Altering the expression of two chitin synthase genes differentially affects the growth and morphology of *Aspergillus oryzae*

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In *Aspergillus oryzae*, one full-length chitin synthase (*chsB*) and fragments of two other chitin synthases (*csmA* and *chsC*) were identified. The deduced amino acid sequence of *chsB* was similar (87% identity) to *chsB* from *Aspergillus nidulans*, which encodes a class III chitin synthase. The sequence obtained for *csmA* indicated that it had high similarity to class V chitin synthases. *chsB* and *csmA* disruption strains and a strain in which *chsB* transcription was controlled were constructed using the nitrite reductase (*niiA*) promoter. The strains were examined during hyphal growth by Northern analysis, analysis of the cell-wall composition and growth in the presence of Calcofluor white (CFW). The *chsB* disrupted strain and the uninduced *p_niiA*–*chsB* strain exhibited hyperbranching, they had a lower level of conidiation than the wild-type and were sensitive to CFW at 50 mg l⁻¹. When *chsB* transcription was induced in the strain containing the *p_niiA*–*chsB* construct, the strain displayed wild-type morphology on solid medium and at sub-maximum growth rates but the wild-type morphology was not fully restored during rapid growth in batch cultivation. The *csmA* disruption strain displayed morphological abnormalities, such as ballooning cells, intrahyphal hyphae and conidial scars. The growth was severely inhibited in the presence of 10 mg CFW l⁻¹. In none of the constructed strains did the cell-wall composition differ from the wild-type. Northern analysis indicated no change in the transcription of the chitin synthase genes *csmA* and *chsC* when *chsB* expression was altered, and there was no change in the transcription of *chsB* and *chsC* when *csmA* was disrupted.

**Keywords:** N-acetylglucosamine, cell wall

**INTRODUCTION**

Filamentous fungi, such as *Aspergillus niger* and *Aspergillus oryzae*, are important in industrial enzyme production, since they are able to synthesize and secrete large amounts of extracellular proteins (e.g. amylases, proteases, phytases and lipases). These organisms grow by hyphal extension and branching, two processes for which regulation is still not completely understood (McIntyre *et al.*, 2001). During submerged cultivation, the growing hyphal elements tend to entangle thereby affecting the rheology of the cultivation medium and the mixing characteristics in an undesirable fashion. The results are poor mixing and poor mass transfer of the substrate. Therefore, further study of hyphal extension and branching is important if we are to obtain fundamental knowledge about these processes. Furthermore, directed genetic alteration of the morphology of filamentous fungi is an obvious, yet remaining, challenge in process optimization.

In this study we examined the roles of three chitin synthase genes in *A. oryzae* during hyphal growth, since chitin is pivotal to the structuring of the hyphal cell wall. Chitin synthases (EC 2.4.1.16) catalyse the formation of β(1→4) glycosidic bonds between N-acetylglucosamine residues to form the unbranched polysaccharide chitin. In fungi, the chitin chains are ordered into long microfibrils, with hydrogen bonds holding adjacent chains together in an antiparallel arrangement (Rudall,
1969; Ruiz-Herrera & Martinez-Espinoza, 1999). The microfibrils are located as a rigid, three-dimensional web at the inner part of the fungal cell wall (Burnett, 1979). In filamentous fungi, a major part of newly formed chitin is deposited within 1 µm of the hyphal apex (Bartnicki-Garcia & Lippman, 1969; Gooday, 1971) and deposition declines rapidly subapically.

The chs genes constitute a multigene family and their gene products have been divided into five groups: classes I to V based on sequence similarities. Several disruption studies (Motoyama et al., 1994, 1996; Horuchi & Takagi, 1999; Culp et al., 2000; Fujiwara et al., 2000) with A. nidulans have revealed that chsA, chsC and chsD (of classes I, II and IV, respectively) all take part in conidiation. These studies further revealed that double disruptions of chsA/chsD and chsA/chsC both severely inhibited conidiation, but only in the chsA/chsC strain was conidiophore morphology altered. The role of chsB is less clear, as A. nidulans chsB disrupted strains have been described with both normal (Horiuchi & Takagi, 1999) and altered (Borgia et al., 1999) conidiation efficiency. It is possible that the role of chsB in conidiation is dependent on the presence of other chitin synthase genes.

In this study, we identified three chitin synthases in A. oryzae, chsB (Yanai et al., 1994; Borgia et al., 1996), and a class V chitin synthase, csmA (chitin synthase with a myosin tail) (Specht et al., 1996; Fujiwara et al., 1997; Horuchi et al., 1999), in hyphal growth. Strains where chsB has been disrupted are disorganized and hyperbranched. Borgia et al. (1996) reported that the chsB disruptant did not form conidiophores or conidia, whereas Horuchi & Takagi (1999) found a 55% reduction in conidiation efficiency upon disruption of this gene. The csmA disruptants had reduced chitin content (Specht et al., 1996), morphological abnormalities involving hyphal walls, tips and septa (Horuchi et al., 1999), and their growth was severely inhibited by chitin-binding dyes. Both the myosin and the chitin synthase domain of csmA were needed for formation of normal-shaped hyphae. These defects were somewhat osmotically remediable in contrast to the defects of a chsB disruptant.

In this study, we identified three chitin synthases in A. oryzae. A class III chitin synthase (chsB) and a fragment of a class V chitin synthase (csmA) were used to construct strains in which chsB or csmA were disrupted and a strain in which chsB transcription could be regulated. The strains were examined during hyphal growth by Northern analysis, cell-wall composition measurements and growth in the presence of the chitin-binding reagent Calcofluor white (CFW).

**METHODS**

**Strains and plasmids.** A. oryzae strains A1560 (originally named IFO 4177, Institute for Fermentation, Osaka, Japan), PaHa101 (a chsB mutant), CM100 (chsB::pyrG) (originally named chsB/G) and HowB101 (a pyrG deletion strain) were donated by Novozymes A/S (Bagsværd, Denmark, and Davis, CA, USA). To construct strain HowB101, A. oryzae IFO 4177 was transformed with a 2048 bp HindIII fragment containing a 922 bp fragment from the region upstream of pyrG and a 1126 bp fragment from the region downstream of pyrG. Transformants resistant to 5-fluoroorotic acid (FOA) were selected (cells that have an intact copy of pyrG are killed by FOA). A transformant, strain HowB101, was shown by Southern blot analysis to be the result of a double-crossover event that deleted the pyrG gene (data not shown).

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<th>Strain</th>
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**Media and transformation procedure.** Investigations of growth of A. oryzae on minimal medium plates were carried out using Cove’s salt solution (Cove, 1966) with 2% (w/v) agar, 1% (w/v) glucose and 10 mM NaNO₃ or 5 mM (NH₄)₂SO₄ in the absence or presence of CFW at concentrations ranging from 10 to 50 µg ml⁻¹. Conidial suspensions were diluted to 6×10⁶ spores ml⁻¹ and 10 µl aliquots were placed onto the solid medium and incubated for 96 h at 30 °C. E. coli liquid cultures were grown in YPG medium [1% (w/v) yeast extract, 2% (w/v) Bacto peptone (Difco), 2% (w/v) glucose]. Batch cultivations of A. oryzae (for chitin determinations) were grown in minimal medium as described by Carlsen et al. (1996). Transformation of A. oryzae and fungal DNA preparations were done essentially as described by Christensen et al. (1988).

**Fluorescence microscopy.** A sample from a shake-flask culture was diluted with PBS (4/623 g KH₂PO₄, 4/414 g Na₂HPO₄ and 4/5 g NaCl in 500 ml distilled H₂O adjusted to pH 9.4 with NaOH) to disperse the hyphal elements. The diluted sample was stained with CFW (0.024–0.036 mg ml⁻¹) for 15 min, before viewing as described previously (Müller et al., 2000). The sample was placed on a microscope slide and viewed under a fluorescence microscope (Nikon Optiphot 2) with a ×100 objective and a total magnification of ×5500 on screen.

**DNA sequencing and sequence alignment.** DNA sequencing was done using the method of Tayer et al. (2000). Sequence alignment was done using gapped BLAST and PSI-BLAST (Altschul et al., 1997).

**Southern hybridization.** Restriction-enzyme-digested A. oryzae DNA was separated by electrophoresis through a 0.8% (w/v) agarose gel. The DNA was transferred to Hybond-N⁺ membranes (Amersham) by capillary blotting in 20 × SSC. Blots were probed with [z-³²P]dCTP-labelled by using the Readiprime II kit (Amersham) and purified using QIAquick (Qiagen). Blots were probed at 65 °C with [z-³²P]dCTP-labelled PCR products. The blots were washed in 2 × SSC/0.1% (w/v) SDS and then in 0.2 × SSC/0.1% (w/v) SDS, and visualized using a PhosphorImager.

**Northern hybridization.** A. oryzae was grown in 5 l batch culture.
cultures as described by Carlsen et al. (1996). Samples were taken approximately 24 h after inoculation, when the cells were growing exponentially. For RNA extraction, biomass was collected by filtration (Whatman GF/C filter), washed with cold deionized water and put in liquid nitrogen within 10 s of sampling. The frozen cells were kept at −80 °C. Sterile (RNase-free) materials, diethyl pyrocarbonate (DEPC)-treated MilliQ water (autoclaved for 30 min at 121 °C) and sterile electrophoresis equipment (incubated for 10 min with 3 %, v/v, H₂O₂) were used throughout the preparation procedure. The mycelium was ground carefully in a mortar with liquid nitrogen and placed into an Eppendorf tube so that the tube was one-third full. RNA was then isolated using the TRIzol method (TRIzol; Life Technologies); the concentration of the RNA was determined and adjusted according to the manufacturer’s instructions. Electrophoresis and blotting were done according to standard protocols (Sambrook et al., 1989). For the hybridization, an ~800 bp probe for the A. oryzae triose-phosphate isomerase (tpiA) gene was used as a loading control (the probe was kindly provided by M. Trier, Novozymes A/S, Bagsværd, Denmark). All probes (chsB, chsC, csmA and tpiA) were prepared as for the Southern analysis. The membranes were hybridized using the ECL Gold kit (Amersham). All membranes were first hybridized to a chsB probe and then reprobed with the tpiA probe to check that equal amounts of RNA had been loaded onto the gel.

Isolation and disruption of chsB. A chsB disruption strain (PaHa101) was found to be a hyperbranched A. oryzae mutant and was produced by the restriction-enzyme-mediated integration method described by Yaver et al. (2000) and Brody et al. (1997). The gene producing this morphological phenotype was rescued back into E. coli and partly sequenced. The two gene fragments, of 1035 bp and 534 bp in size, that flanked the insert were identified; sequence homology data revealed the gene to be homologous to chitin synthase B from A. nidulans.

The primers 5′-CACCAGTGACAGTGTCGTC-3′ and the degenerate primer 5′-GCGICCTTGYGYGAYCCCGA-3′, based on the consensus sequence from A. fumigatus chsB, a 1.9 kb fragment of the gene was amplified by PCR. A disruption construct was made by inserting a 2.9 kb HindIII–EcoRII fragment containing pyrG into a HindIII site located approximately 0.7 kb within the chsB fragment. The construct was amplified and used to transform strain HowB101, resulting in strain CM100 (chsB::pyrG). The primers 5′-TTCTCTCCATCCGTCGACGTGAAAG-3′ and 5′-TATTGAGGCACAGTCTCCTTG-3′ were used to amplify a 629 bp chsB fragment, which was subsequently used as a probe for chsB during isolation and Northern analysis. A clone containing the full-length chsB gene was found using the chsB probe in a Lambda ZipLox genomic library of A. oryzae HowB101, constructed as described by Yaver et al. (2000). A 4975 bp XbaI fragment ligated into pUC13 hybridized to the PCR product and sequencing of the complete insert revealed it to contain the full chsB gene. This sequence has been submitted to GenBank under accession no. AY029621.

Introduction of the p_niiA–chsB insert. Swal and NotI sites were inserted into the N terminus and the C terminus of chsB by using the primers 5′-CCCATTATATACGGCTTCTACCCGTCCTTGTCGACAGTGTCGTC-3′ and 5′-TTCTCTCCATCCGTCGACGTGAAAG-3′, respectively, the ‘overhangs’ are shown in italics and the Swal and NotI sites are underlined. The PCR product was digested with Swal/NotI and ligated into the Swal and NotI sites in pRaM33, after the A. oryzae niiA promoter, to form pChrM01 [pRaM33 is a pUC19-based plasmid that contains the A. oryzae niiA promoter and alp terminator and the bar gene (from pBPIT; Straubinger et al., 1992)] with the A. oryzae pgk promoter and pgk terminator. Single Swal and NotI sites were present at a cloning site after the niiA promoter; subsequently, the construct was transformed with pChrM01 (see Fig. 1), which was sequenced to check that chsB contained no errors due to PCR. Strain CM100 (ΔchsB) was transformed with pChrM01 and the protoplasts were grown in the presence of 1 g glufosinate l⁻¹ (Hoechst). Forty transformants were isolated and subsequently grown in minimal medium containing either 10 mM NO₃⁻ (induced niiA) or 10 mM NH₄⁺ (uninduced niiA). Those that changed morphology depending on the nitrogen source were examined for the presence of the p_niiA–chsB insert by Southern analysis. All transformants examined by Southern analysis contained one or more copies of the p_niiA–chsB insert. We selected one of the strains (NiiA1) that contained a single copy of the insert for further studies.

Obtaining a chsc fragment. A probe for chsC from A. oryzae was produced using degenerate primers based on the sequence of chsC from A. nidulans (Motoyama et al., 1994). A PCR product was amplified using Tag polymerase (Boehringer Mannheim), separated on a 1 % (w/v) agarose gel and purified using a Qiaex II agarose gel extraction kit (Qiagen). The fragment was cloned into the vector pCR-TOPO (Invitrogen) and sequenced. A 641 bp chsc probe for Northern analysis was prepared using the primers 5′-TTACTGCGCCGCTACATTGT-3′ and 5′-CACACATCGCCGCAACAT-3′. The sequence of the probe has been deposited in GenBank under accession no. AF410464.

Isolation of a fragment of csmA and disruption of the gene. The primers 5′-AAGACTCTGCGCTTCTC-3′ and 5′-CAAGACCACCCCGTGTTTT-3′ were designed based on a 537 bp expressed sequence tag (EST) sequence of A. oryzae (provided by Novozymes A/S) that had high homology to csmA from A. nidulans. The primers were used to amplify a 301 bp fragment from A. oryzae A1560 DNA using Tag polymerase. When the fragment was [α-³²P]dCTP labelled (using the Rediprime II kit; Amersham) and used to probe a Lambda ZipLox genomic library of A. oryzae HowB101 (Yaver et al., 2000) and a SuperCos1 library of A. oryzae F1747 (Pedersen et al., 1999), several individual clones were identified. The clones were sequenced but none contained the full-length gene of the A. nidulans csmA homologue. However, even a 3614 bp BsaAI-C-terminal fragment was isolated from a pZL1 clone. This sequence contained the last 712 aa of the apparent A. oryzae csmA gene, including the promoter region (Nagahashi et al., 1995); it has been deposited in GenBank under accession no. AF429307. A disruption cassette consisting of a 2.1 kb XbaI fragment from
pJaL554, containing inverted repeats and pyrG, was cloned into a NheI site between the two essential catalytic domains in the pZL1-csmA clone to yield pZL1-csmApyrG (Fig. 2). This plasmid was used to transform strain HowB101 (AppyG). The csmA disruptants were selected on minimal medium containing 10 mg CFW 1−1, a chitin-binding compound (Maeda & Ishida, 1967; Elorza et al., 1983).

Southern blots were carried out using a 469 bp probe for csmA that was amplified using the primers csmA-3start (5′-CCTTTTCGTGGTGAGG-3′) and csmA-R2 (5′-ACTGACGACGAAAGC-3′), and a 494 bp probe for pyrG that was amplified from the csmA::pyrG disruption cassette using the primers pyrG-F1 (5′-GATGCCACTTACATGTGATC-3′) and pyrG-R1 (5′-TAACCTTCAGACTGAACCTCG-3′). Restriction digests were carried out according to the manufacturer’s instructions (New England Biolabs).

Chitin and glucan measurements. Chitin was determined by a modified method based on that of Dallies et al. (1998). The modifications were as follows. A. oryzae was grown exponentially in 5 l batch cultures using the conditions described by Carlsen et al. (1996). Biomass samples were filtered (Whatman GF/C filters), washed with cold deionized water, put in liquid nitrogen and then frozen at −80 °C. Samples were finely ground in a mortar with liquid nitrogen. Several aliquots (0.05–0.2 g) of each sample were collected and weighed. Some aliquots were dried directly for 24 h at 100 °C for determination of the biomass (g frozen weight)−1. For isolation of the water-insoluble part of the cells, the ground biomass was washed with cold deionized water and pelleted at 5000 g (4 °C) for 12 min in a centrifuge (Hettlich Universal 30RF) until the supernatant became clear (3–4 times). Some aliquots were dried for 24 h at 100 °C after this treatment to allow measurement of the cell-wall content. For measuring the chitin and glucan contents, the washed cell wall was hydrolysed with 375 µl of 72% (w/w) H2SO4 at room temperature for 3 h. The slurry was diluted to 5 ml with 1 ml fucose (10 g l−1) (used as an internal standard) and deionized water to a final concentration of 1 M H2SO4. The mixture was then boiled in a water bath in a thick-walled test tube with glass stopper for 3 h. The tube was cooled on ice and 1 ml of the hydrolysate was transferred to a new test tube, where the sulfate ions were precipitated by the addition of Ba(OH)2; the supernatant was removed as described by Dallies et al. (1998).

The supernatant was used for monosaccharide analysis by liquid chromatography. Stabilities of fucose, galactose, glucose and glucosamine to the acid hydrolysis treatment were examined; recovery for all sugars was >95%.

Cell-wall liquid chromatography analysis. Monosaccharides liberated by hydrolysis were analysed using a Dionex DX500 HPLC system with a CarboPac PA1 anion exchange column (4 × 250 mm) equipped with a CarboPac PA1 (10.32) guard column. Glucose, galactose, glucosamine, mannose and our internal standard, fucose, were detected. For all liquid chromatography analyses, 10 µl of each sample was injected into a 100 µl injection loop on a TSP AS3500 autosampler (Thermoseparator Products). The temperature in the autosampler was 4 °C but elution with 18 mM NaOH was performed at room temperature at a flow rate of 1 ml min−1. After 20 min, the column was eluted with 200 mM NaOH for 8 min. All NaOH solutions were prepared as described by Dallies et al. (1998) and the quantification method used was also the same.

RESULTS AND DISCUSSION

Sequences and sequence alignment

csbB. The A. oryzae chsB gene was identified and isolated following the generation of a hyperbranching mutant by the restriction-enzyme-mediated integration method. The mutant phenotype was reproduced by the insertion of pyrG at the site of the mutation in the chsB gene of the wild-type strain. When the sequence of chsB was compared to gene sequences in the databases, including fungal intron sequences, the results indicated that chsB of A. oryzae contains an ORF with four putative introns. chsB encodes a predicted polypeptide of 920 aa, which has 88 and 90% identity to the A. nidulans chsB and A. fumigatus chsB gene products, respectively; these genes encode class III chitin synthases. The sequenced 981 bp upstream region of chsB contains one putative AbaA-binding site (CATTC/T) (Andrianopoulos & Timberlake, 1994) and one sequence matching the consensus sequence recognized by AbaA and BrlA (C/AG/AAGGG/A) (Chang & Timberlake, 1993). The existence of these sequences has also been demonstrated in the promoter regions of the A. nidulans chsA and chsC genes (Fujiwara et al., 2000), and it has been suggested that MedA may regulate chsB and chsC expression through BrlA and AbaA in asexual development.

chsC. Based on the sequence of chsC from A. nidulans (Motoyama et al., 1994), degenerate primers were designed to amplify a probe for chsC from A. oryzae. The 641 bp PCR product was sequenced (data not shown) and the predicted amino acid sequence for the fragment had 91 and 92% identity to the amino acid sequence of the chsC gene product of A. nidulans (Motoyama et al., 1994) and the chsA gene product of A. fumigatus (Mellado et al., 1996), respectively; these genes encode class I chitin synthases.

csmA. A 537 bp expressed sequence tag (EST) sequence of A. oryzae, which had high similarity to csmA from A. nidulans, was obtained. Primers for the sequence were designed and used to amplify a fragment from A. oryzae A1560, which was used to isolate a 3614 bp BsaAI fragment. The sequence was analysed and compared to existing sequences and the known fungal intron consensus structures. These studies specified a partial ORF.
with one putative intron. The partial A. oryzae csmA gene encoded a predicted polypeptide of 712 aa, which had 66, 62 and 49% identity to the Ustilago maydis chs6, A. nidulans csmA and Pyricularia grisea csm1 gene products, respectively (Xoconostle-Cázares et al., 1997; Fujiwara et al., 1997; Park et al., 1999); these genes encode class V chitin synthases. The partial csmA gene sequence (GenBank accession no. AF303554) contained the putative C-terminal domain and the two essential catalytic domains, LGEDRYL and SQRRRW (Nagahashi et al., 1995), between which the disruption cassette was inserted. Unfortunately, it is not known whether the gene contains an N-terminal myosin motor-like domain similar to that identified in csmA of A. nidulans, or whether it resembles a ‘conventional’ chitin synthase, such as chs6 from U. maydis (Fujiwara et al., 1997; Xoconostle-Cázares et al., 1997).

When the plasmid shown in Fig. 2 was used to transform strain HowB101, 60 transformants were obtained and one (strain CM101) displayed poor growth on CFW. This strain was examined further by Southern blot and PCR analyses, to demonstrate that the integration of the disruption cassette (csmA::pyrG on Fig. 2) only occurred in csmA. The Southern blot (Fig. 3) shows that the fragments from the recipient strain, HowB101, that hybridized with the csmA probe (lanes 1–3) differed in size from the fragments from the transformed strain, CM101, that hybridized with the same probe (lanes 4–6). The restriction digest fragments detected are of the sizes predicted from the known sequence of the csmA locus and the csmA::pyrG disruption cassette. Further Southern analysis using a 494 bp probe for pyrG showed that the recipient strain, HowB101, does not contain pyrG (Fig. 3, lanes 7 and 8). In addition, lanes 9 and 10 of Fig. 3 show that transformant strain CM101 contains only one copy of pyrG. The restriction fragments are of the sizes predicted from the known sequence of csmA. Additionally, when primers pyrG-F1 and csmA-R2 (described in Methods) were used for PCR with the disruption cassette (csmA::pyrG) and genomic DNA from strains CM101 and HowB101, a product was amplified only from the disruption cassette and strain CM101, providing further evidence that the csmA::pyrG disruption cassette had integrated into csmA (data not shown).

In the constructed csmA disruption strain the N-terminal part of csmA might still be expressed. Since the N-terminal end possibly contains a myosin motor-like domain, the mutated csmA gene product could be transported to its correct location. However, the enzyme should be non-functional and disruption of csmA might be expected to result in the same phenotype as that of the null-mutant reported by Horiuchi et al. (1999), who disrupted different domains of the csmA gene in A. nidulans.

Thus, three different classes of chitin synthase were detected in A. oryzae, of which one (class I) has been found in unicellular and filamentous fungi and two have only been found in filamentous fungi (classes III and V) (Horiuchi et al., 1999). However, chitin synthases of all three classes are thought to play a role in chitin synthesis during hyphal growth of A. nidulans (Horiuchi & Takagi, 1999).

Chitin synthase expression analysis

The constructed A. oryzae strains were grown exponentially in batch cultivations and their chitin synthase transcription levels were measured (Fig. 4).
Loading of lane 2 (tpiA, chsB and csmA but not chsC) was low – this was checked by reprobing with tpiA – but this did not affect the overall conclusions. All three chitin synthase genes were transcribed during exponential growth (unless disrupted), suggesting that all three genes function during mycelial growth. Lanes 2, 3 and 4 of Fig. 4 show that chsB transcription can be controlled using the niiA promoter by varying the nitrogen source; lanes 3 and 4 show overexpression of chsB using the niiA promoter. When the chsB transcription level was altered, transcription of chitin synthases chsC and csmA did not change significantly (while there appeared to be a slight increase in chsC expression when chsB expression was increased, this could not be reproduced). Lanes 5 to 7 of Fig. 4 verify the disruption of chsB and csmA, since no mRNA products were detected for these genes in strains CM100 and CM101, respectively. No significant changes in the chsB or chsC expression levels were found for the csmA disruptant strain. For the csmA mRNA, multiple bands were present, indicating that the csmA probe may hybridize with several mRNA species. However, the probe was selected from a putative intron-free region of the gene and the hybridization was carried out at high stringency (wash at 60 °C, 20 min with 1× SSC; 0.1% SDS, followed by 3× 20 min with 0.2× SSC; 0.1% SDS); hence, the additional bands may have arisen from alternative splicing events, alternative transcription initiation sites or from cross-hybridization with another chitin synthase mRNA. Two bands appeared to be present when chsB expression was induced by niiA. Here, the reason for the presence of the two bands could be that a part of the transcript is truncated. That the blots were not overloaded, overexposed or smeared was checked with tpiA-control labelling on the same Northern blot. All blots were reprobed with tpiA after the chitin synthase probes had been stripped off, and sharp bands appeared.

Overexpression of chsB does not necessarily result in more gene product, because ChsB might be degraded during processing in the endoplasmic reticulum. Several albeit failed attempts were made to express chsB using the strong taka (z-amylase) promoter with A. oryzae transformants containing the taka–chsB construct, indicating that a functional taka–chsB construct might be lethal. The lethal phenotype could arise from blocking of the secretion pathway, e.g. accumulation of ChsB in the endoplasmic reticulum. In conclusion, disruption of chsB or csmA or overexpression of chsB appeared to have no significant effect on the mRNA levels of the other chitin synthases examined.

**Morphological studies**

**Characteristics of the chsB disruptant.** Growth on agar with minimal medium indicates that, compared to the wild-type, the chsB disruptant (CM100) forms more compact colonies and has a lower conidiation efficiency (data not shown). The chsB disruptant was more sensitive than the wild-type to a CFW concentration of 50 mg L⁻¹ (data not shown), indicating that alterations in chsB affect cell-wall assembly. Strain CM100 is hyperbranching compared to the wild-type strain A1560 (Fig. 5e compared to Fig. 5a); the length of the hyphal growth unit (µm hyphal length per tip ± 95% confidence interval) was 51 ± 3 µm per tip compared to 97 ± 7 µm per tip for the wild-type after 23 h growth during batch cultivation. Also, the mean hyphal diameter increased 8.3% compared to the wild-type (Müller et al., 2002). None of these effects was remedied by supplementing the medium with 1 M sorbitol (data not shown). The specific growth rate of the chsB disruptant during batch cultivation was approximately the same as that for strain A1560 (A1560, 0.23 ± 0.03 h⁻¹; CM100, 0.22 ± 0.02 h⁻¹; mean ± 95% confidence interval). In comparison, the chsG (class III) disruption strain of A. oryzae (class III) disruption strain of A. oryzae is sensitive to a CFW concentration of 10 µg mL⁻¹ (data not shown). C. Muñoz and others

![Fig. 5. CFW-staining of mycelia showing the pleiotropic morphological defects of strains CM101 (csmA::pyrG) and CM100 (chsB::pyrG). Mycelium was grown submerged in minimal medium with NH₄Cl for 12 h and then stained with CFW. (a) Strain A1560 (wild-type); (b–d) strain CM101; (e) strain CM100. In (b) and (d), ballooning cells were observed; in (b), the arrow points to conidial ‘faults’; in (c), the arrow points to intrahyphal ‘faults’; in (c), the hyperbranching morphology of strain CM100 is apparent.](image-url)
*fumigatus* also hyperbranched and grew as fast as the wild-type (Mellado et al., 1996). In contrast, *A. nidulans* mutants of *chsB* had severe growth defects, indicating that the gene has a more central role in this organism (Borgia et al., 1996).

**Characteristics of strain NiiA1.** Northern analysis (Fig. 4) showed that expression of *chsB* in strain NiiA1 (*chsB::pyrG; p_niiA−chsB*) could be controlled through nitrogen source regulation of the *niiA* promoter. When strain NiiA1 was grown on minimal agar medium containing NO$_3^−$, the morphology of the mutant strain was similar to that of the wild-type grown on NO$_3^−$, with similar levels of conidiation seen in both strains; when the nitrogen source was NH$_4^+$, strain NiiA1 colonies were smaller and more compact with less conidia being produced (data not shown). However, strain NiiA1 extended further on minimal agar medium and produced more conidia than strain CM100 (*chsB::pyrG*) when the nitrogen source was NH$_4^+$ (data not shown). This indicates that *niiA* transcription is not completely silenced during growth on NH$_4^+$ and that the amount of ChsB present is important in the conidiation and colony growth processes of *A. oryzae*.

During batch cultivation with NO$_3^−$ as the nitrogen source, the length of the hyphal growth unit for strain NiiA1 was 90 ± 6 μm per tip compared to 126 ± 6 μm per tip for the wild-type after 23 h growth. When NH$_4^+$ was the nitrogen source, the length of the hyphal growth unit for strain NiiA1 was 60 ± 3 μm per tip compared to 97 ± 7 μm per tip for the wild-type. Examples of the morphologies of strains NiiA1 and A1560 growing with either NH$_4^+$ or NO$_3^−$ as the nitrogen source are shown in Figs 5 and 6. These results suggest that both the nitrogen source and the level of expression of *chsB* affect hyphal branching. They indicate that an approximately 30% increase in the value for the hyphal growth unit was noted when the wild-type was grown in the presence of NO$_3^−$ rather than NH$_4^+$. However, an approximately 50% increase in hyphal growth was noted when strain NiiA1 was grown in the presence of NO$_3^−$ rather than NH$_4^+$. While these results suggest that *chsB* expression affects morphology in *A. oryzae*, we cannot explain why stimulation of *chsB* expression in strain NiiA1 does not restore the phenotype of this mutant to that of the wild-type during batch cultivation.

However, we have found that growth of the mutant under alternative culture conditions, in the presence of NO$_3^−$, closely resembles that of the wild-type. First, as stated above, strains A1560 and NiiA1 exhibit very similar morphologies, levels of conidiation and colony extension rates on solid NO$_3^−$ medium. Second, during chemostat cultivation under glucose limitation with NO$_3^−$ as the nitrogen source, at a specific growth rate of 0.1 h$^{−1}$, strains A1560 and NiiA1 have practically identical branching patterns (Müller et al., 2002). In addition, strain NiiA1 did not differ significantly from the wild-type with regard to its CFW sensitivity, α-amylase production, chitin content, or specific growth rate during batch cultivation when grown with NO$_3^−$ as the nitrogen source (data not shown).

In general, this supports the conclusion that ChsB expression is important for a normal branching pattern, perhaps because it is necessary for polarized cell-wall formation at the hyphal tip.

**Characteristics of the *csmA* disruptant.** When grown on agar with minimal medium, the *csmA* strain (CM101) had a lower colonial growth rate than the wild-type and its conidiation efficiency was severely reduced. Both effects were to some extent remedied by the addition of 1 M sorbitol as an osmotic stabilizer. In the presence of 10 mg l$^{−1}$ of the chitin-binding agent CFW, the growth of strain CM101 was severely inhibited (data not shown), as also observed by Horiuichi et al. (1999) for *csmA*-disrupted strains of *A. nidulans*.

When strain CM101 was grown submerged, its morphology differed considerably from that of the wild-type strain (Fig. 5a–d). However, in batch cultivation the specific growth rate of this strain was close to that of

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Fig. 6. CFW-staining of strains NiiA1 (*chsB::pyrG; p_niiA−chsB*) and A1560 (wild-type). Mycelium was grown submerged in minimal medium with either NH$_4^+$ (no *niiA* induction) or NO$_3^−$ (*niiA* induction) for 12 h and then stained with CFW. (a) Strain NiiA1, NH$_4^+$; (b) strain NiiA1, NO$_3^−$; (c) strain A1560, NO$_3^−$.
Chitin measurements

The amount of cell-wall material and the content of chitin and glucan were estimated by acid hydrolysis of the water-insoluble part of the biomass for the construction strains growing exponentially in batch culture (Table 2). Compared to the wild-type strain (A1560), the cell wall of strain CM100 constituted a higher percentage of the total dry weight, and for strain CM101 the cell wall constituted a lower percentage of the total dry weight. However, in strains CM100 and CM101 the glucan and chitin contents of the cell wall were approximately the same as those of the wild-type. These data suggest that other chitin synthases compensate for the lack of a functional chsB product and/or that ChsB makes very little chitin. The results for strain CM101 (csmA) are in contrast with the findings of Specht et al. (1996), who found a 60% (w/w) reduction in the chitin content of an A. nidulans class V disruption strain. However, the methodology of Specht et al. (1996) was based on enzymatic degradation of the cell wall and not on acid hydrolysis, as used here; this may explain the difference in results.

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REFERENCES


Table 2. Composition of A. oryzae wild-type and disruption strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>N source</th>
<th>Cell wall [mg (g biomass)⁻¹]</th>
<th>Glucosamine [mg (g cell wall)⁻¹]</th>
<th>Glucose [mg (g cell wall)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1560</td>
<td>NH₄⁺</td>
<td>400 ± 50</td>
<td>70 ± 10</td>
<td>330 ± 80</td>
</tr>
<tr>
<td>A1560</td>
<td>NO₃⁻</td>
<td>380 ± 40</td>
<td>70 ± 10</td>
<td>440 ± 110</td>
</tr>
<tr>
<td>CM100</td>
<td>NH₄⁺</td>
<td>500 ± 50</td>
<td>70 ± 10</td>
<td>320 ± 10</td>
</tr>
<tr>
<td>CM101</td>
<td>NH₄⁺</td>
<td>350 ± 10</td>
<td>90 ± 10</td>
<td>370 ± 80</td>
</tr>
<tr>
<td>NiaA</td>
<td>NH₄⁺</td>
<td>460 ± 40</td>
<td>90 ± 20</td>
<td>440 ± 70</td>
</tr>
<tr>
<td>NiaA</td>
<td>NO₃⁻</td>
<td>370 ± 60</td>
<td>80 ± 10</td>
<td>390 ± 120</td>
</tr>
</tbody>
</table>

The ± values represent the 95% confidence limit. Data presented are mg dry weight cell wall (g dry weight biomass)⁻¹ and mg glucosamine (chitin) or glucose (glucan) (g dry weight cell wall)⁻¹.
Chitin synthesis and morphology of A. oryzae


