degrade structural components of plant tissue. The plant’s defence mechanism in the necrotic stage...
includes the increase of the free phenol content and a concomitant upregulation of laccase, polyphenol oxidase and peroxidase activity. The generated monolignol and phenoxy radicals are used for lignin synthesis on cell walls to isolate the invader (Anand et al., 2009) and additionally formed quinones are toxic to fungi (Morpheth, 1991). How can the fungus neutralize the plant’s defence mechanism or reduce its effectiveness? It is possible that this is by an enzyme that reduces generated phenoxy radicals and quinones to minimize lignin formation and toxicity. Such an enzyme would need an abundant co-substrate, such as carbohydrates released by the hydrolysis of the plant cell wall constituents cellulose, xylan and xyloglucan.

In this study we identified, produced and characterized an FAD-dependent glucose dehydrogenase (GDH; EC 1.1.99.10; systematic name, D-glucose : acceptor 1-oxidoreductase) from the plant-pathogenic fungus Glomerella cingulata. In addition to the elucidation of the molecular properties of GDH and its phylogenetic relationship to other GMC oxidoreductases, we focused on its biological function about which very little information exists. In the first report on the existence of a glucose dehydrogenase (from Aspergillus oryzae Ogura, 1951) the author reported that glucose is the substrate and not glucose-6-phosphate and described the interaction with redox indicators like thionine and 2,6-dichloroindophenol (DCIP). Later, the production and biochemical properties of A. oryzae GDH were reported in more detail; the most notable findings were the induction of biosynthesis by hydroquinone or benzoquinone, the glycosylation of the enzyme and the presence of an FAD cofactor and a histidyl residue in its active site (Bak, 1967a, b; Bak & Sato, 1967). Other studies on the few known FAD-dependent GDHs focus on the presence and role in insects (Cavenier & McIntyre 1983; Lovallo & Cox-Foster 1999) and establish FAD-dependent GDH from Drosophila melanogaster as a member of the GMC oxidoreductase family (Cavenier, 1992). The focus for bacterial and fungal GDHs was solely put on the highly successful application in biosensors and biofuel cells (Aiba & Tsuchiya-shi, 2007; Okuda-Shimatani et al., 2008; Omura et al., 2010; Sode et al., 1996; Tsuji et al., 2010). This study is the first, to our knowledge, to elucidate the in vivo role of a fungal FAD-dependent GDH produced by a plant-pathogenic fungus.

**METHODS**

**Strains, media and chemicals.** G. cingulata DSM 62728 was obtained from DSMZ and periodically subcultured on potato dextrose agar (PDA) plates at 25 °C. Media were prepared with tap water. Deionized reverse osmosis water (>16 MΩ cm) was used for buffers and assays. All chemicals for buffer and media were purchased from commercial suppliers in adequate purity. Organic tomato juice for the fermentation medium was from JäNatürlich Naturprodukte, and alkali soluble lignin (Indulin AT) and peroxidase (type II, from horseradish) were from Sigma-Aldrich. Laccase from Trametes pubescens was produced as reported by Galhaup & Haltrich (2001).

**Optimization of medium and production of G. cingulata GDH.** Experiments were performed in 300 ml Erlenmeyer flasks. Supplementing the media with four different carbon sources (cellulose, fructose, glucose and glycerol, final concentration 20 g l−1) and with tomato juice [0, 2 and 10% (v/v)] was investigated. The media also contained 5 g peptone from meat 1−1 and 0.3 ml trace element solution 1−1 (1 g ZnSO4.7H2O, 0.3 g MnCl2.4H2O, 3 g H3BO3, 2 g CoCl2.6H2O, 0.1 g CuSO4.5H2O, 0.2 g NiCl2.6H2O, 4 ml H2SO4; all 1−1) (Sachslehner et al., 1997). The autoclaved media were inoculated with 1 cm2 fine cut agar plugs from freshly overgrown plates and cultivated in temperature-controlled shaking incubators (25 °C, 125 r.p.m., eccentricity 1.25 cm) for several days. Samples were taken periodically and tested for enzymic activity and protein concentration.

Large-scale production of GDH was performed in a 70 l bioreactor (Applikon) filled with 50 l production medium [20 g glucose 1−1, 5 g peptone from meat 1−1, 2% (v/v) tomato juice, 0.3 ml trace elements solution 1−1]. The pH was set to 6.4 before sterilization and was allowed to float freely during cultivation. Fermentation was started by adding 1.2 [2.5% (v/v)] of a 7-day-old pre-culture grown in shaking flasks in the same medium. The cultivation temperature in the bioreactor was 25 °C; the airflow rate was 15 l min−1 and the agitation was 150 r.p.m. (three Rushton turbines; tip speed 0.79 m s−1). Samples were taken regularly and cleared by centrifugation (10 000 g, 5 min). Enzymic activity and extracellular protein concentration were assayed in the supernatant throughout the cultivation.

**Protein purification.** After 78 h, the cultivation of G. cingulata in the bioreactor was stopped and the mycelium was removed from the fermentation broth by centrifugation (6000 g, 30 min). The clear supernatant was concentrated and diafiltered in a hollow fibre cross-flow module (Microza UF module SLP-1053, 10 kDa cut-off, Pall Corporation) to remove low-molecular-mass medium components. The partially deionized enzyme solution (3 mS cm−1) was applied to a column packed with DEAE-Sepharose FF (chromatographic equipment and materials from GE Healthcare), previously equilibrated with 50 mM phosphate buffer, pH 7.5. Proteins were eluted within a linear salt gradient from 0 to 2 M NaCl in 10 column volumes (5 l). Fractions containing GDH activity were pooled and ammonium sulfate was added to a saturation of 60%. After ultra centrifugation (30 000 g, 15 min) the pool was loaded onto a column packed with Source 15 PHE, which was equilibrated with 50 mM phosphate buffer, pH 7.5, containing 60% (saturation) ammonium sulfate. Proteins were eluted within a linear gradient from 60 to 0% ammonium sulfate in 10 column volumes (700 ml). After concentration and rebuffering the pooled fractions to a 50 mM sodium citrate buffer, pH 5.5, with a polyethersulfone flat-stack cross-flow module (VivAflow 50, 10 kDa cut-off; Sartorius), the GDH preparation was split into aliquots and frozen at −70 °C for further use.

**Deglycosylation.** For SDS-PAGE, deglycosylation was performed under denaturing conditions. Homogeneously purified GDH was treated with PNGase F (New England Biolabs) according to the manufacturer’s instructions. For isoelectric focusing (IEF), 0.1 mg GDH was deglycosylated under non-denaturing conditions with 7 µg Endo HF (138 U µg−1; New England Biolabs) and 4 µg z-mannosidase (0.03 U µg−1; from jack bean, Sigma Aldrich) in 100 µl 50 mM sodium citrate buffer pH 5.5 containing 10 mM ZnCl2 for 48 h at 22 °C.

**Electrophoretic analysis.** SDS-PAGE was carried out in a Phast System using precast gels (PhastGel Gradient 8-25 and SDS buffer strips, GE Healthcare) according to the manufacturer’s modifications of the Laemmli procedure. Proteins and the Precision Plus protein dual colour standard (Bio-Rad) were visualized by using Coomassie
brilliant blue staining. For native PAGE, different precast gels were used in the same horizontal electrophoresis system (PhastGel Gradient 8-25 and native buffer strips, GE Healthcare) according to the manufacturer’s recommendations together with the high-molecular-mass native marker kit (GE Healthcare). IEF in the range of pH 3–10 was also performed in the Phast System using precast gels (PhastGel IEF 3-9, GE Healthcare). The broad range pl marker protein kit (pH 3–10; Serva) was used to determine the isoelectric point (pl). GDH bands were visualized by active staining with DCIP (Ludwig et al., 2004).

Spectral characterization. The spectrum of homogeneously purified GDH was recorded at room temperature from 250 to 550 nm in both the oxidized and reduced state using a U-3000 Hitachi spectrometer. GDH was diluted in 50 mM citrate buffer, pH 5.5, to A280=1 and the spectrum was recorded before and shortly after the addition of glucose to the cuvette. The molar absorption coefficient of GDH at 459 nm was determined by precipitation with trichloroacetic acid according to Macheraux (1999) by applying the molar absorption coefficient for free FAD ($\varepsilon_{459}=11.3 \text{ mm}^{-1} \text{cm}^{-1}$).

Activity and protein measurements. Enzymatic activity was spectrophotometrically assayed using 300 $\mu$M DCIP ($\varepsilon_{520}=6.9 \text{ mm}^{-1} \text{cm}^{-1}$), 200 $\mu$M ferrocenium hexafluorophosphate (FcPF6, $\varepsilon_{500}=4.3 \text{ mm}^{-1} \text{cm}^{-1}$) or 200 $\mu$M 1,4-benzoquinone ($\varepsilon_{520}=2.24 \text{ mm}^{-1} \text{cm}^{-1}$) as electron acceptors. The reaction was followed for 180 s at 30 °C in a Lambda 35 UV/Vis spectrophotometer featuring a temperature-controlled 8-cell changer (Perkin Elmer). All assays were measured in 50 mM sodium acetate buffer (DCIP and 1,4-benzoquinone, pH 5.5) and contained 100 mM D-glucose (final concentration).

Oxygen reactivity. A fluorescence-based fibre-optic sensor (PreSens) was used to determine $O_2$ consumption rates. Oxygen-saturated 50 mM sodium acetate buffer, pH 5.5, containing 100 mM glucose (oxygen concentration $\sim$1200 $\mu$M) was magnetically stirred ($\sim$500 r.p.m.) in a gas-tight, temperature-controlled (30 °C) glass vial sealed by a septum (total volume 1867 $\mu$L). The reaction was started by adding 50 $\mu$L enzyme solution. The specific activity of GDH and, as a reference, glucose oxidase from Aspergillus niger (Sigma-Aldrich), was calculated from the oxygen consumption rate and the protein concentration.

Determination of the reaction product and HPLC analysis. A batch conversion (5 ml reaction volume) was carried out under continuous sparging and stirring at 30 °C. The reaction mixture consisted of 50 mM D-glucose, 0.1 mM DCIP, 3 U laccase from Trametes pubescens (Galhaup & Haltrich, 2001) and 3 U GDH in 100 mM citrate buffer, pH 5.0. Carbohydrate samples were analysed by HPLC using a CarboPac PA1 carbohydrate analytical column with the dimensions 4 × 250 mm and a 4 × 50 mm guard column and the ED40 electrochemical detector ( Dionex).

Kinetic measurements. Initial rates to determine pH profiles of various electron acceptors were determined at 30 °C in 100 mM McIlvaine buffer ranging from pH 3.0 to 8.0. All electron acceptors were measured with a final concentration of 100 $\mu$M in the cuvette. The relative activity of GDH for carbohydrates (glucose, methyl-$\alpha$-glucose, methyl-$\beta$-glucose, galactose, mannose, cellobiose, lactose, maltose, trehalose, xylose and arabinose) was measured with a 25 mM substrate concentration in 50 mM sodium acetate buffer pH 5.5 and 300 $\mu$M DCIP as electron acceptor. Catalytic constants for D-glucose, maltose and D-xylose were determined with DCIP at pH 5.5 or ferrocenium as electron acceptor at pH 5.5 and 7.5. Catalytic constants for electron acceptors were measured at pH 5.5. Constants were calculated using non-linear least-squares regression by fitting the observed data to the Michaelis–Menten equation (Sigma Plot 11, Systat).

The inhibition of the laccase-catalysed oxidation of phenols to quinones and phenoxy radicals by GDH was measured in quartz cuvettes containing 0.1 mM of the phenol, 100 mM glucose and GDH in 1 ml of 100 mM sodium citrate buffer pH 5.5. The oxidation of the substrates was started by addition of $T$. pubescens laccase (1.7 $\mu$g ml$^{-1}$). The oxidation reaction without or with GDH (1.9, 5.7, 19 $\mu$g ml$^{-1}$) was performed at 30 °C for 5 min and quantified at the indicated wavelengths.

Peptide sequencing and isolation of genomic DNA and cDNA. Peptide sequences of purified GDH were obtained using LC-ESI-MS/MS analysis as described previously (Harreither et al., 2011). DNA extraction, total RNA isolation and cDNA first strand synthesis was performed according to the method of Harreither et al. (2011). Degenerate primers were derived from peptide sequencing fragments using the program CODEHOP (http://blocks.fhcrc.org/blockscodehop.html). Primers GDHfw2 [5'-GCC-TACTACGT(T/C)CC(AG/CT)TA(C/G)TA-3’, peptide tag: 24AAYFP- YENR205’] and GDHrv1 [5’-CGGGACGTTGTTGCTGG(AGC/AG)- TA(GA)AC-3’], peptide tag: 39VYNTVNR270’] were used to amplify a 950 bp fragment of genomic DNA. For the amplification of the adjacent upstream region, the DNA Walking SpeedUp premix kit (Seegene) was used. Six target-specific forward primers (GDH-TSP1, 5’-AACCAGTCTCGATACGTATTGGG-3’, GDH-TSP2, 5’-TGGCCACCTCCGGAGAGC-3’, GDH-TSP3, 5’-CGTGACGAGTCTAGGTGTGC-3’, GDH-TSP1-2, 5’-TCGTCTGGATGACGTAGTG-3’, GDH-TSP2-2, 5’-TTCGAGGGACCCAGGCTACC-3’, GDH-TSP3-2, 5’-TCTGATGACGACGAGAC-3’) were designed and used together with the DNA walking-annealing control primer and a universal primer provided by the kit to perform the PCRs according to the manufacturer’s guidelines. For amplification of the adjacent 3’ downstream region, the GDH-fw3 primer (5’-TTCATCCGGAGGCTTTGGG-3’) and universal primer (5’-GGGACGCGTCGACTAGTAC-3’) were used together with cDNA as template. To obtain a full-length cDNA encoding the GDH protein, a nested PCR with two forward primers (GDH-fw4, 5’-AGATTCGAAACTCATCAACG-3’, GDH-fw5, 5’-AGCCATGTGTTTAGACGCTCC-3’) from a sequence upstream of the putative start codon, and two reverse primers (GDH-rv1, 5’-TGCACTCTAAGATGTCACC-3’, GDH-rv2, 5’-TCCGAAGCTAGCTGAC-3’) specific for a downstream sequence adjacent to the stop codon and the anchor primer (GDH-rv2, 5’-GCGTACGGCTGACTTGGTTTTTTTTTTTTTTTT-3’) were performed on cDNA as template. The resulting ~1.8 kb PCR product was cloned into the pCR-Blunt II-TOPO vector (Invitrogen) according to the manufacturer’s recommendations and sequenced by a commercial sequencing service (AGOWA). The sequences were submitted to GenBank with the following accession numbers: JF31351, G. cingulata GDH genomic DNA; JF31352, G. cingulata GDH mRNA.

Analysis of gene and protein sequences. Potential N-glycosylation sites were calculated with NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc). Molecular mass, pl and the N terminus of the mature protein were determined with the programs Compute pi/Mw and SignalP hosted by the Expasy Proteomics server (http://www.expasy.ch). Similarities were determined using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/blast.cgi), and the InterPro database (http://www.ebi.ac.uk/interpro/) was used to identify the protein family, domains and functional sites. For the phylogenetic analysis, protein sequences with a high sequence similarity to G. cingulata GDH as well as to members of the GMC oxidoreductase family were
obtained from BLASTP and a tblastn search of available sequences. Evolutionary relationships were inferred by various methods featured in the MEGA5 software (Tamura et al., 2011). The analysis by the maximum likelihood method was based on the JTT + F (Jones, Taylor, Thornton plus frequencies) model and also employed a discrete Gamma distribution to model evolutionary rate differences (+G, five categories). The analysis was based on an alignment of 30 amino acids in MUSCLE. All ambiguous positions were removed for each sequence pair. The initial tree for heuristic search was obtained by using the maximum parsimony method. The tree was searched using close-neighbour interchange. The bootstrap consensus tree is based on 1000 replicates. To test the obtained results, the same sequences were aligned by CLUSTAL and analysed by minimum evolution and maximum parsimony.

RESULTS

GDH production

GDH formation was optimized in shaking flask experiments by combining four different carbon sources with different amounts of tomato juice while keeping the peptone concentration constant. Mycelial pellets with particle diameters from 3 to 10 mm were formed in media containing the soluble carbon sources glucose, fructose or glycerol. In cellulose media, no macroscopic pellets were observed; the hyphae grew around the cellulose particles resulting in a more uniform distribution of the mycelium.

Tomato juice increased the biomass accumulation as well as GDH activity and shortened the cultivation time to reach maximum activity. The maximum volumetric activities were measured in glucose-containing media (no tomato juice, 3400 U l⁻¹ on day 6; 2% tomato juice, 6100 U l⁻¹ on day 7). Media containing fructose (1100 U l⁻¹ on day 9) and glycerol (1100 U l⁻¹ on day 5) worked best with a 10% tomato juice supplement, but produced only a fifth of the activity encountered in the glucose medium. The lowest GDH activity was measured in cellulose-based media supplemented with either 2 or 10% tomato juice (230 U l⁻¹ on day 6).

During cultivation of G. cingulata in an aerated and stirred bioreactor, the pH was allowed to float freely. A strong, immediate acidification of the medium from pH 6.9 to 5.2 was observed within the first 30 h, indicating a viable inoculum and a short lag phase (Fig. 1). During this first acidification phase there was only a low amount of GDH produced (57 U l⁻¹). After 30 h, a second phase was observed where the pH decreased much more slowly (to a final value of 4.85 after 77 h) and GDH activity reached its maximum (860 U l⁻¹) after 69 h. In this phase, most of the glucose in the medium was metabolized. The cultivation ended soon after (77 h, 850 U l⁻¹) to prevent proteolytic degradation of GDH and accumulation of autolysis products. At this time, the extracellular protein concentration was 24 mg l⁻¹. After harvest and centrifugation 40.9 l clear culture supernatant were purified by cross-flow filtration and two chromatographic steps to homogeneity (Table 1). Strict pooling of only the purest fractions led to a moderate yield. The final homogeneous GDH preparation had a specific activity of 840 U mg⁻¹ with the 2,6-dichloroindophenol assay and 565 U mg⁻¹ with the FcPF₆ assay. The homogeneity of the enzyme was verified by SDS-PAGE.

Molecular properties

The molecular mass of GDH was determined by SDS-PAGE, which showed a broad, diffuse band between 95 and 135 kDa (Fig. 2a). After deglycosylation under denaturing conditions with PNGase F, a single, sharp band with an estimated molecular mass of 68 kDa was obtained. Native PAGE showed a similar broad and diffuse band for the glycosylated GDH with a molecular mass range of 100–130 kDa, which indicates a monomeric protein (Fig. 2b). IEF showed that GDH has an acidic pI of 5.6 (Fig. 2c).

Table 1. Purification of G. cingulata glucose dehydrogenase

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity (U)</th>
<th>Total protein (mg)</th>
<th>Specific activity (U mg⁻¹)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clear supernatant</td>
<td>34 800</td>
<td>980</td>
<td>35.5</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>DEAE-Sepharose</td>
<td>23 900</td>
<td>215</td>
<td>111</td>
<td>69</td>
<td>3.13</td>
</tr>
<tr>
<td>Source 15 PHE</td>
<td>14 800</td>
<td>17.6</td>
<td>840</td>
<td>43</td>
<td>23.7</td>
</tr>
</tbody>
</table>
purified enzyme is bright yellow and has a characteristic flavoprotein spectrum with FAD absorption maxima at 370 and 458 nm (384 and 460 nm in the difference spectrum). Upon reduction with glucose, the peaks disappeared and the enzyme became colourless (Fig. 3). Treatment of the enzyme with trichloroacetic acid released FAD from the polypeptide indicating that the FAD is not covalently bound to the protein. The molar absorption coefficient of GDH at 458 nm is 12.1 mM$^{-1}$ cm$^{-1}$.

### Analysis of gene and protein sequences

Peptide sequences were obtained from mass spectrometry of trypsin-digested GDH samples, which showed high similarity to glucose oxidase of *Aspergillus flavus*. In the amplified gene, all tags could be found, confirming the cloning of the gene encoding the target protein. The *G. cingulata gdh* gene is 1880 bp long and contains one intron with a typical splice site. The mRNA of *G. cingulata* GDH contains an open reading frame of 1800 base pairs encoding a 600 amino acid protein. Analysis with SignalP revealed a putative signal peptidase cleavage site (probability=0.996) between amino acid position 16 and 17, which classifies this GDH as a secretory protein. The calculated molecular mass of the mature protein is 62 199 Da and the calculated pi is 4.58. Four potential N-glycosylation sites (N55, N233, N255 and N339) were identified with the program NetNGlyc 1.0. GMC oxidoreductase consensus sequences in the protein sequence were confirmed by comparison with the InterPro database.

The phylogenetic relationship of FAD-dependent GDHs to other GMC oxidoreductases was inferred from protein sequences of well known GMC oxidoreductases and sequences with high similarities (by using *G. cingulata* and other confirmed GDH sequences for BLASTP and tblastn searches). Phylogenetic analysis was performed by using the maximum likelihood method (Fig. 4). The phylogenetic trees obtained by using different alignment methods and phylogenetic algorithms give very similar results (data not shown).

### Catalytic properties

The ability of GDH to oxidize carbohydrates at 50 mM was examined at pH 5.5 with DCIP as an electron acceptor. A strong preference for glucose and the hemicellulose pentose xylose (31 % relative activity compared with glucose) was observed. The relative conversion rates of the disaccharides cellobiose and maltose were very low (1.6 and 2.3 %, respectively) and lactose was not converted at all. For the

![Fig. 2. (a) SDS-PAGE of purified GDH: glycosylated GDH (lane 1), deglycosylated GDH (2) and molecular mass marker (3, Precision Plus unstained, Bio-Rad). (b) Native PAGE of purified glycosylated GDH (1) and molecular mass marker (2, HMW native marker kit, GE Healthcare). (c) IEF of deglycosylated GDH (1) and IEF Marker Liquid Mix (2, Serva). GDH was visualized by active staining with DCIP.](http://mic.sgmjournals.org)

![Fig. 3. Spectral characterization of GDH showing both the oxidized (grey) and reduced (black) spectra. Glucose was used to reduce the enzyme. The difference spectrum (ox-red) is given as an inset; maxima are indicated.](http://mic.sgmjournals.org)
Fig. 4. Bootstrapped phylogenetic tree inferred by the maximum likelihood method using the JTT + F + G model. The analysis involved 30 amino acid sequences of mature GMC enzymes including alcohol dehydrogenase (ADH), alcohol oxidase (AOX), aryl alcohol oxidase (AAOX), cholin dehydrogenase (CHD), cholesterol oxidase (COX, outgroup), cellobiose dehydrogenase (CDH), glucose dehydrogenase (GDH), glucose oxidase (GOX), pyranose dehydrogenase (PDH) and hypothetical proteins (HP).
monosaccharides D-galactose, D-mannose and L-arabinose (0.2, 0.9 and 0.1 % relative activity, respectively) conversion rates were low and therefore they were considered to be poor substrates. Glucose impurities (~0.1–0.3 %) were present in cellobiose, maltose and D-galactose and might cause apparently higher activities.

Apparent catalytic constants of GDH for glucose, xylose and maltose were measured by using the DCIP and ferrocenium assay (Table 2). The catalytic efficiencies are high for both monosaccharides, but glucose is clearly the preferred substrate. The preference for glucose arises from the approximately fivefold higher $k_{\text{cat}}$ values since the apparent $K_m$ values for xylose are, depending on the electron acceptor and pH, only 1.3- to 2-fold higher than for glucose. The disaccharide maltose, which had the third highest activity (2.3 % relative to glucose), has an extrapolated $K_m$ of ~7 M and a catalytic efficiency of ~50 M$^{-1}$ s$^{-1}$, which is 800 times lower than the catalytic efficiency for glucose. The catalytic constants for maltose have to be regarded as approximations. The reaction product of GDH was determined by a batch conversion of glucose using an enzymic regeneration system (Van Hecke et al., 2009). Glucose consumption was accompanied by a steady decrease in pH, indicating an acidic reaction product. HPLC analysis showed the formation of the C$_1$ glucose oxidation product, gluconic acid, in an amount equivalent to that of the glucose used.

To find out which electron acceptors are suitable co-substrates for GDH, pH profiles and turnover numbers were measured for a selection of one- and two-electron acceptors (Table 3). The highest specific activities were observed for un- and methyl-substituted 1,4-benzoquinones. The electron acceptor with the highest catalytic efficiency is 1,4-benzoquinone, which had a $k_{\text{cat}}$ value of 1530 s$^{-1}$, twice as high as for DCIP or FcPF$_6$. In contrast, the turnover of oxygen as an alternative electron acceptor was very low and confirmed that $G.\ cingulata$ GDH is a strict dehydrogenase. Oxygen turnover by GDH (0.27 s$^{-1}$) is 3100 times slower than DCIP turnover at air saturation and pH 5.5. The reduction of phenoxy radical lignin precursors was tested in an inhibition experiment due to the short-lived nature of these intermediates. Laccase from $T.\ pubescens$ was used in place of plant-derived laccases, phenol oxidases or peroxidases to oxidize monolignols and related monophenols. In the presence of glucose, GDH efficiently reduced the accumulation of oxidation products from coniferyl alcohol and ferulic, coumaric, sinapic, vanillic and caffeic acid (Table 4). When GDH was added to the experiment after 3 min, we observed not only a decreased formation rate of the respective phenoxy radical but also complete reduction for guaiacol and 2,6-dimethoxyphenol.

**DISCUSSION**

The high expression of extracellular FAD-dependent GDH by the plant-pathogenic fungus $G.\ cingulata$ indicates an important function for the organism under certain growth conditions. In contrast with other extracellular fungal enzymes such as cellobiose dehydrogenase (Zamocky et al., 2006) or pyranose dehydrogenase (Kittl et al., 2008), which are both involved in lignocellulose breakdown, the presence of its substrate glucose does not suppress GDH formation. In contrast, it results in higher volumetric activities compared with other tested carbon sources. The formation of GDH activity is also triggered by supplementation with tomato juice, a rich source of secondary plant metabolites such as phenols. This and the reported induction of $A.\ oryzae$ GDH production by hydroquinone/benzoquinone suggests that GDH exerts a beneficial activity for the fungus when living on plant material. Tomato-juice-induced extracellular enzyme production had a similar effect on ascomycete laccases (Liers et al., 2007; Mueangtoom et al., 2010). The formation of GDH activity in optimized medium was much higher in shaking flask cultures (7.25 mg l$^{-1}$) than under stirred and well-aerated conditions in the bioreactor (1 mg l$^{-1}$) whereas the

**Table 2. Apparent kinetic constants for electron donors and acceptors**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>pH</th>
<th>$K_m$ (mM)</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$k_{\text{cat}}/K_m$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>5.5*</td>
<td>26 ± 1.5</td>
<td>510 ± 20</td>
<td>19.6 ± 10$^6$</td>
</tr>
<tr>
<td>Xylose</td>
<td>5.5*</td>
<td>38 ± 1.5</td>
<td>78 ± 3.5</td>
<td>2.05 ± 10$^5$</td>
</tr>
<tr>
<td>Glucose</td>
<td>5.5†</td>
<td>10.2 ± 0.2</td>
<td>180 ± 3</td>
<td>17.6 ± 10$^5$</td>
</tr>
<tr>
<td>Xylose</td>
<td>5.5†</td>
<td>21 ± 0.6</td>
<td>40 ± 1.5</td>
<td>1.90 ± 10$^5$</td>
</tr>
<tr>
<td>Glucose</td>
<td>7.5†</td>
<td>19 ± 0.3</td>
<td>380 ± 6</td>
<td>20.0 ± 10$^4$</td>
</tr>
<tr>
<td>Xylose</td>
<td>7.5†</td>
<td>24 ± 1.5</td>
<td>60 ± 2</td>
<td>2.5 ± 10$^4$</td>
</tr>
<tr>
<td>DCIP</td>
<td>5.5‡</td>
<td>0.080 ± 0.006</td>
<td>890 ± 32</td>
<td>11.1 ± 10$^6$</td>
</tr>
<tr>
<td>FcPF$_6$</td>
<td>5.5‡</td>
<td>0.153 ± 0.009</td>
<td>760 ± 15</td>
<td>4.97 ± 10$^6$</td>
</tr>
<tr>
<td>1,4-Benzoquinone</td>
<td>5.5‡</td>
<td>0.078 ± 0.006</td>
<td>1530 ± 42</td>
<td>19.6 ± 10$^6$</td>
</tr>
</tbody>
</table>

* Determined with 0.3 mM DCIP as two-electron acceptor.
† Determined with 0.5 mM FcPF$_6$ as one-electron acceptor.
‡ Determined with 100 mM glucose as electron donor.
maximum activity was reached faster in the reactor. Obviously oxygen saturation and shear stress are important factors for GDH production in *G. cingulata*. The measured glucose consumption during the bioreactor cultivation shows that most of the GDH activity was formed during the phase of highest glucose consumption, indicating an actively growing culture and no repression of GDH synthesis by glucose.

The molecular properties of *G. cingulata* GDH are comparable to those of GDHs from *Aspergillus* or *Penicillium* species, but not to the FAD-dependent GDH isolated from the thermophilic bacterium *Burkholderia cepacia*, a non-glycosylated heterotrimer (Sode et al., 1996). Glucose conversion by *G. cingulata* GDH is very fast and the catalytic efficiency is comparable to glucose oxidases from *A. niger* (31 000 M⁻¹ s⁻¹; Kalisz et al., 1991). The *Kₘ* values for glucose of other fungal GDHs are also in the range of *G. cingulata* GDH (49.7 mM for *A. terreus*, Omura et al., 2010; 13 mM for *Penicillium lilacinoechinulatum* (Aiba, 2007). A lower *Kₘ* value (2.8 mM) was measured only for the bacterial GDH from *B. cepacia* (Yamazaki et al., 1999). The high catalytic efficiencies of *G. cingulata* GDH for glucose and xylose suggest that both substrates can be converted *in vivo*. It is amazing that the enzyme’s *Kₘ* value for the pentose xylose is nearly as low as for the hexose glucose. In comparison, glucose oxidases from *Penicillium* spp. have much higher *Kₘ* values for xylose than for glucose (*P. amagasakiense*, *KₘGlc*=5.7 mM, *KₘXyl*=384 mM, Witt et al., 1998; *P. funiculosum*, *KₘGlc*=14.7 mM, *KₘXyl*=42.9 mM, Semashko et al., 2003). No other hemicellulose-forming monosaccharide, such as

### Table 3. pH-dependent activity of GDH with various electron acceptors

<table>
<thead>
<tr>
<th>Electron acceptor</th>
<th>A (nm)</th>
<th>Absorption coefficient (mM⁻¹ cm⁻¹)</th>
<th>Stoichiometry</th>
<th>pH optimum</th>
<th>pH range of &gt;75% activity</th>
<th>Specific activity at pH optimum (U mg⁻¹)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,4-Benzooquinone</td>
<td>290</td>
<td>2.24</td>
<td>1</td>
<td>6</td>
<td>4–8</td>
<td>1000 ± 70</td>
</tr>
<tr>
<td>Methyl-1,4-benzoquinone</td>
<td>290</td>
<td>3.0</td>
<td>1</td>
<td>6</td>
<td>4–8</td>
<td>1050 ± 80</td>
</tr>
<tr>
<td>2,6-Dimethyl-1,4-benzoquinone</td>
<td>290</td>
<td>2.5</td>
<td>1</td>
<td>6</td>
<td>4–7</td>
<td>1410 ± 42</td>
</tr>
<tr>
<td>2,6-Dimethoxy-1,4-benzoquinone</td>
<td>290</td>
<td>15.5</td>
<td>1</td>
<td>6</td>
<td>4–7</td>
<td>360 ± 28</td>
</tr>
<tr>
<td>3,5-Di-4-tert-butyl-1,2-benzoquinone</td>
<td>420</td>
<td>1.4</td>
<td>1</td>
<td>4</td>
<td>3–6</td>
<td>190 ± 15</td>
</tr>
<tr>
<td>1,4-Naphthoquinone</td>
<td>343</td>
<td>2.84</td>
<td>1</td>
<td>6</td>
<td>4–6.5</td>
<td>125 ± 4</td>
</tr>
<tr>
<td>9,10-Phenanthrenequinone</td>
<td>327</td>
<td>1.6</td>
<td>1</td>
<td>4</td>
<td>3–4.5</td>
<td>400 ± 36</td>
</tr>
<tr>
<td>Ferricyanide</td>
<td>420</td>
<td>0.97</td>
<td>2</td>
<td>3</td>
<td>2.5–3.5</td>
<td>180 ± 12</td>
</tr>
<tr>
<td>Fe(CN)₆⁢⁻⁴</td>
<td>300</td>
<td>5.85</td>
<td>2</td>
<td>≥8</td>
<td></td>
<td>1120 ± 23</td>
</tr>
<tr>
<td>DCIP</td>
<td>520</td>
<td>6.8</td>
<td>1</td>
<td>5.5</td>
<td>5–7</td>
<td>840 ± 35</td>
</tr>
</tbody>
</table>

*Assays performed at 30 °C in 50 mM McIlvane buffer ranging from pH 3.0 to 8.0. The specific activity gives the electron acceptor turnover.

---

### Table 4. Reduction of laccase-catalysed phenoxy radical formation after addition of GDH

<table>
<thead>
<tr>
<th>Substrate*</th>
<th>A (nm)†</th>
<th>Rate reduction by GDH (%)</th>
<th>1.9 µg ml⁻¹</th>
<th>5.7 µg ml⁻¹</th>
<th>19 µg ml⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coniferyl alcohol</td>
<td>265</td>
<td>23</td>
<td>44</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>285</td>
<td>19</td>
<td>33</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>Coumaric acid</td>
<td>285</td>
<td>47</td>
<td>60</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>Sinapic acid</td>
<td>305</td>
<td>20</td>
<td>26</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>310</td>
<td>46</td>
<td>58</td>
<td>66</td>
<td></td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>315</td>
<td>89</td>
<td>90</td>
<td>92</td>
<td></td>
</tr>
<tr>
<td>Guaiacol</td>
<td>465</td>
<td>99</td>
<td>99</td>
<td>99.5</td>
<td></td>
</tr>
<tr>
<td>2,6-Dimethoxyphenol</td>
<td>469</td>
<td>99.9</td>
<td>100</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

*The laccase substrate in its reduced form.
†The laccase (1.7 µg ml⁻¹) catalysed oxidation reactions and their inhibition by GDH were followed spectrophotometrically.
arabinose, mannone or galactose, showed significant activity with 
G. cingulata GDH. It remains to be elucidated how G. cingulata GDH achieves high catalytic efficiencies for a hexose and a pentose and simultaneously discriminates the oxidation of other, structurally close carbohydrates.

The final reaction product of the glucose turnover of GDH is gluconic acid, but a steady pH drop after complete glucose conversion indicates the hydrolysis of gluconolactone, supposedly the primary reaction product. However, for the biological function, the reductive half-reaction seems to be of much greater importance than the final reaction product. Electron acceptors such as DCIP and ferrocenium are efficiently reduced, but they are not present in the natural environment of the fungus. In contrast, quinones are available in plant tissues and are most likely encountered by the enzyme in vivo. The spectrum of reduced quinones is quite broad. While specific activity for 1,4-benzoquinones increases with increasing methyl-substitution, 2,6-dimethoxy-1,4-benzoquinone is converted at a significantly slower rate. Also, the bulky 3,5-di-tert-butyl-1,2-benzoquinone is reduced at a still considerable rate (20 % activity relative to 1,4-benzoquinone). Turnover decreases drastically for naphthoquinone (12 % activity relative to 1,4-benzoquinone). This reduction of the turnover rate most likely stems from the lower redox potential of the two-ring quinone than from its bulkiness, because 9,10-phenanthrenequinone is readily accepted (40 % activity relative to 1,4-benzoquinone). The turnover rates of quinones are high and suggest that several plant quinones with similar size and structure might be suitable electron acceptors. With all measured quinones, GDH activity was observed in a broad pH range, from 3 to 8. Compared with the electron acceptors described above, oxygen turnover is very low (0.02 % of DCIP turnover). This is also evident by comparing the 1350-fold higher oxygen-reducing activity of A. niger glucose oxidase (325 U mg$^{-1}$) with G. cingulata GDH (0.24 U mg$^{-1}$) in identical experiments at air saturation, pH 5.5 and 30 °C. Oxygen can therefore be excluded as a natural electron acceptor of G. cingulata GDH.

The oxidation products of monophenols – phenoxy radicals – were also found to be reduced by G. cingulata GDH. The higher observed reduction rate for guaiacol and 2,6-dimethoxyphenol (a reversible oxidation–reduction behaviour was found for both) than for the structural analogues conifer alcohol, ferulic acid or sinapic acid can be interpreted in two ways: either the propene side chain with the terminal hydroxy or carboxy groups reduces GDH activity or they polymerize faster than the relatively stable phenoxy radicals of guaiacol and 2,6-dimethoxyphenol and would thereby not be accessible for reduction by GDH. A fast dimerization of monolignol radicals would explain the non-linearity of the observed inhibition by increased GDH concentrations.

Analyses of the deduced protein sequence of the gdh gene revealed that GDH belongs to the structural family of glucose–methanol–choline (GMC) oxidoreductases. The highest sequence identities were found in two closely related fungi C. higginsianum (65 %) and C. graminicola (58 %), which are both well-studied plant pathogens. A hypothetical protein (XP_001584680.1) with a sequence identity of 57 % was found during a BLAST search of the genome of the plant pathogen Sclerotinia sclerotiorum. Interestingly, no hypothetical GDH with significant similarities could be identified in the genomes of other known ascomycete plant pathogens such as Neurospora crassa, Magnaporthe grisea or Fusarium and Verticillium species. The rareness of GDH in ascomycete fungi and scarce reports in the literature suggest that it either is not common in ascomycetes or is expressed under very specific conditions.

The phylogenetic relationship of FAD-dependent GDHs and other GMC oxidoreductases shows that FAD-dependent GDHs form a well supported family closely related to their cousins – the glucose oxidases. The fungal GDHs include two hypothetical GMC sequences from G. higginsianum (65 % identity to G. cingulata) and G. graminicola (59 %), two hypothetical protein sequences from A. terreus (58 %) and Sclerotinia sclerotiorum (56 %) and two sequences from A. oryzae (56 %) and Botryotinia fuckeliana (56 %) which are annotated as glucose oxidases, but are much more related to G. cingulata GDH than to A. niger (35 % and 35 % identity, respectively) and P. amagasakiense GOX (35 % and 36 % identity, respectively). These protein sequences should be classified as GDH sequences. However, for a more detailed analysis of GDH evolution, additional sequences would be required.

In conclusion, the results presented here support a role for G. cingulata FAD-dependent glucose dehydrogenase during plant attack. The reduction of plant-generated quinones to protect the fungus against these fungi-toxic substances and the reduction of phenoxy radicals to counteract the plant’s effort to shield itself from the invader are two very likely roles of this extracellular oxidoreductase.

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REFERENCES


