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Cloning and characterization of *chs*D, a chitin synthase-like gene of *Aspergillus fumigatus*

Emilia Mellado [a,1], Charles A. Specht [b], Phillips W. Robbins [b], David W. Holden [a,*]

[a] *Department of Infectious Diseases and Bacteriology, Royal Postgraduate Medical School, Hammersmith Hospital, Du Cane Road, London W12 ONN, UK*

[b] *Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, MA 02139, USA*

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Abstract

A chitin synthase-like gene (*chs*D) was isolated from an *Aspergillus fumigatus* genomic DNA library. Comparisons with the predicted amino acid sequence from *chs*D reveals low but significant similarity to chitin synthases, to other *N*-acetylglucosaminyltransferases (NodC from *Rhizopus* spp., HasA from *Streptococcus* spp. and DG42 from vertebrates. A *chs*D$^-$ mutant strain constructed by gene disruption has a 20% reduction in total mycelial chitin content; however, no differences between the wild-type strain and the *chs*D$^-$ strain were found with respect to morphology, chitin synthase activity or virulence in a neutropenic murine model of aspergillosis. The results show that the *chs*D product has an important but inessential role in the synthesis of chitin in *A. fumigatus*.

Keywords: Chitin synthase; Chitin; *Aspergillus*; Mutation

1. Introduction

Chitin is a major constituent of the cell walls of filamentous fungi and its synthesis is of interest in studies of cell morphogenesis and for the design of antifungal drugs [1]. There are two types of chitin synthases (CHSs): one is a zymogen that is activated in vitro by trypsin treatment, while the other type does not require proteolytic activation. The zymogen form has been further subdivided into three classes based on sequence similarity [2]. Biochemical studies on the biosynthesis of chitin in the opportunistic pathogen *Aspergillus fumigatus* have shown that both zymogen and non-zymogen types of enzyme are present, the non-zymogen type having greater activity [3]. In a previous report we described six different genes, designated *chs*A, *chs*B, *chs*C, *chs*D, *chs*E, and *chs*F, which encode CHS-like enzymes in *A. fumigatus* [4]. Two genes which encode class III zymogen type enzymes were inactivated by gene disruption to investigate their functions: *chs*C$^-$ strains have no obvious phenotypic defects and are not reduced in CHS activity or chitin content, but *chs*G$^-$

* Corresponding author. Tel.: +44 (0181) 383 3487; Fax: +44 (0181) 383 3394; E-mail: dholden@rpms.ac.uk

[1] Present address: Unidad de Micologia, Centro Nacional de Microbiologia, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain.

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strains produce highly branched hyphae and have reduced CHS activity [5]. In this paper we report the nucleotide sequence of *chs*D, the construction of *chs*D$^-$ mutants by gene disruption and their phenotypic characterization.

2. Materials and methods

2.1. Strains and growth media

A. fumigatus strain 237 was originally cultured from an open lung biopsy material from a patient with invasive pulmonary aspergillosis at Hope Hospital, Manchester, UK (obtained as a gift from M. Keaney). The fungus was grown at 37°C in either minimal medium [5] containing 1% glucose, and 5 mM ammonium tartrate, or GYEP (2% glucose, 0.3% yeast extract, 1% peptone). When appropriate, the media were supplemented with 1 M sorbitol or hygromycin B at the appropriate concentration [6]. The drug was filter sterilised and added to the media at 50°C. *Escherichia coli* DH5α was grown in Luria-Bertani (LB) medium [10] for propagation of plasmids, for cloning experiments and for DNA sequencing.

2.2. Molecular cloning and DNA sequencing

The genomic copy of the *chs*D gene was obtained by probing a λ library of genomic DNA from *A. fumigatus* strain 237 as described previously [4]. Phage DNAs were digested with different restriction enzymes, separated on 1% agarose gels and fragments of the appropriate size were excised, electroeluted and purified by passage through elutip-D columns (Schleicher and Schuell). The fragments were then ligated into pUC18. Both strands of insert DNAs of recombinant plasmids were sequenced by the dideoxy chain termination method [8] using Sequenase, Version 2 (US Biochemical Corp.) following the manufacturer's instructions for the sequencing of double-stranded DNA. The deduced amino acid sequence of *chs*D was obtained and analysed using the MacVector 3.5 software package run on a Macintosh LC II computer. Protein similarities were identified and analysed by comparison with those the databases using the FASTA [9] and BESTFIT [10] programs on the network service at the Human Genome Mapping Project Resource Centre, Hinxton, UK. The CLUSTAL [11] program was used to establish regions of amino acid identity shared between different proteins.

2.3. Inactivation of chsD

Plasmid pID97 was constructed for the inactivation of *chs*D. To create this plasmid, part of the polycloning site of pUC18 was deleted by digestion with *Sma*I and *Bam*HI, blunt ending with T_4 DNA polymerase (Gibco-BRL) and religation. A 1.8 kb *Eco*RI fragment comprising most of the coding region of *chs*D was ligated into the *Eco*RI site of this pUC18 derivative. A *Sal*I/*Xho*I fragment (520 bp), which included most of the region encoding the conserved portion of CHSD and related proteins [12], was deleted from the insert DNA and replaced with the 2.4 kb *Sal*I fragment containing the hygromycin phosphotransferase (*hph*) gene cassette [7] to create pID97. A 3.0 kb fragment of pID97, comprising the 2.4 kb *hph* gene cassette flanked by 300 bp of *chs*D sequence on both sides, was amplified by PCR using the primers Dis1 (5′-ATCCACAGAGCATAGACG-3′) and CalC (5′-TTCTCATCCCCTCTGGAAC-3′) which correspond to nucleotides 497–514, and 1589–1607, respectively, as shown in Fig. 2. This linear fragment was used to transform protoplasts of strain 237 as previously described [6].

2.4. Nucleic acid isolation and hybridisation analysis

DNA was isolated from *A. fumigatus* using a rapid extraction procedure [5]. Southern hybridisation analysis was performed as previously described [5]. Probe 1 for detection of homologous integration of *chs*D consisted of a 1.8 kb *Eco*RI/*Hin*dIII fragment obtained by restriction digestion of a plasmid containing *chs*D (Fig. 1). Probe 2 for confirmation of *chs*D inactivation consisted of a 520 bp *Sal*I/*Xho*I fragment of *chs*D (Fig. 1). Restriction fragments were fractionated in 0.7% low melting point agarose gels, and the desired fragments were excised for labelling. A Radprime DNA Labelling System (Gibco-BRL) was used to label DNA probes according to the manufacturer's instructions.

2.5. Chitin content and CHS determinations

The mycelial chitin content of strain 237 and the *chs*D$^-$ strain was measured on three separate occasions by determining the amount of *N*-acetylglucosamine after digestion of the cell wall with chitinase [5]. CHS activity was measured, on three separate occasions, in isolated membranes from fungal strains by the incorporation of UDP-*N*-acetylglucosamine into insoluble chitin, as previously described [5].

2.6. Pathogenicity of the mutant strain

The pathogenicity of the *chs*D$^-$ strain was assessed in neutropenic mice as previously described [6]. Each experimental group consisted of 8 mice inoculated with 5×10^3 spores of the *chs*D$^-$ strain or strain 237. Mice were observed for 15 days, mortality

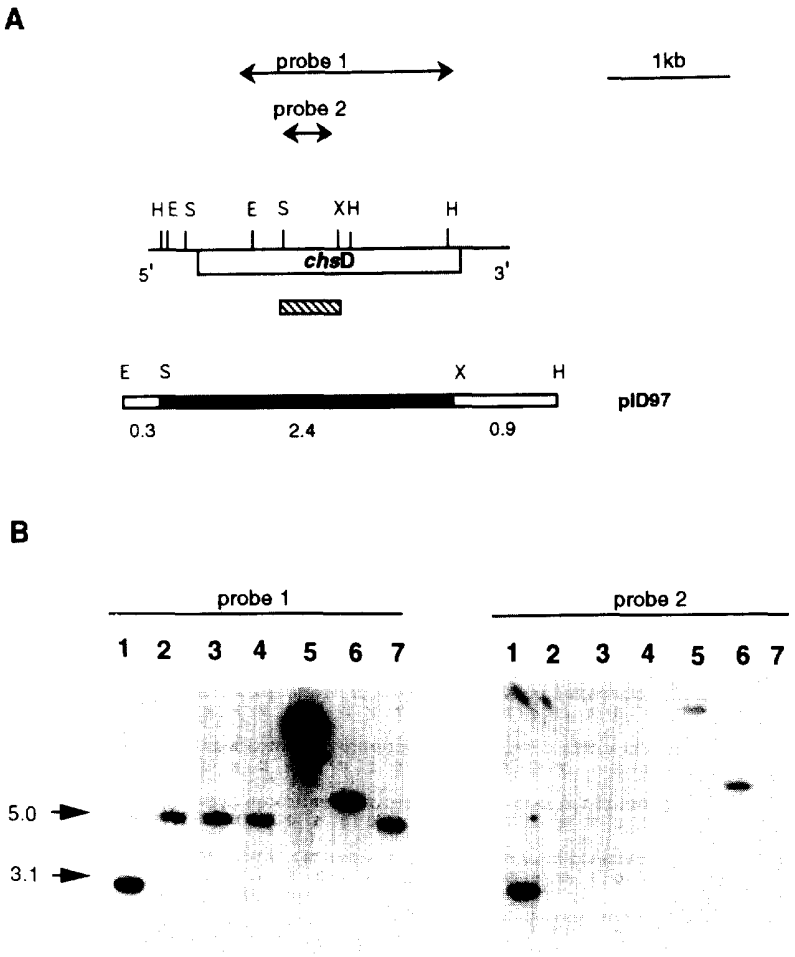
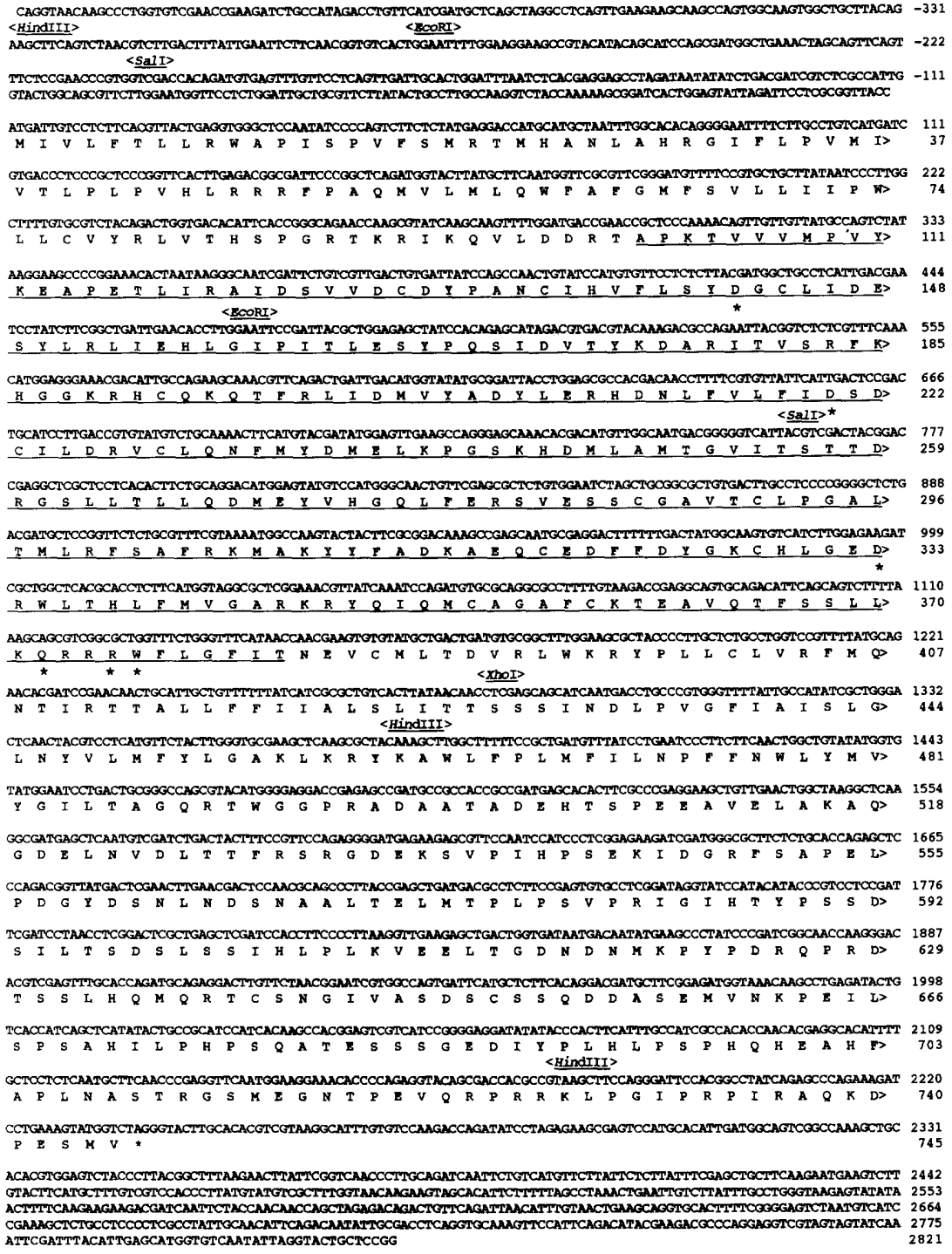


Fig. 1. (A) Restriction map of *A. fumigatus chs*D gene and the plasmid insert from pID97 used for creating the mutant strain. Unfilled boxes indicate coding regions of *chs*D. Black boxes indicate the *hph* gene encoding hygromycin resistance and the hatched box represents the region deleted in the construction of pID97. Probes 1 and 2 were used for Southern analysis and probe 2 was used to isolate *chs*D from a λ DNA library. Sizes of flanking DNA fragments are indicated in kb. Sites for restriction enzymes: E, *Eco*RI; H, *Hin*dIII; S, *Sal*I; X, *Xho*I. (B) Southern hybridisation analysis of genomic DNA of strain 237 (lanes 1) and six transformants (lanes 2–7) after digestion with *Bgl*II. (1) The Southern blot was hybridised with probe 1 in (A). (2) The Southern blot was hybridised with probe 2 in (A). Sizes of hybridising fragments in kb are shown at the side.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<SalI>
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ATGATTGTCCTCTTCACGTTACTGAGGTGGGCTCCAATATCCCCAGTCTTCTCTATGAGGACCATGCATGCTAATTTGGCACACAGGGGAATTTTCTTGCCTGTCATGATC 111
M I V L F T L L R W A P I S P V F S M R T M H A N L A H R G I F L P V M I> 37
GTGACCCTCCCGCTCCCGGTTCACTTGAGACGGCGATTCCCGGCTCAGATGGTACTTATGCTTCAATGGTTCGCGTTCGGGATGTTTTCCGTGCTGCTTATAATCCCTTGG 222
V T L P L P V H L R R R F P A Q M V L M L Q W F A F G M F S V L L I I P W> 74
CTTTTGTGCGTCTACAGACTGGTGACACATTCACCGGGCAGAACCAAGCGTATCAAGCAAGTTTTGGATGACCGAACCGCTCCCAAAACAGTTGTTGTTATGCCAGTCTAT 333
L L C V Y R L V T H S P G R T K R I K Q V L D D R T A P K T V V V M P V Y> 111
AAGGAAGCCCCGGAAACACTAATAAGGCAATCGATTCTGTCGTTGACTGTGATTATCCAGCCAACTGTATCCATGTGTTCCTCTCTTACGATGGCTGCCTCATTGACGAA 444
K E A P E T L I R A I D S V V D C D Y P A N C I H V F L S Y D G C L I D E> 148
<EcoRI> *
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S Y L R L I E H L G I P I T L E S Y P Q S I D V T Y K D A R I T V S R F K> 185
CATGGAGGGAAACGACATTGCCAGAAGCAAACGTTCAGACTGATTGACATGGTATATGCGGATTACCTGGAGCGCCACGACAACCTTTTCGTGTTATTCATTGACTCCGAC 666
H G G K R H C Q K Q T F R L I D M V Y A D Y L E R H D N L F V L F I D S D> 222
<SalI>*
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C I L D R V C L Q N F M Y D M E L K P G S K H D M L A M T G V I T S T T D> 259
CGAGGCTCGCTCCTCACACTTCTGCAGGACATGGAGTATGTCCATGGGCAACTGTTCGAGCGCTCTGTGGAATCTAGCTGCGGCGCTGTGACTTGCCTCCCCGGGGCTCTG 888
R G S L L T L L Q D M E Y V H G Q L F E R S V E S S C G A V T C L P G A L> 296
ACGATGCTCCGGTTCTCTGCGTTTCGTAAAATGGCCAAGTACTACTTCGCGGACAAAGCCGAGCAATGCGAGGACTTTTTTGACTATGGCAAGTGTCATCTTGGAGAAGAT 999
T M L R F S A F R K M A K Y Y F A D K A E Q C E D F F D Y G K C H L G E D> 333
*
CGCTGGCTCACGCACCTCTTCATGGTAGGCGCTCGGAAACGTTATCAAATCCAGATGTGCGCAGGCGCCTTTTGTAAGACCGAGGCAGTGCAGACATTCAGCAGTCTTTTA 1110
R W L T H L F M V G A R K R Y Q I Q M C A G A F C K T E A V Q T F S S L L> 370
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K Q R R R W F L G F I T N E V C M L T D V R L W K R Y P L L C L V R F M Q> 407
* * *
<XhoI>
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Y G I L T A G Q R T W G G P R A D A A T A D E H T S P E E A V E L A K A Q> 518
GGCGATGAGCTCAATGTCGATCTGACTACTTTCCGTTCCAGAGGGGATGAGAAGAGCGTTCCAATCCATCCCTCGGAGAAGATCGATGGGCGCTTCTCTGCACCAGAGCTC 1665
G D E L N V D L T T F R S R G D E K S V P I H P S E K I D G R F S A P E L> 555
CCAGACGGTTATGACTCGAACTTGAACGACTCCAACGCAGCCCTTACCGAGCTGATGACGCCTCTTCCGAGTGTGCCTCGGATAGGTATCCATACATACCCGTCCTCCGAT 1776
P D G Y D S N L N D S N A A L T E L M T P L P S V P R I G I H T Y P S S D> 592
TCGATCCTAACCTCGGACTCGCTGAGCTCGATCCACCTTCCCCTTAAGGTTGAAGAGCTGACTGGTGATAATGACAATATGAAGCCCTATCCCGATCGGCAACCAAGGGAC 1887
S I L T S D S L S S I H L P L K V E E L T G D N D N M K P Y P D R Q P R D> 629
ACGTCGAGTTTGCACCAGATGCAGAGGACTTGTTCTAACGGAATCGTGGCCAGTGATTCATGCTCTTCACAGGACGATGCTTCGGAGATGGTAAACAAGCCTGAGATACTG 1998
T S S L H Q M Q R T C S N G I V A S D S C S S Q D D A S E M V N K P E I L> 666
TCACCATCAGCTCATATACTGCCGCATCCATCACAAGCCACGGAGTCGTCATCCGGGGAGGATATATACCCACTTCATTTGCCATCGCCACACCAACACGAGGCACATTTT 2109
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CCTGAAAGTATGGTCTAGGGTACTTGCACACGTCGTAAGGCATTTGTGTCCAAGACCAGATATCCTAGAGAAGCGAGTCCATGCACATTGATGGCAGTCGGCCAAAGCTGC 2331
P E S M V * 745
ACACGTGGAGTCTACCCTTACGGCTTTAAGAACTTATTCGGTCAACCCTTGCAGATCAATTCTGTCATGTTCTTATTCTCTTATTTCGAGCTGCTTCAAGAATGAAGTCTT 2442
GTACTTCATGCTTTGTCGTCCACCCTTATGTATGTCGCTTTGGTAACAAGAAGTAGCACATTCTTTTTAGCCTAAACTGAATTGTCTTATTTGCCTGGGTAAGAGTATATA 2553
ACTTTTCAAGAAGAAGACGATCAATTCTACCAACAACCAGCTAGAGACAGACTGTTCAGATTAACATTTGTAACTGAAGCAGGTGCACTTTTCGGGGAGTCTAATGTCATC 2664
CGAAAGCTCTGCCTCCCCTCGCCTATTGCAACATTCAGACAATATTGCGACCTCAGGTGCAAAGTTCCATTCAGACATACGAAGACGCCCAGGAGGTCGTAGTAGTATCAA 2775
ATTCGATTTACATTGAGCATGGTGTCAATATTAGGTACTGCTCCGG 2821

Fig. 2. Nucleotide and deduced amino acid sequences of the *A. fumigatus chs*D gene (Genbank accession no. U62614). Amino acids in the conserved region of all CHSs and related genes are underlined. The position of restriction sites shown in Fig. 1 are also indicated. Conserved residues of β-glycosyl transferases [12] are indicated below by asterisks.
←

was recorded and the lungs of each mouse were dissected, homogenised and plated onto Sabouraud medium (Oxoid) to confirm recovery of each of the inoculated strains.

3. Results and discussion

3.1. Cloning and sequencing of the A. fumigatus chsD gene

In a previous report we described the isolation, by polymerase chain reaction (PCR) amplification, of a fragment of the *chs*D gene of *A. fumigatus*. We also showed that the gene is present as single copy in the genome and that it is expressed in actively dividing cells [4]. The full genomic copy of *chs*D was isolated from a λ library of genomic DNA of *A. fumigatus* by using the PCR fragment as a probe. The DNAs from two λ phages containing overlapping inserts were digested with restriction enzymes and fragments hybridising to the PCR probe were subcloned into pUC18. Different plasmid subclones with inserts that hybridised to the PCR probe were used to determine the nucleotide sequence of *chs*D. A restriction map of *chs*D and the position of a 2235 bp open reading frame (ORF) are shown in Fig. 1A. The nucleotide sequence of *chs*D is shown in Fig. 2. The presumptive start codon region is TTACCATGAT which is consistent with the consensus sequence described for eukaryotes by Kozak [13]. The DNA region upstream of the ATG start codon was examined for potential promoter motifs: a possible transcriptional start site (PyAAG) is located at position −52 and is preceded by a pyrimidine-rich sequence [14]. The consensus TATAAA and CAAT sequences were not found, which is not unusual in filamentous fungi, but there is an AT-rich region at position −133 to −141 [14]. The translational stop codon is at position +2236. The *chs*D gene encodes a predicted polypeptide of 745 amino acids with a calculated mass of 84.1 kDa and a p*I* of 7.0.

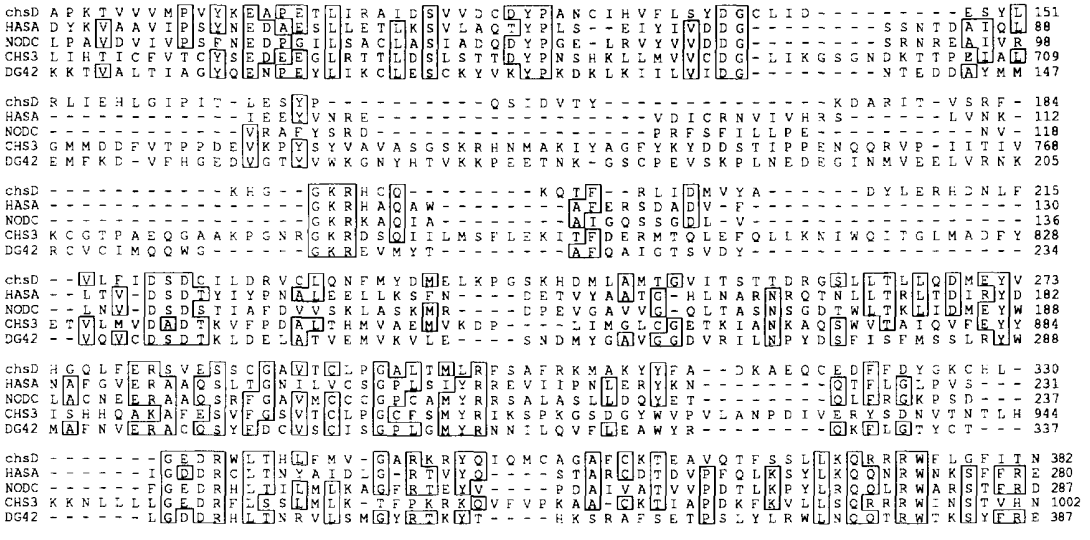

Fig. 3. Comparison of amino acid sequence of CHSD with those of related proteins. Multiple sequence comparisons were compiled using Clustal analysis [11] (PAM 250). Sequences compared to *A. fumigatus* CHSD are HASA (*Streptomyces pyogenes* [24]) NODC (*Rhizobium meliloti* [25]), CHS3 (*Saccharomyces cerevisiae* [21]) and DG42 (*Xenopus laevis* [26]) Boxed amino acids are shared by at least three sequences. Dashes in each sequence represent gaps generated by the alignment algorithm.

3.2. Amino acid comparison

A FASTA search in the Genbank database using the deduced 745 amino acids of the CHSD revealed similarity to CHSs and related enzymes including CHS3 of *Saccharomyces cerevisiae* (23.3% identity over 473 amino acids), *CHS*3 of *Candida albicans* (22.0% identity over 460 amino acids), NODC of different *Rhizobium* spp. (P23% identity over ~400 amino acids), DG42 of *Xenopus laevis* (19.8% identity over 480 amino acids) and HASA of *Streptococcus pyogenes* (26% identity over 400 amino acids). It is noteworthy that although *chs*D was originally isolated using primers specific for CHS genes, the overall level of similarity between the deduced product of *chs*D and other CHSs is relatively low. To determine the significance of these scores, the sequences were optimally aligned using BESTFIT [10]. Statistical analysis [15] revealed that these similarities are statistically significant and suggest common ancestry and/or function. In fact, all the proteins mentioned above are thought to be glycosyl transferases [12]. Although only NODC has a demonstrated *N*-acetylglucosaminyltransferase activity [16], it has also been shown that DG42 catalyses, in vitro, the synthesis of an array of chitin oligosaccharides [17], and it has been suggested that the function of HASA is the β-(1–4) transfer of *N*-acetylglucosamine to glucuronic acid during hyaluronate biosynthesis [18]. Therefore, the structural similarity of these proteins is correlated with a role in the production of β-(1–4)-linked *N*-acetylglucosamine polymers. To establish regions of identity shared among all five proteins, CHSD, HASA, NODC, DG42, and CSD2 were aligned using CLUSTAL [11] (Fig. 3). Analysis of glycosyl transferases by hydrophobic cluster analysis [12] predicts that certain conserved amino acids are involved in catalysis and processivity; these residues are also present in CHSD (Fig. 2). Furthermore, recent work concerning protein domains essential for enzyme activity of CHS2 of *S. cerevisiae*, revealed that most of these residues are essential for the catalytic activity of the enzyme [19].

Table 1
Total mycelial chitin content (nmol mg $^{-1}$ protein) of strain 237 and a *chs*D$^-$ strain

Experiment	Strain 237	*chs*D$^-$ strain
1	455	365
2	255	205
3	225	175

Data were analysed by the paired comparison test [23].
P value = 0.02.

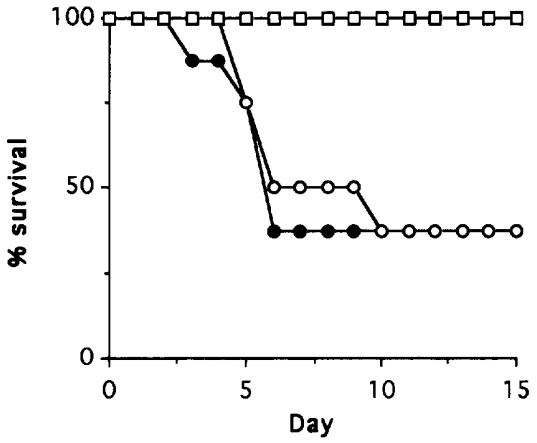

Fig. 4. Survival of groups of neutropenic mice (8 mice/group) inoculated intranasally with 10^3 spores/mouse of either strain 237 (●), and a *chs*D$^-$ strain (○). (□) Control group inoculated with saline.

3.3. Construction and characterization of a *chsD*$^-$ mutant strain

The *A. fumigatus chs*D gene was mutated by excising a 520 bp *Sal*I/*Xho*I fragment from within the coding region of the gene and replacing it with the 2.4 kb *hph* gene [5]. The mutated version was used to transform *A. fumigatus* strain 237 and hygromycin-resistant transformants that had arisen as a result of gene replacement were identified by Southern analysis (Fig. 1B). Homologous integration of the disrupted version of the gene was shown by the shift of a hybridising fragment of 3.1 kb to 5.0 kb, and the loss of a fragment corresponding to the deleted region of the gene. These transformants were designated *chs*D$^-$ (Fig. 1B).

Comparison of growth rates in different solid and liquid media and at different pH values and temperatures did not reveal any differences between *chs*D$^-$ strains and strain 237. No differences were found between strain 237 and the *chs*D$^-$ strains when CHS activity was determined in membrane fractions, either directly of after stimulation with trypsin (re-

sults not shown). However, total mycelial chitin content of a *chs*D$^-$ strain, measured by determining the amount of *N*-acetylglucosamine after digestion of the cell wall with chitinase, was reduced by 20% compared to strain 237 (Table 1). This reduction in chitin content confirms the involvement of CHSD in chitin synthesis despite the failure to detect a decrease in CHS activity (probably masked by the other CHSs activities in *A. fumigatus*). To date, only mutants defective in CHS3 or homologs of this protein have a significant reduction in chitin content [20,21], while mutants defective in the zymogen type of enzyme typically have a normal or a small reduction ($< 10\%$) in chitin content and CHS activity [5,22]. In vitro translation of the *chs*D transcript and demonstration of the ability of the protein to polymerize *N*-acetylglucosamine in vitro would provide proof that it is a CHS. Because of its lack of strong sequence similarity to other CHSs, CHSD represents a new class of CHS.

To determine if inactivation of *chs*D results in an alteration in *A. fumigatus* virulence, spores of the mutant strain and wild-type strains were inoculated into neutropenic mice. Survival curves showed no differences in virulence between these two strains (Fig. 4). Therefore, regardless of the specific role of CHSD in the *A. fumigatus* cell, it is not likely to be a useful target for antifungal drugs.

Acknowledgments

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