

ORIGINAL PAPER

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chs-4, a class IV chitin synthase gene from *Neurospora crassa*

Received: 15 May 1995 / Accepted: 16 August 1995

Abstract In *Saccharomyces cerevisiae*, most of the cellular chitin is produced by chitin synthase III, which requires the product encoded by the *CSD2/CAL1/DIT101/KT12* gene. We have identified, isolated and structurally characterized a *CSD2/CAL1/DIT101/KT12* homologue in the filamentous ascomycete *Neurospora crassa* and have used a “reverse genetics” approach to determine its role in vivo. The yeast gene was used as a heterologous probe for the isolation of a *N. crassa* gene (designated *chs-4*) encoding a polypeptide belonging to a class of chitin synthases which we have designated class IV. The predicted polypeptide encoded by this gene is highly similar to those of *S. cerevisiae* and *Candida albicans*. *N. crassa* strains in which *chs-4* had been inactivated by the Repeat-Induced Point mutation (RIP) process grew and developed in a normal manner under standard growth conditions. However, when grown in the presence of sorbose (a carbon source which induces morphological changes accompanied by elevated chitin content), chitin levels in the *chs-4*^{RIP} strain were significantly lower than those observed in the wild type. We suggest that CHS4 may serve as an auxiliary enzyme in *N. crassa* and that, in contrast to yeasts, it is possible that filamentous fungi may have more than one class IV chitin synthase.

Key words *Neurospora* · Ascomycete · Fungal cell wall

Communicated by C. A. M. J. J. van den Hondel

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Introduction

Chitin, a polymer of $\beta(1,4)$ -linked N-acetylglucosamine (GlcNAc) is an integral component of the cell wall of many fungi. In *Saccharomyces cerevisiae*, chitin is essential for viability; three chitin synthases, each with different functions, account for the production of total cellular chitin (Bulawa 1993; Cabib et al. 1993; Choi et al. 1994). Analyses of DNA fragments from taxonomically diverse fungal species have shown that most fungi have three to six chitin synthases (Bowen et al. 1992; Mehmman et al. 1994; Mellado et al. 1995). The chitin synthase gene fragments which have significant similarity to the *CHS1* and *CHS2* genes of *S. cerevisiae* were grouped into three classes (I, II, III) by Bowen et al. (1992). It is clear that the number of classes needs to be extended to account for those genes with homology to the *CAL1/CSD2/DIT101/KT12* (also referred to as *CHS3*) gene of *S. cerevisiae* (Mellado et al. 1995). For this reason we have designated *CHS3* of *S. cerevisiae* and the homologous gene of the dimorphic yeast *Candida albicans* (*CHS3*; Sudoh et al. 1993) as class IV chitin synthase genes.

Structural and functional analyses of genes and gene products belonging to the chitin synthase classes I–III have been reported for a few filamentous fungal species. Direct involvement in hyphal growth has been demonstrated for the *chs-1* of *Neurospora crassa* (Yarden and Yanofsky 1991) and *chsB* of *Aspergillus nidulans* (Yanai et al. 1994), both of which are class III chitin synthases. In contrast to these results, description of a class III chitin synthase in *Ustilago maydis* has no effect on growth or pathogenicity (Gold and Kronstad 1994). Class I and II chitin synthases have also been analyzed in filamentous fungi, but specific roles for these enzymes have yet to be identified (Beth Din and Yarden 1994; Motoyama et al. 1994a,b; Gold and Kronstad 1994).

Most of the cellular chitin in *S. cerevisiae* (Valdivieso et al. 1991; Bulawa 1992) and *C. albicans* (C. Bulawa,

unpublished) – 90% and 80%, respectively – is produced by the class IV chitin synthase. The enzyme activity (chitin synthase III) in *S. cerevisiae*, however, is determined by three genes: *CHS3*, *CAL2/CSD4*, and *CAL3* (reviewed by Bulawa 1993; Cabib et al. 1993). *CAL2/CSD4* and *CAL3* may encode products essential for regulation or localization of the *CHS3* gene product. *CHS3* is also required for synthesis of chitin that is covalently linked to β 1,3-glucan (Kollar et al. 1995), forming the alkaline-insoluble glucan fraction of the cell wall. In *C. albicans*, only the homologous *CHS3* gene has been characterized (Sudoh et al. 1993; C. Bulawa, unpublished).

Does the class IV chitin synthase from a higher fungus synthesize the major fraction of cellular chitin? We present the first report on the characterization of a class IV chitin synthase-encoding gene in a filamentous fungus, *N. crassa*: *chs-4*. We have cloned, sequenced, and used the repeat-induced point mutation (RIP) process (Selker 1990) to inactivate the chromosomal *chs-4* locus. Strains of *N. crassa* that lack a functional CHS4 chitin synthase did not show any reduction in cell wall chitin or exhibit aberrant features under standard growth conditions. However, the cell wall chitin enrichment typically induced when the fungus is grown on sorbose was significantly impaired in the *chs-4*^{RIP} strain.

Materials and methods

Neurospora strains

Wild-type *N. crassa* strain 74-OR23-1A and *chs-2*^{RIP} (Beth Din and Yarden 1994) strains were used in this study. Cultures were maintained on 1.5% agar slants containing Vogel's minimal medium N (Vogel 1956). Procedures used in growth studies, crosses and other manipulations of *Neurospora* strains are described in Davis and de Serres (1970). DNA transformations of *N. crassa* with constructs derived from the vector pCSN43 (Staben et al. 1989) were carried out as described by Orbach et al. (1986). Chromosome mapping was done by analysis of restriction fragment length polymorphisms (RFLP) from the 'small cross' (FGSC strains 4411-4430), according to the procedure of Metzenberg et al. (1985).

Isolation and analysis of nucleic acids from *N. crassa*

Genomic DNA was purified from *N. crassa* mycelia as described by Beth Din and Yarden (1994). Southern hybridizations using nylon membranes (Hybond N⁺ from Amersham or MagnaCharge from MSI) were as described by Sambrook et al. (1989). Hexamer-labeled probes were prepared with Prime-a-gene kits (Promega) and used for Southern analyses.

Total RNA was isolated by a slight modification of the procedure of Orbach et al. (1990). Mycelia were harvested as described above. Following quick freezing in liquid nitrogen, 25 mg were transferred to a 2 ml screw cap tube (Sarstedt) containing a mixture of 0.75 ml lysis buffer [0.6 M NaCl, 10 mM EDTA, 100 mM TRIS-HCl (pH 8.0), 4% (w/v) SDS], 0.75 ml phenol and 2 g zirconium beads (Biospec Products). The samples were shaken twice for 30 s in a mini bead-beater (Biospec Products). Following a 10 min centrifugation

in a microfuge (10,000 × *g*; 4°C) the aqueous phase was transferred to a new tube and re-extracted with phenol:chloroform (1:1). Following the addition of 0.75 vol of 8 M LiCl to the supernatant, the RNA was precipitated overnight at 4°C and pelleted by centrifugation. The pellet was dissolved in 0.3 ml DEPC-treated H₂O and the RNA re-precipitated in ethanol, washed, dried, and dissolved in DEPC-treated H₂O. Samples of approximately 3 µl of 1 µg/µl poly(A)⁺ RNA (PolyA-tract kit, Promega) were added to 10.5 µl sample buffer (50% formamide, 6% formaldehyde, 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA) and heated at 65°C for 10 min. After cooling, 3.5 µl of the dye marker (50% sucrose, 0.5% bromophenol blue) was added, and the samples electrophoresed on 1% agarose gels prepared in 6% formaldehyde, 20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA and run in 20 mM MOPS, 5 mM sodium acetate and 1 mM EDTA. Northern blots were prepared by capillary transfer (Sambrook et al. 1989). Prehybridization and hybridization were carried out at 60°C in 5 × SSPE, 0.1% SDS, 2.5 × Denhardt's solution, 200 µg/ml yeast tRNA and 100 µg/ml denatured salmon sperm DNA. Standard washes (Sambrook et al. 1989) were carried out at 60–70°C. Probes were labeled with [α -³²P]UTP using phage T3 RNA polymerase and the MaxiScript kit (Ambion), as described by Sambrook et al. (1989). pACTIN (kindly provided by Michael Plamann), a *N. crassa* actin cDNA clone, was used to generate *act-1* RNA probes.

Low-stringency Southern blot and colony hybridizations

A 2.55 kb *Bsa*I restriction fragment that encodes a predicted product which spans amino acids 108-956 of the cloned *S. cerevisiae* *CHS3* gene (Bulawa 1992) was electrophoretically separated in low-melt agarose gel and excised. The fragment in 9 µl of melted agarose was labeled with [α -³²P]dCTP (New England Nuclear) using a random primer labeling kit (Boehringer Mannheim) in a total volume of 20 µl. Unincorporated nucleotides were removed by chromatography on a NICK column (Pharmacia).

DNA from colonies of *E. coli* was bound to reinforced nitrocellulose (Schleicher and Schuell) according to the manufacturer's instructions. Hybridizations were for 16 h at 42°C in 25% formamide, 1 M NaCl, 5 × Denhardt's, 10% dextran sulfate, 1% SDS, and approximately 1 × 10⁶ cpm ³²P-labelled denatured probe per ml of hybridization solution. Filters were rinsed several times, first at room temperature, then at 60°C in 2 × SSC, 1% SDS; and exposed to X-ray film.

Plasmid and cosmid libraries

Two plasmid libraries were constructed with genomic DNA from *N. crassa* strain 74-OR23-1A. The genomic DNA was digested to completion with *Eco*RI or *Hind*III and ligated to pUC18 that had been cut with the appropriate restriction enzyme and treated with calf intestine alkaline phosphatase (Boehringer-Mannheim). Standard methods were used for ligations and transformations of *E. coli* (Sambrook et al. 1989). The Orbach/Sachs *N. crassa* genomic DNA cosmid library (Fungal Genetics Stock Center) was used to isolate a cosmid clone which contains *chs-4*.

DNA sequencing and sequence comparisons

Plasmids containing *N. crassa chs-4* DNA were sequenced by the dideoxynucleotide method of Sanger et al. (1977) using the Sequenase sequencing kit (USB). Several deletions of *N. crassa* DNA were made by removal of restriction fragments contiguous to vector DNA followed by ligation and transformation of *E. coli*. Plasmids with deletions were sequenced with pUC18 sequencing primers.

Additional primers were synthesized (MIT Biopolymers Laboratory) to complete the sequence of both strands of DNA.

The deduced amino acid sequences of the *S. cerevisiae* CHS3 (Bulawa 1992), the *C. albicans* CHS3 gene (Sudoh et al. 1993) and that *N. crassa chs-4* gene products were aligned by the MEGALIGN program (DNASStar).

Fluorescent microscopy, chitin content determination, and chitin synthase activity assays

Samples were viewed with a Zeiss epifluorescence microscope. For chitin visualization, a drop of 10 µg/ml of Calcofluor (Fluorescent Brightener 28, Sigma) was applied to fungal samples prespotted on a microscope slide. The filter combination used was 380–425 nm excitation, a 430 nm dichroic mirror, and a 450 nm barrier filter.

Chitin content was determined by a slight modification of the procedure described by Bulawa et al. (1986). Mycelium that had been washed with H₂O was lyophilized; 2.5–5 mg dry weight was suspended in 1 ml of 6% KOH and heated for 90 min at 80°C. Glacial acetic acid (0.1 ml) was added, and the insoluble material was collected by centrifugation, washed twice with water, resuspended in 0.5 ml sodium phosphate buffer (pH 6.3) and sonicated. A portion of 40–100 µl of a 5 mg/ml partially purified (15% protein by weight) *Streptomyces* spp. chitinase suspension (Robbins et al. 1988) was added, and the tubes were incubated at 37°C for 2–20 h. Following centrifugation, 450 µl of the supernatant were treated with 40 µl of 50 mg/ml β-glucuronidase Type H-5 (Sigma) for 2 h at 37°C. Portions (0.1 ml) of each sample were removed and assayed for GlcNAc content (Reissig et al. 1955).

Chitin synthase was assayed in cell-free extracts prepared from germinating conidia. Germlings were washed with H₂O, resuspended in 100 mM HEPES (pH 7.8) buffer, and disrupted for 1 min in an Ultraturax homogenizer. The homogenate was passed once through a French press and centrifuged for 12 min at 10000 × *g* at 4°C. The 10000 × *g* supernatant was further centrifuged at 100000 × *g* for 40 min (4°C). The pellet contained the fungal microsomal fraction and was used for chitin synthase assays. Protein concentration was determined by the method of Sedmak and Grossbery (1977). All protein preparations and chitin synthase assays were repeated at least twice.

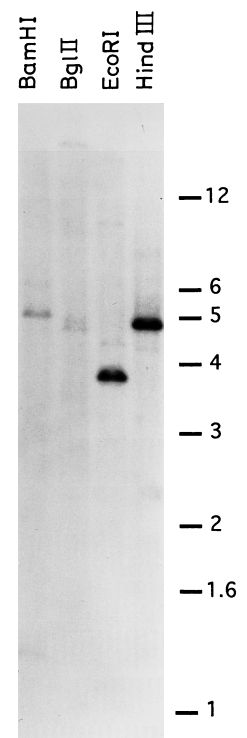
Chitin synthase activity was assayed in 25 µl reaction mixtures containing 100 mM HEPES pH 7.8, 1 mM MgCl₂, 32 mM GlcNAc, 1 mM UDP-GlcNAc and supplemented with 1.5 × 10⁻⁷ mM UDP[1-¹⁴C]GlcNAc (300 mCi/mMol; (Amersham), providing approximately 1 × 10⁵ dpm per mixture). Portions of the fungal cell-free extract used in each reaction mix contained 80 µg protein. Cell-free extracts were trypsin activated; 2 µl of a 0.2 mg/ml trypsin (EC 3.4.21.4, Sigma) solution was added to the extracts, which were then incubated for 15 min at 30°C. Soybean trypsin inhibitor was then added (2 µl of a 0.3 mg/ml solution) to each reaction tube prior to the addition of the chitin synthase assay cocktail. Chitin synthase assay mixtures were incubated at 30°C and reactions were terminated by the addition of 25 µl of glacial acetic acid. Reaction products were separated by paper chromatography using Whatman No. 1 paper and 4:1 ethanol:1 M acetic acid (v/v) as solvent. Air-dried chromatograms were scanned and analyzed with a Fuji Bioimaging BAS1000 analyser.

Results

Cloning and structural analysis of the *N. crassa chs-4* gene

The cloning of an *N. crassa* chitin synthase gene homologous to the *Saccharomyces* CHS3 gene was accomplished by Southern and colony hybridizations under

Fig. 1 Detection of the *Neurospora crassa chs-4* gene by Southern hybridization at low stringency. The 2.55 kb *Bsa*BI fragment from the *Saccharomyces cerevisiae* CAL1/CSD2 gene was used to probe *N. crassa* genomic DNA digested with the restriction enzymes indicated. Size standards are given in kb



conditions of low stringency. A fragment of DNA from the CHS3 gene hybridized to a 3.75 kb *Eco*RI and 4.75 kb *Hind*III fragment in digests of *N. crassa* genomic DNA (Fig. 1). Little or no hybridization was found in *Bam*HI or *Bgl*II digests, possibly because the homologous fragments were either too small or too large for efficient detection. Two genomic libraries were constructed in pUC18 and clones that contained the *Eco*RI and *Hind*III fragments that were identified by Southern hybridizations were isolated. Restriction maps of the cloned *Eco*RI fragment (pHMS1) and *Hind*III fragment (pHMS2) overlap by about 1 kb, as shown in Fig. 2. A clone from the Orbach/Sachs

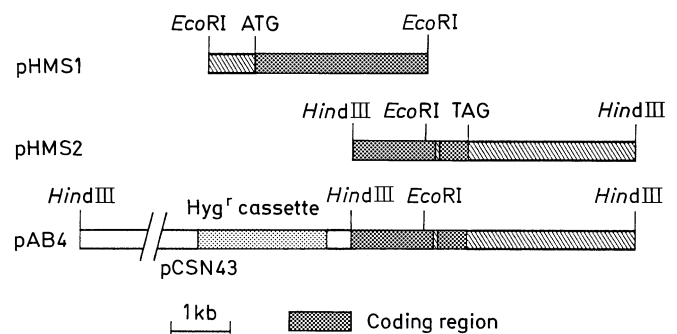
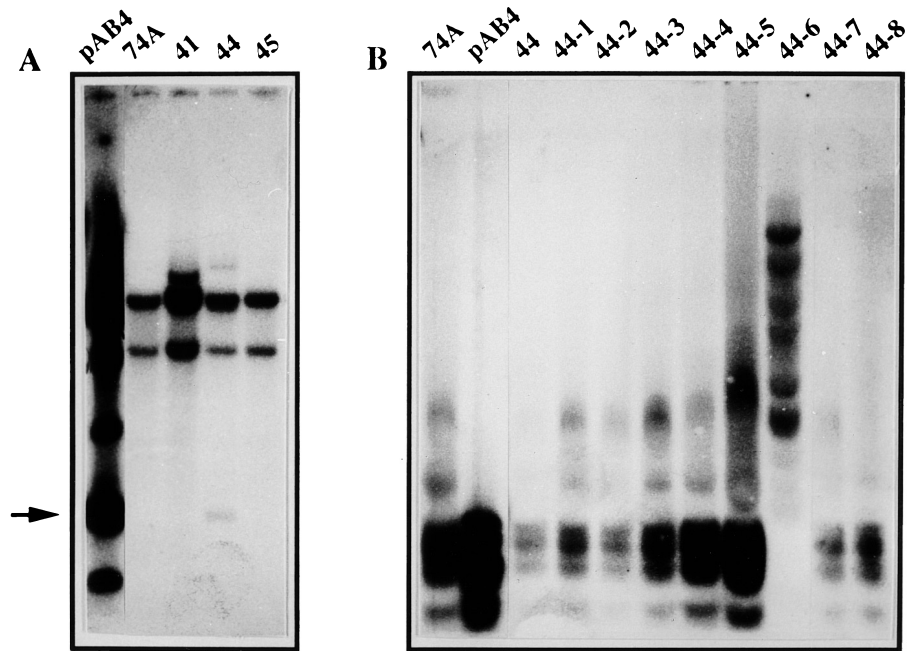


Fig. 2 Clones and constructs used in this study. pHMS1 and pHMS2 represent 3.75 kb *Eco*RI and 4.75 kb *Hind*III, respectively, *chs-4* genomic DNA fragments cloned into pUC18. The 4.75 kb *Hind*III fragment was also cloned into pCSN43, a vector carrying the hygromycin phosphotransferase gene driven by the *Aspergillus nidulans trpC* promoter region, and designated pAB4. The predicted coding regions are indicated by heavy stippling. Flanking regions and the single intron are indicated by hatching

Fig. 5A, B Molecular analysis of *chs-4* DNA in the wild type, a transformed strain, and progeny from a cross between a transformed parent and the wild type. **A** DNA samples from the construct containing the *chs-4* gene in a pCSN43 vector (pAB4), wild type (74A) and the transformed strain (44) were digested with *EcoRI*. **B** Progeny from the cross between the transformed parent and the wild type [(44-1)-(44-8)] were digested with *HpaII*. The blots were probed with the hexamer-labeled 4.7 kb *HindIII* insert from pAB4. The strain 44-6 is a *chs-4^{RIP}* derivative



control RNA probe revealed similar levels of transcript in all three RNA extracts.

The *chs-4^{RIP}* strains were examined both macroscopically and microscopically. No differences were observed between the *chs-4^{RIP}* progeny and wild-type progeny with respect to germination rate, hyphal elongation, conidiation, or hyphal and conidial morphology. In addition, protoperithecium formation and completion of the sexual phase of the life cycle were not affected when *chs-4^{RIP}* strains were mated with the wild-type strain. Cells of the *chs-4^{RIP}* strain (progeny 44-6) were stained with Calcofluor white (a dye which stains $\beta(1,4)$ -linked polysaccharides) and observed by

fluorescent microscopy. No differences in fluorescent intensities of crosswalls or conidial septa, of which chitin is the major component (Hunsley and Gooday 1974), were observed relative to wild-type cells.

Chitin synthesis in the *chs-4^{RIP}* strain

Chitin levels were measured directly in the wild-type and *chs-4^{RIP}* strains. The chitin GlcNAc content of the *chs-4^{RIP}* and wild-type strains were calculated to be $2.1 \pm 0.1\%$ and $2.00 \pm 0.2\%$, respectively, of total cell dry weight. These calculated GlcNAc values are in close agreement with the *N. crassa* wild-type mycelial GlcNAc content reported by Mahadevan and Tatum (1965) and Schmidt et al. (1975). Similar results were obtained in several experiments in which various incubation periods were tested. Chitin synthase activity was measured in cell-free extracts of the wild-type and *chs-4^{RIP}* strains. No significant differences in the incorporation of the labeled precursor could be detected (data not shown).

We analyzed chitin content and chitin synthase activity of *N. crassa* wild-type, *chs-2^{RIP}* and *chs-4^{RIP}* strains grown on sucrose and sorbose. Cultures grown on sorbose have approximately twice as much extractable GlcNAc as cultures grown on sucrose (Burnett 1979). Sorbose is known to shorten the hyphal cell length, increase branching, and induce localized bulges in the walls of *N. crassa* mycelium. Significant differences in chitin content were observed in the cell wall fractions of the different strains (Fig. 7). In contrast to the wild-type and the *chs-2^{RIP}* strains, in which sorbose increased chitin content in the cell wall by approximately 70–80%, the chitin content in the *chs-4^{RIP}* strain

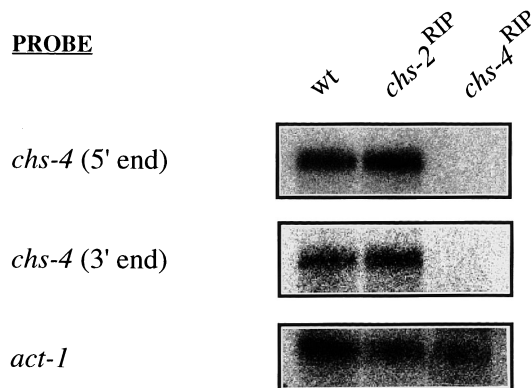


Fig. 6 Expression of *chs-4*. Poly(A)⁺ RNA from 5 h-old germinating conidia from wild-type, *chs-2^{RIP}* and *chs-4^{RIP}* strains was resolved on an agarose gel and blotted onto a nylon membrane. The blot was probed with the *EcoRI* fragment of pHMS1 (5' end of *chs-4*), the *HindIII* fragment of pHMS2 (3' end of *chs-4*) and actin (*act-1*) control RNA probes

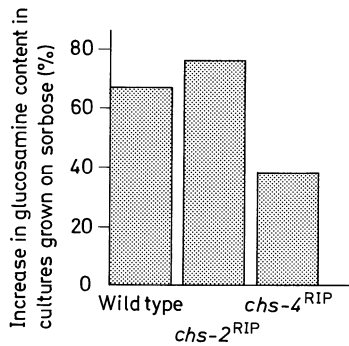


Fig. 7 Increase in chitin glucosamine content (expressed as a percentage of sugar determined by Morgan-Elson assay) measured in cultures of wild type, *chs-2^{RIP}* and *chs-4^{RIP}* grown on sorbose compared to cultures grown on sucrose as carbon source

samples increased by only 30–40%. These relative differences were observed in several experiments. Thus, it is conceivable that CHS4 may be involved in supplementing chitin synthesis under various growth conditions.

Discussion

In this study we present the first functional analysis of a class IV (formerly known as non-zymogen, trypsin-independent or complex-type) chitin synthase in a filamentous fungus. We have cloned, mapped and sequenced the *chs-4* gene of *N. crassa* and have determined that it is homologous to the *CHS3* genes of *S. cerevisiae* and *C. albicans*. Searches of the non-redundant NCBI databases confirmed that the sequence of *N. crassa chs-4* has greatest homology to both the nucleotide and deduced amino acid sequences of the *S. cerevisiae* and *C. albicans CHS3* genes and their products. Two conserved regions were identified for class IV chitin synthases (Fig. 3). The C-terminal region has homology to other chitin synthases and contains the putative catalytic domain. The N-terminal conserved region, whose function remains to be determined, is unique to class IV chitin synthases.

By utilizing the RIP process we inactivated the *chs-4* gene. This was verified both by Southern analyses of progeny in which one parental strain carried a partial duplication of *chs-4*, as well as Northern analyses, which verified the lack of a *chs-4* transcript.

In contrast to the consequences of *CSD2* gene disruption in *S. cerevisiae*, inactivation of the *N. crassa* gene had no observable effect on growth, development or reproduction of the fungus when grown under normal laboratory conditions. Fluorescent microscopy with calcofluor staining provided evidence for the apparently normal distribution of chitin in hyphae and the chitin-rich crosswalls and conidial septa. In addition,

alkali-insoluble sugar levels in the *chs-4^{RIP}* cell wall were not significantly different from those found in the wild type. These results were unexpected, as the yeast *CSD2* gene has been shown to be involved in formation of the chitin ring laid down at early budding (Shaw et al. 1991), and *CHS3* inactivation results in a drastic reduction in cell wall chitin (Bulawa 1992; Pammer et al. 1991; Shaw et al. 1991). The lack of an observable phenotype in the *chs-4^{RIP}* strain could be attributed to several possibilities (or their combination). The standard laboratory growth regime does not represent the natural conditions in which the *CHS4* polypeptide is required and therefore, *CHS4* activity may be crucial under certain conditions (not tested here). Growth conditions have been shown to affect morphology and cell wall chitin production in *N. crassa* (Burnett 1979) and when such conditions (shift from sucrose to sorbose) were applied, a significant reduction in the ability of the *chs-4^{RIP}* strain to accumulate cell wall chitin was observed. Thus, *CHS4* activity is probably linked with changes in environmental conditions and may serve as an auxiliary enzyme when additional chitin is necessary. However, it should be noted that the reduction in cell wall chitin in the *chs-4^{RIP}* strain was not accompanied by any observable morphological consequence, suggesting that at least some of the cell wall chitin is dispensable in *N. crassa*.

It is possible that even though the predicted *CHS4* polypeptide is highly similar to *CHS3*, their functions may not be similar or complementary, which would explain the lack of similar mutant phenotypes. Such a situation has been reported for the *C. albicans CHS1* gene, which could not correct the defects in a *S. cerevisiae* strain in which the structurally related *CHS2* gene had been deleted (Chen-Wu et al. 1992).

Other homologues of *chs-4* may be present in *N. crassa*. To date, chitin synthase gene families have been shown to be larger in filamentous fungi than those found in yeasts. In *N. crassa*, four classes of chitin synthase genes have been identified (including *chs-4*). Recently, Mellado et al. (1995) have found that *Aspergillus fumigatus* has as many as six genes encoding for various chitin synthases. Thus, it is conceivable that other *N. crassa* chitin synthases may either be the functional homologues of *CHS3*, or compensate for the lack of *CHS4* activity in the *chs-4^{RIP}* strain. This possibility is supported by the presence of two genes (*chsE* and *chsF*) in *A. fumigatus* (Mellado et al. 1995) and in *A. nidulans* (C. Specht and C. Bulawa, unpublished data) with homology to the class IV chitin synthases. Thus, even though no clear evidence for the presence of a similar DNA fragment was obtained in low-stringency Southern hybridizations of *N. crassa* genomic DNA with the *S. cerevisiae CHS3* gene, it is still possible that an additional class IV gene that codes for a chitin synthase responsible for the synthesis of a substantial amount of chitin is present in this organism (yet is

sufficiently divergent in DNA sequence to avoid detection by the conditions tested in this study). Alternatively, a gene belonging to yet another class of chitin synthases may be involved in crosswall or conidial septa chitin deposition.

It is possible that under the present study conditions, compensation for single chitin synthase gene inactivation events occurs. If this is the case, masking of the role of a specific chitin synthase in strains where single (or more) chitin synthase genes have been inactivated can be anticipated. Circumventing this possible difficulty requires the identification of all the members of the gene family and generation of multiple mutant strains.

As information concerning the structure and function of fungal chitin synthases accumulates, it is becoming clear that the differences in cell wall architecture which accompany development are orchestrated by a multigene family and that the chitin synthase gene family is apparently larger and more difficult to dissect in filamentous fungi than in yeast.

Acknowledgments We thank M. Plamann (Texas A&M University) for providing the *act-1* clone and C. E. Bulawa for communicating results prior to publication. This research was supported by the Wolfson Research Awards administered by the Israel Academy of Sciences and Humanities and the National Institutes of Health (GM31318 to P.W.R and CA14051 to R. O. Hynes).

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