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The *Aspergillus nidulans* genes *chsA* and *chsD* encode chitin synthases which have redundant functions in conidia formation

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Abstract We previously isolated three chitin synthase genes (*chsA*, *chsB*, and *chsC*) from *Aspergillus nidulans*. In the present work, we describe the isolation and characterization of another chitin synthase gene, named *chsD*, from *A. nidulans*. Its deduced amino acid sequence shows 56.7% and 55.9% amino acid identity, respectively, with Cal1 of *Saccharomyces cerevisiae* and Chs3 of *Candida albicans*. Disruption of *chsD* caused no defect in cell growth or morphology during the asexual cycle and caused no decrease in chitin content in hyphae. However, double disruption of *chsA* and *chsD* caused a remarkable decrease in the efficiency of conidia formation, while double disruption of *chsC* and *chsD* caused no defect. Thus it appears that *chsA* and *chsD* serve redundant functions in conidia formation.

Key words Chitin synthase · Multigene family · Cell wall · Conidia · *Aspergillus nidulans*

Introduction

Chitin, a β-1,4 linked polymer of *N*-acetylglucosamine, is a major component of the cell wall of most filamentous fungi (Bartnicki-Garcia 1968). Hence proper regulation of chitin metabolism is important for the growth of filamentous fungi.

In the filamentous fungus *Aspergillus nidulans*, three chitin synthase genes, *chsA*, *chsB*, and *chsC*, have so far been isolated and characterized by our group (Yanai et al. 1994; Motoyama et al. 1994a). Disruption of *chsA* or *chsC* causes no defects in cell growth or morphology during the asexual cycle, while disruption of *chsB* is lethal. Chitin synthases can be classified into three classes, I, II, and III, on the basis of sequence. In the yeast *Saccharomyces cerevisiae*, three chitin synthase genes, *CHS1*, *CHS2*, and *CAL1*, have been cloned so far (Bulawa et al. 1986; Silverman 1989; Valdivieso et al. 1991), and the amino acid sequence of Cal1 is distantly related to those of other chitin synthases of classes I, II, and III. Disruption of *CAL1* causes a decrease in chitin content to approximately 10% of the parental strain (Shaw et al. 1991). Thus, it would be reasonable to expect that a homolog of *CAL1* in filamentous fungi would have an important role in formation of cell wall, of which chitin is a major component. In filamentous fungi, *CAL1* homologs have been isolated from only one organism, *Aspergillus fumigatus* (Mellado et al. 1995), but their functions have not been examined so far. Here, we report isolation of a *CAL1* homolog from *A. nidulans* and characterization of its function by the gene disruption technique.

Materials and methods

Strains, media and transformation

A. nidulans strains used are shown in Table 1. Complete (CM) and minimal media (MM) for *A. nidulans*, as described by Rowlands and Turner (1973), were used. Transformation was done as described by May (1992). Transformants were grown in MM with appropriate supplements. Plasmids were amplified in *Escherichia coli* MV1190 (Sambrook et al. 1989). *E. coli* was grown in LB medium and transformation was done by the standard method (Sambrook et al. 1989).

Total DNA isolation and Southern analysis

Total DNA was extracted as described by Oakley et al. (1987). For ECL direct nucleic acid labeling and detection the ECL system

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Table 1 Strains used in this study

Strain	Genotype	Source
FGSC89	*argB2 biA1*	FGSC[a]
FGSC773	*pyrG89 pyroA4 wA3*	FGSC[a]
ABPU1	*argB2 biA1 pyrG89 pyroA4 wA3*	This study (FGSC89 × FGSC773)
ABPU/AU	*argB2 biA1 pyrG89 pyroA4 wA3* [pSS1] [pP1]	This study
A-4	*argB2 biA1 pyrG89 pyroA4 wA3 chsA*::*argB*	This study
C2-11, C2-12	*argB2 biA1 pyrG89 pyroA4 wA3 chsC*::*pyr4*	This study
D3-2	*argB2 biA1 pyrG89 pyroA4 wA3 chsD*::*argB*	This study
DA-4	*argB2 biA1 pyrG89 pyroA4 wA3 chsA*::*pyr4 chsD*::*argB*	This study
AD-3	*argB2 biA1 pyrG89 pyroA4 wA3 chsA*::*argB chsD*::*pyr4*	This study
CD-1, CD-2, CD-5	*argB2 biA1 pyrG89 pyroA4 wA3 chsC*::*pyr4 chsD*::*argB*	This study

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(Amersham) was used. DNA was separated by electrophoresis on agarose gels and further manipulation was done as described in the system manufacturer's manual. Low-stringency and high-stringency Southern hybridizations were done at 56° C and 65° C, respectively.

PCR amplification of DNA fragments

PCR amplification was done by using a thermal cycler (Model 480, Perkin-Elmer/Cetus, Norwalk, Conn.) and Taq DNA polymerase (Boehringer Mannheim, Mannheim, Germany). The primers used to amplify a partial fragment of a *CAL1* homolog by PCR were MO-3 [5′-CC(ACGT)GG(ACGT)AA(CT)AGAGG(ACGT)AA(AG)AGAGA-3′] and MO-4 [5′-AT(AG)TA(AG)TA(CT)TC(AG)AA(ACGT)AC(CT)TG(AGT)AT-3′]. Using these two primers, 35 reaction cycles were run consisting of a 1-min melting step at 94° C, a 2-min annealing step at 56° C, and a 3-min extension step at 72° C.

Construction and screening of genomic libraries

Sub-genomic libraries were constructed by cloning digested and size-fractionated (Sambrook et al. 1989) total DNA of *A. nidulans* into pUC118 or pUC119 (Sambrook et al. 1989). Colony and plaque hybridization on membrane filters was done as described in the filter manufacturer's (Amersham) manual.

DNA sequencing

DNA sequencing was done by the method of Sanger et al. (1977). The pUC118 or pUC119 subclones were constructed using a deletion kit (Takara Shuzo, Kyoto, Japan) or by restriction enzyme manipulations. The reactions were done using a Cycle DNA Sequencing kit (Applied Biosystems, Foster City, Calif.). Analysis of reaction products was done on a Model 373A DNA sequencer (Applied Biosystems).

Manipulation of RNA

A conidial suspension of *A. nidulans* FGSC89 was inoculated into liquid MM (200 ml) in a 500-ml Erlenmeyer flask to a final concentration of 2×10^6 conidia/ml. The flask was agitated on a rotary shaker at 30° C for 12 h. Isolation and purification of RNA was done as described previously (Motoyama et al. 1994a). Northern blot analysis was performed as reported previously (Motoyama et al. 1994a). cDNA was synthesized from poly(A)$^+$ RNA using a cDNA synthesis kit (Boehringer Mannheim).

Determination of transcriptional start points

Transcriptional start points were determined using the 5′-AmpliFINDER RACE kit (Toyobo, Tokyo, Japan). cDNA was synthesized from 5 μg of poly(A)$^+$ RNA of *A. nidulans* FGSC89 strain using the primer D-01 (5′-TACTGATGAGACCGATCTTTTC-3′; see Fig. 2). Complementary RNA was degraded, and the AmpliFINDER anchor was ligated to the 3′-end of the first-strand cDNA. PCR amplification was done using D-02 (5′-AAGTCGACAAAGCACTTCAAGTC-3′) and the AmpliFINDER anchor primer, which hybridizes to the AmpliFINDER anchor. The amplified cDNA fragment was cloned into the *Sma*I site of pUC119, and sequenced.

Plasmid constructions

Plasmids for *chsA* disruption were constructed as follows. The 1.7-kb *Bam*HI-*Sph*I fragment of *argB* from pSS1 (Motoyama et al. 1994a) was blunted and ligated with *Eco*RV-digested and alkaline phosphatase (AP)-treated pchsA6.7Sc, to yield pAΔA4. pchsA6.7Sc contains the 6.7-kb *Sac*I fragment (see Fig. 5) of *chsA* in pUC119 (the direction of *chsA* transcription is from the *Hin*dIII site towards the *Eco*RI site in the multiple cloning site). The 1.7-kb *Pst*I-*Sma*I fragment of *pyr4* from pP1 (Motoyama et al. 1994a) was blunted and ligated with *Eco*RV-digested and AP-treated pchsA6.7Sc to yield pAΔP14 (Fig. 5B).

The plasmid for *chsC* disruption was constructed as follows. The 1.7-kb *Pst*I-*Sma*I fragment of *pyr4* from pP1 was blunted and ligated with *Nco*I-digested, blunted, and AP-treated pchsC, to yield pCΔP1 (see Fig. 6A). pchsC contains the entire sequenced region of *chsC* (Motoyama et al. 1994a) inserted between the *Hin*dIII and *Sph*I sites of pUC119 (the direction of *chsC* transcription is from the *Hin*dIII site towards the *Sph*I site in the multiple cloning site).

Plasmids for *chsD* disruption were constructed as follows. The 1.7-kb *Bam*HI-*Sph*I fragment of *argB* from pSS1 was blunted and ligated with *Spl*I + *Xba*I-digested, blunted, and AP-treated pchsD, to yield pDΔA7 (Fig. 7A). The 1.7-kb *Pst*I-*Sma*I fragment of *pyr4* from pP1 was blunted and ligated with *Spl*I + *Xba*I-digested, blunted, and AP-treated pchsD, to yield pDΔP12 (Fig. 6B). pchsD contains the entire sequenced region of *chsD* (see Fig. 1B) inserted at the *Bam*HI site of pUC119 (the direction of *chsD* transcription is from the *Hin*dIII site towards the *Eco*RI site in the multiple cloning site).

Disruption of chitin synthase genes in the genome of *A. nidulans*

All the strains were derived from ABPU1. *argB* and *pyr4* were used as selectable markers to complement arginine and uridine auxotrophy, respectively. A strain, ABPU/AU, which like the double disruptants is prototrophic for arginine and uridine was constructed by introducing pP1 and pSS1 into strain ABPU1.

Disruption of *chsA* by pAΔA4 was carried out as follows. The *chsA* gene of ABPU1 was disrupted by transformation with the 3.3-kb *Hpa*I-*Eco*RI fragment obtained by digesting pAΔA4 with *Hpa*I, *Eco*RI, and *Sca*I (see Fig. 5A). By Southern analysis of the *Sac*I-digested total DNA of twelve transformants probed with the 0.4-kb *Sac*I-HpaI fragment (Fig. 5A, probe 1), integration in the 5′ region of the *chsA* locus was confirmed in strain A-4 by a shift in the size of the labelled fragment from 6.7 kb to 2.1 kb (Fig. 5C, lane 2). By Southern analysis of the *Sac*I-digested total DNA of A-4 with the 6.7-kb *Sac*I fragment (Fig. 5A, probe 2), integration of one copy of the plasmid at the *chsA* locus was confirmed (Fig. 5D, lane 2). The 1.5-kb *Sac*I fragment (Fig. 5A) could not be detected, because the region that can hybridize to probe 2 is very short.

Disruption of *chsA* by pAΔP14 was done as follows. The *chsA* gene in a *chsD* disruptant, D3-2, was itself disrupted by transformation with the 3.3-kb *Hpa*I-*Eco*RI fragment obtained by digesting pAΔP14 with *Hpa*I, *Eco*RI, and *Sca*I (Fig. 5B). By Southern analysis of *Sac*I-digested total DNA of twelve transformants, probed with the 0.4-kb *Sac*I-HpaI fragment (Fig. 5B, probe 1), integration in the 5′-region of the *chsA* locus was confirmed in the strain named DA-4 by a shift in the size of the hybridizing fragment from 6.7 kb to 2.8 kb (Fig. 5C, lane 3). By Southern analysis of the *Sac*I-digested total DNA of DA-4 with the 6.7-kb *Sac*I fragment (Fig. 5B, probe 2), integration of one copy of plasmid at the *chsA* locus was confirmed (Fig. 5D, lane 3). The 0.9-kb *Sac*I fragment (Fig. 5B) could not be detected, because the region hybridizing to probe 2 is very short.

Disruption of *chsC* by pCΔP1 was performed as follows. The *chsC* locus of strain ABPU1, was disrupted by transformation with the 4.1-kb *Bgl*II-*Sph*I fragment obtained by digesting pCΔP1 with *Bgl*II, *Sph*I, and *Sca*I (Fig. 6A). When *Bgl*II and *Eco*RI-digested total DNAs of twelve transformants were tested by Southern analysis with the 1.5-kb *Bgl*II-*Bam*HI fragment (Fig. 6A, probe 1), the integration site in the 5′-region of the *chsC* locus was detected in two strains, named C2-11 and C2-12, by a shift in the signal from 2.6 kb to 4.1 kb (Fig. 6B, lane 2; C2-11). By Southern analysis of the *Bgl*II- and *Eco*RI-digested total DNA of C2-11 with the 6.1-kb *Sse*8387I fragment (Fig. 6A, probe 2), the integration point in the 3′-region of the *chsC* locus was detected by the shift in the signal from 2.6 and 3.1 kb to 4.1 kb (Fig. 6C, lane 2). When probe 2 was used, a common 1.5-kb signal was also detected (Fig. 6C, lanes 1 and 2).

Disruption of *chsD* by pDΔA7 was achieved as follows. The *chsD* gene of ABPU1 was disrupted by transformation with the 4.0-kb *Sca*I-*Kpn*I fragment, which was prepared by digesting pDΔA7 with *Kpn*I, *Sse*8387I, and *Sca*I (Fig. 7A). By Southern analysis of *Sal*I-digested total DNA of five transformants with the 5.5-kb *Sal*I fragment (Fig. 6A, probe 1), the integration site in the 5′-region of the *chsD* locus was confirmed in a strain named D3-2; the fragment detected was altered in size from 5.5 kb to 3.5 kb (Fig. 7C, lane 3). By Southern analysis of *Bam*HI-digested total DNA of the transformants probed with the 1.7-kb *Bam*HI fragment (Fig. 7A, probe 2), the integration site in the 3′-region of the *chsD* locus was localized by a shift the size of the hybridizing fragment from 1.7 kb to 2.0 kb (Fig. 7D, lane 2).

The *chsD* locus in the *chsC* disruptant C2-11 was mutated by transformation with the 4.0-kb *Sca*I-*Kpn*I fragment, prepared by digesting pDΔA7 with *Kpn*I, *Sse*8387I, and *Sca*I (Fig. 7A). By Southern analysis of a *Sal*I-digests of total DNA from six transformants with the 5.5-kb *Sal*I fragment (Fig. 7A, probe 1), integration in the 5′-region of the *chsD* locus was confirmed in three strains, named CD-1, CD-2, and CD-5, by a shift of the size of the fragment 5.5 kb to 3.5 kb (Fig. 7C, lane 2; CD-5). By Southern analysis of *Bam*HI-digested total DNA of the transformants probed with the 1.7-kb *Bam*HI fragment (Fig. 7A), the integration site in the 3′-region of the *chsD* locus was detected by a shift of the signal size from 1.7 kb to 2.0 kb (Fig. 7D, lane 3; CD-5). In the case of CD-1 and CD-2, besides disruption of *chsD*, integration of multiple copies of the transforming plasmid occurred (data not shown), although only one copy of plasmid was homologously integrated as in CD-5.

To disrupt the *chsD* locus in the *chsA* disruptant A-4, the 4.0-kb *Sca*I-*Kpn*I fragment obtained by digesting pDΔP12 with *Kpn*I, *Sse*8387I, *Sca*I was used for transformation (Fig. 7B). By Southern analysis of *Sal*I-digested total DNA of five transformants with the 5.5-kb *Sal*I fragment (Fig. 7B, probe 1), the 5′ integration site in the *chsD* locus was confirmed in a strain named AD-3 by a shift in the signal from 5.5 kb to 3.7 kb (data not shown). By Southern analysis of *Bam*HI-digested total DNA of AD-3 with the 1.7-kb *Bam*HI fragment (Fig. 7B, probe 2), localized the 3′ integration site in the chsD locus by detecting a shift in the signal site from 1.7 kb to 0.8 kb (data not shown).

Analysis of carbohydrate composition

A conidial suspension of *A. nidulans* was inoculated into liquid minimal medium (50 ml) in a 100-ml flask to a final concentration of 2×10^6 conidia/ml. To obtain the hyphal cells, the flask was agitated on a reciprocal shaker at 30° C for 24 h. Cells were collected by filtration. Cell wall composition was analyzed as described previously (Borgia and Dodge 1992).

Analysis of efficiency of conidiation

Approximately 10^5 conidia were spread on a CM plate (90 mm in diam.) supplemented with arginine, biotin, pyridoxine, and uridine. After 3 days of incubation at 37° C, conidia were collected from the plates and counted using a haemocytometer.

Results

Isolation of a *CAL1* homolog from *A. nidulans*

Using the degenerate PCR primers MO-3 and MO-4 (see Fig. 2), designed on the basis of the consensus sequences derived from previously published Cal1-homologous sequences (Valdivieso et al. 1991; Sudoh et al. 1993), we amplified 0.3-kb PCR products from the genomic DNA and from a cDNA library of *A. nidulans* FGSC89. These DNA fragments were cloned into the *Sma*I site of pUC118 (Sambrook et al. 1989). Sequences of the inserts of a plasmid amplified from genomic DNA and of a plasmid amplified from cDNA were determined. Both plasmids had the same insert, the sequence of which showed similarity to *CAL1* and differed from those of *chsA*, *chsB*, and *chsC*. To isolate the whole coding region of this gene, a genomic DNA library cloned in λ 2001 (Yanai et al. 1994) was screened by plaque hybridization with the 0.3-kb fragment amplified from the genomic DNA. One positive phage clone (C4) was isolated (Fig. 1A).

Nucleotide sequence analysis of *chsD*

The nucleotide sequence of the *CAL1* homolog was determined on both strands (Fig. 1B; Fig. 2). The

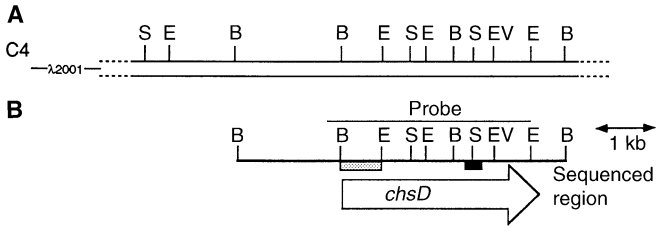

Fig. 1A Restriction map of the clone C4, which contained the *chsD* gene of *A. nidulans*. **B** Sequenced region of the clone C4. The position and direction of the predicted open reading frame of *chsD* are indicated by an *open arrow*. The region used as a probe for Northern analysis is shown as a *stippled box*. The *solid box* represents the PCR-amplified region. The probe used to search for homologs of *chsD* in the genome of *A. nidulans* is also shown by a *bar*. Abbreviations for restriction endonucleases are: B, *Bam*HI; E, *Eco*RI; EV, *Eco*RV; S, *Sal*I

presence of a single intron was deduced (Fig. 2) from the similarity of the predicted amino acid sequence of the cloned DNA to those of the other chitin synthases cloned so far and from the consensus intron sequence for Ascomycetes (Unkles 1992). Because the 5′ upstream region of the *CAL1* homolog showed low similarity to that of *CAL1*, identification of the initiation codon was impossible. Therefore cDNA fragments derived from the 5′-upstream region of the *CAL1* homolog were cloned (see Materials and methods), and sequencing of these cDNA fragments revealed that transcription begins at positions −20, −15 (Fig. 2, short arrows), or (and) a point further upstream. The context of the first ATG (+1 or +3) downstream of the −15 start point, fits the consensus for filamentous fungi (Ballance 1986) to some extent. It was concluded that this ATG functions as the initiation codon. The following additional results support this assignment. First, there is no inframe ATG which fits the consensus in the upstream region (−1756 to −1). Secondly, the deduced size of the polypeptide, 1086 amino acids, is similar to those of the other two Cal1 homologs (1099 amino acids and 1176 amino acids). Lastly, the size determined for the transcript, 3.8 kb (Fig. 3), is sufficient suitable to encode 1086 amino acids. It is therefore deduced that this *CAL1* homolog encodes a polypeptide of 1086 amino acids with a calculated molecular mass of 121.75 kDa. This gene was named *chsD*. ChsD showed high similarity to Cal1 of *S. cerevisiae* (56.7%/991 amino acids) and Chs3 of *C. albicans* (55.9%/1066 amino acids), and less similarity to chitin synthases in classes I, II, and III (for example, it shows only 21.6% similarity over 284 amino acids to the class III chitin synthase of *A. nidulans*). When the deduced amino acid sequences are compared in the region used by Mellado et al. (1995) to compare the deduced amino acid sequences of the Cal1 homologs of *A. fumigatus* with the other Cal1 homologs, ChsD showed highest similarity to ChsF of *A. fumigatus* (Figs. 4A, B).

Disruption of *chsD*

To elucidate the function of *chsD*, the gene in the strain ABPU1 was disrupted using pDΔA7 (Fig. 7A). The *chsD* disruptant D3-2 showed no difference in morphology during the asexual cycle (hyphae, septa and conidiophores) and no significant difference in cell growth from that of the parental strain ABPU1 (data not shown). Because disruption of *chsD* did not cause any decrease in chitin content (Table 2), in contrast to the case of *CAL1* disruption in *S. cerevisiae*, a *chsD* homolog was sought in the genome of *A. nidulans*. High-stringency and low-stringency Southern analyses were done on *Bam*HI-, *Eco*RI- and *Hin*dIII-digested total DNA of strain FGSC89 probed with the 3.5-kb *Bgl*II-*Sna*BI fragment of pchsD, which contains almost all of the *chsD* gene (see Fig. 1). No signals were detected under the low-stringency condition other than those found under high-stringency conditions. It thus appears that a closely related *chsD* homolog is not present in the genome of *A. nidulans*.

Double disruption of *chsA* and *chsD*

To determine whether the effects of loss of ChsD are masked by some other chitin synthase, double disruptants for *chsD* and *chsA* (DA-4 and AD-3; Fig. 5) and *chsD* and *chsC* (CD-1, 2, and CD-5; Fig. 7) were constructed. Single disruptants of *chsA* (A-4; Fig. 5) and of *chsC* (C2-11; Fig. 6) were also constructed for use as control strains.

The four double disruptants of chitin synthase genes, DA-4, AD-3, CD-1, and CD-5, showed no change in morphology during the asexual cycle (hyphae, septa, and conidiophore) and no significant difference in cell growth compared to the parental strain, ABPU1 (data not shown). Single and double disruptants of chitin synthase genes also showed no drastic decrease in chitin content (Table 2). In contrast, the double disruptant for *chsA* and *chsD*, DA4, showed a remarkable decrease in the efficiency of conidia formation, as judged by the number of conidia generated on plates (Table 3). This phenomenon was observed in another double disruptant for *chsA* and *chsD*, AD-3 (Table 3). But none of the single disruptants, A-4, D3-2, and C2-11, showed any significant decrease in the efficiency of conidia formation (Table 3). The lower conidiation efficiency of strains A-4 and D3-2, relative to the ABPU/AU strain, may be caused by the presence of a nutritional marker (*pyrG*$^-$) in these strains which is not present in the ABPU/AU strain, since ABPU1, which has this nutritional marker (*pyrG*$^-$) also produced a reduced number of conidia compared to the strain

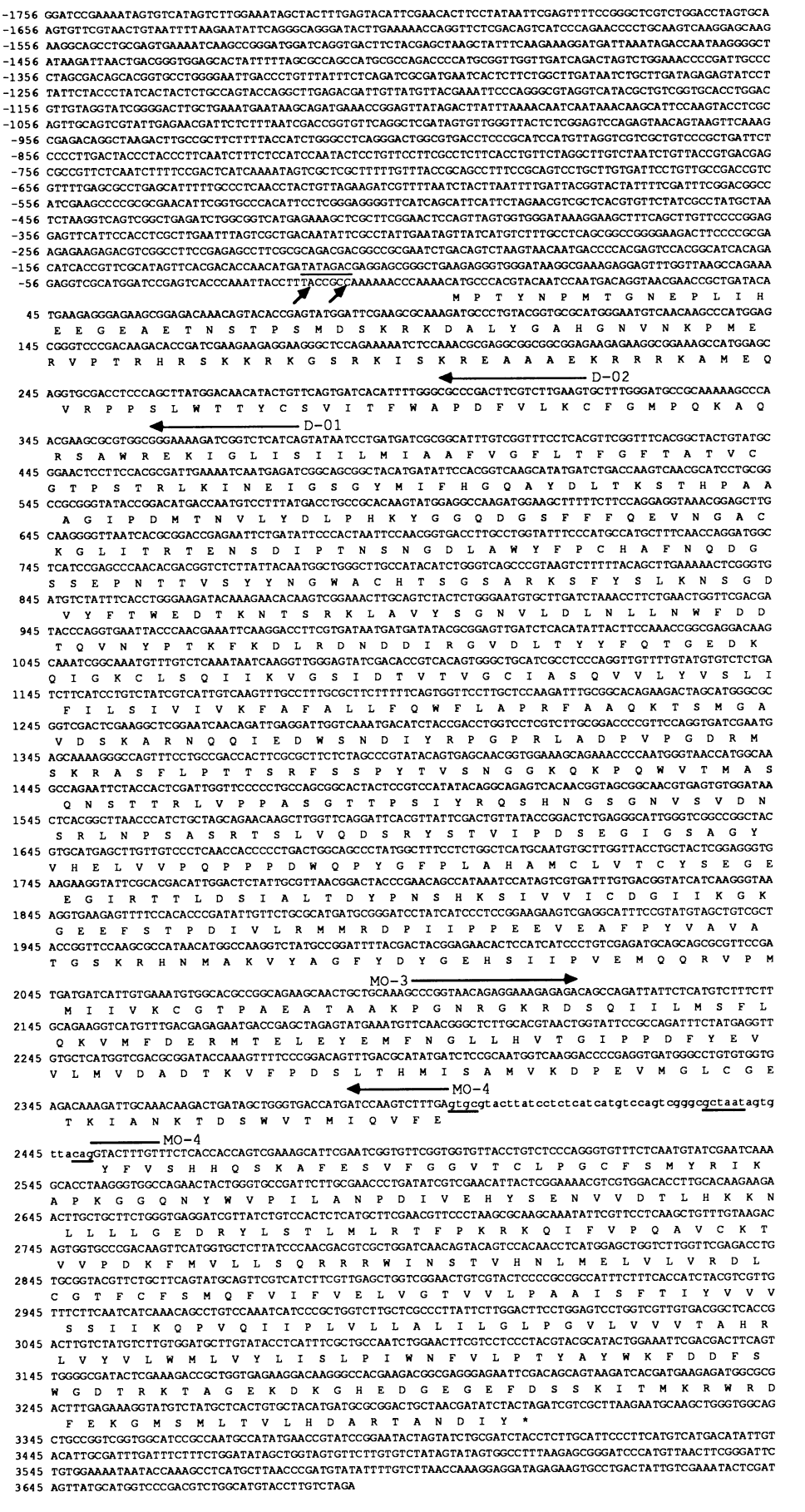

Fig. 2 Nucleotide and deduced amino acid sequences of *chsD*. Putative splicing signal sequences for introns and a putative TATA box are *underlined*. Annealing sites for PCR primers (D-02, MO-3, and MO-4) and for cDNA synthesis (D-01) are shown by *arrows*. The ends of the cDNA inserts are shown by *short arrows*. This sequence is available from DDBJ/EMBL/GenBank under the accession number D83246

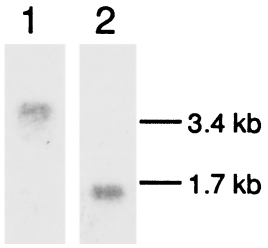

Fig. 3 Northern blot analysis of *chsD* expression in *A. nidulans*. Approximately 4 μg of poly(A)$^+$ RNA from the FGSC89 strain was probed with the 0.8 kb *Bam*HI-*Eco*RI fragment of *chsD* (lane 1, the position of this fragment is shown in Fig. 1B) or the 1.8 kb *Bam*HI-*Sph*I fragment of the *argB* gene of *A. nidulans* from pSS1 (lane 2) as a positive control

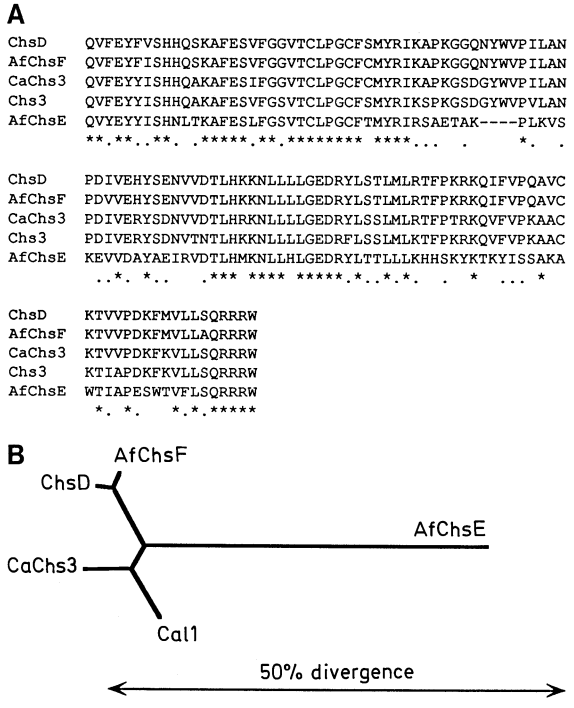

Fig. 4A Alignment of the predicted amino acid sequences of chitin synthases. Alignment was done by the Clustal V program (Higgins et al. 1992). *Dots* indicate similarities; *asterisks* indicate identities between the sequences. **B** Phylogenetic tree of Cal1 homologs constructed by the neighbor-joining method (Saitou and Nei 1987). AfChsE-F, chitin synthase E-F of *A. fumigatus*; CaChs3, chitin synthase 3 of *C. albicans*; ChsD, chitin synthase D of *A. nidulans*

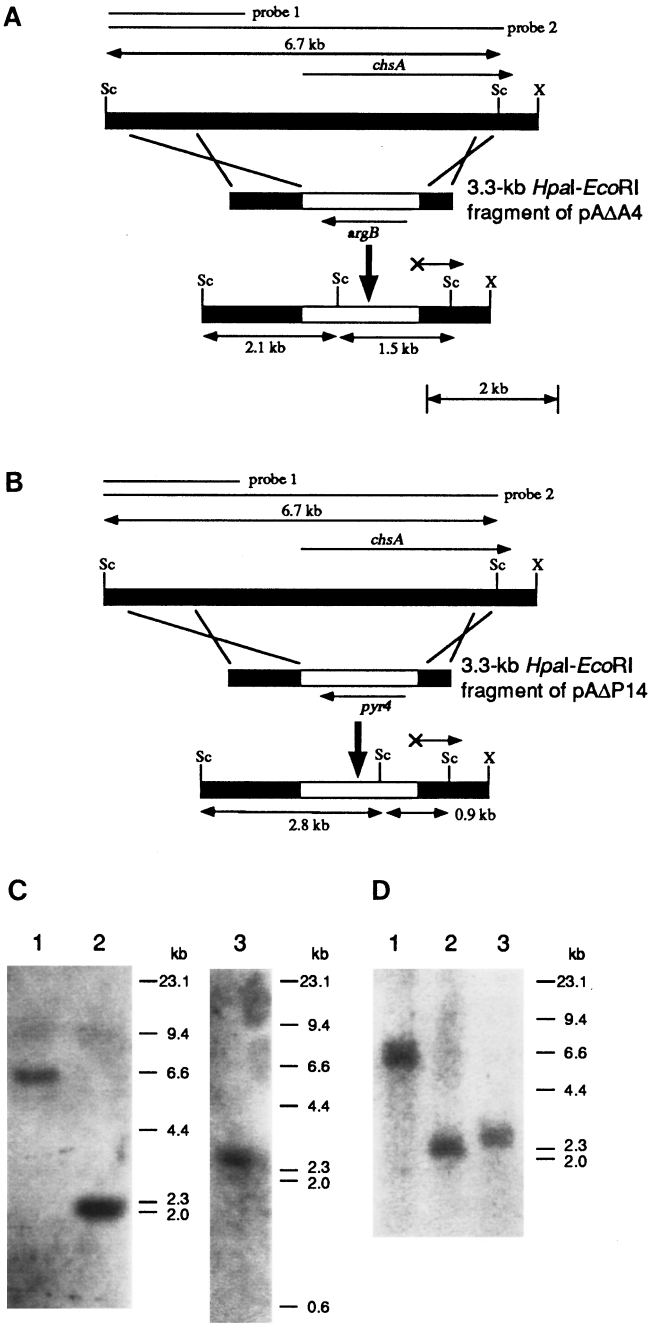

Fig. 5A–D Disruption of *chsA*. **A** Disruption of *chsA* by pAΔA4. **B** Disruption of *chsA* by pAΔP14. The position and direction of the predicted open reading frames for *chsA*, *argB*, and *pyr4* are indicated by *arrows*. The regions used as probes for Southern analyses are shown as *bars*. Abbreviations: Sc, *Sac*I; X, *Xba*I. **C**, **D**. Southern blot analysis of *Sac*I-digested total DNA of strain ABPU1 (**C** and **D**, lanes 1), strain A-4 (**C** and **D**, lanes 2), and strain DA-4 (**C** and **D**, lanes 3) probed with probes 1 (**C**) and 2 (**D**)

ABPU/AU (data not shown). We conclude that ChsA and ChsD of *A. nidulans* have redundant functions in conidia formation.

Discussion

We have cloned a *CAL1* homolog, *chsD*, from the genome of *A. nidulans* and studied its function. Based on the deduced amino acid sequence, ChsD of *A. nidulans* is thought to be a homolog of ChsF of *A. fumigatus* (Fig. 4). We have isolated from *A. nidulans* three other chitin synthase genes, *chsA*, *chsB*, and *chsC* (Yanai et al. 1994; Motoyama et al. 1994a). Using the gene disruption technique, we have characterized the

Table 2 Carbohydrate composition of hyphal cell walls of *A. nidulans*

Strain	Relevant genotype	% Dry weight of purified walls	
		Glucose	GlcNAc
ABPU/AU	Wild type	47.3	20.6
A-4	Δ*chsA*	39.0	22.0
C2-11	Δ*chsC*	38.5	21.6
D3-2	Δ*chsD*	34.3	18.5
DA-4	Δ*chsA* Δ*chsD*	41.5	14.1
CD-1	Δ*chsC* Δ*chsD*	38.5	19.7

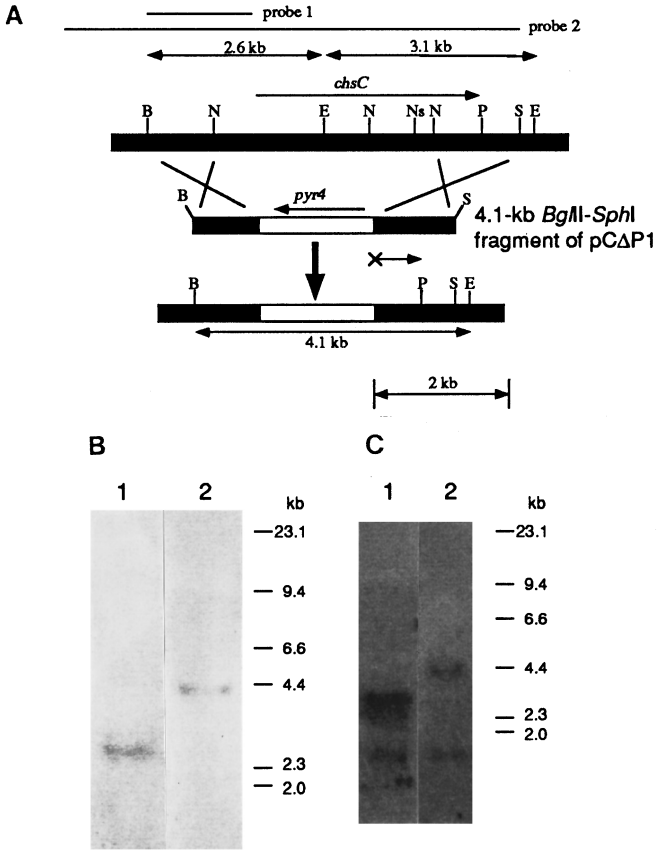

Fig. 6A–C Disruption of *chsC*. **A** Disruption of *chsC* by pCΔP1. The position and direction of the predicted open reading frames for *chsC* and *pyr4* are indicated by *arrows*. The regions used as probes for Southern analyses are shown as bars. Abbreviations: B, *Bgl*II; N, *Nco*I; E, *Eco*RI; Ns, *Nsp*7524V; P, *Pst*I; S, *Sph*I. **B**, **C** Southern blot analysis of *Bgl*II- and *Eco*RI-digested total DNA of strain ABPU1 (**B** and **C**, lanes 1) and strain C2-11 (**B** and **C**, lanes 2) probed with probes 1 (**B**) and 2 (**C**)

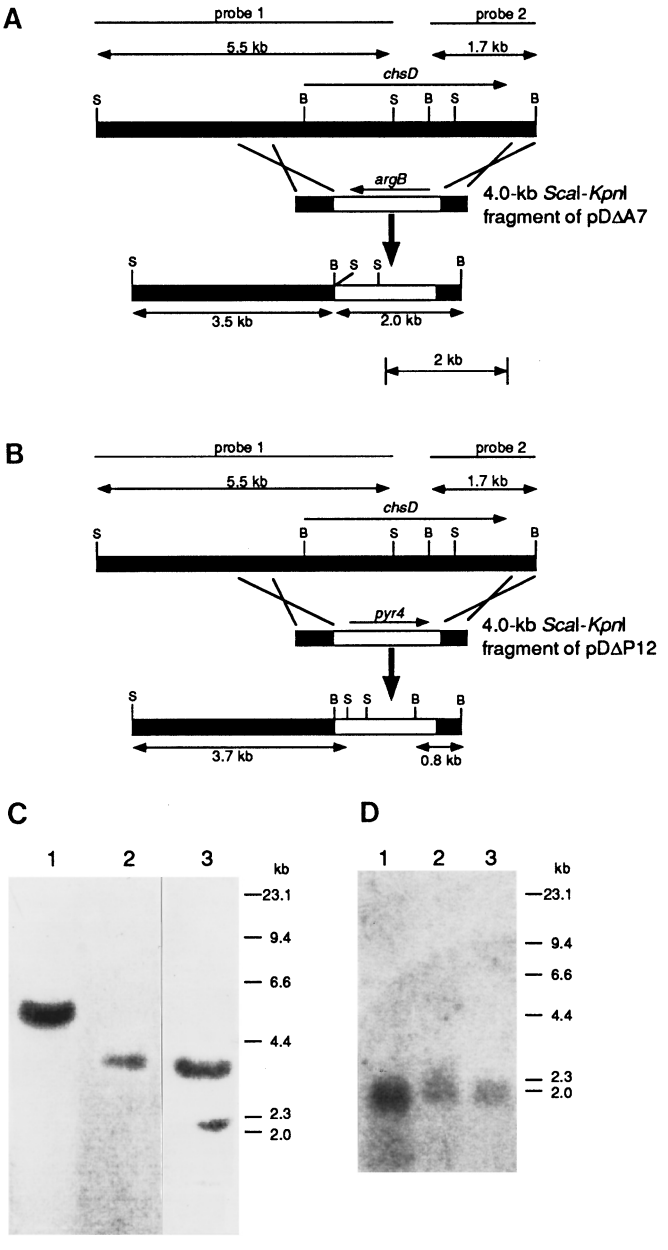

Fig. 7A–D Disruption of the *chsD* gene. **A** Disruptoin of *chsD* by pDΔA7. **B** Disruption of *chsD* by pDΔP12. The position and direction of the predicted open reading frames of *chsD*, *argB*, and *pyr4* are indicated by *arrows*. The regions used as probes for Southern analyses are shown as bars. Abbreviations: S, *Sal*I; B, *Bam*HI. **C** Southern blot analysis of *Sal*I-digested total DNA of strain ABPU1 (lane 1), strain CD-5 (lane 2), and strain D3-2 (lane 3) probed with probe 1. **D** Southern blot analysis of *Bam*HI-digested total DNA of strain ABPU1 (lane 1), strain D3-2 (lane 2), and strain CD-5 (lane 3) probed with probe 2

functions of the four chitin synthases of this organism. The results can be summarized as follows. ChsA, a class II chitin synthase, is non-essential in the asexual cycle (Yanai et al. 1994) but plays a role in conidia formation that can also be filled by ChsD. ChsB, a class III chitin synthase, is required in hyphal growth (Yanai et al. 1994). ChsC, a class I chitin synthase, is a non-essential chitin synthase and its function is unknown (Motoyama et al. 1994a). ChsD, a Cal1 homolog, is non-essential in the asexual cycle and has a function similar to that of ChsA in conidia formation.

Table 3 Conidiation defect of the Δ*chsA* Δ*chsD* mutant

Strain	Relevant genotype	No. of conidia/mm^2 $\times 10^{-4}$ (mean ± S.E.)[a]
ABPU/AU	Wild type	27 ± 3
A-4	Δ*chsA pyrG*$^-$	16 ± 2
C2-11	Δ*chsC argB*$^-$	32 ± 1
D3-2	Δ*chsD pyrG*$^-$	12 ± 1
DA-4	Δ*chsA* Δ*chsD*	2.3 ± 0.5
AD-3	Δ*chsA* Δ*chsD*	1.5 ± 0.0
CD-5	Δ*chsC* Δ*chsD*	29 ± 3

[a] Means (± S.E.) were calculated from at least three independent experiments, except for the D3-2 strain (from two experiments)

The chitin content of hyphae did not decrease in the *chsD* disruptants. This is in clear contrast to *S. cerevisiae*, in which chitin content decreases to approximately 10% of the wild-type level when a *chsD* homolog, *CAL1*, is disrupted (Shaw et al. 1991). ChsD may be required to determine the chitin content not of hyphal but of conidial cell walls. Alternatively, there may be another Cal1 homolog that serves to determine chitin content. In *A. fumigatus*, two *CAL1* homologs, *chsE* and *chsF*, were isolated (Mellado et al. 1995). Although no cross-hybridization with *chsD* of *A. nidulans* was observed under low stringency conditions in the present work, there remains the possibility that other *CAL1* homologs are present in *A. nidulans.*

In *S. cerevisiae*, disruption of *CAL1* is not lethal, but a double disruption of *CHS2* and *CAL1* is lethal (Shaw et al. 1991). In this report, we found that a double disruptant for *chsA* and *chsD* of *A. nidulans* showed a severe defect in conidiation, although each single disruptant showed no marked decrease in formation of conidia. Interestingly, both *CHS2* and *chsA* belong to the class II chitin synthase genes and *CAL1* and *chsD* show high similarity to each other (Fig. 4). Hence, it is possible that the genetic interaction between *CHS2* and *CAL1* of *S. cerevisiae* during cell growth is conserved in *A. nidulans* as a genetic interaction between *chsA* and *chsD* in conidia formation.

Because disruption of the class III chitin synthase of *A. nidulans* and of *Neurospora crassa* causes similar defects (Yanai et al. 1994; Yarden and Yanofsky 1991), there is a possibility that chitin synthases in the same class have similar functions. A class II chitin synthase gene (*chs-2*) was also isolated from *N. crassa* (Din and Yarden 1994). Because inactivation of *chs-2* caused increased sensitivity to an inhibitor of phosphatidyl choline synthesis (edifenphos), which is known to be a non-competitive inhibitor of chitin synthase in several fungal species (Binks et al. 1993), the product of *chs-2* is thought to be expressed during hyphal growth. As ChsA of *A. nidulans* is also a class II chitin synthase, it may have some function not only in conidia formation but also in hyphal growth.

The function of ChsC in *A. nidulans* is currently not known. In this report, it was shown that simultaneous deletion of *chsC* and *chsD* has no effect on the asexual cycle. There remains the possibility that ChsC may have a similar function to ChsA or ChsB.

Transcriptional regulation of chitin synthase genes has been reported in *S. cerevisiae* (Choi et al. 1994; Pammer et al. 1992), in *C. albicans* (Chen-Wu et al. 1992; Sudoh et al. 1993), and in *Rhizopus oligosporus* (Motoyama et al. 1994b). Because ChsA and ChsD have redundant functions in conidia formation, it is possible that their expression is regulated by a transcription factor required for conidiophore development. Interestingly, putative recognition sites for Br1A (Chang and Timberlake 1993) and AbaA (Andrianopoulos and Timberlake 1994) are present in the promoter regions of *chsA* (AbaA) and *chsD* (AbaA and BrlA). We are now investigating whether these genes are actually regulated by these factors.

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