Repression of *chsB* expression reveals the functional importance of class IV chitin synthase gene *chsD* in hyphal growth and conidiation of *Aspergillus nidulans*

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The functions of two previously identified chitin synthase genes in *Aspergillus nidulans*, *chsB* and *chsD*, were analysed. First, a conditional *chsB* mutant was constructed in which the expression of *chsB* is under the control of a repressible promoter, the *alcA* promoter, of *A. nidulans*. Under repressing conditions, the mutant grew slowly and produced highly branched hyphae, supporting the idea that *chsB* is involved in normal hyphal growth. The involvement of *chsB* in conidiation was also demonstrated. Next, double mutants of *chsB* and *chsD* were constructed, in which *chsB* was placed under the control of the *alcA* promoter and *chsD* was replaced with the *argB* gene of *A. nidulans*. These double mutants grew more slowly than the *chsB* single mutant under high-osmolarity conditions. The hyphae of the double mutant appeared to be more disorganized than those of the *chsB* single mutant. It was also found that ChsD was functionally implicated in conidiation when the expression of *chsB* was limited. These results indicate the importance of the ChsD function in the absence of *chsB* expression. The roles of ChsB and ChsD in hyphal growth and in conidiation were supported by the analysis of the spatial expression patterns of *chsB* and *chsD*, using *lacZ* of *Escherichia coli* as a reporter gene.

Keywords: cell wall, conidiation, filamentous fungi

INTRODUCTION

Chitin is a major component of the cell walls of most filamentous fungi and a minor component of the cell walls of many yeasts. Chitin synthase genes have been isolated from many filamentous fungi and yeasts, and have been classified into at least five classes, I–V, according to sequence similarity (Bowen *et al.*, 1992; Beth Din *et al.*, 1996; Specht *et al.*, 1996; Motoyama *et*

al., 1996; Aufauvre-Brown *et al.*, 1997; Xoconostle-Cázares *et al.*, 1997). In *Aspergillus nidulans*, four chitin synthase genes, *chsC*, *chsA*, *chsB* and *chsD*, which belong to class I, class II, class III and class IV, respectively, and a gene, *csmA*, which encodes chitin synthase with a myosin motor-like domain, have been cloned in our laboratory (Yanai *et al.*, 1994; Motoyama *et al.*, 1994a, 1996; Fujiwara *et al.*, 1997). Our *chsD* is also referred to as ‘*chsE*’ by Specht *et al.* (1996). The chitin synthase domain of CsmA, which belongs to class V, is similar to the product of ‘*chsD*’ as reported by Specht *et al.* (1996).

The functions of chitin synthases in filamentous fungi have been analysed by gene disruptions or gene deletions. The respective null mutants of class I (Motoyama *et al.*, 1994a), class II (Beth Din & Yarden, 1994; Yanai *et al.*, 1994) and class IV (Beth Din *et al.*, 1996; Motoyama *et al.*, 1996; Specht *et al.*, 1996) chitin

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Abbreviations: AP, alkaline phosphatase; RACE, rapid amplification of cDNA ends.

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synthase genes of filamentous fungi exhibited no obvious phenotypic defect under standard conditions with the exception of that studied by Beth Din & Yarden (2000). Recently, it was reported that a class II chitin synthase gene (*CHS1*) of the dimorphic yeast *Candida albicans* was essential even for the growth of the yeast form (Sudoh *et al.*, 2000; Munro *et al.*, 2001). Disruptions or deletions of class III (Yarden & Yanofsky, 1991; Yanai *et al.*, 1994; Borgia *et al.*, 1996; Mellado *et al.*, 1996) or class V (Specht *et al.*, 1996; Aufauvre-Brown *et al.*, 1997; Horiuchi *et al.*, 1999) chitin synthase genes, with the exception of two class III genes (Gold & Kronstad, 1994; Mellado *et al.*, 1996), caused some phenotypic changes. These observations indicate that the respective chitin synthase genes (not all genes) alone do not bear essential functions for hyphal growth and conidiation, and that the class of chitin synthase is not necessarily related to the function and importance of these genes among different fungal species.

To reveal the function of individual chitin synthase genes, double and triple disruption mutants were constructed and analysed. We previously found that the class II and class IV chitin synthases of *A. nidulans* have some overlapping function(s) in conidiation (Motoyama *et al.*, 1996), and that the class I and class II chitin synthases of *A. nidulans* share important roles in hyphal growth and conidiation (Fujiwara *et al.*, 2000). A partial redundancy of function of class II and class IV chitin synthases was also reported by another group (Culp *et al.*, 2000). From these results, we speculated that chitin synthases in class I, class II and class IV may play important roles in growth or differentiation, and that these synthases cooperate with each other. In the yeast *Saccharomyces cerevisiae*, the class IV chitin synthase is involved in most of the cellular chitin synthesis and serves redundant functions with class II chitin synthase in growth (Valdivieso *et al.*, 1991; Shaw *et al.*, 1991). These results suggest that fungal chitin is synthesized by the overlapping and cooperative function of multiple chitin synthase genes.

In our previous study, we showed that *A. nidulans chsD*, which encodes a class IV chitin synthase, is expressed in the growing hyphae (Motoyama *et al.*, 1996), and *chsB*, which encodes a class III chitin synthase, plays essential roles in normal hyphal growth. These results suggest that ChsD and ChsB have some related functions in chitin synthesis and other activities of growing hyphae. In this study, we focused on the functional relationship between ChsB and ChsD in hyphal growth and in conidiation. The construction of double deletion mutants of *chsB* and *chsD* seemed to be difficult because null mutants of *chsB* are heavily damaged in hyphal growth (Yanai *et al.*, 1994; Borgia *et al.*, 1996). Therefore, we constructed conditional double mutants in which *chsD* was replaced with the *argB* gene of *A. nidulans*, and *chsB* was placed under the control of a repressible promoter (the *alcA* promoter). We then analysed the functional relationship between the two chitin synthases.

METHODS

Strains, media and transformation. The *A. nidulans* strains used are shown in Table 1. Complete medium (CM) and minimal medium (MM) for *A. nidulans* described by Rowlands & Turner (1973) were used. YGU medium [0.5% yeast extract (Difco), 1% glucose, 0.1% trace elements (Rowlands & Turner 1973), 10 mM uridine] was also used. When the expression of the *alcA* promoter was induced, we used 100 mM threonine and 0.1% fructose as carbon sources instead of glucose (MMFT). Transformation was done as described by May (1992). When the expression of the *alcA* promoter was induced in the transformation experiments, we used 1.2 M sorbitol as an osmotic stabilizer instead of sucrose. Transformants were grown in MM with appropriate supplements.

Plasmids were amplified in *Escherichia coli* MV1190 [$\Delta(lac-proAB)$ *thi supE* $\Delta(srl-recA)306::Tn10$ (Tet^r) (F' *traD36 proAB lacIⁿ ZAM15*)]. *E. coli* was grown in LB and transformation was done by standard methods (Sambrook *et al.*, 1989).

Total DNA isolation and Southern analysis. Total DNA of filamentous fungi was extracted as described by Oakley *et al.* (1987). Southern analysis was done with ECL direct nucleic acid labelling and detection systems (Amersham Pharmacia Biotech). DNA was separated by electrophoresis on agarose gels and further manipulation was done as described in the manufacturer's instructions.

Total RNA isolation and Northern analysis. Total RNA was prepared from mycelia by using the RNeasy Mini Kit (QIAGEN). Five micrograms of total RNA were separated by electrophoresis on a formaldehyde-agarose gel, and blotted onto Hybond-XL membrane (Amersham Pharmacia Biotech) according to the manufacturer's instructions. Blots were hybridized to ³²P-labelled probes prepared by using Random Primer DNA Labelling Kit Version 2 (TaKaRa). As a probe for *chsB* expression, the 1.6 kb *NcoI* fragment from *pchsB* was used.

PCR-amplification of DNA fragments. PCR-amplification was done by using a thermal cycler (model 480, Perkin-Elmer/Cetus) and *Taq* DNA polymerase (Boehringer Mannheim) or the Expand High Fidelity PCR system (Boehringer Mannheim).

RT-PCR amplification. Twenty micrograms of total RNA were treated with deoxyribonuclease (RT Grade) (NIPPON GENE), then reverse transcriptions were performed by using 3 µg DNA-free RNA and ReverTra Ace reverse transcriptase (TOYOBO) according to the manufacturer's instructions. The primer used for reverse transcriptions was *chsB.3072as* (5'-GGCATCCAGTCTAGGTTGC-3'). For PCR amplifications, 5 µl samples from 20 µl of reverse transcription reactions were used. The primers used were *chsB.2055s* (5'-CATCATGGATCTGGTAGG-3') and *chsB.3075as* (5'-ATACATACAATTGCAAGGC-3'). Since we designed the primer set to amplify the region that contains an intron, the products of RT-PCR were expected to be 57 bp smaller than the PCR products amplified from genomic DNA by using the same primer set. PCR products and 100 bp DNA ladder (New England Biolabs) were electrophoresed on a 1.0% agarose gel.

Plasmid constructions. Plasmids used in this paper are shown in Table 2. To make the strain in which the expression of *chsB* is under the control of the *alcA* promoter, the plasmid p5BP-

Table 1. *A. nidulans* strains used in this study

Strain	Genotype	Source
AU1	<i>pyrG89 wA3 argB2</i>	Motoyama <i>et al.</i> (1994a)
AU/A	<i>pyrG89 wA3 argB2</i> [pSS1]	This study
ABPU1	<i>biA1 pyrG89 wA3 argB2 pyroA4</i>	Motoyama <i>et al.</i> (1996)
ABPU/A	<i>biA1 pyrG89 wA3 argB2 pyroA4</i> [pSS1]	This study
ABPU/AU	<i>biA1 pyrG89 wA3 argB2 pyroA4</i> [pSS1][pP1]	Motoyama <i>et al.</i> (1996)
BM-3	<i>biA1 pyrG89 wA3 argB2 pyroA4 ΔchsB::pyr-4::alcA(p)::chsB</i>	This study
BM-3/A1	<i>biA1 pyrG89 wA3 argB2 pyroA4 ΔchsB::pyr-4::alcA(p)::chsB</i> [pSS1]	This study
BM-4	<i>biA1 pyrG89 wA3 argB2 pyroA4 ΔchsB::pyr-4::alcA(p)::chsB</i>	This study
BM-12	<i>biA1 pyrG89 wA3 argB2 pyroA4 ΔchsB::pyr-4::alcA(p)::chsB</i>	This study
BB-3	<i>biA1 pyrG89 wA3 argB2 pyroA4 ΔchsB::pyr-4::alcA(p)::chsB</i> [pSS-chsB]	This study
BD-2	<i>biA1 pyrG89 wA3 argB2 pyroA4 ΔchsB::pyr-4::alcA(p)::chsB ΔchsD::argB</i>	This study
D3-2	<i>biA1 pyrG89 wA3 argB2 pyroA4 ΔchsD::argB</i>	Motoyama <i>et al.</i> (1996)
DB-13	<i>biA1 pyrG89 wA3 argB2 pyroA4 ΔchsB::pyr-4::alcA(p)::chsB ΔchsD::argB</i>	This study
DB-19	<i>biA1 pyrG89 wA3 argB2 pyroA4 ΔchsB::pyr-4::alcA(p)::chsB ΔchsD::argB</i>	This study
BL-1	<i>pyrG89 wA3 argB2</i> [pB-LAC]	This study
DL-4	<i>biA1 pyrG89 wA3 argB2 pyroA4</i> [pD-LAC]	This study

Table 2. Plasmids used in this study

Plasmid	Construction	Reference
pchsB	5.5 kb <i>HindIII</i> fragment containing the <i>chsB</i> gene cloned into the <i>HindIII</i> site of pUC118	This study
pchsBR	Identical to pchsB except that the direction of the <i>chsB</i> transcription is opposite	This study
pchsB5.5H	Plasmid derived from pchsBR by introducing a <i>HindIII</i> site just in front of the <i>chsB</i> initiation codon	This study
pDJB1	Plasmid containing the <i>pyr-4</i> gene of <i>N. crassa</i>	Ballance & Turner (1985)
pDJB-ALC-BA	1.7 kb <i>HindIII</i> – <i>EcoRI</i> fragment of the <i>chsB</i> N-terminal coding sequence downstream of the <i>alcA</i> promoter cloned into pDJB1	This study
pP1	Plasmid containing the <i>pyr-4</i> gene of <i>N. crassa</i>	Motoyama <i>et al.</i> (1994a)
p5BP	0.9 kb <i>EcoRI</i> – <i>HindIII</i> fragment containing the <i>chsB</i> promoter and 5' noncoding region cloned into the <i>XbaI</i> site of pP1	This study
p5BP-ALCB	1.7 kb <i>ClaI</i> fragment from pDJB-ALC-BA containing <i>alcA(p)::chsB</i> cloned into the <i>Sse8387I</i> site of p5BP	This study
pSS1	1.8 kb <i>Sall</i> – <i>SphI</i> fragment containing the <i>argB</i> gene cloned into the <i>Sall</i> – <i>SphI</i> site of pUC118	Motoyama <i>et al.</i> (1994a)
pSS-chsB	5.5 kb <i>HindIII</i> fragment containing the <i>chsB</i> gene cloned into the <i>BamHI</i> site of pSS1	This study
pMC1871	Plasmid containing the <i>lacZ</i> gene of <i>E. coli</i>	Casadaban <i>et al.</i> (1983)
pSS-LZ	3.0 kb <i>BamHI</i> <i>lacZ</i> -containing fragment from pMC1871 cloned into the <i>BamHI</i> site of pSS1	This study
pSS-TB-LZ	Plasmid derived from pSS-LZ by introducing the terminator region of <i>chsB</i> into the <i>XbaI</i> site downstream of the <i>lacZ</i> gene	This study
pB-LAC	Construct to express the ChsB–LacZ fusion protein under the control of the <i>chsB</i> promoter; 1.8 kb <i>HindIII</i> – <i>NaeI</i> fragment containing the <i>chsB</i> promoter and leader sequence was cloned into the <i>SmaI</i> site of pSS-TB-LZ	This study
pchsD	7.3 kb <i>BamHI</i> – <i>Sall</i> fragment containing the <i>chsD</i> gene cloned into the <i>BamHI</i> – <i>Sall</i> site of pUC119	Motoyama <i>et al.</i> (1996)
pD-LAC	Construct to express the ChsD–LacZ fusion protein under the control of the <i>chsD</i> promoter; 1.8 kb <i>NspV</i> fragment containing the <i>chsD</i> promoter and leader sequence was cloned into the <i>SmaI</i> site of pSS-TB-LZ	This study
pDΔA7	Plasmid derived from pchsD by replacing the 3.6 kb <i>SplI</i> – <i>XbaI</i> fragment containing the entire coding region of <i>chsD</i> with the 1.8 kb <i>argB</i> -containing fragment from pSS1	Motoyama <i>et al.</i> (1996)

ALCB was constructed as follows. The *alcA* promoter was amplified from the genomic DNA of strain AU1 by the PCR method using the two primers, M16-1 (5'-AAAATCGATGGCGGGGCGGAAATTGACA-3') and M16-2 (5'-AAAAAGCTTTGAGGCGAGGTGATAGGAT-3'), according to the sequence reported by Kulmburg *et al.* (1992). Using these two primers, 35 cycles were run consisting of a 94 °C 1 min melting step, a 56 °C 2 min annealing step, and a 72 °C 3 min extension step. The 5.5 kb *Hind*III fragment containing the *chsB* gene was prepared from the DNA purified from the phage clone that contains the *chsB* gene (Yanai *et al.*, 1994) and ligated with *Hind*III-digested and bacterial alkaline phosphatase (AP)-treated pUC118 (Takara Shuzo) to yield pchsB and pchsBR. The direction of *chsB* transcription was from the *Hind*III site to the *Eco*RI site on the multiple cloning site of pchsB, and the direction of the *chsB* insert of pchsBR was reverse compared to pchsB.

The *Hind*III site (indicated by underlining, see below) was introduced on the plasmid pchsBR just in front of the initiation codon (indicated by boldface, see below) by a site-directed mutagenesis kit (Bio-Rad) using the primer KY-X (5'-GCCATGGTTAAGCTTGTATGTGC-3') to yield pchsB5.5H. The *Cla*I- and *Eco*RI-digested and AP-treated pDJB1 (Ballance & Turner, 1985), the *Cla*I- and *Hind*III-digested 0.5 kb PCR fragment which contains the *alcA* promoter, and the 1.7 kb *Hind*III-*Eco*RI fragment which contains a region of *chsB*, from its initiation codon to about half of ORF, derived from pchsB5.5H, were ligated to yield pDJB-ALC-BA. The blunted 0.9 kb *Hind*III-*Eco*RI fragment, which contains the upstream region of the *chsB* promoter, derived from pchsB5.5H, was ligated with *Xba*I-digested, blunted, and AP-treated pP1, a plasmid bearing the *Neurospora crassa pyr4* gene (Motoyama *et al.*, 1994a), to yield p5BP. The direction of *chsB* transcription was from the *Eco*RI site to the *Hind*III site on the multiple cloning site. The blunted 1.7 kb *Cla*I fragment, which contains the *alcA* promoter and part of the *chsB* ORF, derived from pDJB-ALC-BA, was ligated with *Sse*8387I-digested, blunted, and AP-treated p5BP to yield p5BP-ALCB.

The plasmid pSS-*chsB*, for *chsB* expression, was constructed by ligating the 5.5 kb *Hind*III fragment, which contains the *chsB* gene derived from pchsB, and *Bam*HI-digested, blunted, and AP-treated pSS1 (Motoyama *et al.*, 1994a).

Plasmids for the analysis of promoter activities in *A. nidulans* with *lacZ* of *E. coli* as a reporter gene were constructed as follows. The 3.0 kb *Bam*HI fragment which contains *lacZ* of *E. coli* derived from pMC1871 (Casadaban *et al.*, 1983) was ligated with *Bam*HI-digested and AP-treated pSS1 to yield pSS-LZ. The blunted 0.9 kb *Sph*I fragment, which is expected to contain the terminator region of *chsB*, derived from pchsB5.5H, was ligated with *Xba*I-digested, blunted, and AP-treated pSS-LZ to yield pSS-TB-LZ. The 1.8 kb *Hind*III-*Nae*I fragment, which contains the *chsB* promoter (1.3 kb) and a part of the *chsB* ORF (0.5 kb), derived from pchsB, was blunted and ligated with *Sma*I-digested and AP-treated pSS-TB-LZ to yield pB-LAC. The 1.8 kb *Nsp*V fragment, which contains the *chsD* promoter (1.4 kb) and a part of the *chsD* ORF (0.4 kb), derived from pchsD, was blunted and ligated with *Sma*I-digested and AP-treated pSS-TB-LZ to yield pD-LAC. Linkage points were confirmed by sequencing with a primer, prm-LZ2 (5'-TTCTGGTGCCGGAACCA-3'). We found some sequencing errors in the *chsD* and the new version of the *chsD* nucleotide sequence is registered with accession no. D83246. Consequently, total amino acid residues of ChsD were changed from 1086 to 1184 due to the extension of 96 aa residues in its N-terminus. From the results of 5'-RACE

Fig. 1. Constructions of conditional *chsB* mutant strains. (a) Construction of strains BM-3, BM-4 and BM-12 by inserting the 3.9 kb *Dra*I-*Cla*I fragment from p5BP-ALCB into the promoter region of *chsB*. Abbreviations: H, *Hind*III; P, *Pst*I. (b) Southern analysis of transformants. *Hind*III-digested total DNA of strain BM-3 (lane 1), strain BM-4 (lane 2), strain BM-12 (lane 3) and strain ABPU1 (lane 4) was hybridized with the 1.3 kb *Hind*III fragment (Fig. 1a, probe) from pchsB5.5H. *Pst*I-digested total DNA of strain BM-3 (lane 5), strain BM-4 (lane 6), strain BM-12 (lane 7) and strain ABPU1 (lane 8) was hybridized with the same 1.3 kb *Hind*III fragment.

experiments, the transcriptional start point(s) were estimated approximately 530 bp upstream of the revised ATG initiation codon and an intron of 151 bp was identified from -6 to -157 when the first A of the revised initiation codon was designated +1.

Construction of *A. nidulans* strains by transformation. All the strains originated from AU1 or ABPU1. The *argB* gene of *A. nidulans* and the *pyr4* gene of *N. crassa* were used as selectable markers to complement arginine- and uridine-auxotrophy, respectively.

Conditional mutants of *chsB* were made as follows. The promoter of *chsB* in the genome of *A. nidulans* strain ABPU1 was interrupted with the *alcA* promoter by transformation with the 3.9 kb *Dra*I-*Cla*I fragment from p5BP-ALCB. By Southern analysis of *Hind*III-digested or *Pst*I-digested total DNA of ABPU1 transformants probed with the 1.3 kb *Hind*III fragment (Fig. 1a, probe) from pchsB5.5H, homologous recombination at the *chsB* locus was confirmed in strains

Fig. 2. Construction of strains to analyse promoter activities of *chsB* and *chsD*. (a) Construction of strain BL-1, which produces β -galactosidase under the control of the *chsB* promoter. Strain AU1 was transformed with BglII-digested pB-LAC. Abbreviations: B, BglII; X, XbaI. (b) Southern blot analysis of XbaI-digested (lanes 1–3), StuI-digested (lane 4) and HindIII-digested (lane 5) total DNAs from strain AU1 (lane 1) and from strain BL-1 (lanes 2–5) probed with the 1.1 kb StuI–HindIII fragment from pSS1 (lanes 1 and 2) or probed with the 3.0 kb BamHI fragment of *lacZ* from pMC1871 (lanes 3–5). (c) Construction of strain DL-4, which produces β -galactosidase under the control of the *chsD* promoter. Strain ABPU1 was transformed with NcoI-digested pD-LAC. Abbreviations: N, NcoI; X, XbaI. (d) Lanes 1 and 2, Southern blot analysis of XbaI-digested total DNA from strains ABPU1 (lane 1) and DL-4 (lane 2) probed with the 1.1 kb StuI–HindIII fragment from pSS1. Lanes 3–5, Southern blot analysis of BamHI- (lane 3), HindIII- (lane 4) and XbaI- (lane 5) digested total DNA of strain DL-4 probed with the 3.0 kb fragment from pSS-TB-LZ.

named BM-3, BM-4 and BM-12 by a shift of the signal size from 5.5 kb to 1.9 kb and from 1.6 kb to 3.8 kb, respectively (Fig. 1b, lanes 1–8). By the same strategy, the promoter of *chsB* in the genome of the *chsD* null mutant D3-2 (Table 1, Motoyama *et al.*, 1996) was exchanged with the *alcA* promoter to yield strains DB-13 and DB-19. The exchange of these promoters was confirmed by Southern analysis (data not shown). Strain BB-3 was created by transformation of strain BM-3 with pSS-*chsB*. The strains ABPU/A and BM-3/A1 were constructed by transformation of strains ABPU1 and BM-3, respectively, with pSS1. *chsD* of strain BM-3 was replaced with the *argB* gene by using pDAA7 as described previously (Motoyama *et al.*, 1996), generating a strain designated as BD-2.

The strain to analyse the promoter activity of *chsB* was constructed as follows. AU1 was transformed with BglII-digested pB-LAC. By Southern analysis of XbaI-digested total DNA of a transformant, BL-1, probed with the 1.1 kb HindIII–StuI fragment (Fig. 2a, probe) from pSS1, homologous recombination at the *argB* locus was confirmed by a shift of the signal size from 3.5 kb to 14 kb (Fig. 2b, lane 2). Only one signal was found by Southern analysis of total DNA

of this transformant digested with three different restriction enzymes and probed with the 3.0 kb BamHI fragment which contains *lacZ* from pMC1871, indicating that one copy of the plasmid was integrated into this transformant (Fig. 2b, lanes 3–5). The strain to analyse the promoter activity of *chsD* was constructed as follows. ABPU1 was transformed with NcoI-digested pD-LAC. By Southern analysis of XbaI-digested total DNA of a transformant, DL-4, probed with the 1.1 kb HindIII–StuI fragment (Fig. 2c, probe) from pSS1, homologous recombination at the *argB* locus was confirmed by a shift of the signal size from 3.5 kb to 6.6 kb and 7.2 kb (Fig. 2d, lane 2). Only one signal was found by Southern analysis of total DNA of this transformant digested with three different restriction enzymes and probed with the 3.0 kb BamHI fragment which contains *lacZ* from pSS-TB-LZ, indicating that one copy of the plasmid was integrated into this transformant (Fig. 2d, lanes 3–5).

Calcofluor white staining. Conidia were spread on a YGU plate, and incubated for 24 h at 37 °C. Hyphae on a piece of agar (approx. 15 mm × 15 mm) were cut off, stained with 0.01% calcofluor white (fluorescent brightener 28, Sigma), and observed under a fluorescent microscope.

Carbon source exchange experiment. Conidial suspensions of *A. nidulans* were inoculated into liquid MMFT (200 ml, fructose and threonine as carbon sources) in a 500 ml Erlenmeyer flask to a final concentration of 2×10^6 conidia ml⁻¹. After agitating the flasks on a rotary shaker for 17 h at 37 °C, mycelia were collected by filtration through G3 glass filter, washed by liquid MM (glucose as a carbon source), and spread on MM plates (glucose as a carbon source). After 25 h incubation at 37 °C, conidia were collected from the plates and the numbers were counted using a haemocytometer. All the MMFT and MM used were supplemented with 0.20 mg arginine ml⁻¹, 0.02 µg biotin ml⁻¹, 0.50 µg pyridoxine ml⁻¹ and 10 mM uridine. To examine the abundance of the *chsB* transcript, total RNA was prepared from BM-3/A1 (*chsB* mutant) mycelia taken at 0, 5, 15 and 25 h after transfer onto a MM plate.

Chitin content determination. Chitin content was determined as described previously (Fujiwara *et al.*, 2000), except that strains were grown in 100 ml YGU medium for 18 h at 37 °C, and that the cell-wall-containing pellets were sonicated in distilled water to a fine suspension before enzymic treatment. The protein levels, by which we normalized the amount of N-acetylglucosamine, were not very different between the cell extracts from different strains.

In situ staining of the β-galactosidase activity. β-Galactosidase activity was stained *in situ* by a modification of a method reported previously (Adams & Timberlake, 1990; Aguirre *et al.*, 1990). In brief, 20 µl of a conidial suspension (10^8 conidia ml⁻¹) was spread on a MM plate (supplemented with biotin, pyridoxine and uridine). After incubating for 13 or 36 h at 37 °C, hyphae on a piece of agar (approx. 15 mm × 15 mm) were cut off, and treated with chloroform vapour for 20 min at room temperature. The piece was transferred into a staining solution (0.05 M sodium phosphate, pH 7.5, 0.02% X-Gal), incubated for 2 h at 37 °C, and cells were observed under a microscope.

Microscopy. Samples were observed and photographed by using a light microscope (model BHS-RFK, Olympus) equipped with an automatic camera (model PM-10ADS, Olympus) attachment.

RESULTS

Construction of conditional mutants of *chsB*

We constructed *chsB*-conditional mutants in which *chsB* was placed under an inducible promoter, namely the alcohol dehydrogenase gene (*alcA*) promoter of *A. nidu-*

lans (Kulmburg *et al.*, 1992), and we analysed the functions of ChsB using these mutants. In these mutants, the expression of *chsB* is expected to be induced by ethanol or threonine and repressed by glucose. By introducing the 3.9 kb *DraI*-*Clal* fragment from p5BP-ALCB into strain ABPU1, we obtained three transformants in which the promoter of *chsB* in the genome was interrupted by the *alcA* promoter. We designated these three transformants BM-3, BM-4 and BM-12 (Fig. 1, Table 1). We used strain BM-3 for further experiments.

Effect of *chsB* repression on growth and differentiation

We analysed the effect of the repression of *chsB* expression on the growth of *A. nidulans* by comparing the radial growth rate of the *chsB*-repressed strain with that of the wild-type strain. Since auxotrophic markers could affect the growth rate, BM-3/A1 was constructed by the transformation of BM-3 with pSS1, a plasmid containing the *argB* gene, and the growth rate of BM-3/A1 was compared with that of the wild-type strain ABPU/AU. Conidia of each strain were point-inoculated on solid medium and incubated for 72 h at 37 °C. The colony diameter was then determined (Table 3). On MM plates [*alcA*(p)-repressing condition], the colony diameter of BM-3/A1 (15.5 ± 4.3 mm) was much smaller than that of ABPU/AU (34.5 ± 2.9 mm). Under this condition, dark brown pigmentation of hyphae in the agar was observed in BM-3/A1. Borgia *et al.* (1996) reported that the *chsB* disruptant colonies became brown. Significant reduction in the growth rate of BM-3/A1 was also observed on YGU plates [*alcA*(p)-repressing condition]. Since the growth defects of some cell-wall-deficient mutants can occasionally be remedied by the addition of osmotic stabilizers into the media, we examined the effects of osmotic stabilizers. The growth defect of strain BM-3/A1 on YGU plates was not suppressed by the addition of 0.6 M KCl. On MMFT plates [*alcA*(p)-inducing condition], the colony diameter of BM-3/A1 (31.8 ± 0.8 mm) was slightly smaller than that of ABPU/AU (36.7 ± 0.9 mm). Taken together, these results suggest that the growth defect of BM-3/A1 on MM and YGU plate was caused by the repression of *chsB* expression.

Table 3. Comparison of growth rate of the wild-type strains and mutants

Strain	Relevant genotype	Colony diameter (mm) (means ± SD)*			
		MMFT	MM	YGU	YGU+0.6M KCl
ABPU/AU	Wild type	36.7 ± 0.9	34.5 ± 2.9	35.7 ± 2.6	47.3 ± 0.9
BM-3/A1	<i>alcA</i> (p):: <i>chsB</i>	31.8 ± 0.8	15.5 ± 4.3	12.1 ± 2.7	12.3 ± 2.5
DB-13	<i>alcA</i> (p):: <i>chsB</i> Δ <i>chsD</i>	37.2 ± 1.4	7.3 ± 0.2	10.2 ± 0.2	5.9 ± 0.3
ABPU/A	<i>pyrG</i>	31.7 ± 1.2	29.0 ± 0.8	29.7 ± 1.2	40.3 ± 0.9
D3-2	Δ <i>chsD pyrG</i>	34.7 ± 0.9	35.2 ± 0.6	33.8 ± 0.2	44.5 ± 0.4

* Strains were grown for 72 h at 37 °C. Means ± SD were calculated from three independent experiments.

We next analysed the morphology of BM-3/A1 under the *chsB*-repressing condition. BM-3/A1, ABPU/AU and BB-3, an *alcA(p)::chsB* strain bearing an extra copy of the intact *chsB* gene, were incubated for 24 h on YGU plates. Hyphal growth and conidiophore development were then observed (Fig. 3). BM-3/A1 displayed defects in hyphal growth (Fig. 3d, e); specifically, its hyphae branched more frequently than those of ABPU/AU, and the positions of branch formation were aberrantly close to the tips of the hyphae or to each other. The lateral walls lacked smoothness. Hyphal tips, branching points close to tips, and septa, which appeared to be formed normally, were intensely stained with calcofluor white, which is known to bind to chitin (Fig. 3e). Conidiophores developed rather normally on BM-3/A1 colonies, although fewer of them and of the conidia produced on each of them were produced than in ABPU/AU (Fig. 3f, and data not shown). The morphology of BB-3 was almost the same as that of ABPU/AU (Fig. 3i, j, k). These results suggest that the defects of BM-3/A1 were caused by the repression of the *chsB* expression.

Involvement of *chsB* in conidiation

In the previous section, we showed that BM-3/A1 has some defects in asexual development. It is possible that ChsB is directly involved in conidiophore development and conidia formation, or that ChsB is involved only in hyphal growth. In the latter case, the defect in conidiophores and conidia formation could be caused by the partial inability to produce conidiation-competent hyphae (Miller *et al.*, 1991). To clarify which possibility is the case, we performed a carbon source exchange experiment (see Methods). Conidiation-competent hyphae of ABPU/AU, BM-3/A1 and BB-3, cultured in *alcA(p)*-inducing liquid MMFT, were transferred onto *alcA(p)*-repressing MM plates on which conidiation was induced. The numbers of conidia, which were expected to be produced without additional *chsB* expression, were then counted (Table 4). BM-3/A1 could form only 45% of the number of conidia produced by ABPU/AU. The efficiency of the conidiation of BB-3 was recovered up to 75% of that of ABPU/AU, suggesting that a part of the reduction in the conidiation efficiency of BM-3/A1 could be due to the absence of *chsB* expression during developmental induction. The reason why the conidiation efficiency of BB-3 could not reach the same level as that of the wild-type strain is not clear. It is possible that the expression of the extra copy of *chsB* is not sufficient due to the effect of the position where *chsB* was integrated.

In comparison with the previous report by Borgia *et al.* (1996), in which they showed that the *chsB* disruptants did not conidiate, the reduction in conidiation efficiency of BM-3/A1 was rather moderate. It is possible that the repression of *chsB* transcription is incomplete on MM plates, and the incompleteness could relieve the conidiation defect to some extent. We examined the abundance of the *chsB* transcripts after the shift to MM plate by Northern analysis (Fig. 4a). *chsB* mRNA was easily

Table 4. Conidia formation efficiency of the wild-type strain and mutants

Strain	Relevant genotype	$10^{-4} \times$ No. of conidia mm^{-2} (mean $\pm$ SD)*
ABPU/AU	Wild-type	100
BM-3/A1	<i>alcA(p)::chsB</i>	45 $\pm$ 4.9
BB-3	<i>alcA(p)::chsB chsB</i>	75 $\pm$ 3.9
DB-13	<i>alcA(p)::chsB ΔchsD</i>	26 $\pm$ 1.8

* Means $\pm$ SD were calculated from at least three independent experiments.

detectable in the sample prepared from the mycelium cultured for 17 h in liquid MMFT (Fig. 4a, 0 h). After shift to a MM plate, *chsB* mRNA decreased drastically by 5 h after shift, and was almost undetectable at 15 h after shift (Fig. 4a). At 25 h after shift, a low level of *chsB* expression was again detected (Fig. 4a). This resumption of *chsB* expression may be due to the decrease of glucose level and the concomitant derepression of the *alcA* promoter. By performing RT-PCR, we obtained a clearer result that shows the presence of the *chsB* transcripts at 5 h and 25 h after shift (Fig. 4b). These results imply that the conidiation defect of BM-3/A1 was underestimated in the carbon source exchange experiment because of the stability of *chsB* mRNA and the leaky expression of *chsB*.

Functional importance of ChsD in the *chsB*-conditional mutant

To examine the functional importance of ChsD under the ChsB-repressing condition, we generated double mutants of *chsB* and *chsD*. We transformed the *chsD* null mutant, D3-2 (Motoyama *et al.*, 1996), by using the same strategy as the BM-3 construction, generating strains DB-13 and DB-19 (see Methods). Since DB-19 showed almost the same phenotype as DB-13 (data not shown), data on DB-13 are given for the following experiments. We also generated a double mutant by deleting the *chsD* gene of BM-3. The resulting strain, BD-2, exhibited almost the same phenotype as DB-13 under the conditions tested (data not shown). We first examined the growth rate of the double mutant (Table 3). On MMFT plates, the colony diameter of DB-13 was almost identical to that of the wild-type strain ABPU/AU and was larger than that of BM-3/A1. The colony diameter of the double mutant was about half that of BM-3/A1 on MM plates, whereas it was almost the same as that of BM-3/A1 on plates of YG, another *alcA(p)*-repressing medium. We found the osmolarity of YGU medium to be lower than that of minimal medium (data not shown). Supplementing YGU plates with 0.6 M KCl caused the colony diameter of DB-13 to decrease to half of that of BM-3/A1. The colony diameters of the *chsD* null mutant (D3-2) were slightly larger than those of the relevant wild-type strain (ABPU/A) under all conditions tested. These results indicate that the importance of the ChsD function in

Fig. 4. Expression of *chsB* after the shift from inducing conditions to repressing conditions. (a) Northern blot analysis of *chsB*. Total RNA was prepared from the mycelia of the *chsB* mutant (BM-3/A1) cultured for 0, 5, 15 and 25 h after the shift onto MM plates. Equal loading of total RNA was evaluated by ethidium bromide staining (rRNA). (b) RT-PCR amplification of *chsB*. The RNA samples used for Northern analysis were treated with DNase, and subjected to RT-PCR. To assure the amplification from cDNA, PCR was also performed by using total DNA from strain ABPU1 (wild-type) as a template (lane D). The DNA standard (100 bp ladder) is in lane M.

hyphal growth increased under high osmolarity conditions when the expression of *chsB* was repressed.

When the hyphal morphology of DB-13 on YGU plate was observed microscopically, the hyphae were seen to be even more disorganized than those of BM-3/A1 (Fig. 3g, h). The number of conidiophores was much smaller than that of BM-3/A1 (data not shown). The number was not greatly increased by 24 h of additional incubation (data not shown).

In the carbon source exchange experiment, it was shown that DB-13 generated approximately 60% of the number

Fig. 5. Spatial expression patterns of *chsB* and *chsD*. Conidia from strains BL-1 (a, b) and DL-4 (c, d) were incubated for 13 h (a, c) or for 36 h (b, d) on MM plates, and mycelia were stained with X-Gal and observed under a microscope. Bar, 25 µm.

of conidia generated by BM-3/A1 (Table 4). Taking into account that the *chsD* single mutants show no significant reduction in conidiation efficiency (Motoyama *et al.*, 1996; Culp *et al.*, 2000, in which *chsD* is referred to as *chsE*), it is suggested that the importance of the ChsD function in conidiation also increased under the *chsB*-repressing condition.

We measured the chitin content of BM-3/A1 and DB-13 (Table 5). The chitin contents of BM-3/A1 in YGU and in YGU containing 0.6 M KCl were both approximately 70% of those of the wild-type strain (ABPU/AU), whereas those of BB-3 were almost the same as those of the wild-type strain. The chitin contents of DB-13 were approximately half those of BM-3/A1. Since the chitin contents of D3-2 under these conditions were not very different from those of the relevant wild-type strain

Fig. 3. Morphology of the *chsB* single mutant and the *chsB chsD* double mutant. The wild-type strain (ABPU/AU) (a–c), the *chsB* mutant (BM-3/A1) (d–f), the *chsB chsD* double mutant (DB-13) (g, h) and the *chsB* mutant transformed with the *chsB* gene (BB-3) (i–k) were grown for 24 h on YGU plates. Hyphae were stained with calcofluor white to visualize chitin distribution (b, e, h and j). Bar, 30 µm.

Table 5. Chitin contents of the wild-type strains and mutants

Strain	Relevant genotype	Chitin content [mg GlcNAc (mg protein) ⁻¹]*	
		YGU	YGU + 0.6 M KCl
ABPU/AU	Wild-type	1.09 (100) ± 0.090	0.79 (100) ± 0.031
BM-3/A1	<i>alcA(p)::chsB</i>	0.73 (67) ± 0.038	0.59 (75) ± 0.031
DB-13	<i>alcA(p)::chsB ΔchsD</i>	0.37 (34) ± 0.004	0.31 (39) ± 0.022
BB-3	<i>alcA(p)::chsB chsB</i>	1.04 (95) ± 0.040	0.77 (97) ± 0.064
ABPU/A	<i>pyrG</i>	0.80 (100) ± 0.053	0.74 (100) ± 0.043
D3-2	<i>ΔchsD pyrG</i>	0.74 (93) ± 0.031	0.63 (85) ± 0.008

* Means ± SD were calculated from the results of triplicate samples. Similar results were reproducibly observed in several experiments. The ratio of chitin content of each strain to that of each of the wild-type strain is shown in parentheses.

(ABPU/A), the effect of the *chsD* deletion on chitin content was considered to be larger in the *chsB*-conditional mutant than in the wild-type strain. Taking these results together, we concluded that the *chsB* repression increased the importance of the ChsD functions in hyphal growth, conidiation and chitin content maintenance.

Expression of *chsB* and *chsD*

Transcriptional regulation of chitin synthase genes has been shown in other fungi (Chen-Wu *et al.*, 1992; Pammer *et al.*, 1992; Sudoh *et al.*, 1993; Choi *et al.*, 1994; Motoyama *et al.*, 1994b; Xoconostle-Cázares *et al.*, 1996, 1997; Munro *et al.*, 1998; Wang & Szaniszló, 2000), and seems to be one of the regulatory mechanisms for chitin synthesis. To investigate the involvement of ChsB and ChsD in hyphal growth and conidiation in another way, we analysed the expression pattern of *chsB* and *chsD* using *lacZ* of *E. coli* as a reporter gene. We constructed strains BL-1 and DL-4 using plasmids pB-LAC and pD-LAC, respectively (Fig. 2, see Methods). In strain BL-1, it was predicted that the ChsB-LacZ fusion protein, which contains a 10 aa linker between the N-terminal 132 aa of ChsB and LacZ, would be expressed under the control of the *chsB* promoter. In strain DL-4, it was predicted that the ChsD-LacZ fusion protein, which contains a 10 aa linker between the N-terminal 126 aa of ChsD and LacZ, would be expressed under the control of the *chsD* promoter (see Methods). To avoid a position effect on expression, both pB-LAC and pD-LAC were integrated into the same *argB* locus. The growth rates of strains BL-1 and DL-4 were almost the same as those of the relevant wild-type strains AU/A and ABPU/A, respectively, indicating that expression of the fusion proteins is not toxic (data not shown). We analysed the spatial expression pattern of these genes by staining β -galactosidase activity using X-Gal as a substrate (Fig. 5). In strain BL-1, blue staining was observed in the hyphae (Fig. 5a), as well as in the conidiophore, especially in the metulae, phialides and conidia (Fig. 5b). A similar staining pattern was observed in strain DL-4 (Fig. 5c, d), while no staining was detected in the wild-

type strains, AU/A and ABPU/A (data not shown). These expression data further support the proposition that ChsB and ChsD function not only in hyphal growth, but also in conidiation.

DISCUSSION

In this study, we found by using a conditional mutant of *chsB* that ChsB functions not only in hyphal growth but also in conidiation. By using double mutants of *chsB* and *chsD*, we found evidence that the functional importance of ChsD increases in hyphal growth and in conidiation when the expression of *chsB* is repressed. The involvement of ChsB and ChsD in both hyphal growth and conidiation was supported by the expression analysis using *lacZ* of *E. coli* as a reporter gene.

Our laboratory reported previously that a *chsB* disruptant could grow only as a heterokaryon containing both wild-type and disrupted *chsB* alleles (Yanai *et al.*, 1994). The hyphae of a haploid *chsB* disruptant stopped growing immediately after the germination of conidia, with their hyphal tips swelling. Borgia *et al.* (1996) obtained viable homokaryons carrying the disrupted *chsB* allele. Their mutants grew very slowly with a high degree of branching, and contained normal septa. The colonies did not form conidiophores and conidia, and became brown after about 4 days incubation. These phenotypes of the Borgia group's *chsB* disruptants are very similar to those of the repressed *chsB* conditional mutant generated in this study (Fig. 3, and our unpublished result). These results strongly suggest that *chsB* is necessary not only for hyphal growth but also for conidiation. The lethality of the *chsB* disruptant reported by Yanai *et al.* (1994) might be caused not only by the disruption of *chsB*, but also by the production of the C-terminally truncated ChsB, which could be expressed from the disrupted *chsB* locus. As shown in Fig. 4 and will also be mentioned below, the repression of *alcA(p)::chsB* is imperfect in nature, and the viability of the conditional *chsB* mutant BM-3/A1 may depend on this imperfection. While Borgia *et al.* (1996) reported that the chitin content of their *chsB* disruptant was almost the same as that of the parental strain, the

chitin contents of the mutant BM-3/A1 in YGU and in YGU containing 0.6 M KCl were both approximately 70% of those of the wild-type strain (Table 5). It is possible that these differences were simply derived from the differences in the genetic backgrounds of the strains employed.

The results in Table 4 clearly show the defect of *chsB* mutants in conidiation, though the reduction in conidiation efficiency seems rather moderate. When we use repressible promoters for the analysis of the functions of gene products, we should pay attention to two points. First, when the repression of the transcription of the gene is leaky, effects milder than gene disruptions or deletions would be observed under the repressing condition. It has been reported that the expression from the *alcA* promoter is not completely repressed in minimal medium (Som & Kolaparthi, 1994; McGoldrick *et al.*, 1995). The results in Fig. 4 show that the *chsB* transcripts are actually present even at 25 h after the shift from MMFT to MM plate. Second, when transcripts and/or products of the gene are stable after the shift to the repressive condition, the effects of transcriptional repression would be observed only after prolonged cultivation. As shown in Fig. 4 (5 h lane), this was also the case. Taking these precautions into account, it is possible that the contribution of ChsB to conidiation was underestimated in the carbon source exchange experiment (Table 4).

The defects in the hyphal growth of BM-3/A1 after the shift to a *chsB*-repressing MM plate might have resulted in a reduction of the amount of conidiation-competent hyphae. However, in combination with the *chsB* expression in conidiophores and conidia (Fig. 5), we take our result to suggest that the function of ChsB is not limited to the generation of conidiation-competent hyphae but is directly involved in conidiation.

We noticed that the colony diameter of BM-3/A1 on MMFT plates is smaller than that of the wild-type strain. This finding might be caused by the dominant negative effect of the *chsB* overexpression. However, the *chsB chsD* double mutant DB-13 showed almost the same colony diameter as the wild-type strain. Therefore, it is likely that the *chsB* overexpression *per se* does not cause growth inhibition, but the simultaneous expression of *chsD* disturbs the fully wild-type growth.

A reduction in the growth rate of DB-13 compared to that of BM-3/A1 was observed on MM plates and YGU plates containing 0.6 M KCl, but occurred only slightly on YGU plates (Table 3). This result indicates that, when *chsB* expression is repressed, the importance of the ChsD function in hyphal growth increases under conditions of higher osmolarity. ChsD seems to be dispensable for hyphal growth under conditions of low osmolarity in the absence of *chsB* expression. It is possible that ChsD either does not function or its loss can be compensated for by other chitin synthases when the osmolarity of the culture media is low. However, the hyphal morphology of DB-13 was more disorganized

than that of BM-3/A1 on YGU plates (Fig. 3g, h), suggesting that ChsD functions and contributes to hyphal morphology even under low-osmolarity conditions. To measure the growth rates on solid media, we inoculated many conidia at one point on a plate. It is possible that, as the colony diameter and hyphal density increase and glucose is consumed, leaky expression of *chsB* occurs around the centre of a colony. Thus, it is likely that we have underestimated the effect of the *chsD* deletion on the growth rate on YGU. Supplementation of 0.6 M KCl did not greatly affect the ratio of the chitin content between DB-13 and BM-3/A1 (Table 5), suggesting that chitin content does not necessarily correlate with the degree of physiological disorder.

The conidiation efficiency of DB-13 was approximately 60% that of BM-3/A1 in the carbon source exchange experiment (Table 4). In this experiment, we used as media MMFT and MM, which have higher osmolarity than YGU. Since the deletion of *chsD* affected hyphal growth under these conditions (Table 3), the defect in conidiation may have been derived from some defects in hyphal growth. Furthermore, since we have obtained evidence that ChsD is present in conidiophore structures of the wild-type strain (Fig. 5), we propose that ChsD may also be directly involved in conidiation.

Our analyses of DB-13 revealed the importance of the *chsD* function under *chsB*-repressing conditions. Beth Din *et al.* (1996) suggested that Chs4, the class IV chitin synthase of *N. crassa*, may serve as an auxiliary enzyme that supplements chitin synthesis when additional chitin is necessary. ChsD, also a class IV chitin synthase, may contribute to chitin synthesis in response to various conditions, including the *chsB*-repressing condition. Since the *chsD* deletion in the *chsA* mutants reduced the conidiation efficiency of the mutants (Motoyama *et al.*, 1996; Culp *et al.*, 2000), it is possible that the *chsA* deletion may also be the condition that increases ChsD activity. Alternatively, ChsD may have some redundant functions with ChsA and ChsB.

The phenotypes of the conditional *chsB* mutants have some similarity to the phenotypes of the null mutants of the class III chitin synthase genes of other filamentous fungi, namely *chs-1* of *N. crassa* and *chsG* of *A. fumigatus*. Further analysis of the chitin synthase functions using the conditional mutation in combination with mutations of other related genes will help to elucidate their coordination for fungal morphogenesis and differentiation, and to develop anti-fungal reagents effective against filamentous fungi.

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