Synthesis *in vitro* of crystalline chitin by a solubilized enzyme from the cellulosic fungus *Saprolegnia monoica*

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Enriched preparations of chitin synthase were obtained from cell homogenates from *Saprolegnia monoica*. Chitin synthase was solubilized from a mixed membrane fraction by two successive digitonin treatments. Glycerol gradient centrifugation of the solubilized proteins separated the chitin synthase activity from the majority of proteins and from β -1,3 and β -1,4 glucan synthases. The properties of chitin synthase from this Oomycete fungus are similar to those reported for the enzymes of chitinous fungi. The solubilized enzyme catalysed the synthesis *in vitro* of spindle-like crystals of chitin. Biophysical analysis (by electron and X-ray diffractometry and infrared spectroscopy) demonstrated that the polymer synthesized *in vitro* was α -chitin. These results and those previously reported demonstrate unambiguously that chitin synthase and chitin are normal components of the cell wall of the cellulosic fungus *S. monoica*.

Introduction

In several species of Oomycetes, which have been traditionally regarded as cellulosic fungi, small quantities of hexosamines have been detected in hydrolysed cell wall materials but the cell wall components they may constitute have not been characterized (Sietsma, 1969; Aronson, 1965; Aronson & Bertke, 1987). Biophysical or cytochemical studies have revealed that chitin is present in Leptomitales (Aronson & Lin, 1978; Bertke & Aronson, 1980) and in *Achyla* (Dietrich, 1975; Campos-Takaki *et al.*, 1982). Recently we demonstrated the presence of chitin in the cell wall of *Saprolegnia monoica* by chemical extraction of the polymer, X-ray and electron diffraction patterns and infrared spectroscopy. Moreover, chitin synthesis *in vivo* was characterized in regenerating protoplasts and chitin synthase activity of cell-free extracts from hyphae was demonstrated (Bulone *et al.*, 1992). The crude enzyme preparation exhibited properties such as zymogenicity, Mg^{2+} stimulation, apparent K_m for UDP-GlcNAc and inhibition with polyoxin D similar to those reported for other fungi (Gooday, 1990).

The aim of this study was to solubilize and to partially

purify the chitin synthase from *S. monoica* and to characterize the polymer synthesized *in vitro*.

Methods

Enzyme extraction. Cultures of *Saprolegnia monoica* Pringsheim (no. 53967 Dick from CBS, Baarn, The Netherlands) were grown for 3 d in Petri dishes (14 cm diam.) containing 100 ml of the liquid medium of Machlis (1953). The mycelium was collected and homogenized at 4 °C in extraction buffer (10 mM-Tris/HCl pH 7.4; 500 mM-sorbitol) with a blade homogenizer (Virtis 45) for three 30 s periods. The homogenate was centrifuged at 2000 *g* for 10 min and the supernatant was then centrifuged at 48000 *g* for 30 min at 4 °C. The mixed membrane fraction (48000 *g* pellet) was resuspended in extraction buffer and the membrane-bound enzymes were solubilized with digitonin (10 mg ml⁻¹) at 4 °C for 30 min. The detergent extracts were centrifuged at 48000 *g* for 30 min and the supernatant was used as the solubilized enzyme preparation. In order to determine the effect of detergent on enzyme solubilization, the pelleted extracted membranes were resuspended in extraction buffer for determination of residual activity.

The solubilized enzymes (3 ml samples) were layered onto a 30 ml 12–45 % (w/w) glycerol gradient (in 10 mM-Tris/HCl buffer, pH 7.4). The gradient was centrifuged for 4 h at 120000 *g* in a Beckman SW27 rotor at 4 °C. Sixteen fractions were collected and assayed for chitin synthase activity or for β -glucan synthase activities (Girard & Fèvre, 1984). Fractions were also analysed by SDS-PAGE on 7.5 % (w/v) slab gels (Laemmli, 1970). Samples were denatured at 100 °C for 5 min in 2 % (w/v) SDS, 5 % (v/v) mercaptoethanol and Tris/HCl buffer pH 6.8. Proteins separated by SDS-PAGE were silver stained.

Chitin synthase assay. Membrane-bound or solubilized enzymes (10–100 μ g protein) were assayed in 10 mM-Tris/HCl buffer pH 7.4

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with 0.5 mM-UDP-GlcNAc, 25 nCi UDP-[14 C]GlcNAc (Amersham, 8.7 GBq mmol $^{-1}$), 20 mM-GlcNAc, 10 mM-MgCl $_2$ and 1.25 μ g trypsin ml $^{-1}$ in a total volume of 200 μ l. The assay mixture was incubated at 25 °C for 30 min and the reaction was stopped with 2 ml 95% (v/v) ethanol. The reaction mixture was filtered on a 2.5 cm diameter glass-fibre filter (GF/C, Whatman) and washed successively with 5 ml 0.5 M-NaOH, 2 $\times$ 10 ml 95% (v/v) ethanol/1 M acetic acid (2:8; v/v) and 2 $\times$ 5 ml 95% ethanol. The discs were dried and their radioactivity was measured by scintillation spectrometry (Gay *et al.*, 1989).

Protein content was determined by the Lowry method using BSA as a standard. All experiments were repeated a minimum of three times and results are means $\pm$ SD.

Biophysical analysis of chitin. The reaction mixture without radioactive substrate was scaled up to 6 ml and incubated as above. The reaction was stopped with 95% ethanol and the material was centrifuged at 48 000 *g* for 30 min. The pellet containing the chitin was stirred overnight in 5% (w/v) aqueous KOH solution at room temperature. The polymer was rinsed with distilled water and then treated for 2 h with a sodium chlorite solution at 70 °C. The sodium chlorite solution was prepared by mixing 9.6 ml water with 3.2 ml of solution A (1.7 g NaClO $_2$ ·H $_2$ O in 100 ml water) and 3.2 ml of solution B (2.7 g NaOH, 7.5 ml concentrated acetic acid, adjusted to 100 ml). The chlorite treatment was followed by thorough washing and the treatments with KOH and chlorite were repeated three more times. The samples were then washed thoroughly and stored in methanol.

For X-ray diffraction analysis, samples were inserted in thin-wall X-ray capillary tubes, mounted in a flat-film Wharfedale vacuum camera and subjected to Cu K α radiations. The resulting X-ray patterns were compared with those of a standard of α -chitin extracted from lobster tendon.

For electron microscopy, samples were deposited on carbon-coated EM grids. The samples were observed under low-illumination conditions using a Philips EM 400 T electron microscope operated at 120 kV. Low-dose images and electron diffractograms were recorded on a Mitsubishi MEM emulsion processed with Ilford PQ Universal developer (diluted with water 150:2000; v/v). Purified samples were also observed after negative staining with uranyl acetate and recorded at 80 kV on Kodak 4489 EM film developed in D 19 Kodak developer (2:1).

For infrared spectroscopy, thin films of chitin were prepared by allowing aqueous suspensions of the prepared chitin fraction to evaporate. Fourier transform infrared (FTIR) spectra were obtained in transmission mode with a Perkin Elmer 1720 X FTIR instrument equipped with a microfocus accessory (Sugiyama *et al.*, 1991).

Results

Solubilization of chitin synthase and properties of the solubilized enzyme

Chitin synthase was released by permeabilizing the mixed membrane fraction (MMF) with detergents. Various detergents such as digitonin, octyl glucoside, CHAPS [3-((3-cholamidopropyl)dimethylammonio), 1-propane-sulphonate] or combined mixtures of these last two detergents solubilized proteins from the membranes. The chitin synthase activity detected following the extraction with CHAPS and (or) octylglucoside was lower than that in digitonin extracts (Table 1). Thus digitonin extraction was used for further experiments. Crude membrane fractions were incubated in increasing concentrations of digitonin and enzyme solubilization

was monitored by the presence of enzyme activity in the supernatant. The highest activity in the soluble fraction was recovered at 10 mg digitonin ml $^{-1}$ (Fig. 1). The digitonin treatment also increased the enzymic activity of treated membranes, i.e. detergent-insoluble residue, perhaps by unmasking active enzymes enclosed in vesicles.

As only part of the enzyme present in the mixed membrane fraction was recovered in the detergent extract (SF1) and as high enzymic activity remained in the detergent-insoluble residue (MMF1) (Table 2) the pelleted extracted membranes were treated a second time with 10 mg digitonin ml $^{-1}$. The soluble fraction obtained by this second treatment with digitonin (SF2) had a specific activity 3.6 times higher than that of the enzyme recovered after the first step of detergent extraction (SF1). In contrast, the second digitonin treatment did not increase the chitin synthase activity still associated with the insoluble membrane residue (MMF2). The specific activity of MMF2 (1.68 nmol mg $^{-1}$ min $^{-1}$) was similar to that of MMF1 (1.70 nmol mg $^{-1}$ min $^{-1}$). Sonication during detergent action did not improve enzyme solubilization.

Table 1. *Solubilization of chitin synthase with detergents*

The mixed-membrane fraction (500 μ l) was incubated with detergents for 30 min at 4 °C and then centrifuged at 48 000 *g* for 30 min. The supernatant was used as the solubilized enzyme.

Detergent (mg ml $^{-1}$)			Specific activity (nmol mg $^{-1}$ min $^{-1}$)
CHAPS	Octylglucoside	Digitonin	
5	2	—	0.76 $\pm$ 0.18
10	4	—	0.92 $\pm$ 0.23
10	—	—	1.02 $\pm$ 0.27
—	4	—	0.35 $\pm$ 0.05
—	—	10	1.66 $\pm$ 0.29

Fig. 1. Recovery of solubilized chitin synthase following digitonin treatment. Membrane-bound enzymes were treated with digitonin for 30 min at 4 °C, and the enzymic activity was assayed in the solubilized fraction (●) and in the pellet (○) (means from three experiments).

Table 2. Solubilization of chitin synthase from *S. monoica*

Enzyme was solubilized in a two-step procedure. Mixed-membrane fraction (MMF, from 3×100 ml cultures) was treated with digitonin (10 mg ml^{-1}) and centrifuged to obtain the solubilized fraction (SF1) and the pellet (MMF1). This treatment was repeated on the MMF1 fraction giving a second soluble fraction (SF2) and the detergent-insoluble membrane ghosts (MMF2).

Fraction	Protein (mg)	Total activity (nmol min ⁻¹)	Specific activity (nmol mg ⁻¹ min ⁻¹)
MMF	15.9 ± 2.04	0.96 ± 0.20	0.06 ± 0.01
SF1	6.8 ± 0.65	6.25 ± 1.11	0.92 ± 0.09
MMF1	9.1 ± 0.36	15.47 ± 1.08	1.70 ± 0.06
SF2	2.9 ± 0.40	9.51 ± 0.88	3.28 ± 0.30
MMF2	6.2 ± 0.50	10.42 ± 2.04	1.68 ± 0.31

Fig. 2. Effect of divalent cations on chitin synthase activity. ●, Mg^{2+} ; ○, Co^{2+} (means from three experiments).

Table 3. Effect of trypsin treatment on the activity of the solubilized chitin synthase

Trypsin ($1.8 \mu\text{g ml}^{-1}$) was added prior to the assay or during the assay as described in text.

Time of treatment with trypsin (min)	Activity (c.p.m.)	Percentage activity
<i>Before assay</i>		
0	851 ± 25	33
10	879 ± 75	34
20	1224 ± 44	48
30	1438 ± 83	56
<i>During assay</i>		
30	2552 ± 85	100

Properties of the solubilized chitin synthase

Digitonin-solubilized chitin synthase had an apparent temperature optimum at about 30°C and exhibited optimal activity at pH 7.4 (results not shown). The

Fig. 3. Effect of trypsin on chitin synthase activity. Solubilized extracts were subjected to proteolysis during the assay. Results are given as percentages of the control (831 ± 54 c.p.m., enzyme activity without proteolytic treatment).

activity of digitonin-solubilized chitin synthase was tested in the presence of various amounts of divalent cations. MgCl_2 stimulated the enzyme activity and the presence of 12.5 mM-Mg^{2+} in the assay medium resulted in a 6.3-fold increase in activity (Fig. 2). Mg^{2+} could be substituted by Co^{2+} but this cation was less efficient.

The zymogenicity of the solubilized enzyme was revealed by adding various amounts of trypsin to the preparation. The enzyme activity was increased when trypsin ($1.8 \mu\text{g ml}^{-1}$) was added during the assay or when trypsin treatment was done before the assay. In this case, enzyme was incubated with trypsin for 10–30 min at 25°C and the reaction was stopped by adding $3 \mu\text{g}$ soybean trypsin inhibitor ml^{-1} . The incubation of the enzyme with trypsin prior to the assay was less efficient than the addition of trypsin during the assay (Table 3). Enzyme activity was stimulated at low protease concentration and maximal activity was observed at $1.8 \mu\text{g ml}^{-1}$ (Fig. 3).

Partial purification of the enzyme

Glycerol gradient centrifugation of the solubilized proteins has proved efficient in the separation of glycosyltransferases (Bulone *et al.*, 1990) and was used to purify the chitin synthase. The behaviour of this enzyme during velocity sedimentation in a glycerol gradient is shown in Fig. 4. Chitin synthase activity sedimented as a sharp peak ahead the bulk of solubilized proteins. After 4 h centrifugation this activity was also separated from β -1,3 and β -1,4 glucan synthases which were collected at higher densities in the gradient.

The polypeptide composition of the glycerol gradient fractions was determined following silver staining of SDS-PAGE gels. Significant qualitative differences were found between the protein profile of the chitin synthase

Fig. 4. Glycerol-gradient centrifugation of digitonin-solubilized chitin and glucan synthases. The membrane-bound enzyme preparation that had been treated with digitonin (10 mg ml^{-1}) was layered onto a linear glycerol gradient and centrifuged for 4 h at $120\,000 \text{ g}$. Fraction 1, bottom of the gradient (density, 1.087); fraction 17, top of the gradient (density, 1.011). ●, Chitin synthase activity; ■, β -1,3 glucan synthase activity; □, β -1,4 glucan synthase activity; ○, protein; ▲, density of the fractions.

fractions and that of the original extract. As no enzyme activity remained after SDS treatment, chitin synthase activity could not be shown on gels. There was a correspondence between polypeptide bands and chitin synthase activity shown by comparing the enzymic activity of the glycerol gradient fractions with the presence and intensity of the various polypeptides of the fractions on the gels. The chitin synthase fraction was enriched in subunits with apparent molecular masses of 39, 42, 46, 53 and 95 kDa (Fig. 5), the 39 kDa band being

Fig. 6. Low-dose electron micrograph of the polymer synthesized by the solubilized enzyme. Inset: electron diffraction diagram of the circled area of the specimen. In the diagram, the three diffraction rings at 0.943, 0.466 and 0.341 nm are characteristic of crystalline α -chitin.

the most abundant. These proteins were well separated from the polypeptides (34, 48 and 50 kDa) characterizing the β -1,3 glucan synthase activity (Girard *et al.*, 1992).

Biophysical characterization of chitin

The insoluble polymer produced by the chitin synthase was treated with KOH and sodium chlorite. Electron microscopic analysis revealed that it was made of spindle-like structures of about $15 \times 30 \text{ nm}$ (Fig. 6). Electron diffraction analysis of this material gave a diagram with three strong diffraction lines at 0.943, 0.466 and 0.341 nm, which are characteristic of α -chitin (inset in Fig. 6). The electron diffraction data were confirmed by X-ray analysis of the material, which displayed reflections at 0.943, 0.516, 0.466 and 0.341 nm (Fig. 7A) corresponding

Fig. 5. SDS-PAGE analysis of the proteins separated by glycerol-gradient centrifugation. Each lane contained $5 \mu\text{g}$ of the proteins. Numbering of lanes corresponds to fraction numbers shown in Fig. 4. The gel was stained with silver. Proteins that correlate with chitin synthase activity are indicated by arrows. Proteins that correlate with β -1,3 glucan synthase are shown by arrowheads.

Fig. 7. X-ray diagrams of the polymer synthesized by the solubilized enzyme of *Saprolegnia monoica* (A) and of a test specimen of purified α -chitin from lobster tendon (B). The strong diffraction lines of α -chitin at 0.943, 0.516, 0.466 and 0.341 nm can be seen clearly.

Fig. 8. Infrared spectrum of the polymer synthesized by the solubilized enzyme. The three C=O bands at 1657, 1623 and 1559 cm^{-1} are characteristic of α -chitin.

to the strongest reflections of diagrams of purified lobster tendon α -chitin (Fig. 7B).

Enzymically synthesized α -chitin from *S. monoica* was also identified by infrared analysis. Its infrared spectrum displayed strong absorption bands at 1657, 1623 and 1559 cm^{-1} (Fig. 8) which are characteristic of α -chitin (Michell & Scurfield, 1970).

Discussion

The properties (pH requirement, optimal temperature, zymogenicity, effect of divalent cations) of the solubilized enzyme were similar to those of the crude enzyme in cell-free extracts. The soluble fraction obtained following a two-step detergent extraction had a specific activity 55 times higher ($3.28 \text{ nmol mg}^{-1} \text{ min}^{-1}$) than the mixed-membrane fraction ($0.06 \text{ nmol mg}^{-1} \text{ min}^{-1}$) from which it

was extracted. This large difference in specific activity between membrane-bound and solubilized enzyme may have been related to the accessibility of the substrate to the enzyme. When mixed-membrane fractions were permeabilized with digitonin, the specific activity reached $1.7 \text{ nmol mg}^{-1} \text{ min}^{-1}$, which was in the range of activities observed with typical chitinous fungi (Goody, 1990; Gay, 1991).

Glycerol-gradient centrifugation of solubilized enzyme showed that the peak of chitin synthase activity correlates with the distribution of the polypeptides of 39, 42, 46, 53 and 95 kDa. However this separation procedure is not a probe for demonstrating the real association of these peptides with the chitin synthase complex. Machida & Saito (1993) have purified the chitin synthase from *Absidia glauca* to homogeneity and the SDS-PAGE of the purified enzyme revealed a single band with a molecular mass of 30 kDa. This molecular mass is not very different from the value of the main band (about 39 kDa) found in the enriched chitin synthase fraction. However, these polypeptides are smaller than the polypeptides encoded by the chitin synthase genes cloned from *Saccharomyces* (Bulawa *et al.*, 1986; Valdivieso *et al.*, 1991), *Candida* (Au-Young & Robbins, 1990) or *Neurospora* (Yarden & Yanofsky, 1991) which predict molecular masses of 130, 124, 88 and 107 kDa respectively.

Solubilized chitin synthase incubated with substrate synthesized chitin in the form of short spindle-like particles. This polymer was different in morphology from the long interweaving chitin microfibrils produced by regenerating protoplasts or from the small round granules observed in hyphal cell walls (Bulone *et al.*, 1992). This polymorphism may be related to the different organization of the chitin synthase complex in the detergent micelles and in the cell membranes (Gay *et al.*, 1992). The insoluble polymer synthesized by the solubilized enzyme was unequivocally characterized as chitin by electron diffraction and X-ray analysis. The diffraction rings were characteristic of α -chitin (Giraud-Guille *et al.*, 1990). The presence of a ring at 0.516 nm indicates that the chitin synthesized *in vitro* was more crystalline than the polymer produced *in vivo*, which lacked this diffraction ring (Bulone *et al.*, 1992). The crystalline form of the product was confirmed by the infrared spectrum, which was identical to that of chemically purified crab α -chitin.

These results and those previously reported demonstrate unambiguously that α -chitin is a normal component of cell walls of hyphae and regenerating protoplasts from *S. monoica* and that the chitin synthase from this cellulosic fungus exhibits properties which are similar to those reported for the enzymes obtained from chitinous fungi.

References

- ARONSON, J. M. (1965). The cell wall. In *The Fungi: An Advanced Treatise*, vol 1, pp. 49–76. Edited by G. C. Ainsworth & A. S. Sussman. New York: Academic Press.
- ARONSON, J. M. & BERTKE, C. C. (1987). Isolation and analysis of cell walls. In *Zoospore Fungi in Teaching and Research*, pp. 175–185. Edited by M. S. Fuller & A. Jaworski. Athens, GA: Southeastern PC.
- ARONSON, J. M. & LIN, C. C. (1978). Hyphal cell wall chemistry of *Leptomitius lacteus*. *Mycologia* **70**, 363–369.
- AU-YOUNG, J. & ROBBINS, P. W. (1990). Isolation of a chitin synthase gene (*CHS1*) from *Candida albicans* by expression in *Saccharomyces cerevisiae*. *Molecular Biology* **4**, 197–207.
- BERTKE, C. C. & ARONSON, J. M. (1980). Hyphal wall composition in *Apodachlyella completa*. *Current Microbiology* **4**, 235–238.
- BULAWA, C. E., SLATER, M., CABIB, E., AU-YOUNG, J., SBURLATI, A., ADAIR, W. L., JR & ROBBINS, P. W. (1986). The *S. cerevisiae* structural gene for chitin synthase is not required for chitin synthesis *in vivo*. *Cell* **46**, 213–225.
- BULONE, V., GIRARD, V. & FEVRE, M. (1990). Separation and partial purification of 1,3- β -glucan and 1,4- β -glucan synthases from *Saprolegnia*. *Plant Physiology* **94**, 1748–1755.
- BULONE, V., CHANZY, H., GAY, L., GIRARD, V. & FEVRE, M. (1992). Characterization of chitin and chitin synthase from the cellulosic cell wall fungus *Saprolegnia monoica*. *Experimental Mycology* **16**, 8–21.
- CAMPOS-TAKAKI, G. M., DIETRICH, S. M. C. & MASCARENHAS, Y. (1982). Isolation and characterization of chitin from the cell walls of *Achyla radiosa*. *Journal of General Microbiology* **128**, 207–209.
- DIETRICH, S. M. C. (1975). Comparative study of hyphal wall components of Oomycetes: *Saprolegniaceae* and *Phythiaceae*. *Anais da Academia Brasileira de Ciências* **47**, 155–162.
- GAY, L. (1991). Chitin content and chitin synthase activity as indicators of the growth of three different anaerobic rumen fungi. *FEMS Microbiology Letters* **80**, 99–102.
- GAY, L., HEBRAUD, M., GIRARD, V. & FEVRE, M. (1989). Chitin synthase activity from *Neocallimastix frontalis*, an anaerobic rumen fungus. *Journal of General Microbiology* **135**, 279–283.
- GAY, L., BULONE, V., GIRARD, V., FEVRE, M. & CHANZY, H. (1992). Chitin pleomorphism in the cellulosic cell wall fungus *Saprolegnia*. *FEMS Microbiology Letters* **100**, 405–410.
- GIRARD, V. & FEVRE, M. (1984). β -1,4 and β -1,3 glucan synthases are associated with the plasma membrane of the fungus *Saprolegnia*. *Planta* **160**, 400–406.
- GIRARD, V., BULONE, V. & FEVRE, M. (1992). Separation and partial characterization of β 1-3 glucan synthase from *Saprolegnia*. *Plant Science* **82**, 145–153.
- GIRAUD-GUILLE, M. M., CHANZY, H. & VUONG, R. (1990). Chitin crystals in arthropod cuticles revealed by diffraction contrast transmission electron microscopy. *Journal of Structural Biology* **103**, 232–240.
- GOODAY, G. W. (1990). Inhibition of chitin metabolism. In *Biochemistry of Cell Walls and Membranes in Fungi*, pp. 61–79. Edited by P. J. Kuhn, A. P. J. Trinci, M. J. Jung, M. W. Goosey & L. G. Coppin. Berlin: Springer Verlag.
- LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.
- MACHIDA, S. & SAITO, M. (1993). Purification and characterization of membrane-bound chitin synthase. *Journal of Biological Chemistry* **268**, 1702–1707.
- MACHLIS, L. (1953). Growth and nutrition of water molds in the subgenus *Euellomyces*. II. Optimal composition of the minimal medium. *American Journal of Botany* **40**, 449–460.
- MICHELL, A. J. & SCURFIELD, D. (1970). The assessment of infrared spectra as indicators of fungal cell wall composition. *Australian Journal of Biological Sciences* **23**, 345–360.
- SIETSMAN, J. H. (1969). *Protoplast formation and cell wall composition of some Oomycetes species*. PhD thesis, University of Amsterdam.
- SUGIYAMA, J., PERSSON, J. & CHANZY, H. (1991). Combined infrared and electron diffraction study of the polymorphism of native celluloses. *Macromolecules* **24**, 2461–2466.
- VALDIVIESO, M. H., MOL, P. C., SHAW, J. A., CABIB, E. & DURAN, A. (1991). *Call*, a gene required for activity of chitin synthase 3 in *Saccharomyces cerevisiae*. *Journal of Cell Biology* **114**, 101–109.
- YARDEN, O. & YANOFSKY, C. (1991). Chitin synthase 1 plays a major role in cell wall biogenesis in *Neurospora crassa*. *Genes and Development* **5**, 2420–2430.