A 1,3-β-glucanosyltransferase isolated from the cell wall of Aspergillus fumigatus is a homologue of the yeast Bgl2p

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A 1,3-β-glucanosyltransferase which introduces intrachain 1,6-β linkages into 1,3-β-glucan was isolated from the cell wall of Aspergillus fumigatus. The biochemical and molecular characterization of the A. fumigatus transferase showed it was homologous to the Saccharomyces cerevisiae and Candida albicans transferase Bgl2p. A null mutant constructed in A. fumigatus by gene replacement did not show a distinct phenotype from the parental strain. The putative function of this major cell-wall-associated protein is discussed.

Keywords: Aspergillus fumigatus, BGT1, cell wall, 1,3-β-glucan, BGL2

INTRODUCTION

The hyphal wall of the human pathogen Aspergillus fumigatus is composed predominantly of polysaccharides (1,3-β-glucan, chitin, 1,3-z-glucan and galactomannan) and glycoproteins (Hearn & Sietsma, 1994; Latgé et al., 1991, 1994; Fontaine et al., 1996). The only enzymes involved in cell wall synthesis characterized until now are the synthase complexes responsible for the synthesis of the skeletal polysaccharides, 1,3-β-glucan and chitin (Beauvais et al., 1993; Mellado et al., 1995, 1996). However, cell wall expansion during growth requires not only synthases, but also hydrolases to break the existing bonds and transglycosidases to form new covalent bonds (Wessels, 1988). Chitinases and 1,3-β-glucanases, isolated from the cell wall of A. fumigatus, could play the hydrolytic role during morphogenesis (Hearn & Sietsma, 1994). In contrast, our knowledge of the transglycosidases and repair enzymes is very limited. One 1,3-β-glucanosyltransferase responsible for the elongation of 1,3-β-glucan chains has been characterized to date (Hartland et al., 1996). During the search for nonhydrolytic enzymes using 1,3-β-glucan as a substrate, a homologue of the Bgl2p β-glucanosyltransferase of Saccharomyces cerevisiae (Klebl & Tanner, 1989) and Candida albicans (Sarthy et al., 1997) was identified. In yeast, this enzyme releases a free disaccharide unit from the reducing end of a laminarioligosaccharide substrate and transfers the newly generated reducing end to the nonreducing end of another 1,3-β-glucan molecule, forming a new intrachain 1,6-β linkage. This paper describes the purification of the homologue of this glucanosyltransferase in A. fumigatus, the characterization of its enzymic activity, the cloning of the encoding gene and the phenotype of null mutants.

METHODS

Strains and growth conditions. A. fumigatus CBS 144.89 was used throughout this study and was maintained on 2% malt agar. Cultures grown in Sabouraud liquid medium (2% glucose + 1% mycpeptone; Biokar) were used for DNA and protein extraction. Mycelial cultures of A. fumigatus were grown in flasks or in fermenters as previously described (Hartland et al., 1996). For growth kinetic studies in fermenters, sequential aliquots of the culture slurry were filtered

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Abbreviations: DP, degree of polymerization; DQF-COSY, two-dimensional double-quantum-filtered correlated spectroscopy; HPAEC, high-performance anion-exchange chromatography; NOESY, two-dimensional nuclear Overhauser effect spectroscopy; PAHBAH, p-hydroxy benzoic acid hydradize; r, reduced; RELAYH, two-dimensional relayed coherence transfer correlated spectroscopy; TOCSY, total two-dimensional correlated spectroscopy.

The GenBank accession number for the sequence reported in this paper is AF038596.
under vacuum, extensively washed with water, dried at 105 °C and weighed. For transformation experiments, minimal medium (per litre: glucose, 10 g; ammonium tartrate, 0.92 g; KCl, 0.52 g; MgSO\(_4\).7H\(_2\)O, 0.52 g; KH\(_2\)PO\(_4\), 1.52 g; trace elements solution (Cove, 1966), 1 ml; pH adjusted to 6.8 with NaOH) was used.

The MIC values for drugs were determined by microbroth dilution in 96-well microtitre plates using a 1% yeast extract medium (Difco) with or without 2% glucose or 0.6 M mannitol. Media (150 µl per well) were inoculated with 10\(^5\) conidia ml\(^{-1}\) in yeast extract medium. MICs were taken as the lowest drug concentration at which no growth was detected after 2 d incubation at 37 °C. Kinetics of conidial germination were measured after deposition of a conidial suspension (2 µl of a suspension of 10\(^8\) conidia ml\(^{-1}\)) on 2% malt agar and incubation at 37 °C.

**Preparation of enzyme extracts.** Preparation of a cell wall autolysate from a 42 h culture was performed as previously described (Hartland et al., 1996). Briefly, mycelium grown in a 15 litre fermenter in Sabouraud medium was collected by filtration and broken with the presence of glass beads. The precipitated cell wall material was suspended in 50 mM sodium acetate, 5 mM Na\(_2\)PO\(_4\), pH 5.6, and incubated for 3 d at 37 °C. After several centrifugation, dialysis and concentration steps, the autolysate contained 250–300 mg protein and was used for the DEAE-Sepharose step.

The culture filtrate was concentrated to 11 l by ultrafiltration through a Pellicon cell fitted with a Pellicon cassette with a 10 kDa cut-off. The filtrate was dialysed for 2 d against running tap water, and after centrifugation (8000 g, 15 min), the supernatant was dialysed against 10 mM sodium acetate, pH 5.6, and filtered before being used for the first chromatography step.

**Enzyme purification.** Fractions collected during every chromatography step were assayed for enzyme activity using the nonradioactive transferase assay (see below). The concentrated, dialysed autolysate was applied to a column (4 × 18 cm) of DEAE-Sepharose Fast-Flow (Pharmacia) equilibrated in 5 mM sodium acetate, pH 5.6, and eluted with a linear gradient of 0–1 M NaCl (1 l) at a flow rate of 240 ml h\(^{-1}\). Fractions containing the transferase activity were pooled, dialysed against 10 mM β-mercaptoethanol, 5 mM EDTA, 10 mM sodium acetate buffer, pH 4.0, applied to a Mono S column (HR 5/5; Pharmacia) and eluted with a linear NaCl gradient (0–300 mM in 40 min) at a flow rate of 0.8 ml min\(^{-1}\). The fractions containing the transferase activity were pooled, dialysed against 10 mM Tris/Cl, pH 7.0, deposited on a DEAE-5PW column (8 × 75 mm; Tosohaas) and eluted with a linear NaCl gradient (0–300 mM in 60 min) at a flow rate of 0.75 ml min\(^{-1}\). Fractions containing the transferase activity were pooled, dialysed against 10 mM β-mercaptoethanol, 5 mM EDTA, 10 mM sodium acetate, pH 4.0, deposited on a Chang column (8 × 75 mm; Tosohaas) and eluted with a linear NaCl gradient (0–300 mM in 60 min) at a flow rate of 0.8 ml min\(^{-1}\). Fractions containing the purified transferase were pooled, dialysed against 5 mM sodium citrate, pH 5.0, concentrated under vacuum and stored at −20 °C until used.

**Enzyme assays.** β-Glucosidase activity was quantified using p-nitrophenyl glucoside as a substrate and measuring the amount of p-nitrophenol liberated (Fontaine et al., 1997), 1,3-β-Glucanase activity was measured by a reducing-sugar assay using p-hydroxybenzoic acid hydrazide (PAHBH) with borohydride-reduced laminarin as a substrate (Fontaine et al., 1997; Hartland et al., 1996). Labelled 3H-reduced laminari-oligosaccharides were obtained as described previously (Hartland et al., 1996). Transferase activity was assayed qualitatively by incubating enzyme samples in 50 mM sodium acetate buffer, pH 5.6, at 37 °C with 7.5 mM borohydride-reduced laminarioligosaccharides containing at least five glucose units (assay volume of 20 µl). Sequential samples taken over time were diluted 20 × with 50 mM NaOH and analysed by high-performance anion-exchange chromatography (HPAEC) on a CarboPac PA1 column using chromatographic conditions previously developed (Hartland et al., 1996). The preparation of reduced substrates, necessary to avoid the peeling reaction of 1,3-β-glucan substrates in alkaline conditions during the HPAEC analysis was done as previously reported (Hartland et al., 1996). The pH optimum and K\(_m\) value of the enzyme were determined with 3H-labelled G, as substrate using HPAEC and an on-line Radiomatic flow scintillation analyser (model 150TR; Packard). Gentiooligosaccharides were prepared as previously described (Hartland et al., 1996) and cellopentoase was obtained from Sigma.

**Analysis of the reaction product.** The number of glucose units per oligosaccharide was determined by comparison of the elution times of the reaction products with standard laminarioligosaccharides of known degree of polymerization (DP) and confirmed by mass spectrometry using a MALDI-TOF apparatus (matrix-assisted desorption/ionization time-of-flight mass spectrometry). The MALDI mass spectrum was measured on a reflectron-type Vision 2000 time-of-flight mass spectrometer (Finnigan MAT). Samples were mounted on an x, y moveable stage, allowing irradiation of selected sample areas. A nitrogen laser with an emission wavelength of 337 nm and 3 ns pulse duration was used. The spectrum was recorded in the positive-ion mode and accelerated to an energy of 3 keV before entering the flight tube. Ions were post-accelerated to an energy of 5 keV for detection. Samples were prepared by mixing directly on the target 1 µl of the oligosaccharide solution (about 25 pmol) and 1 µl 2,5-dihydroxybenzoic acid matrix solution (12 mg ml\(^{-1}\) dissolved in 90% methanol) (Faugeron et al., 1997).

The product of a transferase reaction, purified by HPAEC after incubation of reduced G, with the enzyme fraction, was analysed by 1H-NMR spectroscopy. Samples were deuterium-exchanged by freeze-drying a solution in D\(_2\)O (99.95 atom %) (Solvants Documention Synthese) and then dissolved in D\(_2\)O. The concentration of oligosaccharides was approximately 1.5 mM. All NMR spectra were obtained at 27 °C on a Varian Unity 500 spectrometer operating at a proton frequency of 500 MHz. Spectra were referenced to external 3-(trimethylsilyl)propionic acid (sodium salt). Proton signal assignments were done by DQF-COSY (Rance et al., 1983), TOCSY (Griesinger et al., 1988) and RELAYH with single and two-step relayed coherence transfer (Wagner, 1983). A NOESY experiment was used for sequential assignment and branching point determination (Mucra et al., 1981). All two-dimensional data, except for RELAYH, were collected in the phase-sensitive mode using the States–Haberkorn method (States et al., 1982). Coupling constants were measured from one-dimensional spectra recorded with a digital resolution of 0.033 Hz/pixel or from the DQF-COSY experiment with a digital resolution of 0.43 Hz per point after zero-filling.

**Protein analysis.** Protein samples were analysed by SDS-PAGE (Laemmli, 1970) using 4% stacking and 10% separating gels. De-N-glycosylation was performed using recombinant N-glycosidase F (Oxford Glycosystems) according to the manufacturer’s instructions.

Internal peptides were obtained after endolysin digestion. Internal and N-terminal peptide sequencing was performed by
Cloning procedures and DNA manipulation. Approximately 50000 recombinant plaques of a zEMBL3 genomic library of A. fumigatus (Monad, 1994) were immobilized on nylon membranes (Zeta-Probe; Bio-Rad). Filters were probed with 32P-labelled degenerate oligonucleotides, deduced from amino acid sequencing and from the cDNA usage of A. fumigatus, in a solution containing 5 x SSC, 7% SDS, 10 x Denhardts and 20 mM Na2HPO4 (pH 7) at 50 °C for 24 h. The membranes were exposed to X-ray film after two washes at 42 °C in a solution of 3 x SSC, 5% SDS, 10 x Denhardts, 25 mM Na2HPO4 (pH 7) and two washes in a solution of 1 x SSC, 1% SDS. Positive plaques were purified and the DNA was isolated using the Qiagen Lambda kit. Agarose gel electrophoresis of restricted recombinant bacteriophage zEMBL3 DNA, Southern blotting and subcloning of genomic DNA fragments into plasmids were performed according to standard protocols (Sambrook et al., 1989). Escherichia coli DH5α was used for plasmid propagation. pUC19 was used in subcloning procedures. Double-stranded DNA was sequenced using the Sequenase 2.0 sequencing kit (USB) according to the supplier’s instructions. A. fumigatus chromosomal DNA was isolated according to the procedure of Girardin et al. (1993).

A. fumigatus transformation. pAN7.1 (Punt et al., 1987) carrying the E. coli hygromycin B phosphotransferase gene (bph) was used for transformation of A. fumigatus. The transformation of A. fumigatus was done as previously described (Paris et al., 1993). After overnight expression of bph, the transformants were selected on minimal medium containing 200 µg hygromycin B ml−1 for 7 d at 25 °C.

Analysis of transformants
Preparation of cell walls and immunoblotting. After 24 h growth at 37 °C in Sabouraud medium, the mycelia of the mutant and parental strains were collected by vacuum filtration on a Büchner funnel and extensively washed with water. The mycelia were disrupted for 3 min with 1 mm diameter glass beads in a Dyno-Mill cell breaker (WAB) in 50 mM Tris/HCI, pH 7-5. The cell wall pellet was washed three times by centrifugation with water and stored at −20 °C.

For Western blot analysis, cell wall proteins were extracted with Zymolyase 100T (2 ml of 0:1 mg Zymolyase ml−1 per 100 mg cell walls in 50 mM KH2PO4/NaOH, pH 6-5, 2 mM DTT, 5 mM sodium azide, 2 mM PMSF) overnight at room temperature and transferred overnight (30 V) to nitrocellulose membranes (cellulose nitrate, 0.2 mm pore size; Schleicher & Schuell) in 50 mM Tris, 200 mM glycine, 20% methanol (Towbin et al., 1979). After blocking with TBS (10 mM Tris/HCI, pH 7-2, 150 mM NaCl, 10 mM EDTA), 0.05% Tween 20 plus 5% defatted milk powder (Reglait) for 1 h at room temperature, the membranes were incubated with anti-transferase antiserum (diluted 1:3000 in TBS containing 0.1% Tween 20) for 2 h at room temperature. To obtain the anti-transferase antibodies, 20 µg purified protein in 0.9% NaCl (w/v) were mixed with an equal volume of Freund’s complete adjuvant and injected intradermally at multiple sites into female Balb/C mice. Two booster injections of the transferase in Freund’s incomplete adjuvant (1:1, v/v) were administered at 2 week intervals. The blots were washed five times for 3 min in TBS, 0.05% Tween 20, incubated in TBS with peroxidase-conjugated antimouse IgG (Sigma) (1:3000 dilution) for 45 min at room temperature, then washed three times in TBS, 0.05% Tween 20, twice in PBS-Tween 20 followed by a 10 mM Tris/HCI, pH 7-2, 150 mM NaCl final wash.

Cell-wall composition analysis. For analysis of the cell-wall composition, 250 µg cell walls of each strain were extracted with 1 ml 1 M NaOH for 30 min at 65 °C and then centrifuged at 3500 g for 15 min. The alkali-soluble supernatant was adjusted to pH 5-0 by addition of glacial acetic acid, dialysed against water for 3 d, freeze-dried and stored. The alkali-insoluble pellet was extracted again for 30 min with 1 M NaOH at 65 °C and centrifuged. The pellet was washed five times with water and freeze-dried. Total hexose determination was done by the anthrone method (Lunt & Setchiffe, 1953). Monosaccharides were determined by GLC after hydrolysis, reduction and peracylation of total cell wall, alkali-soluble and alkali-insoluble fractions (Sawardeker et al., 1967). GLC was performed on a Delta 200 instrument with a flame-ionization detector using a capillary column (25 m x 0.32 mm) filled with DB-1, and a temperature gradient from 100 to 240 °C at 2 °C min−1. Analysis of interglycosidic linkage, and specifically the presence of 1,6-glucosidic bonds, in the alkali-insoluble fraction was done by methylation according to the procedure described by Paz Parente et al. (1985) and modified by Fontaine et al. (1991). Permethylated products were analysed by GLC/MS after methanolysis and acetylation according to Fourier et al. (1981). Quantzyme degradation of the alkali-soluble and -insoluble fractions was performed over 24 h at 37 °C in aliquots containing 600 µl 50 mM Tris/HCI, pH 7-4, 40 mM β-mercaptoethanol, 1 µl Quantzyme (50 U µl−1), 100 µl alkali-soluble or -insoluble fractions. Release of reducing sugars was measured after 1, 2, 5, 8 and 24 h incubation on 50 µl of the reaction mixed with 950 µl PAHBAH buffer (10 mM CaCl2, 250 mM NaOH, 25 mM sodium citrate, 2.5 mM Na2SO4, 3.2 mM PAHBAH) using laminaripentaose as the standard (Seikagaku).

RESULTS

Purification of the 37 kDa protein
A 1,3-β-glucanosyltransferase (Bgt1p, for 1,3-1,6-β-glucanosyltransferase 1) was purified from a cell wall autolysate of A. fumigatus using four conventional ion-exchange chromatography steps. The activity was also present in the culture filtrate. The activity was associated with a 37 kDa protein. The MonoS step fractionated glucanosyltransferase, 1,3-β-glucanase and β-glucosidase activities: the 49 kDa glucanosyltransferase previously characterized eluted with the laminarinase and β-glucosidase activities (Hartland et al., 1996); the second glucanosyltransferase Bgt1p eluted late in the salt gradient (Fig. 1). Further purification on DEAE-5PW and CM-5PW columns showed that a minor protein of 35 kDa remained associated with the major protein of 37 kDa (Fig. 2). N-terminal amino acid sequence analysis of the first 10 residues of the 35 kDa protein showed an identical sequence to that of the 37 kDa protein (data not shown). Digestion of the 35/37 kDa protein doublet with N-glycosidase F resulted in the formation of a band at 34 kDa and another band at 32 kDa containing N-glycosidase F. This result bands were visualized by using 0.4% (w/v) diaminobenzidine in 50 mM phosphate buffer, pH 7-4, containing 0.01% (v/v) H2O2. The reaction was stopped by extensive washing with distilled water.
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Fig. 1. Fractionation of the pool of the β-D-glucosidase laminarinase positive fractions recovered from DEAE-Sepharose on a MonoS column (10 mM β-mercaptoethanol, 5 mM EDTA, 10 mM sodium acetate, pH 4, 0–300 mM NaCl gradient). Fractions labelled B contained Bgtlp; fractions labelled A contained the β-D-glucosidase, laminarinase and the other 1,3-β-glucanosyltransferase identified in A. fumigatus by Hartland et al. (1996). The UV absorbance is indicated by the solid line, the NaCl gradient by the dotted line.

Fig. 2. SDS-PAGE analysis of 1,3-β-glucanosyltransferase-containing fractions. Lanes: A, purified 37 kDa protein (1.5 μg); B, purified 37 kDa protein (1.5 μg) after treatment with N-glycosidase F; C, N-glycosidase F only. The N-terminal amino acid sequences and the deglycosylation experiments suggested that the 35 kDa band was two species of the same protein with different glycosylation levels.

Enzyme activity of Bgtlp

HPAEC profiles of products obtained with reduced (r) oligosaccharides of sizes G₃₃, G₆, G₁₁ or G₁₈ incubated with Bgtlp are shown in Fig. 3. Bgtlp specifically cleaved a biose unit from the reducing end of rGₘ and transferred the remainder to an rGₘ acceptor molecule to form rGₘ+n-2 in a reaction consistent with the following scheme:

\[ E + rGₘ \rightarrow E \cdot Gₘ-n + rG \]
\[ E \cdot Gₘ-n + rG \rightarrow E + rGₘ+n-2 \]

where E is the enzyme and rGₘ+n-2 represents a reduced laminarioligosaccharide n or m glucosyl units long.

Analysis of the linkages in the newly synthesized oligosaccharide was performed with a reduced G₁₀ transferase product obtained from the reaction with reduced G₆. GLC analysis of the permethylated reduced G₁₀ after hydrolysis, reduction and acetylation showed that a single 1,6 linkage was present in the reaction product (data not shown). The one-dimensional "H-NMR spectrum of the G₁₀ transferase product presented six doublets in the anomic region at 4.55, 4.68, 4.73, 4.75, 4.77 and 4.80 p.p.m., each corresponding to one anomic proton except for the signal at 4.80 p.p.m., which corresponds to four protons (Table 1). These chemical shifts were in good agreement with those published for a linear 1,3-β-glucan containing an intrachain 1,6-β linkage (Yu et al., 1993). Since glucitol does not give any signal between 4.5 and 4.8 p.p.m., the results confirmed that the oligosaccharide contained 10 glucose residues. The coupling constants J₁,₂ measured from the one-dimensional spectrum were between 7.8 and 8 Hz, which is typical of β-linked units. The presence of six different anomic protons indicated that the transfer product did not contain only 1,3-β linkages. The RELAYH experiment was performed to identify unambiguously H3 proton resonances of the glucose units to confirm sequential assignment and to establish linkage characterization. The chemical shifts, reported in Table 1, showed that four glucose units (glucose F) are indistinguishable by their proton resonances, as would be expected for a homo-oligosaccharide. The NOESY experiment indicated that all residues were linked via a 1,3-β linkage, except for the glucose C residue, which was substituted via a 1,6-β linkage with the glucose A residue (data not shown). The analysis of inter-residue NOE interactions showed the absence of disubstituted glucose residues and the presence of only one terminal nonreducing-end glucose residue (residue D), excluding the possibility of a branching glucan chain. The NMR data and the GLC data were in agreement, showing that the rG₁₀ transferase product was a linear 1,3-β-glucan chain with a single 1,6-β intrachain linkage.

The presence of a single glucose unit at the nonreducing end of the newly synthesized oligosaccharide indicated that Bgtlp transferred the donor chain onto the nonreducing end of the acceptor. From NMR and GLC data, the exact position of the 1,6-β linkage in the glucan chain could not be identified (see formula in Table 1). Since Bgtlp only released an rG₆ from the laminarioligosaccharide, an rG₁₀ product resulted inevitably from the transfer of the nonreduced G₆ to the rG₆ substrate. As a
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Fig. 3. HPAEC analysis of 37 kDa transferase products from the incubation with reduced laminarioligosaccharides. Purified 37 kDa protein (0.4 μg) was incubated with 8 mM of a reduced laminarioligosaccharide of size G2 (left), G3 (centre left), G11 (centre right) or G18 (right) in 100 mM sodium acetate, pH 5.6, at 37°C (10 μl assay volume). The HPAEC profiles obtained from 3 μl samples taken at zero time and 30 min are shown, along with the size of the major products.

Table 1. Proton chemical shifts in p.p.m. (upper line) and coupling constants in Hz (lower line) of the oligosaccharide transfer product

The nonequivalent germinal proton resonating at lower field is denoted H'. The glucose residues were labelled A–F in order of increasing chemical shift of their anomeric protons of the following deduced structure: Glcβ1-3(Glcβ1-3)5Glcβ1-3Glcβ1-6Glcβ1-3(Glcβ1-3)5-Glcβ1-3glucitol, where x = 1, 2 or 3.

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result, the only position possible for the 1,6-β linkage was at the 6th linkage from the reducing end. These results showed that the activity of A. fumigatus Bgt1p was similar to the yeast Bgl2p transferases.

Analysis of the transfer reaction was easily performed by HPAEC since the injection of the 'kinked' transferase product with a linear laminarioligosaccharide with the same number of glucose units resulted in the appearance
of two peaks on HPAEC (data not shown). The minimum substrate size was G₄, and oligosaccharides at least as large as laminarin also formed substrates. The presence of 1,6-β linkages in the transferase products of the reaction of laminarin with Bgtlp resulted in the transferase products of laminarin also formed substrates. The formation of soluble oligosaccharides of DP 3). Bgtlp was specific for 1,3-β linkages as no reaction product was seen with 1,4-β- and 1,6-β-linked oligosaccharides with or without the addition of a laminarinoligosaccharide substrate. The 37 kDa enzyme had an optimum pH of 5.5. The N-deglycosylated form of the enzyme was also active (data not shown).

Lowering the concentration of the acceptor molecule promoted hydrolysis, which produced rG₄ and G₂ from rG₆; at concentrations lower than 3 μM, no transfer products were seen (Fig. 4). In contrast, 100% transfer was evident at 1 mM, as confirmed by the absence of formation of oligosaccharides with a new reducing end during the reaction, as determined by the PAHBAH method. The transferase reaction rate was determined with 8 mM [3H]-reduced laminarioligosaccharide up to rG₁₅.

Measuring the formation of rG₂, the initial rate was 0.24, 0.46, 0.76, 1.12, 1.25, 1.26, 1.21, 1.29, 1.37 and 1.24 for DP 5, 6, 7, 8, 9 10, 11, 12, 13 and 15, respectively. These data showed that the transferase reaction rate increased with the DP of the laminarioligosaccharide used as a substrate. Bgtlp catalysed its transferase reaction via a bi-reactant (two-step) mechanism with initial hydrolysis of a donor substrate, specifically releasing a biose unit from the reducing end, followed by transfer of the remainder to the nonreducing end of an acceptor. The problem with determining a Kₘ for the donor site is that donor molecules can also act as acceptors in the transferase reaction. To determine the Kₘ for reduced rG₄ as the donor, a range of concentrations of [3H]rG₄ (kept below 8 mM) was used in the presence of a high concentration (64 mM) of rG₄ acceptor (not a donor). Such experimental conditions were sufficient to force Bgtlp to specifically use rG₄ as the donor and rG₄ as the acceptor. By measuring the formation of the [3H]rG₄ product, an apparent Kₘ of 58 mM was obtained from the double-reciprocal plot (r² value 0.999) (data not shown).

**Molecular characterization of the gene (BGT1) encoding the 37 kDa glucanosyltransferase**

Based on the sequences of the N-terminal (SKNGTL-GFALGNKNEGKCKVKQVSDYETDFDTLKEVTSLV) and internal (SLTPQALLARIQQV) peptides, two oligonucleotide probes were designed based on part of these sequences: probe A, GGYAACAAAGCAGGGYGGYAA GTGYAAGGGT(C/T)/CA (encodes GNNKNEGGKCKVKQV), and probe B, TG(T/A)/AC(T/C/)/TG(T/A/G)/AT(C/A)/CG(C/A)/GC (encodes ARIQVQ).

Probe A was used to screen the genomic library. Three positive clones were identified. Restriction enzyme analysis of purified bacteriophage DNA revealed that the three clones had a common SacI–EcoRI fragment hybridizing with both probes A and B. This 3 kb genomic DNA fragment containing the BGT1 gene was isolated and sequenced. An ORF of 939 nucleotides, which encodes a 313 amino acid polypeptide with a theoretical molecular mass of 34 kDa, was detected. The genomic DNA contained three introns of 42, 60 and 63 bp, starting at nucleotides 306, 587 and 809, respectively. Two potential N-glycosylation sites were found situated at amino acid residues 21 and 196. The hydropathy profile showed the presence of one hydrophobic domain at the N terminus. The first 18 amino acids (MRVSTLLPLAAGTAAA) of the polypeptide predicted by the BGT1 sequence were absent from the mature protein. This hydrophobic domain represented a signal peptide, confirming that the protein was secreted.

A FASTA search of the GenBank and SWISS-PROT databases (Pearson & Lipman, 1988) revealed that the protein encoded by the BGT1 gene was 40% identical to the proteins encoded by the S. cerevisiae and C. albicans BGL2 genes (Klebl & Tanner, 1989; Sarthy et al., 1997) (Fig. 5). Although FASTA searches did not identify obvious similarities with other proteins, hydrophobic cluster analysis (Gaboriaud et al., 1987) showed that several regions of the Bgtlp sequence displayed similarities with glycoside hydrolases family 17 (Henrissat, 1991; Henrissat & Bairoch 1993, 1996). These regions are shown in Fig. 6.

**Disruption of BGT1 in A. fumigatus**

The strategy described in Fig. 7 (a) was used to produce an A. fumigatus strain with a nonfunctional disrupted copy of BGT1.
A 1 kb BamHI–NaeI fragment of \( BGT1 \) containing about 50\% of the coding region was replaced by the 3.65 kb \( StuI – BsmHI \) fragment of \( pAN7.1 \) containing the \( hph \) gene flanked by the \( trpC \) terminator and \( gpd \) promoter of \( Aspergillus nidulans \)(Punt et al., 1987). The plasmid DNA linearized by Smal was used to transform protoplasts of \( A. fumigatus \). Twelve transformants resistant to hygromycin were obtained. Southern blot analysis confirmed the gene replacement at the \( BGT1 \) locus in two transformants and the integration of \( hph \) at the \( BGT1 \) locus (Fig. 7b). Western blot analysis of total protein extracts with anti-Bgtlp antibodies con-
firmed the absence of Bgt1p in the two transformants (Fig. 7c). In addition, no Bgt1p transferase activity was detected in a cell wall autolysate of the BGT1 null mutant (data not shown).

No phenotypic difference was seen between the BGT1 null mutant and the parental strain. Growth rates in a 2% glucose + 1% mycoprotein medium, or in a 1% yeast extract medium supplemented with 2% glucose or 0.6 M mannitol, or in the absence of a carbon source, were identical in both strains. In a 1% yeast extract medium, MIC values for Nikkomycin Z, Polyoxin D, SDS and calcofluor white were 0.015–0.02; > 2; 0.78–1.56; and 0.2–0.3 mg ml⁻¹, respectively, at 37 or 41 °C. Hyphal morphology and germination rate were not influenced by the disruption of BGT1. Total hexose and hexosamine contents of the different cell wall extracts of the parental and mutant strains were comparable (Table 2). The amount of 1,3-β-glucan in the different cell wall extracts (alkali-soluble or -insoluble) was estimated after Quantazyme digestion. The results were similar in the mutant and parental strains. No 1,3-β-glucan was found in the alkali-soluble fraction and Quantazyme digestion released 1,3-β-glucan accounting for 33% of the alkali-insoluble fraction. Monosaccharide composition, analysed by GLC, showed the presence of glucose, galactose, mannose and N-acetylglucosamine in similar proportions in all cell wall fractions. Analysis of the glycosidic linkages in the alkali-insoluble cell wall fraction by methylation confirmed the presence of methyl 2,3,4-tri-O-methyl-6-O-acetyl glucoside, corresponding to glucose residues linked through a 1,6 linkage. In the alkali-insoluble fraction of both strains (wild-type and mutant), this 1,6-linked glucose residue was found in the same ratio of 0.5 residues per 100 residues of 1,3-glucose (data not shown).

TABLE 2. Composition of wall fractions of BGT1 mutant and parental A. fumigatus strains, expressed as percentage of the dry weight of the alkali-soluble and -insoluble fractions of the cell wall extract

<table>
<thead>
<tr>
<th>Strain</th>
<th>Hexose</th>
<th>Alkali-insoluble</th>
<th>Alkali-soluble</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>77</td>
<td>72</td>
<td>85</td>
</tr>
<tr>
<td>BGT1 mutant</td>
<td>70</td>
<td>65</td>
<td>80</td>
</tr>
<tr>
<td>Hexosamine</td>
<td>19</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Wild-type</td>
<td>21</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>BGT1 mutant</td>
<td>21</td>
<td>4</td>
<td>2</td>
</tr>
</tbody>
</table>

DISCUSSION

A glucanosyltransferase homologous to the yeast Bgl2p (Hartland et al., 1991; Goldman et al., 1995) was found in A. fumigatus. As in yeast, null mutants of A. fumigatus behave like the parental strains with respect to growth, osmotic stability, sporulation and cell wall composition. The only phenotype seen in strains of C. albicans lacking the BGL2 gene is an increase in the sensitivity to chitin synthesis inhibitors (not tested in S. cerevisiae) (Klebl & Tanner, 1989; Mrsa et al., 1993; Sarthy et al., 1997). In contrast, in A. fumigatus, the same sensitivity to these inhibitors was seen in both the mutant and parental strain.

The protein displayed the same physico-chemical and enzymic characteristics among the different species: it is a molecule slightly glycosylated (10%), with a molecular mass around 30 kDa, that is extremely resistant to heat. In A. fumigatus, after boiling for 10 min in a 2% SDS-containing buffer, transferase activity can still be detected (data not shown). All transferases from Aspergillus, Candida and Saccharomyces use G₆ as a minimal substrate size, and decreasing the concentration of substrate promoted the hydrolytic activity of the molecule. The use of low substrate concentration by Mrsa et al. (1993) may be the reason why this protein was initially but wrongly identified as a 1,3-β-glucanase.

Analysis of sequencing data has shown that Bgt1p and the two homologous yeast Bgl2 proteins can be assigned to family 17 of the glycoside hydrolases (Henrissat, 1991; Henrissat & Bairoch 1993, 1996). Interestingly, these three proteins are more related to each other than to any other member of family 17, where they clearly form a subfamily. The assignment of these proteins to family 17 is in agreement with the retention of the anomeric configuration which prevails in this family and which is a prerequisite for transglycosylation reactions. Based on the sequence alignment shown in Fig. 6, the acid/base and nucleophilic residues of BGT1 were putatively identified as E128 and E229 respectively. By analogy with the three-dimensional structure of the barley enzymes, the two catalytic glutamic residues will flank the deep substrate-binding groove of the β/α₈ barrel structure reported for the members of family 17 (Varghese et al., 1994; Henrissat et al., 1995; Jenkins et al., 1995). Bgt1p is the first glucanosyltransferase clearly identified in glycoside hydrolase family 17. This indicates that the enzymic reaction in hydrolases and transferases of this family is catalysed by proteins of similar three-dimensional structure and catalytic machinery. The presence of transferases in glycoside hydrolase families is a situation already well documented in other families such as family 13, which contains cyclodextrin glucanotransferases and starch branching enzymes along with a large number of hydrolytic enzymes, or family 16, which contains xyloglucan endotransglycosylases along with 1,3-β-glucanases (Henrissat & Bairoch, 1996).

Although present as a major cell-wall-associated protein in several species of yeast and filamentous fungi (Herrero et al., 1987), the physiological role of these glucanosyltransferases is presently unknown. Since the gene is present as a single copy in the genome, the absence of phenotype for the null mutant suggests that this enzyme does not play a major role in cell-wall morphogenesis.
and in particular in cross-linking of cell wall polysaccharides as suggested by Goldman et al. (1995).

The Bgtlp enzyme activity requires the presence of laminarioligosaccharides with free reducing ends. Since such substrates could only be formed by the action of endoglucanases, it was also speculated that this enzyme may play a role in conjunction with B-glucanases to keep the cell wall plastic during cell expansion and branching. No modification of cell-wall morphogenesis was seen in the mutants, arguing against this hypothesis.

A possible role for these enzymes would be an involvement in the nutrition of the fungus, as they may be responsible for the release of high concentrations of laminaribiose into the culture supernatant, as shown during the exponential growth of S. cerevisiae (Coen et al., 1994). The laminariobiose is then hydrolysed to glucose, which can be metabolized by the cell (Coen et al., 1994). In addition, kinked laminarioligosaccharides may serve as a soluble-carbon storage source. A putative role of Bgl2p/Bgtlp in the nutrition of the fungus would be in agreement with the absence of phenotype for a null mutant since other fungal hydrolases may substitute for Bgtlp.

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REFERENCES


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