Proteomic and immunological analysis of the *Neurospora crassa* cell wall

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C. Alexander Valencia

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ABSTRACT

The fungal cell wall has a complex, laminate structure composed primarily of polymeric, mixed glucans and chitin, as well as a minor fraction of proteins and glycoproteins. To study this proteinaceous component, an antiserum (NcCW) has been raised against a cell wall extract from the filamentous fungus, *Neurospora crassa*. Immuno-gold labelling confirmed that this NcCW antiserum binds primarily to the cell envelope and cleavage with endo-proteinases and chemical reagents confirmed that the reacting antigens are proteinaceous. Western blot analysis revealed that five prominent proteins and more than 20 minor proteins are recognized by the NcCW antiserum. Extractions of the cell wall fraction with detergents, urea, sodium hydroxide, hydrochloric acid and trifluoromethanesulfonic acid suggest that several of the antigens are covalently linked to the cell wall through an O-glycosyl linkage. A comparison of these multi-species profiles of NcCW antiserum binding and lectin-binding supports the conclusion that this antiserum primarily recognizes polypeptides. The NcCW antiserum was utilized to identify 6 cDNA clones, encoding putative cell wall-associated proteins, from an expression library. A similar approach using antiserum raised against *Schizophyllum commune* cell walls (ScCW) resulted in the identification of 14 clones, encoding cell wall-associated and novel proteins, and provides evidence that the ScCW antiserum mainly recognized proteins.

Complementary approaches, namely, bioinformatic and proteomic-based methods, were also employed to further identify wall-associated proteins of *N. crassa*. To characterize the surface proteins, intact hyphae were treated with trypsin and the tryptic

fragments were compared to proteins released by chemical treatments from purified cell walls. To obtain amino acid sequence information to allow protein identification, proteins extracted with i) urea plus 2-mercaptoethanol, from purified walls, were separated by two-dimensional polyacrylamide gel electrophoresis (2-D SDS PAGE) before mass spectrometric analysis, whereas, ii) the trypsin extracts, from intact cells, were directly examined by on-line liquid chromatography tandem mass spectrometry (CapLC-MS/MS). Furthermore, proteins secreted into the growth media and released by Novozyme™ treatments from purified cell walls were identified by mass spectrometry. The proteins identified in the datasets included homologs of yeast wall proteins, glycolytic enzymes, mitochondrial proteins, heat shock proteins, translation factors and novel proteins.

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“In the advance of civilization, it is new knowledge which paves the way, and the

pavement is eternal". W.R. Whitney

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LIST OF ABBREVIATIONS

TBS	Tris buffered saline
TBST, TBST2	Tris buffered saline with Tween-20
TFMS	Trifluoromethanesulfonic acid
HRP	Horseradish peroxidase
ConA	Concanavalin A
NCS	N-chlorosuccinimide
endo-Glu-C	Endoproteinase Glu-C
ME	2-mercaptoethanol
CWP	Cell wall protein
1-D	One-dimensional
2-D	Two-dimensional
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
CapLC-MS/MS	On-line liquid chromatography tandem mass spectrometry
FLB	Fungal lysis buffer
NcCW	<i>Neurospora crassa</i> cell wall
ScCW	<i>Schizophyllum commune</i> cell wall

CHAPTER 1

GENERAL INTRODUCTION AND LITERATURE REVIEW

Introduction

Ascomycetes and *Neurospora crassa*

N. crassa belongs to the phylum of fungi called Ascomycota whose defining feature is the production of sexual spores in sac-like asci (Springer 1993; Fig. 1.1; Fig. 1.2). There are more than 60,000 species in this phylum and they can be found in a wide variety of habitats (Kendrick 1992). They range in size and complexity from unicellular yeasts to the elaborate cup fungi and truffles (Campbell, 1993; Fig. 1.1). Ascomycetes include the most devastating plant pathogens and about half of this division live with algae in the symbiotic association called lichens (Kendrick 1992). Some ascomycetes form mycorrhizae with plants (Campbell 1993). Others live on leaves on the surface of mesophyll cells, where the fungi apparently help protect these plant tissues from insects by releasing toxic compounds.

N. crassa has been a useful organism in genetic, biochemical and developmental research (Mishra 1977). *N. crassa* has many advantages including: i) a haploid genome, ii) well investigated biochemistry and cytology, iii) it is a eukaryotic organism, iv) tetrad and heterokaryon analysis, v) selective and other techniques of microbial genetics apply, vi) it also undergoes a certain degree of differentiation and developmentally controlled structures can be analyzed genetically and biochemically and vii) a large number of mutants have been isolated for biochemical or developmental analyses (Mishra 1977; Perkins *et al.*, 1982; Horowitz 1991; Perkins 1992; Radford and Parish 1997).

Fig. 1.1 Multi-kingdom phylogeny, demonstrating the evolutionary relation between *N. crassa* and other organisms, inferred from one of 18 equally parsimonious trees based on the analysis of small ribosomal rRNA, actin and translation elongation 1 α genes (Gehrig *et al.*, 1996; Voigt and Wostemeyer 2001). *Bold letters*, genus of species used in several experiments to test cross-reactivity of NcCW and ScCW antisera (Chapters 2 and 3).

Fig. 1.2 Life cycle of *Neurospora crassa* (Springer 1993).

***Neurospora crassa* and its cell wall**

Filamentous structure maintenance and the cell wall

Several observations agree with the assumption that the *N. crassa* cell wall is important for determining morphology (deTerra and Tatum 1963; Scott 1976; Mishra 1977). This idea is supported by the fact that wild-type tubular hyphae form osmotically sensitive, spherical protoplasts when treated with a mixture of cell wall-degrading hemicellulases (Bachman and Bonner 1959; Reyes and Lahoz 1976). It was noted that treatment with lower concentrations of these enzymes cause the wild-type hyphae to have a colonial form of growth (Bachman and Bonner 1959). It was also observed that the cell wall composition of morphological mutants is different when compared to the wild-type (deTerra and Tatum 1963; Mahadevan and Tatum 1965; Scott 1976; Mehta and Mahadevan 1975; Mishra 1977). For example, Wrathall and Tatum (1974) were able to show mutant strains with abnormal growth patterns that had reduced quantities of wall peptides. In addition to giving form to fungal cells, other functions of the cell wall are: preventing mechanical or turgor injury, serving as a scaffold for nutrient assimilation and mediating cell/cell interactions (Cabib *et al.*, 1988; Stratford 1994).

The morphogenetic aspect of cell wall synthesis has been of particular interest because of its role in imparting form to the hypha (Cabib *et al.*, 1998). If cell wall expansion is continually restricted to a small area at the apex, the result will be a tube-like hyphae (Harold, 1997). In contrast, the opposite extreme of isometric cell wall expansion results in an enlarging spherical shape such that of yeasts (Cabib *et al.*, 1988). In budding yeast, all three modes of growth are observed during the cell cycle: growth is initially

restricted to one area at bud emergence, more generalized growth occurs during expansion, and septum formation complete the final stages of the cycle.

Regulatory mechanisms both at the subcellular and molecular levels are responsible for this precise localization of growth. An accumulation of vesicles, called 'SpitzenKorper', near the tips of the hyphae have been found which fuse with the plasma membrane (Grove and Bracker 1970). Cell wall components, including proteins and enzymes are delivered to the apical growth zone by exocytosis (Cabib *et al.*, 1988). From the periplasmic space, these molecules must still traverse a nascent, primary cell wall to be incorporated into the mature cell envelope.

Components of the *Neurospora crassa* cell wall

Some aspects of the chemical composition of the cell walls of several fungi have been analysed to understand the relationship between the hyphal wall and fungal morphology (Mishra 1977). For example, to determine the composition of the *N. crassa* cell wall, Mahadevan and Tatum (1967) removed the components of the cell wall by chemical or enzymatic degradation. They found that the *N. crassa* cell wall contains four components, denoted as fraction I, II, III and IV. Fraction I is an alkali-soluble peptide-polysaccharide complex containing glucose glucan, amino acids and galactosamine. Fraction II consists primarily of glucan which is alkali-insoluble but acid-soluble. Fraction III is a β -(1,3) glucan which can easily be digested by laminarase. Fraction IV was determined to be chitin. In summary, these early fractionation experiments revealed that the *N. crassa* cell wall contains glucan (Cardemil and Pincheira 1979), chitin and proteins (Mishra 1977; Wrathall and Tatum 1974).

Using a combination of various lytic enzymes and scanning electron microscopy, Hunsley and Burnett (1970) initially showed that the *N. crassa* cell wall is composed of four distinct layers: i) The outermost layer are composed of fibrillar structures and is divisible into inner and outer layers. The outer fibrils are composed of β -(1,3) glucan-peptide-galactosamine complexes which were found in fraction I and II by Mahadevan and Tatum (1967). ii) The middle layers consisting of substantial amounts of protein and glycoprotein. iii) The inner fibrils are made up β -(1,3) glucan (fraction III). iv) The innermost layer which is made up of chitin (fraction IV). The chitin, seems to be distributed throughout the inner cell-wall layer as fine fibrils, embedded in the β -(1,3) glucan or fraction II and III (Mahadevan and Tatum 1967). Most of the glucosamine is in this chitin fraction (Schmit *et al.*, 1975). The following layers (from outside to inside) therefore are currently thought to make up the *N. crassa* wall: i) a layer of mixed α and β -glucans, ii) a glycoprotein reticulum merging into a proteinaceous layer, iii) a distinct layer of protein, and iv) an innermost chitinous region in which chitin microfibrils are embedded (Fig. 1.3; Trinci 1978; Ballou *et al.*, 1980; Wessels 1986).

Chemically, chitin is a linear polysaccharide made of GlcNAc and is the skeletal component of cell walls (Bartnicki-Garcia 1968; Farkas 1979; Ruiz-Herrera and Xoconostle-Cazares 1995). Its selective enzymatic dissolution leads to cell wall lysis, but when other cell wall components are removed, leaving chitin unaffected, hyphae retain their shape (Ruiz-Herrera and Xoconostle-Cazares 1995). Chitin microfibrils are positioned in random orientations and are only seen when the β -glucans are removed (Wessels 1986).

Fig. 1.3 Cell wall composition of *N. crassa* (Trinci 1978).

The most abundant glucans of fungal cell walls are those with the β -configuration (Wessels, 1986). β -glucans have been characterized as polymers of glucose moieties joined through β -(1,3) linkages (Ruiz-Herrera and Sentandreu 1989). Some of the polymers have branches joined through (1,6)- β linkages. The degree of branching, the molecular weight and their macromolecular structure are variable (Ruiz-Herrera and Sentandreu 1989). Glucan synthesis is catalyzed by enzymes known as glucan synthases (Mishra and Tatum 1972; Enderlin and Selitrennikoff 1994).

The hyphal structure is made up of a primary wall next to the plasma membrane and an outer secondary wall. The mature wall may be divided into a primary wall containing microfibrils which is largely formed during hyphal extension and a secondary wall which lacks microfibrils and is formed sub-apically upon the outside of the primary wall (Trinci 1978). Therefore, the primary wall which forms at the tip will eventually be the inner layer at maturity (Trinci 1978).

The primary wall of *N. crassa* is 50 nm thick and is made up of chitin microfibrils and thought to be covered by a layer of proteins (Trinci 1978; Fig. 1.3). These microfibrils increase in thickness and 'packing' with distance from the tip, which indicates that additional fibrils are added to the wall at maturity (Trinci 1978). The primary wall of *N. crassa* appears to be overlaid subapically by a protein/glycoprotein layer (40-50 nm) thick containing a coarse glycoprotein reticulum which in turn is overlaid by a layer of amorphous glucose containing polymers with β -(1,3), β -(1,6) and α -(1,6) linkages (Trinci 1978). The chitin layer of the wall has an almost identical thickness as the primary cell wall.

Cell wall expansion

Two general hypotheses have been forwarded to explain the difference in physicochemical properties of growing and nongrowing cell wall regions and to explain cell wall elongations. Bartnicki-Garcia (1973) proposed a model which suggested a balance between wall synthesis and lysis. According to his ideas, invagination of new wall material at the apical growing region requires softening of the preformed microfibrillar structure by lytic enzymes. The evidence for this model comes from studies of the bursting of hyphal apices induced by different types of solutions which suggests a biochemical reaction, rather than a purely physical phenomenon (Bartnicki-Garcia and Lippman 1972a; Bartnicki-Garcia and Lippman 1972b).

Based on the different structural properties of the hyphal apices, Wessels and his colleagues have proposed a steady-state model of apical wall growth (Wessels 1986; Wessels 1987; Fig. 1.4). According to the steady-state model, lytic enzymes play no role; it is suggested instead that chitin and β -glucans extruded into the cell wall are hydrated and organized in the form of a viscoelastic structure which yields to the turgor pressure of the cell, stretching and displacing continuously to subapical regions during wall expansion (Ruiz-Herrera and Sentandreu 1989). During this process, the cell wall becomes rigidified by establishment of covalent bonding between the different wall components and by crystallization of the wall polymers (Wessels 1987). In a series of papers, Wessels and colleagues have also established that newly-synthesized β -(1,3) glucans present in the apical region are water-soluble and that after some time they become insoluble in water and then insoluble in alkali (Mol and Wessels 1987; Sietsma

Fig. 1.4 The steady-state model of apical growth. For the extending apex (vesicles fusing with the plasma membrane after transport on the actin cytoskeleton) the schematic drawing shows the stretching of the wall and the addition of new wall material from the cytoplasmic side, maximally at the extreme tip. Newly added wall consists of chitin chains (straight lines) and β -(1,3) glucan chains (wavy lines). Other wall components are omitted because they are less important as structural components. With time, these two polymers interact to form chitin microfibrils and β -glucan chains which also become branched. At the very tip (A) the wall is minimally crosslinked and supposed to be most plastic. Subapically (B) a wall volume added at the very tip is stretched and partially cross-linked (increased shading) while new wall material is being added from the inside to maintain wall thickness. Note that wall material moves from the inside to the outside and that the wall at the outside is always oldest. Areas with the same shading in (A), (B) and (C) represent equal volumes of wall but note that in reality there are no discontinuities in the wall. At (C) the average crosslinking has proceeded to the point that the wall hardly yields to turgor pressure but it has not yet maximal strength. Crosslinking continues in the tubular part of the wall until completion far behind the apex (D). In a non-extending apex (no vesicle present), the steady state breaks down because the steady-state amount of plastic wall material is no longer pushed out of the zone of rigidification. Crosslinking between the wall polymers now occurs over the whole apex (D) (Wessels 1993).

et al., 1985). The latter process depends on their covalent bonding to chitin, since it is inhibited by polyoxin D. Moreover, after digestion with chitinase, all the glucan becomes alkali-soluble. In line with this model it may be added the possibility that self-assembly of the glycoprotein matrix may be an organizer for the correct establishment of the microfibrillar mesh into the newly synthesized wall (Ruiz-Herrera and Sentandreu 1989).

Which of the two hypotheses is closer to the mechanism operating *in vivo* is difficult to discern at the present time (Ruiz-Herrera and Sentandreu 1989). As it normally happens, perhaps both mechanisms are substantial, and the real process involves both hypotheses (Ruiz-Herrera and Sentandreu, 1989). It is a fact that lytic enzymes are involved in wall metabolism at certain times such as fusion of hyphae during mating (Wessels 1987). It is possible that under normal conditions wall growth occurs, as suggested by the steady-state model, and that lytic enzymes play a regulatory role to increase or reduce wall growth rates and to induce changes in the direction of hyphal growth (Ruiz-Herrera and Sentandreu 1989).

Cell wall proteins in *N. crassa*

The existence of proteins in the cell wall of *N. crassa* was initially reported by deTerra and Tatum (1963) and Mahadevan and Tatum (1965). Mahadevan and Tatum (1965) showed the presence of amino acid in acid hydrolysates of cell wall extracts. Based on electron microscopy it was concluded that proteins form a discrete layer within the wall (Hunsley and Burnett 1970). The peptides of the hyphal cell wall of the wild-type *N. crassa* were shown to make up 10% of the hyphal dry weight (Wrathall and Tatum 1974). Wrathall and Tatum (1973) proposed the existence of two types of cell wall

proteins; some of these proteins are functional or enzymatic, such as invertase, and others fulfilling a structural role which determines the morphology of the fungus (Chang and Trevithick, 1972; Ruiz-Herrera and Sentandreu, 1989).

Cell wall bound-proteins

Some of the cell wall proteins are believed to be covalently bound to the carbohydrate portion of the wall by O-glycosyl-serine linkages, which are labile to strong alkali (Wrathall and Tatum 1974). Mahadevan and Mahadkar (1970) were able to show that cell wall-bound enzymes had the capacity to lyse cell wall polymers during periods of growth. Sukamara and Mahadevan (1975) showed the presence of one wall-bound protease and two β -(1,3) glucanases capable of hydrolyzing the structural polymers of the cell wall in *N. crassa*. Similarly, Mehta and Mahadevan (1975) were able to detect a cell wall-bound protease by using antibodies raised against cell wall bound enzymes. Since this protease is evident during 24 hr and 72 hr growth stages it was concluded that wall-bound autolytic enzymes acting on the protein (proteases) and glucan (glucanases) portion of the cell walls are activated and participate in the process of branching (Mahadevan and Mahadkar, 1970; Mehta and Mahadevan 1975). Based on evidence from the yeast *S. cerevisiae*, chitinases which hydrolyze β -(1,4) glucosidic bonds of chitin to yield chitin oligomer (by endo-activity) and diacetylchitobiose (by exo-activity) are also thought to be bound to the cell wall (Gooday and Gow 1990).

Other proteins with enzymatic activity have also been found to be associated with the *N. crassa* cell wall. For example, Chung and Trevithick (1970) used osmiophilic staining in combination with indirect immunofluorescence to showed that 20-30% of the

invertase was bound to the cell wall of *N. crassa* (Chang and Trevithick 1972).

Cell wall-associated proteins

Various enzymes involved in metabolism and cell wall synthesis have been found to be associated with the cell wall. For instance, trehalase has been found to be associated with the cell walls in all stages of the life cycle of *N. crassa* (Hecker and Sussman 1973a). Both lyophilization and immunofluorescent experiments indicated that trehalase is associated with the innermost ascospore wall (endosporium) (Hecker and Sussman 1973a; Hecker and Sussman 1973b). Trehalase is probably absorbed by or entrapped within purified walls or other protective substance during lyophilization, because of the ease with which it can be removed from this association in aqueous solution (Chang and Trevithick 1972; Hecker and Sussman 1973a).

Another cell wall-associated protein involved in copper detoxification was isolated by Suresh and Subramanyam (1996). The culturing of *N. crassa* in the presence of high levels of copper produced blue coloured mycelia and cell walls due to the presence of copper in them. The treatment of these blue cell walls with ethylenediamine tetracetate (EDTA) and 8-hydroxyquinoline (8-HQ) solubilized a copper binding protein (MW=14 kDa) which was associated with phosphate and carbohydrate moieties required for metal detoxification.

Other wall-associated proteins, namely, chitin and glucan synthases, are believed to be involved in synthesizing components of the cell wall (Wessels 1993). Several biochemical and cytological studies have shown the association of chitin synthesis activity within microvesicles, designated chitosomes (Ruiz-Herrera 1992). Based on these

observations, a model was proposed suggesting that chitin synthase is transported to the growing zone or hyphal tip of the cell in a compartmentalized form, packed in chitosomes, which prevents it from interacting with the sugar substrate in the cytoplasm (Bracker *et al.*, 1976). Afterwards, the chitosome fused with the plasma membrane and the enzymes are inserted into the wall (Ruiz-Herrera 1992). On the other hand, the glucan synthase enzymes have been found to be anchored to the plasma membrane, since the isolation of this membranous fraction lead to the *in vitro* synthesis of β -glucan (Ruiz-Herrera 1992). Even though, glucan synthesis occurs in the apical/subapical portion of the plasma membrane glucan cross-linking occurs in the subapical region of the wall (Gooday and Gow 1990). Whether the glucan synthases are localized on the edges of the membrane and close or in the intramural region, is still not known. Nevertheless, these enzymes are thought to be associated with the cell wall and have been studied at a molecular level.

Structural and functional analyses of genes and gene products belonging to the chitin synthase classes I-III have been reported (Din *et al.*, 1996). Direct involvement in hyphal growth has been demonstrated for *chs-1* of *N. crassa* (Yarden and Yanofsky 1991). In addition, the *chs-2* was mutated by the repeat-induced point mutation (RIP) to see the effects on the growth of the fungus. No effects were found. The mutant and the wild-type under standard growth conditions had the same morphology. The function of *chs-2* is not clear, but it was suggested by the authors that it may have repair functions like *CHS1* of *S. cerevisiae*. Similarly, *N. crassa* strains in which *chs-4* had been inactivated by the RIP process grew and developed in a normal manner under standard

conditions (Din *et al.*, 1996). However, when grown in the presence of sorbose (induced elevated chitin content), chitin levels in the *chs-4^{RIP}* strain were lower than those observed in the wild type. Din *et al.*, (1996) suggested that CHS4 serves as an auxiliary enzyme in *N. crassa*.

Several mutants of *N. crassa* are defective in β -(1,3) glucan synthase activity, and the gene that complemented one group of mutants was cloned and designated synthase-1 (*gs-1*) (Enderlin and Slitrennikoff 1994). Importantly, mutants of *gs-1* were defective for β -(1,3) glucan synthase activity and for cell wall formation. When the amount of cell wall β -(1,3) glucan was reduced (either by drug treatment or by mutations that alter the level of substrate or enzyme activity), the resulting cell wall assembly and morphogenesis were abnormal, and cells either grew poorly or lysed (Tentler *et al.*, 1996). Thus, β -(1,3) glucan synthase activity is essential for cell wall assembly (Tentler *et al.*, 1996).

Wall proteins with structural non-enzymatic functions in *N. crassa*

Proteinaceous extracellular coat

The presence of these high molecular weight proteins in fungal cell walls was suggested by analyzing the proteinaceous material secreted by the *slime* mutant of *N. crassa*. This variant is able to grow as a protoplast and it secretes into the medium a proteinaceous extracellular coat (PEM) which takes the form of organized lamellae able to bind to the lectin ConA which recognizes α -D-mannosyl and α -D-glucosyl residues (Martinez *et al.*, 1989). SDS-PAGE revealed the presence of high molecular weight proteins which did not stain with Coomassie blue, but were detected when stained with ConA-peroxidase indicating that the carbohydrate is bound through – and O-glycosidic

linkages (Casanova *et al.*, 1989; Ruiz-Herrera, 1992). Comparative electrophoretic analysis and immunological analysis suggested that PEM represent a normal proteinaceous layer present in the wild type cell wall which in the absence of structural polymers is secreted into the medium (Martinez *et al.*, 1990) These high molecular weight glycoproteins might have a structural function.

Hydrophobins

Hydrophobins are abundant components of the cell surface of filamentous fungi; as structural components of the cell wall and by virtue of their role in environment sensing (Ebbole 1996). These small, cysteine-rich and generally hydrophobic polypeptides are able to self-assemble into rodlet structure at hydrophobic-hydrophilic interfaces (Ebbole 1996; Wessels 1996). Rodlets can be found to decorate the conidia and aerial hyphae where they alter the hydrophobic characteristics of the cell surface of *N. crassa* (Beever *et al.*, 1979; Dempsey and Beever 1979; Ebbole 1996). Aerial hyphae and conidia coated with rodlets are difficult to wet but hydrophobin mutants are easily wettable (*eas*) (Lauter *et al.*, 1992). In *N. crassa* and *Aspergillus nidulans* the hydrophobin rodlet layer aids in aerial dispersal of conidia (Ebbole 1996).

***Saccharomyces cerevisiae* cell wall architecture**

The cell wall of *S. cerevisiae* has been extensively explored and it serves as a model from which to start to explore the cell wall composition of filamentous fungi. Many of the principles and methods have been developed for yeasts, but may be also applied to filamentous fungi with the objective of understanding the cell wall composition of filamentous fungi since this area of research remains largely unexplored.

The ultimate goal of understanding the cell wall composition differences between yeast and filamentous fungi is underway and will be possible with the increased availability of proteomic tools and rapid accumulation of genomic data. Therefore, it is of importance to be aware of the advances that have been made in understanding the cell wall composition of *S. cerevisiae*.

Saccharomyces cerevisiae cell wall structure

The cell wall constitutes some 15-25% of the dry weight of the cell (Stratford, 1994). The yeast cell wall is composed largely of carbohydrate, some of which is covalently linked to protein (Fleet 1991). Cell wall carbohydrates comprise: β -glucans, fractions of β -(1,3) and β -(1,6)-linked N-acetyl glucosamine and α mannan, and highly glycosylated, secreted mannoproteins (Stratford 1994).

The current model of yeast cell-wall structure resembles concrete with steel reinforcing rods being analogous to alkali-insoluble β -(1,3) glucan fibrils, comprising 35-50% of the cell wall (Stratford 1994; Fig. 1.5). The secreted mannoproteins, analogous to pebbles (25%-50% of the wall), are encased in a matrix of amorphous β -glucan and chitin (Stratford 1994; Fig. 1.5).

Although both are fungi, significant differences between the *S. cerevisiae* and *N. crassa* cell walls exist (Wrathall and Tatum 1973; Standford 1994). For example, the proteins of the *Neurospora* cell wall were shown to comprise only 10 % of the hyphal wall by weight (Wrathall and Tatum 1974; Gozalbo *et al.*, 1993). In *S. cerevisiae*, the cell-wall surface is normally smooth, but glucanase (zymolyase) treatment makes the surface rough and exposes the sub-surface structures where the glucan matrix has been

Fig. 1.5 Composition and structure of the cell wall of *Saccharomyces cerevisiae*. The cell wall, which is located outside the plasma membrane, consists of two layers. The inner layer provides cell wall strength, and is made of β -(1,3) and β -(1,6) glucan that is complexed with chitin. The outer layer consists of mannoproteins, and determines most of the surface properties of the cell. The majority of mannoproteins are covalently linked to the inner glucan layer. Periplasmic enzymes are trapped between the plasma membrane and the inner skeletal layer (Schreuder *et al.*, 1996).

removed (Stratford 1994; Klis, 1994).

Extraction of yeast cells with hot alkali leaves an insoluble fraction consisting of β -(1,3), β -(1,6) glucan and chitin (0.6%-1%) (Klis 1994; Iranzo *et al.*, 2002). Since β -(1,3), β -(1,6) glucan is in itself soluble in alkali, this suggests that chitin is responsible for the insolubility of this fraction (Klis 1994). This current evidence suggests that glucan and chitin are covalently linked (Klis 1994).

Electron microscopy of the cell wall of *S. cerevisiae* reveals a two-layered structure: an electron-dense, dark-staining, outer layer and a non-staining inner layer (Zlotnik *et al.*, 1984). It was shown that when cells were incubated with a protease, this resulted in the loss of the electron-dense outer layer (Zlotnik *et al.*, 1984). These results indicated that the inner layer is the glucan layer and the electron-dense is the mannoprotein layer (Zlotnik *et al.*, 1984). In contrast, chemical and enzymatically treated *N. crassa* walls were shown, through electron microscopy, to possess four layers including an amorphous glucan (β -1,3 and β -1,6) layer, a glycoprotein reticulum, a distinct layer of protein, and an innermost chitinous region (Fig. 1.3; Hunsley and Burnett 1970; Trinci 1978). The distinct features of the *N. crassa* cell walls need to be elucidated, but the *S. cerevisiae* cell wall work serves as a model from which to start.

Cell wall proteins found in *Saccharomyces cerevisiae*

Yeast wall mannoproteins are highly glycosylated (Van der Vaart *et al.*, 1995; Fig. 1.5). In *C. albicans*, glycosylation maximizes adherence to host cells and contributes to virulence in models of systemic infection (Cutler 2001). Many of them carry N-linked glycans with a core structure of $\text{Man}_{10-14}\text{GlcNAc}_2\text{-Asn}$, structures very similar to

mammalian high-mannose N-glycan chains. Outer chains present on yeast N-glycans consist of 50 to 200 additional α -linked mannose units, with a long α -(1,6)-linked backbone decorated with short α -(1,2) and α -(1,3)-linked side chains (Lipke and Ovalle 1998). Phosphorylation of the mannosyl side chains gives yeast its anionic surface charge (Lipke and Ovalle 1998).

The mannoprotein fraction can be divided into two populations. One population is extractable with a detergent such as SDS and the remainder are released after incubation of cell walls with a β -glucanase (Valentin *et al.*, 1984; Zlotnik *et al.*, 1984). The glucanase extractable mannoproteins are released after an incubation with β -(1,3) or β -(1,6) glucanase which indicates that they are tightly bound to the glucan layer (Pastor *et al.*, 1984).

The glucanase-extractable mannoproteins so far identified have three characteristics in common: they contain a secretion signal at their N-termini, they are rich in serine and/or threonine and they all contain a putative glycosylphosphatidylinositol (GPI)-attachment signal at their C-termini (Table 1.1; Fig. 1.5). Serine and threonine residues are often clustered within the sequences of wall proteins (Van der Vaart *et al.*, 1995). Where O-linked saccharides have been mapped, most or all of the clustered residues are O-glycosylated (Van der Vaart *et al.*, 1995). O-mannosylation is important for proper cell wall biogenesis. Disruption of O-glycosylation causes not only aberrant processing of wall mannoproteins, but leads also to significant reduction in wall component of the branched β -(1,6) linker glucan (Lussier *et al.*, 1997). Van Risum *et al.*, (1991) isolated a β -(1,6) glucan chain on the cell wall proteins and is involved in cell wall

anchorage (Kapteyn *et al.*, 1996). N-terminal sequencing identified two open reading frames (ORFs) (Van der Vaart *et al.*, 1995). One is the YKL096w gene product, which was sequenced by Pallier *et al.*, (1993) and was named Cwp1p (cell wall protein 1). The other was identified as the gene product which had already been identified by Kondo and Inouye (1991) as a temperature-inducible protein. A third ORF that was identified was *CWP2* (Van der Vaart and Verrips 1998). Several mutants depleted for a cell wall protein were created (Van der Vaart *et al.*, 1995). Phenotypical analysis of yeast cells depleted for Cwp2p revealed an increased sensitivity for the cell wall disturbing compounds Congo Red and Calcofluor White, and for Zymolyase. Furthermore, the creation of fusion constructs consisting of the coding region of the reporter enzyme α -galactosidase and C-terminal parts of several proteins with the characteristics of cell wall proteins confirmed the cell wall localizations and β -(1,6) glucosylation of several potential cell wall proteins. The C-terminal regions of Sed1p (multicopy suppressor of *erd2* gene) (Hardwick *et al.*, 1992), Flo1p (involved in flocculation), which was already identified as a cell surface protein (Bidard *et al.*, 1995) and YCR89w, an α -agglutinin homolog, are all capable of immobilizing α -galactosidase on the cell wall and these fusion proteins all contain a β -(1,6) glucan chain in their cell wall bound forms (Van der Vaart *et al.*, 1996).

The incorporation of *Saccharomyces cerevisiae* proteins in the cell wall

Biotinylation has also been used to tag and identify cell surface proteins (Casanova *et al.*, 1992). Using this method, Mrsa *et al.*, (1997) identified two classes of detergent-extractable cell wall proteins: i) *GIP proteins (GPI-CWPs)*. Genes encoding GPI-CWPs correspond to serine and threonine rich proteins that possess an N-terminal

Table 1.1 Glucanase-extractable cell wall proteins in *Saccharomyces cerevisiae* (Van der Vaart *et al.*, 1995; Van der Vaart *et al.*, 1996a; Van der Vaart and Verrips 1998).

Gene	Protein	(1,6)- β -glucan ^a	Ser/Thr (%) ^b	N-glycosylation ^c	GPI-anchor ^d	Comments
<i>CWP1</i>	Cwp1p	+	44	+	nd	Detection of GPI-signal leads to secretion
<i>CWP2</i>	Cwp2p	+	48	-	+	Mature protein consists of 54 aa
<i>TIP1</i>	Tip1p	+	50	-	nd	Temperature inducible
<i>SED1</i>	Sed1p	+	41	+	nd	Multicopy repressor of <i>erd2</i>
<i>TIR1</i>	Tir1p	+	45	-		Anaerobically expressed
<i>TCR89w</i>		+	47	+	nd	Homolog of α -agglutinin anchor protein
<i>FLO1</i>	Flo1p	+	48	+	nd	Involved in flocculation
<i>AGal</i>	α -agglutinin	+	41	+	+	Involved in mating, deletion of GPI-signal of GPI leads to secretion
<i>AGAl</i>	a-agglutinin	nd	52	-	nd	Involved in mating

^a Proteins are recognized by an anti (1,6)- β -glucan antiserum.

^b (Number of Ser-Thr res/ total number of res.) X 100% of C-terminal half of protein.

^c Presence of potential N-glycosylation sites.

^d Biochemical evidence for GPI-anchor. nd, no data.

signal peptide, often contain internal repeats and terminate in a hydrophobic region in the endoplasmic reticulum that is replaced by a GPI anchor (Caro *et al.*, 1997; Fig. 1.5). GPI-CWPs are linked to other cell wall components through a remnant of their GPI anchor and can be released by β -glucanase digestion of the cell wall (Caro *et al.*, 1997). As expected, truncated proteins in which the carboxy-terminal hydrophobic region has been removed by genetic engineering and which therefore do not receive a GPI anchor, are secreted into the medium (Shimoi *et al.*, 1995). ii) *Pir proteins*. PIR proteins encode a family of proteins sharing the following characteristics: an N-terminal peptide, a Kex2 protease recognition site and one or more repeats (Mrsa *et al.*, 1997; Fig. 1.5). They do not contain a C-terminal GPI anchor addition sequence. Pir proteins can be released from the cell wall by overnight incubation in mild alkali or by β -glucanase digestion (Mrsa *et al.*, 1997). As the glycopeptide linkage of O-linked side chains is cleaved under mild alkali conditions, a possible interpretation is that Pir proteins might be linked to other cell wall components through their O-linked side chains.

GPI proteins in the cell wall

The identified cell wall proteins of yeast share several characteristic features. They are β -(1,6) glycosylated in their cell wall-bound forms, they contain an N-terminal signal, are rich in serine and/or threonine and contain a C-terminal GPI attachment signal (Vaart and Verrips 1998; Fig. 1.5). Van Berkel *et al.*, (1994) showed that a fusion protein consisting of the invertase signal sequence, the α -galactosidase coding sequence, and the C-terminal thirty amino acids of α -agglutinin, which includes the GPI-attachment signal, is incorporated into the cell wall and becomes β -(1,6) glycosylated. This result indicates

that, while the serine/threonine-rich region of this protein is not involved in cell wall anchorage, GPI-anchoring is required (Van Berkel *et al.*, 1994). Moreover, a temperature-sensitive mutant of *CWH6* (*GP13*, *SPT14*) a gene involved in GPI-synthesis, displays a large decrease in the amount of mannan in the cell wall, which indicates a lower amount of incorporated cell wall proteins in this mutant (Vossen *et al.*, 1995). Many open reading frames in the yeast genome, such as Tip1 and Cwp1, have the sequence characteristics of GPI-anchored wall proteins (Caro *et al.*, 1997). After secretion of the GPI-anchored mannoprotein to the outer leaflet of the plasma membrane, the anchor is cleaved within the C-terminal glycan and the remnant is transferred to form a glycosidic linkage with the branched β -(1,6) glucan (De Nobel and Lipke 1994). The β -(1,6) glucan is, in turn, glycosidically linked to the β -(1,3) glucan-chitin complex that makes up the fibers of the inner wall (Kapteyn *et al.*, 1996).

Not all GPI-anchored proteins become incorporated into the cell wall. Gas1p and Yap3p are GPI-anchored proteins and have the plasma membrane as their final destination (Nuoffer *et al.*, 1993; Popolo *et al.*, 1993; Ash *et al.*, 1995). The mechanism by which a cell discriminates between plasma membrane and cell wall localization is unknown. A dibasic motif, N-terminal of the GPI-attachment site, is found in plasma membrane proteins but not in cell wall proteins (Vossen *et al.*, 1997). This motif could act as a plasma membrane residence signal or could facilitate cleavage of plasma membrane proteins resulting in the complete loss of their GPI-moieties (Vossen *et al.*, 1997). This would imply rapid turnover of plasma membrane proteins as loss of the GPI-anchor would free them from the plasma membrane and move on to the cell wall where

they become linked through β -(1,6) linkages (Van der Vaart and Verrips 1998). It is not known which enzymes are involved in the cleavage and anchorage processes.

Pir proteins in the cell wall

Another group of at least six covalently linked CWPs has been identified in yeast, which could be released from SDS-extracted yeast cell walls under very mild alkali conditions (Mrsa *et al.*, 1997; Fig. 1.5). Four proteins solubilized by this mild alkali treatment have been N-terminally sequenced and found to be members of the *PIR* gene family (a gene family consisting of proteins with internal repeats) (Toh-E *et al.*, 1993). Further analysis revealed that, like the GPI-CWPs, the Pir-CWPs are highly O-glycosylated, but a striking difference is that *PIR* genes do not encode a GPI anchor additional signal. It is possible that Pir-CWPs are cross-linked via their O-linked saccharides to an as yet unknown cell wall component (Kapteyn *et al.*, 1999).

Although the identity of the cell wall components to which Pir proteins are linked is still unknown, β -(1,3) glucan is a possible candidate. As mentioned, β -(1,3) glucanase digestion of the cell wall results in the release of (almost) all CWPs, whereas β -(1,6) glucanase digestion is less efficient and releases 84% of the CWPs. This might mean that β -(1,6) glucanase-resistant protein fraction corresponds to the Pir proteins, in agreement with the speculative structure: Pir-CWP-Ser/Thr-O-chain- β -(1,3) glucan (Kapteyn *et al.*, 1999).

Other mannoproteins

Other mannoproteins are wall associated by other mechanisms. Invertase and other enzymes are physically entrapped in the wall (Cid *et al.*, 1995). Flo1p, a protein

involved in flocculation in yeast, is initially GPI anchored but may remain unlinked to glucan (Bony *et al.*, 1997). In addition, mild treatments (30 mM NaOH, 16 h, at 4°C) liberates four mannoproteins that have no GPI anchor signal in the corresponding sequence (Mrsa *et al.*, 1997). Some mannoproteins are disulfide bonded to GPI-anchored lattice proteins (Cappellaro *et al.*, 1994).

The attachment site of the β -(1,6) glucan chain to GPI-CWP of *Saccharomyces cerevisiae*

The wall proteins are β -glucosylated in their wall bound forms. Recently, it was shown that the β -(1,6) glucan chains links cell wall mannoproteins to β -(1,3) glucan in the cell wall (Kapteyn *et al.*, 1996) The exact attachment site of this chain on to a cell wall protein is unknown. Potential attachment sites are: amino acid residues, N-linked carbohydrate side chains, O-linked carbohydrate chains, or the GPI-moiety (Van der Vaart and Verrips 1998). Kapteyn *et al.*, (1996) suggested that the GPI- is the attachment site of the β -(1,6) glucan chain (Van der Vaart and Verrips 1998). The findings that the β -(1,6) glucan chain of several cell wall proteins can be removed by phosphodiesterases indicates that a phosphate group is present between the protein and the β -(1,6) glucan chain (Van der Vaart and Verrips 1998). Fujii *et al.*, (1996) showed the presence of ethanolamine and the loss of glucosamine from the GPI-moiety of the cell wall bound form of Tirp1. These results indicate that the GPI-anchor of the plasma membrane bound form of cell wall protein is processed in such a way that the β -(1,6) glucan chain becomes bound to the GPI-anchor (Fig. 1.6A). Van de Vaar and Verrips (1998) have proposed a complete mechanism of anchorage of GPI-proteins and it is summarized on Fig. 1.6B.

Fig. 1.6 A) Schematic representation of the cell wall of *Saccharomyces cerevisiae*. The movement of a glucanase-extractable cell wall protein from the plasma membrane form to the cell wall bound form is displayed. The exact mechanism by which this movement takes place is unknown but the represented attachment of the β -(1,6) glucan chain to the remnant of the GPI-moiety is essential for cell wall anchorage. B) Potential cell wall anchoring mechanism for cell wall proteins in *Saccharomyces cerevisiae*. In this model the plasma membrane bound form of a cell wall protein is first cleaved by a phospholipase (1, optional) and secondly by a mannase (2). The resulting terminal mannose residue of the GPI-moiety will subsequently act as an acceptor of the β -(1,6) glucan chain (3), which will be bound to the cell wall glucan layer (4). Step 2 and 3 might be carried out by the same transglycosydase (Van der Vaart and Verrips 1998).

Regulation of cell wall protein expression

The cell wall is a dynamic structure: its composition and architecture and internal stimuli. For example, the deposition of chitin in the cell wall during the cell cycle is both temporary and spatially controlled (Cabib *et al.*, 1997). One of the reasons chitin deposition has been studied more extensively than other cell wall components is its easy visualization by the dye Calcofluor White. Two recently developed techniques promise rapid progress in our understanding of the contribution of CWPs to the dynamics of cell wall architecture: (i) the introduction of CWPs tagged with green fluorescent protein (GFP-CWPs), enabling the visualization of specific cell wall proteins in living cells (Ram *et al.*, 1998), and (ii) the use of DNA chips, which allow monitoring gene expression on a genomic scale (DeRisi *et al.*, 1997).

Cell cycle controlled expression

Ram *et al.*, (1998) have constructed two fusion proteins, GFP-Cwp1p and GFP-Cwp2p, both regulated by their own promoter, and demonstrated that both fusion proteins were incorporated in the cell wall and became linked to β -(1,6) glucan. This approach will make it possible to answer the question when and where specific cell wall proteins are deposited during the cell cycle and how this is regulated. Indeed, several GPI-CWP encoding genes are expressed periodically during the cell cycle. For example, the expression of *EGT2* is confined to a short period of the cell cycle between the end of mitosis and early G1 (Kovacech *et al.*, 1996). In mutant *egt2*, cell separation is delayed. This is not due to a delay in mitotic events or because chitin degradation in the septum is inhibited. This indicates that Egt2p is active after cytokinesis and might be involved in

the degradation of non-chitinous cell wall components (Kapteyn *et al.*, 1999). Another CWP-encoding gene that is periodically expressed in G1 is *TIP1* (Caro *et al.*, 1998). *CWP1*, *CWP2* and *TIR1* are also cell cycle regulated in S/G2 phase (Caro *et al.*, 1998).

Environmentally induced changes in cell wall protein expression

There is ample evidence for the effects of growth conditions on transcriptional activation or repression of specific CWPs. For example, *TIR1* transcription is induced at low and high temperatures and under anaerobic conditions (Donzeau *et al.*, 1996). *TIR1* transcription also increased under anaerobic conditions; in line with this, Tir1p is a major cell wall protein in standing yeast cultures, whereas it is absent in shaken cultures (Kitagaki *et al.*, 1997). TIR transcription is also stimulated by glucose (Marguet *et al.*, 1988). Other well-known examples are the mating type specific sexual adhesion proteins, Aga1p and Ag α 1p, the transcription and expression of which are induced by sexual pheromones from close cells of the opposite mating type (Lipke and Kurjan 1992). Moreover, Flo1p, which is involved in flocculation, induced by nutrient depletion (Teunissen and Steensma 1995), and Cwp1 is highly expressed under stress conditions resulting from reductions in the β -(1,3) glucan content of the cell wall (Kapteyn *et al.*, 1997). Transcript profiling using DNA chips has confirmed the dynamic nature of the cell wall with respect to CWPs (DeRisi *et al.*, 1997; Wodicka *et al.*, 1997). Wodicka *et al.*, (1997) have compared the expression levels of yeast genes in cells grown to mid-log phase in rich and in minimal glucose-containing medium. The transcript levels of putative CWP-encoding genes *UTR2* were 5-fold more abundant in rich medium than in minimal medium, whereas *SED1* was expressed at intermediate levels in rich medium, but became

highly expressed in minimal medium (Caro *et al.*, 1997). The transcript level of *GSC3* (*FKS2*), which is believed to encode the catalytic subunit of β -(1,3) glucan synthase, increased 11-fold in minimal medium (Kapteyn *et al.*, 1999). In batch-grown cells supplied with rich medium, the transcript levels of several GPI-CWPs are affected in the final phases of growth (DeRisi *et al.*, 1997). The expression levels of *SED1*, *TIP1*, and in particular *YER150W*, rose at the end of the growth period, whereas the expression levels of *EGT2* and *UTR2* decreased (Kapteyn *et al.*, 1999). Thus, it is clear that the cell prefers specific CWPs in its wall, not only during various phases of the cell cycle but also dependent on growth conditions. To date, clear functions are known only for α -agglutinin, α -agglutinin, the flocculins encoded by *FLO9* and *FLO11* (Kapteyn *et al.*, 1999).

Cell wall repair mechanisms

Fungal cells often reside in environments in which they are regularly confronted with cell wall degrading enzymes (Kapteyn *et al.*, 1999). It is therefore not surprising that fungal cells have developed compensation mechanisms that become active when the cell wall is damaged or stressed. The following observations are consistent with the existence of such a compensation mechanism. Deletion of *FKS1*, which encodes the putative catalytic subunit of β -(1,3) glucan synthase, results in large, swollen cells which have lost a large part of their β -(1,3) glucan, but which also show strongly increased chitin deposition in their lateral walls (Kapteyn *et al.*, 1997). This is accompanied by a dramatic change in molecular architecture of the cell wall. Whereas in wild-type cells only 2% of all CWPs are directly linked to chitin through β -(1,6) glucan, this linkage becomes more abundant in *fks1* cells (Kapteyn *et al.*, 1997). In the cells of *gas1* strain, which also has

much lower β -(1,3) glucan content, similar changes occur (Kapteyn *et al.*, 1997). Further analysis of *gas1* strains showed that the levels of Cwp1p and Fks2p, which is the alternative catalytic subunit of β -(1,3) glucan synthase, increased (Kapteyn *et al.*, 1999). It is tempting to predict that these changes contribute to the strength of the cell wall as part of a general compensation mechanism activated by cell wall damage. Further support for the hypothesis that increased deposition of chitin in the lateral walls is part of a compensation mechanism comes from the observation that *fks1* and *gas1* cells become very sensitive to nikkomycin, an inhibitor of chitin synthesis (Nonet *et al.*, 1987). Increased deposition of chitin in the lateral walls is regularly observed in cell wall mutants (Kapteyn *et al.*, 1999). This is frequently described as “delocalized” chitin deposition due to disorganization of the actin cytoskeleton (Kapteyn *et al.*, 1999). However, after cytokinesis in wild-type cells, chitin is also deposited in the lateral walls. An alternative explanation in at least some of these cases might therefore be that chitin synthesis in the lateral walls has been activated as part of a rescue mechanism (Kapteyn *et al.*, 1999).

A clear picture of the molecular architecture of the cell wall is beginning to emerge. The four major components of the cell wall are covalently linked to each other and several of these cross-links are now known in more detail. The next step, therefore, will be to identify their cross-linking reactions. It is also clear that the structure is highly dynamic, and regulated depending on internal and external cues. The discovery of potential compensation mechanisms activated by cell wall damage is another illustration of the dynamic nature of the cell wall.

Research purpose and objectives

The objective of this thesis was to identify putative cell wall proteins of the filamentous fungi *Neurospora crassa* and *Schizophyllum commune*. These fungi were chosen for several reasons. *N. crassa* was chosen since it offered many research advances such as the availability of the genome and well established genetic techniques. The availability of genomes data was crucial in the identification of wall proteins by proteomic and immunological techniques. Moreover, the use of well established genetic technique will enable the confirmation of the proteomic and immunological findings through gene knock-out experiments. In contrast, *S. commune* was chosen because there is very limited information on the protein component of the cell wall. In fact, only hydrophobins, a class of hydrophobic cell wall proteins, have been studied in detail. The aim of this research was to expand the list cell wall proteins known in this organism. Finally, *N. crassa* and *S. commune* were chosen because they are both filamentous fungi and any information obtained about their walls would complement the yeast work. This would permit cell wall comparative analyses to be performed and would further advance our understanding of the differences and similarities between yeast and filamentous fungi, as well as, ascomycetes and basidiomycetes.

Several approaches have been used to analyze and identify the proteinaceous component of the cell wall of *N. crassa* and *S. commune*. First, a NcCW polyclonal antiserum has been raised against cell wall extracts and recognizes several abundant cell wall-associated proteins of *N. crassa*. In chapter 2, I report on the characterization of this NcCW antiserum and some of the wall-associated proteins which it recognizes. In

addition, the NcCW antiserum was used to screen a λ ZAP expression library in order to identify clones encoding putative cell wall proteins. Second, in chapter 3 a similar approach was followed to define wall associated proteins in the basidiomycete *S. commune* by the use of a ScCW antiserum. In chapter 4, I describe the use of bioinformatic and proteomic approaches to catalogue the large set of proteins associated with the *Neurospora* cell wall. Specifically, I have used 2-D gel electrophoresis to resolve and isolate individual proteins for sequence analysis by mass spectrometry after chemical and enzymatic release. Moreover, many putative cell wall proteins of *N. crassa* were identified by comparing known *S. cerevisiae* wall proteins, that affect the structure of the wall directly or indirectly, against the *Neurospora* database.

CHAPTER 2

IMMUNOLOGICAL CHARACTERIZATION OF THE *NEUROSPORA CRASSA* CELL WALL

Introduction

The growth and maintenance of the characteristic, tubular hyphae of fungi such as *Neurospora crassa* depends in part, on the structure of the cell wall (Bachman and Bonner 1959; deTerra and Tatum 1963; Reyes and Lahoz 1976; Mishra 1977; Cabib *et al.*, 1988). Historically, the composition of fungal cell walls has been investigated by selectively removing the constituents of the cell wall by chemical and enzymatic treatments (Mahadevan and Tatum 1967; Ruiz- Herrera 1992). The two most abundant polymers that make up the *N. crassa* cell wall are glucans (primarily in β -1,3 and β -1,6 linkages) and chitin, in both alkali-insoluble and soluble fractions (Wrathall and Tatum 1974; Gardemil and Pincheira 1979). The *Neurospora* cell wall also consists of approximately 10 % dry weight protein (Wrathall and Tatum 1974; Gozalbo *et al.*, 1993).

Polypeptides associated with the cell wall of *N. crassa* were first detected by deTerra and Tatum (1963) and Mahadevan and Tatum (1965) during alkali and acid solubilization experiments. These proteins can be either covalently bound or more loosely associated with the cell wall and plasma membrane. Some previously identified, cell wall-bound proteins include (1, 3)- β glucanase, invertase, trehalase, chitin synthases, a copper detoxification protein and the *eas* hydrophobin (Chung and Trevithick 1970; Mahadevan and Mahadkar 1970; Sukumaran and Mahadevan 1975; Mehta and Mahadevan 1975; Hecker and Sussman 1973a; Ruiz-Herrera 1992; Wessels 1993; Suresh and Subramanyam 1996). More recently, a number of cytoplasmic and organelle proteins have also been found to be associated with the cell wall of *N. crassa* (Valencia *et al.*, 2003; manuscript submitted) and the cell walls of other fungi and yeasts (Angiolella *et*

al., 1996; Lopez-Ribot and Chaffin 1996; Lopez-Ribot *et al.*, 1996; Alloush *et al.*, 1997; Eroles *et al.*, 1997; Pardo *et al.*, 1999; Pitarch *et al.*, 1999; Edwards *et al.*, 1999; Pardo *et al.*, 2000b; Delgado *et al.*, 2001; Lim *et al.*, 2001; Pitarch *et al.*, 2001).

In the yeasts *Saccharomyces cerevisiae* and *Candida albicans*, many wall-bound or wall-associated proteins are post-translationally modified by addition of a glycosyl phosphatidylinositol (GPI) anchor, and by – and O-linked glycosylation (Orlean 1997). A bioinformatic study, based on a *S. cerevisiae* and *C. albicans* GPI algorithm, predicted the existence of GPI proteins in *Schizosaccharomyces pombe* and *N. crassa* (De Groot *et al.*, 2003). In *S. cerevisiae*, the mating factor α -agglutinin is cross-linked to cell wall β -glucans via its GPI anchor (Lu *et al.*, 1995). For other yeast proteins, sulfhydryl linkages appear to mediate their association with the cell wall.

For our studies of the fungal cell wall, we have raised a polyclonal antiserum (NcCW antiserum) which recognizes several abundant cell wall-associated proteins of *N. crassa*. Here we report on our characterization of this NcCW antiserum and some of the wall-associated proteins which it recognizes.

Methods and Materials

Strains and growth conditions

Escherichia coli strains BB4 and XL1 blue were grown in Luria broth (LB) or on LB plates (containing 1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.5 with 1.5% agar) at 37°C overnight. LB+Amp plates were prepared as LB plates with the addition of ampicillin to a final concentration of 0.1 mg/ml. To prepare LB+Amp+X-gal plates, 10 μ l of a 100 mM IPTG and 50 μ l of a 2% X-gal solution were added to each plate.

N. crassa wild type strain (74-OR23-1VA; FGSC 987), *Aspergillus nidulans* (FGSC A4), *Aspergillus niger* N400 (FGSC A732), and *Fusarium oxysporum* (FGSC 6607) were obtained from the Fungal Genetics Stock Center. *Schizophyllum commune* (strains 4-40) was obtained from M. Raudaskoski (University of Helsinki) and *Saccharomyces cerevisiae* (strain S288C) and *Agaricus bitorquis* (ATCC 24666) were obtained from the American Type Culture Collection. *Coprinus lagopus*, *Phycomyces blakesleeanus* (+ mating type), *Candida albicans* (+ mating type), and *Mucor rouxii* were obtained from Ward's Biology.

Cultures of *N. crassa*, *A. nidulans*, *A. niger* and *A. bitorquis* were grown on Vogel's minimal medium (Vogel 1964) in 250 ml flasks containing 50 ml of liquid media, with agitation (180 rpm) overnight at room temperature. *C. lagopus* was grown on DMY medium as described by Rao and Niederpruem (1969). Slant cultures were incubated in the dark at 37°C for 4 days and then transferred to 25°C until further growth developed. Liquid cultures were incubated at 30°C for 3 days to hasten growth and transferred to room temperature in ordinary light. *P. blakesleeanus* (grown at 25°C), *C. albicans* (grown at 37°C), and *M. rouxii* (grown at 25°C) cultures were grown overnight on Sabouraud-Dextrose medium (40 g/L dextrose, and 10 g/L peptone). For all of the above filamentous fungi, mycelium was harvested by vacuum filtration, washed with distilled water, pressed dry between paper towels, cut into 1 cm² pieces and frozen at -70°C overnight.

S. commune mycelium was grown at room temperature on cellophane membranes on the surface of complete medium containing 5 g/L MgSO₄·H₂O, 4.6 g/L KH₂PO₄, 10

g/L K_2HPO_4 , 20 g/L tryptone, 2 g/L yeast extract and 200 g/L of dextrose. The cellophane membranes were peeled off the plate and stored at -70°C . The yeast, *S. cerevisiae*, was grown at 30°C on YPD medium containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L dextrose and cells were collected by centrifugation at 5000 g for 5 min and frozen at -70°C overnight. Frozen cells were lyophilized for 8 h in a Savant SS2 SpeedVac and stored at -70°C until required.

Isolation of cell walls and preparation of the NcCW antiserum

The *N. crassa* crude cell wall was isolated essentially according to the procedure of Martinez *et al.*, (1991) except that the final pellet was extracted once with acetone and once with absolute ethanol. Two female, New Zealand, white rabbits were immunized with 10 mg (dry weight) in an emulsion of Freund's incomplete adjuvant (Huet 1994) followed by 2 booster injections of the same mixture at two week intervals.

Transmission electron microscopy

Transmission electron microscopy was performed by Dorothy McColl (1997). In short, *N. crassa* mycelium from overnight cultures were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2 at room temperature for 2 h, washed two times for 15 min with 0.1 M cacodylate buffer, dehydrated twice in 30% ethanol for 15 min and then dehydrated in 50%, 70% and 90% ethanol for 50 min each (McColl 1997). The tissue was infiltrated in two washes of 90% ethanol/LR White (1:1) for 45 min, submerged in LR White at 4°C for 24 h and then placed in gelatin capsules filled with LR White with polymerization stimulated by incubation at 60°C for 36 h.

Tissue sections were cut on a Reichert Ultracut E, placed on 400-mesh nickel

grids, and incubated in a drop of 1% bovine serum albumin (BSA) in a solution containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl and 0.05% Tween-20 (TBST). After washing twice with TBST, grids were incubated for 45 min in one of the following preparations: TBS (no Tween-20), pre-immune NcCW antiserum diluted 1:100 in TBS, or NcCW antiserum diluted 1:100 in TBS. The sections were washed three times in TBST and incubated in diluted (1:1 v/v) goat anti-rabbit IgG-10 nm gold-conjugated secondary antibodies (Bioad) for 45 min. The sections were washed four times with TBST, stained in lead citrate for 5 min, rinsed with distilled water and air dried. Post-staining was done in 2.5% (w/v) uranyl acetate in 50% methanol for 10 min. The sections were then rinsed in 50% (v/v) methanol, air-dried, and examined with a Philips 201C transmission electron microscope at 60 kV.

One-dimensional SDS-PAGE

For one-dimensional (1-D) SDS-PAGE, proteins were extracted from lyophilized, *N. crassa* mycelium by grinding in a microcentrifuge tube using a plastic pestle. The mycelial powder was resuspended in a ratio of powder: SDS sample buffer of 1:4 (Laemmli 1970), vortexed for 20 seconds and then was incubated for 10 min at room temperature. The extraction mixture was heated up on a hot plate to 100°C for 5 min. After boiling, the mixture was centrifuged at 13000 g for 20 min. Proteins were separated using the discontinuous buffer system of Laemmli (1970). Separatory gels contained 10% acrylamide, while stacking gels contained 4%. Staining of proteins was done overnight in a solution containing 0.25% Coomassie brilliant blue R-250, 40% (v/v) methanol, and 10% (v/v) acetic acid. Gels containing low amounts of proteins were silver stained

following the manufacturer's instructions (Biorad). The concentration of protein in the extracts was determined by spectrophotometry (Stoscheck, 1990) using a diode array spectrophotometer (Hewlett-Packard).

Two-dimensional SDS-PAGE

Cell extracts for two-dimensional (2-D) SDS-PAGE were prepared by grinding lyophilized mycelium in Cold Extraction Buffer (10 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.1% Triton X-100, 10 µg/ml, pepstatin, 10 µg/ml, leupeptin, 0.05 µg/ml, aprotinin, 10 µg/ml, TPCK, and 0.1 µg/ml PMFS) (Roche) and clarification by centrifugation at 13000 g for 20 min at 4°C. An equal volume of 1-D sample buffer, containing 9.5 M urea, 2% (w/v) Triton® X-100, 5% (v/v) 2-mercaptoethanol, 1.6% Bio-Lyte® 5/7 (Biorad), 0.4% (v/v) Bio-Lyte® 3/10 (Biorad) and distilled water was added to a final volume of 10 ml. The samples were incubated at room temperature for 10 min, centrifuged at 13000 g for 20 min and assayed for protein concentration as determined previously.

2-D SDS-PAGE was performed following the procedure of O'Farrell (1975) with a Mini-PROTEAN II 2-D cell apparatus (Biorad) according to the manufacturer's instructions.

Following isoelectric focusing, the gel tubes were extruded and placed on the 4% stacking gels and equilibrated for 10 min in the second dimension sample buffer containing 0.5 M Tris-HCl (pH 6.8), 12.5% (v/v) glycerol, 10% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 0.05% (w/v) bromophenol blue and electrophoresed as described above.

Immunoblotting and lectin blotting

After SDS-PAGE, the gels were equilibrated for 30 min in a transfer buffer containing 25 mM Tris-HCl, 192 mM glycine and 20% (v/v) methanol. Subsequently, proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) at 15 V for 30 min by using the Transblot^R SD Semi-Dry Electrophoretic Transfer Cell (Biorad).

Membranes were blocked and probed essentially as described by Towbin (1979) in 25 mM Tris-HCl pH 8.0 containing 150 mM NaCl, 0.1% Tween-20 (TBST2) and 50 g/L nonfat milk for 1 h at room temperature with gentle rocking. After washing twice with TBST2 for 5 min, the membranes were incubated in a solution containing 2.5% nonfat milk in TBST2 and 1:2000 or 1:5000 dilution of the NcCW antiserum. The membranes were washed three times with TBST2 for 5 min and then submerged in a solution containing 2.5% nonfat milk in TBST2 and 1:5000 dilution of goat anti-rabbit HRP-conjugated secondary antibody (Promega) for 1 h at room temperature. The membranes were washed 3 times with TBST2 and then incubated with the Enhanced Chemiluminescent substrate system (Roche). The membranes were then exposed to XAR Scientific Imaging Film (Kodak) for 1-60 sec.

The lectin blotting procedure was essentially as described by Millette and Scott (1984). The biotinylated lectins (Sigma), from *Phytolacca americana* (5 µg/ml), Succinylated *Canavalia ensiformis* (ConA; 1 µg/ml) and *Bauhinia purpurea* (5 µg/ml) were used at the indicated final concentrations and detected with a 1:2000 dilution of streptavidin-horse radish peroxidase (HRP) conjugate (BioRad) and the Enhanced Chemiluminescent substrates.

Lectins were stripped from blots according to a modification of the procedure of Montreuil *et al.*, (1986) for Western blots. Membranes were wetted in TBST2 for 15 min, equilibrated in sodium acetate buffer, pH 5.2 (0.1 M NaCl, 1 mM CaCl₂, 1 mM MnCl₂, 1 mM MgCl₂, 0.02% (w/v) sodium azide, 5 mM sodium acetate, pH 5.2) for 10 min, and then washed in a descending series of solutions of methyl- α -D-glucopyranoside at 0.5 M, 0.3 M, 0.1 M, 0.05 M, and 0.01 M (Sigma) in sodium acetate buffer. The 0.5 M wash was for 90 min and all others were for 30 min. Between each wash, the membranes were rinsed twice with sodium acetate buffer, pH 5.2 for 5 min each. Finally, the membranes were washed seven times with TBST2 for 5 min each at room temperature. The membranes were ready to be blocked and probed with the NcCW antiserum as previously described in the immunoblotting section.

Chemical and enzymatic protein cleavage

The proteolytic digestions were performed according to the procedures of Lischwe and Ochs (1982) and Sambrook *et al.*, (1989). SDS-extracted proteins from *N. crassa* were incubated at 100°C for 2 min, cooled on ice and then centrifuged at 13000 g for 2 min before adding endo-proteinase Glu-C (endo-Glu-C) (Roche) or trypsin (Sigma). Each 20 μ l endo-Glu-C reaction contained *Neurospora* crude cell extract (50 μ g of protein), 10 mM Tris-Cl (pH 6.8), 0.5%(v/v) of SDS and either 0, 10, 100, or 1000 μ g/ml of the protease. Similarly, the 20 μ l trypsin reaction contained crude extract (100 μ g of protein), 10 mM Tris-Cl (pH 8.0), 20 mM CaCl₂, 0.05% SDS and 10 μ g/ml of trypsin (Sambrook *et al.*, 1989). These reactions were incubated at 37°C for 30 min and were stopped by adding 40 μ l of 2X SDS sample buffer.

The chemical cleavage with N-chlorosuccinimide (NCS, a tryptophan-specific reagent)/urea (Sigma) was according to the procedure developed by Lischwe and Ochs (1982). The 20 μ l reaction contained 100 μ g of protein and 0.15 M NCS in a solution prepared from 1 g of urea, 1 ml of 17 M acetic acid and 1 ml of distilled water (1:1:1 ratio). The reactions were incubated at 37°C for 30 min. To neutralize the acidity of each reaction, proteins were precipitated by adding 6 volumes of acetone for 10 min on ice and recovered by centrifugation at 13000 g for 4 min. The precipitates were washed and centrifuged with a solution containing 1 volume of 0.5 M Tris-HCl (pH 6.8) and 6 volumes of acetone and once with absolute ethanol. The pellets were resuspended in a solution containing 1 volume of 0.5 M Tris-Cl (pH 6.8) and 2 volumes of the SDS sample buffer. The chemical and enzymatic digestion products were separated by SDS-PAGE (10% separatory gel; 4% stacking gel) and transferred onto nitrocellulose membranes by semi-dry blotting. Finally, the fragments were probed with the NcCW antiserum and the HRP-conjugated antibodies, respectively, as previously mentioned in detail.

Chemical extractions of cell wall matrix

The fungal cell walls were prepared according to the procedure of Martinez *et al.*, (1991). Essentially, spores of two slant tubes were resuspended in 5 ml of sterile water and were used to inoculate two 125 ml flasks containing VMM. The cultures were grown for 16 h at room temperature with shaking at 180 rpm. The cultures were harvested and washed 5 times with water. Fresh mycelia was ground in 10 ml of ice cold fungal lysis buffer (FLB) containing 10 mM Tris-HCl, pH 7.2, and 1 mM PMSF. The mixture was centrifuged at 1000 g for 5 min. The pellet was washed again with FLB and centrifuged.

The washes were repeated 10 times. The final pellet was resuspended in 10 ml of FLB containing 10 µg/ml, pepstatin, 10 µg/ml, leupeptin, 0.05 µg/ml, aprotinin, 10 µg/ml, TPCK, and 0.1 µg/ml PMFS. Aliquots (0.5 ml) of this cell wall mixture were centrifuged and the drained cell wall pellets were re-suspended in 0.5 ml in different extraction reagents: control (FLB), 1% SDS, 100 mM 2-mercaptoethanol + 8 M urea (ME), a 2:1 mixture of trifluoromethanesulfonic acid and anisole (TFMS), 0.5 M NaOH and 1 M HCl.

The extractions in FLB, ME, HCl or NaOH were for 10 min at 20°C and repeated once, essentially according to the procedure of Wrathall and Tatum (1973). The TFMS treatment was for 5 h at 0°C and proteins were recovered from the supernatant by precipitation according to the B method of Edge *et al.*, (1981). The residual wall matrix in each extraction was pelleted by centrifugation at 13000 g for 15 min, washed twice with distilled water and resuspended in SDS sample buffer with the pH adjusted to 6.8 as required. Proteins solubilized by the various extraction procedures were precipitated by adding trichloroacetic acid (TCA) to 10% (w/v) and with 1 µg of BSA added as a carrier. The samples were left on ice for 1 h, centrifuged at 13000 g for 15 min, washed twice with a solution 95% ethanol containing 100 mM Tris-HCl, 8.0. The pellets were dried briefly and then resuspended in SDS sample buffer. The pellet and supernatant proteins were separated by SDS-PAGE (10% separatory; 4% stacking), transferred to nitrocellulose membranes by semi-dry blotting and probed with the NcCW and HRP-conjugated antibodies as previously described.

Novozyme™ extraction of wall proteins

A ml of purified cell wall mixture was thawed and aliquots were distributed to as many mini centrifuge tubes as necessary. The tubes were centrifuged at 14000 RPM for 5 min and drained. The cell walls were resuspended in a 99 µl buffer containing 10 mM Tris-HCl, pH 7.2 and 10X strength protease cocktail mix (Roche Diagnostics, Palo Alto, CA). An appropriate amount (0.001 to 100 µg) of Novozyme™ 234 (Novo Biolabs, Danbury, CT), in a 1 µl volume, was added to the cell wall mixture. One µl of Tris buffer was added to the control reaction instead of the Novozyme™. The reactions were incubated for 1 or 5 h at 37°C. Following incubation, the samples were centrifuged at 14000 RPM for at least 30 min or until the cell walls formed a solid pellet. The supernatants containing the released proteins were transferred to new tubes and the protein concentrations were measured by spectrophotometry as previously described. The appropriate amount of released proteins (5-25 µg) were separated by 1-D SDS PAGE, transferred onto nitrocellulose membranes by semi-dry blotting and membranes were probed with the NcCW antiserum.

Protein secretion

Flasks containing 50 ml of VMM were inoculated with 1×10^6 spores and grown at room temperature for 12, 18 or 24 h. Mycelium was harvested by vacuum filtration and the media containing the secreted proteins was collected. The media was dialyzed in a 10 ml Spectra/Por® Floalyzer membrane (cut off MW 1000 Da, Spectrum, Laguna Hills, CA) overnight with 4 changes of deionized water. The protein concentrations were determined by spectrophotometry. Proteins resuspended (5-15 µg) in 1-D SDS PAGE

sample buffer and separated by gel 1-D SDS PAGE and transferred onto nitrocellulose membranes by semi-dry blotting. Membranes were probed with the NcCW antiserum and the HRP-conjugated antibodies, respectively, as previously mentioned.

Biotinylation of surface proteins

Cell surface proteins were biotinylated by following the procedure of Mrsa *et al.*, (1997). In summary, 50 ml *N. crassa* cultures were grown overnight, harvested by centrifugation at 5000 RPM for 10 min, and washed twice with 40 ml of 50 mM-K-phosphate buffer pH 8. Cells were then resuspended in 3 ml of the same buffer containing 1.5 mg of NHS-LC-Biotinylation reagent (sulfosuccinimidyl-6-(biotinamido)hexanoate; Pierce, Rockford, USA) and incubated for 90 min on ice, centrifuged and washed twice with 10 ml of 50 mM-Tris-HCl buffer pH 7.5 + 50 mM MgCl₂ followed by another wash with 10 ml of 50 mM-K-phosphate buffer pH 8. The biotinylated proteins were extracted from the mycelia by grinding in a material with a mortar and pestle in SDS sample buffer. The mixture was centrifuged at 5000 RPM for 20 min and the supernatants were transferred to new tubes. The appropriate amount of protein (5-15 µg) were separated on a 1-D SDS-PAGE gels and transferred onto a nitrocellulose membrane using a transfer tank filled with 10 mM CAPS in 10% methanol. Electroblotting was done at 30 V for 16 h.

Biotinylated proteins were detected by using a streptavidin conjugated to HRP (Pierce). Membranes were washed in TBST for 10 min and blocked with BSA blocking solution (0.9% NaCl, 3% BSA, 10 mM Tris-HCl, pH 7.4). Subsequently, membranes were incubated with Streptavidin conjugated-HRP (1 µg/ml) in the BSA blocking

solution for 1 h. The membranes were washed with TBST for 5 min and detected with the ECL substrates.

Probing wall extracts with phospho-antibodies

Proteins extracted with U+ME (8 M urea, 100 mM 2-mercaptoethanol) were separated by 1-D SDS-PAGE, transferred onto nitrocellulose membranes and probed with various phospho-specific antibodies (Cell Signalling Technology™) according to the manufacturer's instructions. Membranes were washed with 1X TBS (To prepare 1 L of 10X TBS: 24.2 g Tris base, 80 g NaCl; adjusted to pH 7.6) for 5 min at room temperature. The membranes were incubated in 25 ml of blocking buffer (1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk) for 1 h at room temperature. Membranes were washed 3 times for 5 min each with 15 ml of TBS/T (1X TBS, 0.1% Tween-20). Membranes and appropriate dilution of primary -phospho-antibodies (PKC 1:1000, Akt 1:1000, PKA 1:1000, Tyr 1:2000), which recognize the substrates of PKC, Akt and PKA, were incubated in a primary antibody dilution buffer (1X TBS, 0.1% Tween-20 with 5% BSA). Following the overnight incubation, membranes were washed for 5 min with 15 ml of TBS/T. Membranes were incubated with HRP-conjugated antibody (1:2000) in 10 ml of the blocking buffer for 1 h at room temperature with gentle agitation. Finally, membranes were washed 3 times with 15 ml of TBS/T and detected with the Enhanced Chemiluminescent substrates (Roche).

Cellular fractionation

Preparation of membranes was done as described by Bruneau *et al.*, (2001). Mycelium was resuspended in a 200 mM Tris-HCl buffer, pH 8, containing 50 mM

EDTA, 1 mM PMSF, and disrupted in an ice-cold bead beater (Bead Beater™, Biospec Products, Bartlesville, OK) for 3 min. Cell walls were removed by centrifugation at 4000 g for 15 at 4°C. The supernatant was centrifuged at 100000 g for 1 h at 4°C. The membrane pellet was homogenized in a 50 mM Tris-HCl buffer, pH 8.5, containing 5 mM EDTA, 0.5 mM PMSF, 1 mM DTT and immediately frozen at -70°C until required. Prior to gel electrophoresis, membranes were thawed and β -n-octylglucopyranoside and PMSF were added to reach a final concentration of 1% and 0.5 mM, respectively.

Total cell lysates, nuclei and mitochondria were prepared according to the methods previously reported (Schwerdtfeger and Linden 2000; Ahting *et al.*, 2001). The mycelial pads (3-15 g, wet weight) were homogenized in a bead beater in 6 mL buffer A (1 M sorbitol, 7% (w/v) Ficoll, 20% (v/v) glycerol, 5 mM magnesium acetate, 5 mM EGTA, 3 mM CaCl₂, 3 mM dithiothreitol, 50 mM Tris-HCl, pH 7.5) with glass beads for 2 min. The chamber temperature was maintained below 4 °C. The crude extract was filtered through cheesecloth and 2 vol. of buffer B (10% (v/v) glycerol, 5 mM magnesium acetate, 5 mM EGTA, 25 mM Tris/HCl, pH 7.5) were slowly added to the filtrate while stirring. The diluted homogenate was layered over 15 ml of a solution consisting of a 1 : 1.7 mix of buffers A and B in a 50 ml tube and centrifuged at 3000 g for 7 min at 4 °C to remove cell debris. The supernatant was removed and aliquots were taken (referred to here as total cell lysate). The supernatant was then layered onto a 5 ml step gradient (1 M sucrose, 10% (v/v) glycerol, 5 mM magnesium acetate, 1 mM dithiothreitol, 25 mM Tris/HCl, pH 7.5) and centrifuged at 9400 g for 15 min at 4 °C. The resulting nuclear pellets were gently resuspended in ice-cold storage buffer (25% (v/v) glycerol, 5 mM

magnesium acetate, 3 mM dithiothreitol, 0.1 mM EDTA, 25 mM Tris-HCl, pH 7.5) and stored at -70 °C. All buffers contained leupeptin (1 μ M), pepstatin (1 μ M), and phenylmethanesulfonyl fluoride (50 μ M). The supernatant (nuclear-free cytoplasmic fraction) was collected and used to subsequently isolate mitochondria. Mitochondria were sedimented by centrifugation of the supernatant for 50 min at 17700 g, washed by resuspending them in SM buffer (0.2 M sucrose, 10 mM MOPS, 1 mM PMSF, complete protease inhibitor cocktail; Roche). After a second centrifugation step for 50 min at 17700 g, mitochondria were resuspended in SM buffer and stored in aliquots at -20°C.

For Western blot analysis of the cellular fractions, appropriate amount of proteins were resolved by 1-D SDS PAGE and transferred to nitrocellulose membranes by semi-dry blotting. The membranes were probed with the NcCW antiserum at a dilution of 1:5000.

cDNA library

A library (titer 4×10^9 PFU/ml) made from mRNA of wild type *N. crassa* mycelia (strain 74-OR23-1VA) was obtained from the Fungal Genetics Stock Center at University of Kansas Medical Center (Orbach *et al.*, 1990). The library was prepared in λ ZAPII (Stratagene) and propagated in *E. coli* strain XL-1 Blue or BB4 (Short *et al.*, 1988).

cDNA library screening

Screening of a cDNA library was carried out by plating on LB as previously described (Sambrook *et al.*, 1989). Briefly, phage lysates diluted to 1×10^4 PFU/ml were used to infect *E. coli* XL-1 Blue or BB4 and were plated on LB plates and allowed to grow at 37°C for 4 h before being overlaid with IPTG-soaked (10 mM) filters and

incubated overnight at 37°C. Membranes were blocked in 25 mM Tris-HCl pH 8.0 containing 150 mM NaCl, 0.1% Tween-20 (TBST2) and 5% nonfat milk for 1 h. After washing twice with TBST2 for 5 min, the NcCW antiserum (1:5000 in TBST2 containing 2.5% nonfat milk) was allowed to react with the membrane for 1 h at room temperature. Membranes were then washed three times with TBST2 for 5 min and developed using a goat anti-rabbit HRP-conjugated secondary antibody (Promega) (1:5000 in 2.5% nonfat milk in TBST2) for 1 h at room temperature with the Enhanced Chemiluminescent substrate system (Roche). The membranes were then exposed to XAR Scientific Imaging Film (Kodak) for 1-60 sec. Alternatively, positive clones were determined by employing the naphthol method of detection. In short, 60 mg of 4-chloro-1-naphthol was dissolved in 20 ml of methanol was added to TBST2 (without Tween-20) containing 0.015% of hydrogen peroxide. The membranes were submerged in this solution for 20 min or until positive clones were visible.

Isolation of cDNA clones

pBluescript plasmids were excised from positive phage clones as outlined by Short *et al.*, (1988). Briefly, λ ZAPII clones (titer 1×10^5 PFU/ml) were combined with R408 helper phage (titer 1×10^{11} PFU/ml) and *E. coli* XL1-Blue cells and allowed to co-infect. The lysate was used to infect *E. coli* strain XL1-Blue and cells harbouring the plasmid were selected on LB+Amp plates. Plasmids were purified and inserts characterized by restriction enzyme analysis in 0.8% agarose gels.

Sequence analysis

Dideoxy sequence analysis was performed on positive clones according to the

method of Sanger *et al.*, (1977) using T7 Sequenase v2.0 sequencing kit (Amersham). Alternatively, positive clones were sequenced using the Li-Cor 4200L automated DNA sequencer (Canadian Molecular Research Services, Inc., Ottawa). DNA and protein homology searches were performed using the BLAST (Altschul *et al.*, 1997) network service of the National Center for Biotechnology Information, *Saccharomyces* Genome Database (<http://genome-www2.stanford.edu/cgi-bin/SGD/>) and *Neurospora* Genome Database (<http://www-genome.wi.mit.edu/cgi-bin/annotation/neurospora/>). Typically, BLASTX were used because DNA to protein searches are more discriminatory due to the larger number of different amino acids. The BLASTX matches were divided into groups according to statistical significance values (E values): highly significant (E values $< 10^{-20}$), moderately significant (E values 10^{-5} to 10^{-19}), weakly significant (10^{-2} to 10^{-4}), and not statistically significant (E values $> 10^{-2}$).

Cloning of putative cell wall genes

Genes encoding putative cell wall proteins were amplified by PCR. Typically a 46.5 μ l PCR mix (containing 39 μ l H₂O, 5 μ l 10X PCR buffer, 1.5 μ l 50 mM MgCl₂ and 1 μ l 10 mM dNTPs) was prepared per PCR reaction. A μ l of template genomic DNA (25 ng) and 1 μ l of each of the primers (primer 1: 25 pmol and primer 2: 25 pmol) were added to the PCR mix (Table 2.1). The mixture was heated for 5 min at 95°C in a thermocycler (Perkin-Elmer). Following the hot-start, 0.5 μ l of Taq polymerase was added to the reaction and amplification was performed for 30 cycles as follows: denaturation at 95°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 90 sec. PCR products were resolved on 0.8% agarose gels (Sambrook *et al.*, 1989).

Table 2.1 Primers used to amplify the putative cell wall encoding genes.

Gene	Protein	Forward primer	Reverse primer
<i>Eno</i>	Enolase	5'-GCGGACCTGACAATAATGCAC-3'	5'-AACAAGACCAAGCTCGGCG-3'
<i>Chit</i>	Chitinase	5'-TCGAATGCTGGAGACGATTGT-3'	5'-AGGTCCCTCGATCGTCCTCA-3'
<i>GlcS</i>	Glucan synthase	5'-TGTAGACAGCTTTGCGATCCG-3'	5'-CAGGAGCCACGTGTGTTTCGT-3'

Typically, 0.8% agarose gels were generally run. Usually, 0.24 g of agarose (Boehringer Mannheim) was dissolved by heating 30 ml of 0.5X TBE (45 mM Tris-borate, 1 mM EDTA). After the solution was less than 60°C, 1.5 µl of ethidium bromide [0.05% (w/v)] was added. The solution was mixed thoroughly and poured on the casting plate. The electrophoresis unit was filled with enough 0.5X TBE to just cover the surface of the gel. DNA concentrations were determined by comparing the intensity of the marker bands to the PCR products.

Cloning of PCR products

Following separation by gel electrophoresis, PCR products were cut from gels and gel cleaned (Sambrook *et al.*, 1989). Ligation of PCR products into the pDrive cloning vector was done by following the instruction provided by the manufacturer (Qiagen). In short, the proper concentration of vector to insert were calculated (usually 1:10 or 50 ng to 500 ng), ligations were set up (10 µl reaction: 1 µl of pDrive, 3.61 µl of PCR product, 0.39 µl H₂O, 5 µl ligation master mix) and incubated at 16°C for 2 h. *E. coli* cells were transformed by adding 2 µl of the ligation mix, incubating them on ice for 5 min and then at 42°C for 30 sec. The cells were immediately placed on ice for 2 min and 250 µl of SOC media was added per tube. For blue-white screening, 100 µl and 200 µl of the ligation reactions were plated on LB+Amp+X-gal.

The positive colonies (white) containing the cloned DNA were picked, grown overnight and plasmids were extracted by the alkaline-lysis method. Approximately, 1.5 ml of the ampicillin resistant cultures was transferred to microfuge tubes. Each tube was spun at 14000 RPM for 5 min at room temperature. Each pellet was resuspended in 100

μl of $T_{10}E_1$. A 200 μl of fresh 0.2 M NaOH and 1% SDS was added to the resuspended culture. The microfuge tube was placed on ice for 5 min. About 150 μl of 3 M K-acetate, pH 4.8 was added to the tubes. The microfuge tube was placed on ice for 5 min. The tube was centrifuged at 14000 RPM for 5 min and the pellet contained the chromosomal DNA. The supernatant containing plasmid DNA was purified by phenol:chloroform:isoamyl alcohol (P:C:I) extraction followed by ethanol precipitation. A volume of P:C:I was added to the tube and it was spun at 14000 RPM for 5 min. The aqueous layer was removed and it was transferred to a new tube. A volume of C:I (24:1) was added to the new tube and it was spun at 14000 RPM for 5 min. The top layer was transferred to a new tube. 1/10 volume of 3 M sodium acetate (NaOAc, pH 5.2) was added. The solution was gently mixed. Then, 2 volumes of cold absolute ethanol were added. The solution was gently vortexed. The tube was placed on ice for 1 hour. The tube was spun at 14000 RPM for 5 min. The pellet was washed with cold 70% ethanol, vacuum-dried for 15 min and then resuspended in 20 μl of $T_{10}E_1$. For the removal of RNA, 1 μl of a RNase A (10 mg/ml) was added to the tube. For pure plasmid preparations the PlasmidPURE™ miniprep kit (Sigma) was used according to the manufacturer's instructions.

Restriction digests were performed on the extracted plasmids to check whether the PCR product had been cloned in the pDrive vector. Normally, 20 μl restriction digestion reactions were prepared. It involved mixing together 3 μl of the extracted plasmid DNA, 1 μl of a restriction enzyme, 1/10 volume of the specific buffer, 1/10 volume of bovine serum albumin or BSA (if required by the enzyme used), and the volume was made up to

20 μ l with dH₂O. The enzymes were purchased from New England Biolabs (NEB), Gibco/BRL or Boehringer Mannheim (BM) and the appropriate buffering system was used according to the manufacturer's recommendations. The reactions were incubated at 37°C for 2 hours or overnight. The reactions were stopped by adding 5 μ l of loading buffer I (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 40% (w/v) sucrose in dH₂O) containing 10 mM EDTA. The digested DNA along with a molecular marker (λ HindIII, Stratagene) were separated on a 0.8% agarose gel run at 70 volts. After the gel had been run to completion, the gel was photographed by using the Fotodyne Inc. transilluminator which was equipped with a polaroid camera.

Results

Antigen localization

For the initial assessment of the specificity of the NcCW antiserum, transmission electron microscopy with immuno-gold labelling was employed. In Fig. 2.1 (A and B), the cell wall is visible as a slightly opaque layer immediately outside the convoluted plasma membrane bilayer. Irregular blebs of extracellular matrix were also apparent in some of the sections. In conjunction with the pre-immune NcCW serum, only a handful of gold particles could be detected on the cell sections, none of which were at the cell envelope (Fig. 2.1A).

In contrast, gold particles were primarily localized to the external envelope of the fungal cells following incubation with the NcCW antiserum (Fig. 2.1B). Two sites were preferentially recognized: the region between the cell membrane and the outer boundary surface of the wall and the outer surface of the wall which included some diffuse patches

Fig. 2.1 Immuno-gold labelling of *N. crassa* hyphae with NcCW antiserum. Goat anti-rabbit IgG conjugated to 10 nm gold particles was used as the secondary antibody.

Hyphae were labelled with either pre-immune (A), or NcCW antiserum (B and C).

Arrows indicate the presence of some representative gold spheres in the cytoplasm (panel A), in the cell wall (panel B, black arrow for the outer wall; white for the inner wall) and associated with the extracellular matrix (panel C). Scale bars = 0.25 μm .

of extracellular matrix still attached to the outer wall surface (Fig. 2.1C). Within the cytoplasm, the periphery of electron-dense bodies and small cytoplasmic granules were also recognized by the NcCW antiserum.

Antigen analysis by 2-D SDS-PAGE

N. crassa cell extracts were also analyzed by 2-D SDS-PAGE and Western blotting to better resolve proteins of similar size (Fig. 2.2). The first dimension, isoelectric separation revealed that several of the more prominent antigens most likely consist of multiple, size and charge variants of the same protein. For example, there are two, bead-like, arrays of spots, between the 31-43 kDa size markers, each consisting of four to six resolvable variants. These electrophoretic patterns are similar to known proteins which exist in multiple, post-translationally modified forms of the same protein (Casanova and Chaffin 1991a; Packer *et al.*, 1997). The 2-D pattern of the abundant 41 kDa and 64 kDa antigens may also be attributable to charge variation of a common protein. In total, the NcCW antiserum recognized approximately 30 antigens that were primarily neutral or slightly charged.

Biochemical characterization of wall-associated antigens

Previously, antibodies have been generated which recognize cell wall polysaccharides such as β glucan (Ruiz-Herrera 1991; Kapteyn *et al.*, 1994; Marshall *et al.*, 1997; Ram *et al.*, 1998). To demonstrate that the NcCW antiserum recognized proteins or the polypeptide component of glycoproteins, protein digestion experiments were performed using the proteases endo-Glu-C and trypsin and the tryptophan-specific reagent N-chlorosuccinimide (Fig. 2.3). The digested proteins were analyzed by

Fig. 2.2 Western blot analysis of *N. crassa* proteins separated by two-dimensional gel electrophoresis. The nitrocellulose membranes were probed with the NcCW antiserum at a 1:5000 dilution. The arrow indicates one of the protein patterns which resembles the isoelectric signature of a protein with multiple sites which undergo post-translational modification.

Fig. 2.3 Biochemical characterization of the *N. crassa*, immuno-reactive antigens. A)
Western blot of cell extracts which were first treated with endo-Glu-C protease, trypsin or
N-chlorosuccinimide at the indicated concentrations. Both blots were probed with a
1:2000 dilution of NcCW antiserum. The arrows indicate some of the immuno-reactive
peptide fragments generated by each of the treatments.

Western blotting and probing with the NcCW antiserum. At moderate protease concentrations (10 and 100 ug/ml), the abundance of all of the antigens was reduced significantly and new proteolytic fragments are apparent in all lanes (Fig. 2.3A). Higher concentrations of protease resulted in the loss of almost all immuno-reactive antigens. A few proteolytic fragments could still be detected by prolonged exposure to the chemiluminescent substrate. Very similar results were observed with trypsin and the chemical reagent NCS. In addition, the NcCW antiserum did not recognize commercial preparations of glucan or chitin (results not shown). All of these experiments confirmed that the antigens recognized by the NcCW antiserum were proteinaceous.

The two dimensional gel results suggested that at least some of the NcCW antibodies were recognizing proteins which undergo multiple post-translational modifications. To test this possibility, *N. crassa* cell extracts were subjected to treatments with a battery of deglycosylating enzymes (results not shown). However, only two minor antigens, at 43 kDa and at 29 kDa, were sensitive to the glycosidase mixture following β -galactosidase treatment.

An alternative method for the analysis of glycosylated proteins is blotting with various lectin-conjugates, proteins from plant and animal sources that have binding sites for specific mono or oligosaccharides in cell walls or membranes (Millette and Scott 1984). The 1-D profiles generated by lectins from *P. americana*, *C. ensiformis* (ConA) and *B. purpurea* were compared to a Western blot probed with NcCW antiserum. Although there were some very clear differences, a number of the immuno-reactive proteins appeared to be recognized by all three lectins (Fig. 2.4). For example, 45 kDa

Fig. 2.4 Comparison of one-dimensional antigen and lectin binding profiles of the *N. crassa* cell extract. The antiserum lane was probed with a 1:5000 dilution of the NcCW antiserum. The lectin blots were probed with the biotinylated lectins from *Phytolacca americana* (Pa) (5 µg/ml), *Canavalia ensiformis* (ConA) (1 µg/ml) and *Bauhinia purpurea* (Bp) (5 µg/ml). Proteins at electrophoretic positions recognized by the NcCW antiserum and all three lectins (black arrows), by the lectins only (grey arrows) and by the antiserum alone (black arrowhead).

and 65 kDa bands present in all four lanes. The *P. americana* lectin is specific for N-acetyl- β -D-glucosamine while ConA lectins recognize α -D-mannosyl and α -D-glucosyl residues. Therefore, these common antigens are likely to be N-linked mannoproteins. The *B. purpurea* lectin has an affinity for N-acetyl-D-galactosamine and D-galactose, residues which are characteristic of O-linked chains. Since the three, prominent proteins labelled by the *B. purpurea* lectin co-migrate with proteins recognized by the other two sugar binding proteins, they may be indicative of proteins modified at multiple positions.

Several chemical extractions were also employed to determine the type of binding between the antigens and the cell wall matrix (Fig. 2.5). Since many cell wall proteins of *C. albicans* and *S. cerevisiae* have been reported to be released by extraction with 1mM 2-mercaptoethanol, which reduces disulfide bonds (Casanova and Chaffin 1991b; Moukadiri *et al.*, 1999), we tested whether the NcCW antiserum could detect any comparable proteins from the *N. crassa* wall. However, even the combination 100 mM 2-mercaptoethanol and 8 M urea failed to solubilize any antigens from the cell wall preparation (Fig. 2.5, ME lanes). In contrast, the brief treatments with 1% SDS at 20°C or 1 M HCl were effective in the selective solubilization of several proteins from the cell wall preparation (Fig 2.5, SDS and HCl S lanes) although a number of other proteins were equally abundant in both the pellet and supernatant fractions.

In the experiments reported by Wrathal and Tatum (1973), cell wall proteins were liberated from the carbohydrate matrix by NaOH extraction. Our studies confirmed the effectiveness of NaOH extraction in removing many of the antigens from the wall matrix. However, there was a decrease in amount of antigen recognized by the NcCW antiserum

Fig. 2.5 Antigenic proteins released by several chemicals from the cell wall matrix. The cell walls were extracted with fungal lysis buffer (FLB), 100 mM 2-mercaptoethanol (ME), 66% (v/v) trifluoromethanesulfonic acid (TFA), 0.5 M sodium hydroxide (NaOH), 1 M hydrochloric acid (HCl), and 1% (w/v) sodium dodecyl sulphate (SDS) and the proteins left in cell wall pellet and supernatant are denoted by lanes P and S, respectively. The HMW, antigenic material generated by NaOH treatment (arrowheads), and the two prominent antigens released by NaOH and TFMS (arrows) are indicated.

and a corresponding increase in HMW, cross-reacting material of heterogeneous size, in both the pellet and supernatant fractions. The prominent 66 kDa and 97 kDa antigenic bands were also more diffuse (Fig. 2.5, NaOH, Lane P and S).

The *Neurospora* cell wall extract proved to be resistant to enzymatic digestion by the glycosidases PNGaseF and a purified β -glucosidase (not shown) but it was sensitive to chemical extraction by TFMS. The 5 h, TFMS treatment effectively liberated all of the antigens from the pellet (TFMS, Lane P). However, the resulting immuno-reactive profile of solubilized antigens, particularly in the LMW range, were also quite different from the SDS or HCl treated samples. As with the NaOH extraction, this may indicate some undesirable modification such as cross linking, of the liberated antigens.

Cross-species comparison of NcCW antiserum

To assess the species specificity of the NcCW antiserum, Western and lectin blots were prepared with cell extracts from several representative ascomycetes, yeasts, basidiomycetes and zygomycetes (Fig. 2.6). The evolutionary relation between *N. crassa* and the fungi tested is shown on Fig. 1.1. All of the species tested reacted to the NcCW antiserum, although the intensity of the chemiluminescent signal was approximately 10-fold stronger for the *N. crassa* extract. This suggests that a small fraction of antibodies in the NcCW antiserum are responsible for the interspecies cross-reactivity. Some of the immuno-reactive proteins, such as the 45 kDa molecule (arrow) in *A. nidulans* and *A. niger*, may be a conserved homolog of the *Neurospora* protein since these fungi are closely related (Fig. 1.1). Some related fungi, such as the basidiomycetes *C. lagopus*, *S. commune* and *A. bitorquis*, had very different immuno-reactive profiles (Fig. 2.6A).

Fig. 2.6 Cross-reactive properties of the NcCW antiserum and comparison to ConA binding. The Western blot (A) and lectin blot (B) were prepared identically with the cell extracts from *Neurospora crassa*, *Aspergillus nidulans*, *Aspergillus niger*, *Fusarium oxysporum*, *Saccharomyces cerevisiae*, *Candida albicans*, *Coprinus lagopus*, *Schizophyllum commune*, *Agaricus bitorquis*, *Mucor rouxii* and *Phycomyces blakesleeanae*. For the *N. crassa* lane, 2.5 µg of total protein while 5 µg was loaded in all of the other lanes. The western blot (A) was probed with a 1:5000 dilution of the NcCW antiserum and the lectin blot (B) was probed with 2 µg/ml of biotinylated ConA. In A and B, the position of the 45 kDa antigen in *Neurospora* and the *Aspergilli* is shown with a black arrow.

Similarly, the two zygomycetes we tested also had very divergent profiles. At least some of this binding in the more distant fungi, namely, basidiomycetes and zygomycetes, may be due to non-specific interactions and no epitope conservation should be inferred or similar epitopes in distinct proteins (Fig. 1.1).

The corresponding lectin blot revealed very diverse patterns of ConA binding for each fungal species (Fig. 2.6B). Both the *Neurospora* and *Saccharomyces* cell extracts contain more than 10 putative glycoproteins whereas none were detectable in *C. lagopus* or *P. blakesleanus* extracts with the concentrations of ConA employed in this study. There was no significant correlation between binding by the NcCW antiserum and ConA lectin.

Novozyme™-extraction, secreted protein and phospho-proteins

To determine if antigens could be extracted from purified walls by enzymatic methods Novozyme™ (mixture of glucanase and chitinase) experiments were performed. Western blot analysis revealed the release of proteins from the cell wall matrix by Novozyme™ (Fig. 2.7A). Seven proteins were clearly release by enzymatic digestions of purified walls. Interestingly, two of these antigens have a high molecular masses of approximately 56 and 97 kDa, respectively. Two antigens were seen in the untreated samples, indicative of a loose association with the wall. In contrast, only two proteins were recognized by the NcCW antiserum in growth media after 18 h, but only one antigen was clearly visible at growth time of 24 h (Fig. 2.7B). Many of antigens recognized by the NcCW antiserum are associated with the wall and not readily secreted (Fig. 2.2).

It was important to determine if there were any phosphorylated proteins present in

Fig. 2.7 Western blot analysis showing proteins recognized by the NcCW antiserum in NovozymeTM and secretion-extracts, as well as, proteins recognized by phospho-specific antibodies in U+ME wall extracts. A) Proteins extracted from the cell wall by NovozymeTM were recognized by the NcCW antiserum. In addition, proteins antigens, found in the growth media, recognized by the NcCW antiserum are also illustrated. B) U+ME extracts (8 M urea + 100 mM 2-mercaptoethanol) probed with various phospho-specific antibodies. The phospho-specific antibody PKC recognized the largest number of antigens (Black arrows).

U+ME cell wall extracts since it has been reported that there are cell wall-associated kinases in plants (Sivaguru *et al.*, 2003). Several antigens were detected by the various phospho-specific antibodies (Fig. 2.7B). Intriguingly, 5 antigens were recognized by phospho-PKC substrate antibody (Fig. 2.7B, Black arrows). PKC is known to be involved in the cell wall integrity pathway (Schmelzle *et al.*, 2002).

Cellular fractionation

To determine the antigen in the major cellular compartments of *N. crassa* fractionation by differential centrifugation was performed (Fig. 2.8). As expected, a large number of proteins were recognized by the NcCW antiserum in SDS sample buffer-extracts of the cell wall, but only a few were recognized in the plasma membrane fraction. Surprisingly, the cell lysate and nuclear fractions contained a similar pattern of proteins recognized by the NcCW antiserum. Furthermore, several antigens were also seen in mitochondrial fraction. Moreover, the 55 kDa and 66 antigens were found in all and may represent abundant proteins.

Biotinylation and TA cloning of putative cell wall genes

Since cell wall proteins from intact *S. cerevisiae* cells were reported to have been successfully biotinylated with the non-permeable reagent (Mrsa *et al.*, 1997), a similar experiment was performed in *N. crassa* (data not shown). Although proteins were recognized by streptavidin conjugated-HRP in the biotinylated samples, this experiment was unsuccessful in *N. crassa* because many non-biotinylated proteins were bound by streptavidin conjugated-HRP.

In an effort to genetically characterize genes encoding cell wall proteins, three

Fig. 2.8 Western blot analysis of cell fractionation experiments. Determination of antigens recognized by the NcCW antiserum in different cell fractions. *CW*, cell wall fraction; *PM*, plasma membrane fraction; *Nu*, nuclear fraction and *Mt*, mitochondrial fraction.

genes were selected and cloned into a vector (data not shown). The selection of genes, to clone and eventually disrupt, was based on the presence of the gene product in chemical and enzymatic extracts (Chapter 4) and/or extensive documented involvement in cell wall biogenesis. For example, enolase has been found by mass spectrometry to be associated with the wall and is a good candidate for gene disruption (Chapter 4). The selected genes, encoding enolase, chitinase and glucan synthase, were PCR amplified and successfully cloned into the pDrive vector. The long term goal of creating knock-out mutant strains and examining their phenotype in comparison to the wild-type strain are currently being pursued by other students in Dr. Vierula's laboratory.

Isolation and identification of cDNA clones

To confirm that the NcCW antiserum recognizes proteins, it was used to screen a *N. crassa* cDNA library expressed in an *E. coli* host. In our initial probing, 12 positive clones were isolated from the expression library that were specifically recognized by the NcCW antibodies. Plasmids from each of clones were excised from λ ZAPII bacteriophage vectors and digested with *Pvu*II to estimate insert sizes (Short *et al.*, 1988). The cDNAs ranged in size from 500 to 1900 bp.

DNA sequence analysis revealed that 3 of the clones were derived from the *Neurospora* homolog of the *nmt1* (no message in thiamine) gene (McColl *et al.*, 2003; Table 2.2, clone DM1). Two of the cDNA clones encoded part of the *N. crassa* mitochondrial ATP synthase alpha subunit. This gene is strongly conserved within the fungi and even with other eukaryotes such as *Drosophila melanogaster* (62% identity, 78% similarity) and *Homo sapiens* (59% identity, 77% similarity). Two other clones are

Table 2.2 Identification of *N. crassa* cell wall immunoreactive clones through BLAST searches. The DNA sequences of each clone was searched against the *Neurospora crassa* database through BLASTX. The matching parameters of the proteins to which the clones are closest to are shown below.

Clone ID	<i>Neurospora</i> database Accession Number	Protein name	E value	Identity (%)	Similarity (%)
DM1	NCU09345.1	No message in thiamine (NMT1) ^{b, c}	0	300/300 (100)	300/300 (100)
DM6	NCU02514.1	ATP synthase alpha subunit ^{b, c}	E-104	135/135 (100)	135/135 (100)
DM2	NCU07733.1	Electron transfer flavoprotein beta subunit ^{b, c}	5E-96	180/190 (94)	180/190 (94)
AV2	NCU10051.1	Flavohemoglobin ^{b, c}	1E-59	110/110 (100)	110/110 (100)
AV3	NCU04156.1	C8 sterol isomerase ^{a, c, d}	2E-12	29/29 (100)	29/29 100
AV6	NCU02289.1	Ubiquitin-conjugating enzyme ^{b, c}	1E-61	111/111 (100)	111/111 (100)

^aModerately significant BLASTX match (refer to Methods and Materials).

^bHighly significant BLASTX matches.

^cMatches between the query and subject occurred in one location instead of being distributed throughout the protein sequence. In this E value is mainly dependent on the length of the match.

^dHigh E value is due to short DNA sequence obtained and match was also a short stretch of amino acids.

cDNAs which encoded carboxy terminal segments of the *Neurospora* mitochondrial electron transfer flavoprotein beta subunit.

Two other immunoreactive clones were identified as a partial flavohemoglobin cDNA and a cDNA encoding a C8 sterol isomerase. This isomerase, encoded by the *Neurospora erg1* gene, is required for ergosterol biosynthesis. Amino acid sequence conservation between C8 sterol isomerases is also notable among fungi. For example, *Ustilago maydis*, *Saccharomyces cerevisiae*, and *Schizosaccharomyces pombe* shared 47%, 42%, 40% identity with the *N. crassa* C8 sterol isomerase, respectively. Finally, clone AV6, was found to encode a hypothetical protein with similarity to ubiquitin-conjugating enzymes. This hypothetical, *N. crassa* ubiquitin-conjugating enzyme is also highly conserved between species. For example, it shares 93% identity and 97% similarity to a ubiquitin-conjugating enzyme of *Schizosaccharomyces pombe* (Damagnez *et al.*, 1995).

Discussion

As part of our study of the proteins associated with the *Neurospora* cell wall, we have raised a high titer, polyclonal antiserum against *N. crassa* cell wall extracts. Transmission electron microscopy was used to demonstrate that this NcCW polyclonal antiserum binds principally to the cell envelope. Unlike other cell wall antisera described previously (Behamou *et al.*, 1990; Wycoff and Ayers 1990; Marshall and Gull 1997), the NcCW antiserum does not recognize purified glucans or chitin. Several endo-proteolytic digestion experiments confirmed that the antigens are proteins (Fig. 2.3). Finally, immunochemical screening of a cDNA library with the

NcCW antiserum identified 12 cDNAs encoding six distinct proteins (Table 2.2). The fact that these clones were expressed in bacterial host cells confirms that the NcCW antiserum recognizes polypeptide sequences, not simply eukaryotic, post-translational modifications.

The 2-D PAGE and Western blotting demonstrated that several of the immuno-reactive, 1-D proteins are most likely to be multiple, electrophoretic variants of a common core protein. The string of polypeptides, each with slightly different isoelectric mobilities, is characteristic of proteins which are known to be post-translationally modified, either by phosphorylation or glycosylation (Danzo *et al.*, 1989; Packer *et al.*, 1997). In addition, a large range of molecular mass proteins were characteristically recognized by the NcCW antiserum. Consistent with these observations, a wide range of molecules of apparent molecular masses (20 to greater than 100 kDa) showed specific binding to antibodies raised in rabbits to *Aspergillus fumigatus* wall (Hearn *et al.*, 1990).

The lectin blotting experiments revealed that some of the proteins recognized by the NcCW antiserum co-migrate with *P. americana*, ConA and *B.purpurea* lectin binding activity (Fig. 2.4). However, each of the lectins and the NcCW antiserum recognizes a unique subset of proteins. This result, as well as the cross-species binding experiments (Fig. 2.6) provide further evidence that the NcCW antiserum does not primarily recognize common carbohydrate residue.

Our conA lectin blot showed the recognition of many antigens in *N. crassa* extracts, but only one and two antigens in *A. niger* and *A. nidulans*, respectively (Fig. 2.6B). To date, lectin experiments have not been performed in *A. niger* and *A. nidulans*,

but they have been done in *Aspergillus fumigatus* (Ste-Marie *et al.*, 1990). Western blotting with the lectin conA or BS-1 from the seeds of *Bandeirea simplicifolia* three mannose- and galactose-containing components (80, 62, and 49 kilodaltons) of a *A. fumigatus* mycelial homogenate (Ste-Marie *et al.*, 1990). A monoclonal antibody (MAb 1) raised against *A. fumigatis* walls also recognized these three carbohydrate-containing components. Not surprisingly, only several conA antigens were recognized in *A. niger* and *A. nidulans* in whole cell, SDS extracts (Fig. 2.6B).

To investigate the nature of the interaction between the proteins and cell wall matrix, various chemical extractions were employed for solubilization (Fig. 2.5). For example, ME is known to extract non-covalently or soluble cell wall and disulphide-linked proteins from *S. cerevisiae* and *C. albicans* (Casanova and Chaffin 1991b; Moukadiri *et al.*, 1999; Edwards *et al.*, 1999). In contrast, reduction by ME did not result in a significant solubilization of proteins from the *Neurospora* cell wall. This may be indicative of differences in cell wall architecture between filamentous fungi, such as *N. crassa*, and the yeasts. The room temperature extractions with SDS and HCl were more effective and displayed some selectivity, but a surprising number of proteins were subsequently present in both pellet and supernatant fractions (Fig. 2.5). The boiling of cell wall extracts in SDS sample buffer (1% SDS, 0.5% 2-mercaptoethanol) has been reported to extract both non-covalently- and covalently linked proteins from *S. cerevisiae* cell wall, presumably by reduction of exposed disulphide bonds following denaturation (Mrsa *et al.*, 1997; Mrsa and Tanner 1999). The broad distribution of proteins in both pellet (P) and supernatant (S) fractions suggest that SDS (and HCl treatments) may

accomplish this for *N. crassa* as well.

TFMS has been reported to be a particularly effective reagent for the cleavage of O-glycosyl bonds (Edge *et al.*, 1981). In the experiments described here, TFMS was the most effective reagent for the release of all of the SDS/ 2-mercaptoethanol extractable antigens from the cell wall pellet (Fig. 2.5, TFMS, Lane P). Therefore, at least some of the *N. crassa*, wall-associated proteins are likely to be attached through an O-glycosyl bond to the polysaccharide matrix of the cell wall. The fact that no further immuno-reactive material was released by boiling the TFMS-extracted, cell wall pellet in the 2% SDS sample buffer also supports the proposition that SDS is capable of extracting covalently linked proteins. The 66 and 97 kDa proteins were most prominent in the TFMS-extracted, supernatant fraction (Fig. 2.5, TFMS S lane). The loss of resolution and or reactivity of some of the liberated proteins may be the result of different degrees of deglycosylation or some additional reactivity of this reagent against the antigenic sites of the proteins (Edge *et al.*, 1981).

O-glycosyl linkages to serine or threonine are also reputed to be susceptible to concentrated NaOH (Wrathall and Tatum 1973). The experiments reported here indicate that NaOH is less useful for extraction of cell-wall associated proteins since treatment with the strong base resulted in the formation of antigenic complexes which were resistant to subsequent solubilization by boiling in SDS-PAGE sample buffer (Fig. 5, NaOH, lanes P and S).

Most secreted and externally disposed membrane, membrane-associated, wall-associated, and wall-bound proteins are believed to be post-translationally modified by

processes such as glycosylation, acetylation, prenylation, phosphorylation and ubiquitination (Coding 1986; Chaffin *et al.*, 1998). In fungi and yeasts, these modifications are believed to target proteins to specific cellular locations (Chaffin *et al.*, 1998). Some of these proteins are modified by the addition of a GPI anchor for targeting initially to the plasma membrane and are then released into the cell wall by cleavage of the GPI anchor (Terashima *et al.*, 2003). Some *C. albicans* cell wall proteins are initially secreted in an O-glycosylated form with short, unbranched mannose chains which become cross-linked with β -glucan and/or other N-glycosylated proteins after incorporation into the cell wall structure (Chaffin *et al.*, 1998). The N-linked proteins are complexed with mannosyl groups that are universally attached through two N-acetylglucosamine residues and an N-linked asparagine (Ballou 1976). *N. crassa*, mycelial cell walls are also thought to contain both N-linked and O-linked proteins such as those found in *S. cerevisiae* (Nakajima *et al.*, 1984).

NovozymeTM was used in an attempt to release proteins from purified walls enzymatically (Fig. 2.7A). Indeed, several antigens were released by these NovozymeTM treatments. Classically, QuantazymeTM, a highly purified β -1,3 glucanase, has been used to digest the wall and release proteins entrapped or covalently linked to the cell wall matrix of the yeasts *C. albicans* and *S. cerevisiae* (Pitarch *et al.*, 2002). Unfortunately, such QuantazymeTM treatments were unsuccessful in *N. crassa* and may indicate a difference in the composition of the cell wall. In contrast, NovozymeTM contains β -glucanase and chitinase activities and both may be required for the successful digestion of the wall. This is in agreement with the model of wall growth (Wessels 1993). In this case,

both polymers, glucan and chitin, become gradually insoluble as covalent linkages between them form. The use of Novozyme™ will prove to be useful for the identification of proteins that are either covalently linked or entrapped in the polymeric matrix.

Filamentous fungi are characterised by efficient synthesis and secretion of hydrolytic enzymes (Pakula *et al.*, 2003). The fact that only a few antigens were detected by the NcCW antiserum in the growth media could be explained by two reasons (Fig. 2.7A). First, it is possible that these proteins are secreted into the media because they have been found in the media of regenerating protoplasts of *S. cerevisiae* and in media of *N. crassa* of non-regenerating cells (Pardo *et al.*, 2000a; Table 4.2). In this case, a large number of proteins was not detected because the NcCW antiserum was raised against a cell wall fraction not against secreted proteins. Alternatively, with the assumption that these proteins are wall proteins that were dislodged into the media, the low number of recognized proteins could be explained by suggesting that these are low abundant proteins that do not induce a strong humoral immune response.

Since protein kinases have been found to be associated with the wall and the protein kinase C (PKC) is involved in the cell wall integrity pathway, it was of interest to examine the phosphorylation patterns in cell wall extract using phospho-specific antibodies (Schmelzle *et al.*, 2002; Sivaguru *et al.*, 2003; Fig. 2.7B). Intriguingly, phosphorylated substrates of PKC, Akt, PKA and Tyr were found through Western blot analysis of U+ME cell wall extracts. It is possible that these phosphorylated proteins are native to the cell wall or they are just insignificant contaminants of the cell wall preparation and were readily detected by the highly sensitive antibodies. To resolve these

issues the identity of these kinase substrates should be addressed by mass spectrometric-based methods.

As previously reported in yeasts, typical wall associated proteins include heat shock and mitochondrial proteins (Pardo *et al.*, 2000; Pardo *et al.*, 2000b, Pitarch *et al.*, 2002; Chapter 4). Evidence that supports this hypothesis is the cellular fraction experiment (Fig. 2.8). Here, several antigens were recognized by the NcCW antiserum in the mitochondrial fraction. Numerous antigens were recognized in the nuclear fraction. This may be due to non-specific binding because no nuclear proteins were identified by mass spectrometry (Chapter 4, Table 4.2).

To prevent the criticism of contamination in the cell wall fraction, cell surface labelling of intact cells with a non-permeable (does not penetrate plasma membranes) biotinylating reagent was carried out in *N. crassa* by following the experimental procedures of *S. cerevisiae* (Casanova *et al.*, 1992; Mrsa *et al.*, 1997). This experiment was unsuccessful in *N. crassa*, because the streptavidin conjugated-HRP supplied by the labelling kit was detecting proteins in the non-biotinylated samples after SDS sample buffer extraction. A recent publication revised this data by testing the specificity of the biotin and streptavidin interactions (Pierce labelling kit) and reached the same conclusions (Msra 1997; Masuoka *et al.*, 2002).

The identities of the 6 proteins recognized by the NcCW antiserum were initially surprising since all of them were thought to function exclusively in the cytoplasm or within mitochondria. Since ergosterol is a component of the plasma membrane, an attachment to the cell surface may not be very surprising for the C8 sterol isomerase

(Lees *et al.*, 1996). A possible function for an extracellular electron transfer flavoprotein is the reduction of external nutrients, including glucose and iron, and it could be the electron transfer protein that is external to the plasma membrane and wall-associated as proposed by Ponitz and Roos (1994). Similarly, flavohemoglobin at the surface of fungal cells may provide protection from oxidative stress either as signalling molecules or the detoxification of reactive oxygen species. Such antioxidant defence systems against environmental toxins have been found in plant cell walls and at the outermost skin layer (Cross *et al.*, 1998).

A role at the cell surface for NMT-1 and ATP synthase alpha subunit are less obvious. However, a growing body of evidence suggests that many proteins have multiple functions and reside in more than one location within or outside of cells. For example, immunoscreening of a *Candida albicans* cDNA library with a monoclonal antibody which recognizes an epitope of high-molecular weight mannoproteins in the mycelial cell walls resulted in the isolation of a clone encoding a mitochondrial cytochrome c haem lyase (Cervera *et al.*, 1998). Western blot analysis of subcellular fractions from wild-type and null mutant strains demonstrated that the *CaCYC3* gene encodes two proteins, one targeted to the mitochondria and the other to the cell wall (Cervera *et al.*, 1998). Furthermore, some cytoplasmic proteins such as enolase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase and members of the Hsp70 family have been found in *S. cerevisiae* and *C. albicans* walls (Angiolella *et al.*, 1996; Lopez-Ribot and Chaffin 1996; Lopez-Ribot *et al.*, 1996; Alloush *et al.*, 1997; Eroles *et al.*, 1997; Pardo *et al.*, 1999; Edwards *et al.*, 1999; Delgado *et al.*, 2001).

In higher eukaryotes, ubiquitin plays important roles in protein modification, protein degradation, gene transcription, organization of chromatin structure, and stress resistance (Finley and Chau 1991, Hershko 1991). It is also associated with some cell surface proteins, including receptor molecules (Leung *et al.*, 1987; Saxena and Calderone 1990; Cenciarelli *et al.*, 1992, Mori *et al.*, 1992; Paolini and Kinet 1993). Clones coding for polyubiquitin have been isolated by screening a *Candida albicans* cDNA library with antibodies generated against the candidal 37 kDa laminin-binding protein (Sepulveda *et al.*, 1996). In addition, the presence of several proteins including receptor-like molecules, have been detected in the cell wall of *C. albicans* that bear ubiquitin-like epitopes (Saxena and Calderone 1990; Sepulveda *et al.*, 1996). In *C. albicans*, there are two functions of cell surface ubiquitination have been proposed: adhesion and receptor-mediated signalling (Calderone 1993; Bailey *et al.*, 1995; Sepulveda *et al.*, 1996).

Several recent studies using a proteomic approach have shown that a number of cytoplasmic and mitochondrial proteins are associated with plant, yeast and fungal cell walls (Pardo *et al.*, 1999; Pitarch *et al.*, 1999; Pardo *et al.*, 2000b; Lim *et al.*, 2001; Pitarch *et al.*, 2001, Chivasa *et al.*, 2002). In agreement with proteomic data, the *SUN* (*SIM1*, *UTH1*, *NCA3* and *SUN4*) protein family is involved in a variety of cellular functions and several members have been found in the mitochondria as well as in the cell wall of yeast (Velours *et al.*, 2002). Dithiothreitol-treatment of intact cells induced the release of Uth1p, Sun4p and Sim1p from the cell wall. Two of these glycosylated proteins, Uth1p and Sun4p, have also been found in the mitochondria. (Velours *et al.*, 2002). Our own proteomic analysis of the *Neurospora* cell wall is also consistent with the

immunoscreening experiments reported here (manuscript submitted).

The utility of monoclonal antibodies for the study of fungal cell walls has been amply demonstrated in several studies (Benhamou *et al.*, 1990; Kapteyn *et al.*, 1994; Marshall *et al.*, 1997). The polyclonal NcCW antiserum described here has also proven to be a very useful reagent for exploring the architecture of the *Neurospora* cell wall. On two dimensional blots, it recognizes more than 30 proteins or multiple isoelectric forms of a more limited set of proteins. Since immunological recognition does not appear to involve sugar residues, it was also useful for probing bacterial cDNA expression libraries which recognized genes encoding putative cell surface proteins. Its binding to proteins in other fungal species may also be a useful property in identifying surface proteins which are conserved in related fungal species. The effort to clone and characterize the genes encoding these surface proteins has begun and will further advance our understanding of fungal cell wall architecture and function.

CHAPTER 3

IMMUNOLOGICAL CHARACTERIZATION OF THE *SCHIZOPHYLLUM*

COMMUNE CELL WALL

Introduction

When one looks at fungal hyphae, what one sees is shaped by the cell wall (Cabib *et al.*, 1988). The myriad of forms that fungi adopt, such as mycelia, budding cells, fruiting bodies, spores, all owe their shape to this exoskeleton that supports them (Cabib *et al.*, 1988). In addition to morphogenesis, fungal cell walls have other functions that are equally important including providing protection against mechanical injury and turgor pressure, environmental sensing and the mediation of cell-cell interactions (Zlotnik *et al.*, 1984).

The cell wall of filamentous fungi such as the basidiomycete *Schizophyllum commune* have three major components. The wall is composed of β -(1,3)/ β -(1,6) glucan-chitin complex, α -(1,3) glucans and proteins (Sietsma and Wessels 1994). The outer α -(1,3) glucan layer is alkali soluble whereas the inner layer forms an insoluble residue composed of β -(1,3)/ β -(1,6) glucan and chitin following alkali extraction (Wessels 1986). The polymers of the inner layer are extruded from the cell as a water soluble β -(1,3) glucan and chitin and become cross-linked together following deposition. The β -glucan also becomes modified by the introduction of (1,6)- β -linkages during maturation to an alkali-insoluble β -glucan-chitin complex (Sonnenberg *et al.*, 1982; van Wetter *et al.*, 2000). Wessels (1986) proposed that this maturation process results in cell wall plasticity at the growing hyphal tip and an increasingly rigid wall subapically.

Recent research efforts have focused on identifying the proteins of the cell wall of yeast as well as filamentous fungi. Wessels and coworkers have identified a class of abundant, hydrophobic cell wall proteins, named, hydrophobins (Wessels *et al.*, 1995). To

date, four genes that encode hydrophobins have been characterized in *S. commune* (Wessels *et al.*, 1995). They play a role in the formation of aerial structures and in attachment of hyphae to hydrophobic surfaces (Wosten and Wessels 1997; van Wetter *et al.*, 2000). Other *S. commune* cell wall proteins have yet to be discovered. In contrast, research of the cell wall *S. cerevisiae* and *C. albicans* has yielded the identification of various classes of wall proteins (Klis 1994; Stratford 1994).

In yeast, electron-microscopic studies have revealed that mannoproteins form a fibrillar layer extending radially from the inner skeletal layer which is formed by the polysaccharide fraction of the cell wall (Tokunaga *et al.*, 1986). Two fractions of wall mannoproteins have been identified through chemical and enzymatic extraction procedures (Pastor *et al.*, 1984; Frevert and Ballou 1985). The majority of proteins are SDS-extractable, are O-glycosylated and are of low molecular weight. The high molecular weight remainder are generally N-glycosylated, and extractable by glucanases. For instance, Montijn *et al.*, (1994) reported eight glucanase-extractable proteins from the cell wall of *mnn9* (a gene required for the synthesis of N-linked outer-chain mannan in yeast) cells, indicating a covalent attachment of these proteins to the wall. In contrast to the O- and N-linked wall proteins, other proteins are attached to the wall by a glycosylphosphatidylinositol (GPI) anchor (Herscovics and Orlean 1993; Stratford 1994).

The best studied examples of wall proteins are sexual agglutinins that mediate sexual recognition between cells of opposite mating type (Cappellaro *et al.*, 1991). The presence of a GPI anchor in intracellular α -agglutinin precursor forms has been confirmed by radioactive labelling with myoinositol and palmitic acid (Wocjciechowicz *et al.*,

1993). In addition, C-terminal deletions resulted in the appearance of active α -agglutinin in the medium, indicating that its GPI-anchor is involved in the incorporation of this protein into the wall (Wocjciechowicz *et al.*, 1993; Klis *et al.*, 1997).

Compared to Ascomycetes, such as *S. cerevisiae*, little is known about the identity of proteins in the walls of Basidiomycetes including *S. commune*. Hydrophobins have been identified and studied in great detail in *S. commune*, but the identity of the remaining proteinaceous component of the wall remains to be determined (Wessels *et al.*, 1995). The molecular architecture of *S. commune* cell wall has been investigated using an immunological approach. For our studies of the *S. commune* cell wall, we have raised a polyclonal antiserum (ScCW antiserum) which recognizes several abundant cell wall-associated proteins of *S. commune*. Here we report on our initial characterization of this ScCW antiserum and some of the wall-associated proteins identified by screening an *S. commune* λ ZAP expression library.

Methods and Materials

Strains and growth conditions

Escherichia coli strains BB4 and XL1 blue (from the -70°C freezer in 15% glycerol) were grown in Luria broth (LB) or on LB plates (containing 1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.5 with 1.5% agar) at 37°C overnight. LB+Amp plates were prepared as LB plates with the addition of ampicillin to a final concentration of 0.1 mg/ml.

N. crassa wild type strain (74-OR23-1VA; FGSC 987), *Asperillus nidulans* (FGSC A4), *Aspergillus niger* N400 (FGSC A732), *Fusarium oxysporum* (FGSC 6607)

were obtained from the Fungal Genetics Stock Center. *Schizophyllum commune* (strains 4-40) was obtained from M. Raudaskoski (University of Helsinki) and *Saccharomyces cerevisiae* (strain S288C) and *Agaricus bitorquis* (ATCC 24666) were obtained from the American Type Culture Collection. *Coprinus lagopus*, *Phycomyces blakesleeanus* (+ mating type), *Candida albicans* (+ mating type), and *Mucor rouxii* were obtained from Ward's Biology.

Cultures of *N. crassa*, *A. nidulans*, *A. niger* and *A. bitorquis* were grown on Vogel's minimal medium (Vogel 1964) in 250 ml flasks containing 50 ml of liquid media, with agitation (180 rpm) overnight at room temperature. *C. lagopus* was grown on DMY medium as described by Rao and Niederpruem (1969). Slant cultures were incubated in the dark at 37°C for 4 days and then transferred to 25°C until further growth developed. Liquid cultures were incubated at 30°C for 3 days to hasten growth and transferred to room temperature in ordinary light. *P. blakesleeanus* (grown at 25°C), *C. albicans* (grown at 37°C), and *M. rouxii* (grown at 25°C) cultures were grown overnight on Sabouraud-Dextrose medium (40 g/L dextrose, and 10 g/L peptone). For all of the above filamentous fungi, mycelium was harvested by vacuum filtration, washed with distilled water, pressed dry between paper towels, cut into 1 cm² pieces and frozen at -70°C overnight.

S. commune mycelium was grown at room temperature on cellophane membranes on the surface of complete medium containing 5 g/L MgSO₄·H₂O, 4.6 g/L KH₂PO₄, 10 g/L K₂HPO₄, 20 g/L tryptone, 2 g/L yeast extract and 200 g/L of dextrose. The cellophane membranes were peeled off the plate and stored at -70°C. The yeast, *S. cerevisiae*, was

grown at 30°C on YPD medium containing 10 g/L yeast extract, 20 g/L (w/v) peptone, 20 g/L dextrose and cells were collected by centrifugation at 5000 g for 5 min and frozen at -70°C overnight. Frozen cells were lyophilized for 8 h in a Savant SS2 SpeedVac and stored at -70°C until required.

Purification of cell walls and preparation of ScCW antiserum

The *S. commune* crude cell wall was isolated essentially according to the procedure of Martinez *et al.*, (1991) except that the final pellet was extracted once with acetone and once with absolute ethanol. Two female, New Zealand, white rabbits were immunized with 10 mg (dry weight) in an emulsion of Freund's incomplete adjuvant (Huet 1994) followed by 2 booster injections of the same mixture at two week intervals.

One-dimensional SDS-PAGE

1-D SDS PAGE and Western blots were performed by Joanne Burbidge (NSERC summer student). For one-dimensional (1-D) SDS-PAGE, proteins were extracted from all lyophilized fungi by grinding in a microcentrifuge tube using a plastic pestle. The mycelial powder was resuspended in a ratio of powder: SDS sample buffer of 1:4 (Laemmli 1970), vortexed for 20 seconds and then was incubated for 10 min at room temperature. The extraction mixture was heated up on a hot plate to 100°C for 5 min. After boiling, the mixture was centrifuged at 13000 g for 20 min. Typically, 10 to 20 µg of proteins were separated using the discontinuous buffer system of Laemmli (1970). Separatory gels contained 10% acrylamide, while stacking gels contained 4%. Staining of proteins was done overnight in a solution containing 0.25% Coomassie brilliant blue R-250, 40% (v/v) methanol, and 10% (v/v) acetic acid. Gels containing low amounts of

proteins were silver stained following the manufacturer's instructions (Biorad). The concentration of protein in the extracts was determined by spectrophotometry (Stoscheck 1990) using a diode array spectrophotometer (Hewlett-Packard).

Western and Lectin blotting

After SDS-PAGE, the gels were equilibrated for 30 min in a transfer buffer containing 25 mM Tris-HCl, 192 mM glycine and 20% (v/v) methanol. Subsequently, proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) at 15 V for 30 min by using the Transblot^R SD Semi-Dry Electrophoretic Transfer Cell (Biorad).

Membranes were blocked and probed essentially as described by Towbin (1979) in 25 mM Tris-HCl pH 8.0 containing 150 mM NaCl, 0.1% Tween-20 (TBST2) and 50 g/L nonfat milk for 1 h at room temperature with gentle rocking. After washing twice with TBST2 for 5 min, the membranes were incubated in a solution containing 2.5% nonfat milk in TBST2 and 1:1000 or 1:2000 dilution of the ScCW antiserum. The membranes were washed three times with TBST2 for 5 min and then submerged in a solution containing 2.5% nonfat milk in TBST2 and 1:5000 dilution of goat anti-rabbit HRP-conjugated secondary antibody (Promega) for 1 h at room temperature. The membranes were washed 3 times with TBST2 and then incubated with the Enhanced Chemiluminescent substrate system (Roche). The membranes were then exposed to XAR Scientific Imaging Film (Kodak) for 1-60 sec.

The lectin blotting procedure was essentially as described by Millette and Scott (1984). The biotinylated lectin (Sigma) from Succinylated *Canavalia ensiformis* (ConA; 1 µg/ml) was used at the indicated final concentrations and detected with a 1:2000

dilution of streptavidin-horse radish peroxidase (HRP) conjugate (BioRad) and the Enhanced Chemiluminescent substrates.

Lectins were stripped from blots according to a modification of the procedure of Montreuil *et al.*, (1986) for Western blots. Membranes were wetted in TBST2 for 15 min, equilibrated in sodium acetate buffer, pH 5.2 (0.1 M NaCl, 1 mM CaCl₂, 1 mM MnCl₂, 1 mM MgCl₂, 0.02% (w/v) sodium azide, 5 mM sodium acetate, pH 5.2) for 10 min, and then washed in a descending series of solutions of methyl- α -D-glucopyranoside at 0.5 M, 0.3 M, 0.1 M, 0.05 M, and 0.01 M (Sigma) in sodium acetate buffer. The 0.5 M wash was for 90 min and all others were for 30 min. Between each wash, the membranes were rinsed twice with sodium acetate buffer, pH 5.2 for 5 min each. Finally, the membranes were washed seven times with TBST2 for 5 min each at room temperature. The membranes were ready to be blocked and probed with the ScCW antiserum as previously described in the immunoblotting section.

cDNA library

A cDNA library (titer 4×10^9 PFU/ml) made from mRNA of wild type *S. commune* mycelia was obtained from Marjatta Raudaskoski, Department of Biosciences, Division of Plant Physiology, University of Helsinki, Finland. The library was prepared in λ ZAPII (Stratagene) and propagated in *E. coli* strain XL-1 Blue or BB4 (Short *et al.*, 1988; Orbach *et al.*, 1990).

cDNA library screening

Screening of a cDNA library was performed by Joanne Burbidge by plating on LB as previously described (Sambrook *et al.*, 1989). Briefly, phage lysates diluted to 1×10^{-4}

PFU/ml were used to infect *E. coli* XL-1 Blue or BB4 and were plated on LB plates and allowed to grow at 37°C for 4 h before being overlaid with IPTG-soaked (10 mM) filters and incubated overnight at 37°C. Membranes were blocked in 25 mM Tris-HCl pH 8.0 containing 150 mM NaCl, 0.1% Tween-20 (TBST2) and 5% nonfat milk for 1 h. After washing twice with TBST2 for 5 min, the ScCW antiserum (1:5000 in TBST2 containing 2.5% nonfat milk) was allowed to react with the membrane for 1 h at room temperature. Membranes were then washed three times with TBST2 for 5 min and developed using a goat anti-rabbit HRP-conjugated secondary antibody (Promega) (1:5000 in 2.5% nonfat milk in TBST2) for 1 h at room temperature with the Enhanced Chemiluminescent substrate system (Roche). The membranes were then exposed to XAR Scientific Imaging Film (Kodak) for 1-60 sec.

Isolation of cDNA clones

pBluescript plasmids were excised from positive phage clones as outlined by Short *et al.*, (1988). Briefly, λ ZAPII clones (titer 1×10^5 PFU/ml) were combined with R408 helper phage (titer 1×10^{11} PFU/ml) and *E. coli* XL1-Blue cells and allowed to co-infect. The lysate was used to infect *E. coli* strain XL1-Blue and cells harbouring the plasmid were selected on LB+Amp plates. Plasmids were purified and inserts characterized by Alice Lee (NSERC summer student) by restriction enzyme analysis in 0.8% agarose gels.

Sequence analysis

Dideoxy sequence analysis was performed on positive clones according to the method of Sanger *et al.*, (1977) using T7 Sequenase v2.0 sequencing kit (Amersham).

Alternatively, positive clones were sequenced using the Li-Cor 4200L automated DNA sequencer (Canadian Molecular Research Services, Inc., Ottawa). DNA and protein homology searches were performed using the BLAST (Altschul *et al.*, 1997) network service of the National Center for Biotechnology Information (NCBI). In general, BLASTX searches were used instead of BLASTN because DNA to protein searches are more discriminatory, give more biologically significant matches (amino acid comparisons) and E values tend smaller (more significant) than BLASTN searches, because of the existence of a larger number of different amino acids. The BLASTX matches were divided into groups according to statistical significance values (E values): highly significant (E values $< 10^{-20}$), moderately significant (E values 10^{-5} to 10^{-19}), weakly significant (10^{-2} to 10^{-4}), and not statistically significant (E values $> 10^{-2}$).

Results

Characterization of antigens

As part of our study of the proteins associated with the *S. commune* cell wall, we have raised a high titer, polyclonal antiserum against crude *S. commune* cell wall extracts. The 1D *S. commune* Western blot profile demonstrated the recognition of approximately 10 antigens with diverse size differences (Fig. 3.1A, lane *S. commune*). The most prominent antigens had molecular weights of less than 37 kDa but several higher molecular weight antigens at 49 kDa and 55 kDa and above were clearly detected by the ScCW antiserum.

Cross-reactivity of ScCW antiserum

To assess the species specificity of the ScCW antiserum, Western and lectin blots

Fig. 3.1 Cross-reactive properties of the ScCW antiserum and comparison to ConA binding. The Western blot (A) and lectin blot (B) were prepared identically with the cell extracts from *Schizophyllum commune*, *Neurospora crassa*, *Aspergillus nidulans*, *Aspergillus niger*, *Saccharomyces cerevisiae*, *Saccharomyces octosporus*, *Candida albicans*, *Coprinus lagopus*, *Agaricus bitorquis*, *Mucor rouxii* and *Phycomyces blakesleeana*. Approximately 10 µg of total protein was loaded in all lanes. The western blot (A) was probed with a 1:2000 dilution of the ScCW antiserum and the lectin blot (B) was probed with 1 µg/ml of biotinylated ConA.

were prepared with cell extracts from several representative ascomycetes, basidiomycetes and zygomycetes, whose evolutionary relation to *S. commune* varies depending on species (Fig. 1.1; Fig. 3.1). All of the species tested reacted to the ScCW antiserum, although the intensity of the chemiluminescent signal was approximately 5-fold stronger for the *S. commune* extract. This suggests that a small subset of antibodies in the ScCW antiserum are responsible for the interspecies, cross-reactivity. Some of the immuno-reactive proteins, such as the 43 kDa molecule (arrow) in the closely related species, namely, *C. lagopus* and *A. bitorquis*, may be a conserved homolog of the *S. commune* protein (Fig. 1.1). Some evolutionarily distant fungi, such as the ascomycetes, *N. crassa*, *A. nidulans* and *A. niger*, had very different immuno-reactive profiles (Fig. 1.1; Fig. 3.1A). Similarly, the zygomycete *P. blakesleanus* had a very divergent profile, as indicated by the lack of extensive reactivity.

Protein antigens

To demonstrate that the ScCW antiserum was primarily recognizing proteins and not sugar residues, cross-comparisons were made between Western and lectin blots (Fig. 3.1). Glycosylated proteins have been detected with ConA lectins, a protein known to recognize α -D-mannosyl and α -D-glucosyl residues (Millette and Scott 1984). The corresponding lectin blot revealed very diverse patterns of ConA binding for each fungal species (Fig. 3.1B). Both the *Neurospora* and *Saccharomyces* cell extracts contain more than 10 putative glycoproteins whereas none were detectable in *C. lagopus* or *P. blakesleanus* extracts with the concentrations of ConA employed in this study. The lectin blotting experiments revealed that some of the proteins recognized by the ScCW

antiserum co-migrate with *P. americana* ConA lectin binding activity. However, ConA and the ScCW antiserum recognizes a unique subset of proteins. There was no significant correlation between binding by the ScCW antiserum and ConA lectin.

cDNA library screen

Several Petri dishes of the *S. commune* cDNA phage expression library were screened with the ScCW antiserum; 14 positive clones were isolated, 12 of which were distinct based on the insert size differences (Table 3.1; Appendix A). All of the clones were purified and tested for their reactivity with the ScCW antiserum. The reactivity of each clone was higher as the number of purification steps increased.

Plasmids from the 12 clones which bound the ScCW antibodies were excised from the λ ZAPII bacteriophage vector for further characterization. The sizes of the inserts, after *Pvu*II digestion, varied from 950 to 2050 bp. The nucleotide sequence of each was determined. The smallest number of nucleotides for a specific clone was 634 bp (clone JB-SC-3B). In contrast, the longest cDNA clone, JB-SC-5A, was 1692 bp.

Identity of clones

Nine clones showed significant homology to other proteins in public databases, as suggested by the low expected values, while three clones did not (Table 3.1). For example, nucleotide sequence analysis of clone JB-SC-6A revealed a protein with 71% similarity with ubiquitin-conjugating enzyme from *N. crassa* (Table 3.1). Two well documented cell wall proteins were identified, namely, elongation factor 1-alpha and heat shock protein 60 kDa (Maneu *et al.*, 1996; Thomas *et al.*, 1997). In addition, JB-SC-1A has a weak similarity to chitinase 3 precursor from *C. albicans* (Kuranda and Robbins

Table 3.1 Identification of *S. commune* cell wall immunoreactive clones using BLAST searches. The DNA sequence of each clone was searched against the GenBank database using BLASTX. The matching parameters of the proteins to which the clones are closest to are shown below.

Clone ID	GenBank Accession Number	Protein name	Species	E value	Identity (%)	Similarity (%)
JB-SC-1A	1168933	Unknown protein with chitinase 3 domain ^d	<i>C. albicans</i>	0.38	21	34
JB-SC-2A	19112922	Translation initiation factor eIF-5A ^{a,e}	<i>S. pombe</i>	4E-63	77	91
JB-SC-2B	19113203	Putative saccharopine dehydrogenase ^a	<i>S. pombe</i>	E-101	48	68
JB-SC-3A	6322593	Xanthine phosphoribosyl transferase ^a	<i>S. cerevisiae</i>	6E-41	46	63
JB-SC-3B	28917011	Probable ribosomal protein 10 ^{a,e}	<i>N. crassa</i>	1E-75	77	80
JB-SC-4A	11277955	Probable heat shock protein hsp60 ^e	<i>N. crassa</i>	E-116	78	89
JB-SC-5A	2780181	Unknown proteins with Orf2 domain ^d	<i>M. tuberculosis</i>	0.29	28	38
JB-SC-5B	16415985	Conserved hypothetical protein ^a	<i>N. crassa</i>	3E-49	55	69
JB-SC-6A	6320947	Ubiquitin-conjugating enzyme ^{a,e}	<i>S. cerevisiae</i>	1E-50	55	71
JB-SC-6B	730569	60S ribosomal protein L2 ^{a,e}	<i>S. cerevisiae</i>	E-102	73	88
JB-SC-7A	19112113	Weak similarity to putative transcriptional activator ^c	<i>S. pombe</i>	0	28	47
JB-SC-7B	6015061	Elongation factor 1-alpha ^{a,e}	<i>S. commune</i>	0	98	98

^aHighly significant BLASTX matches (refer to Methods and Materials).

^bModerately significant BLASTX matches.

^cWeakly significant BLASTX matches.

^dNot statistically significant BLASTX matches.

^eProteins identified as cell wall or cell surface proteins in other organisms (Table 4.2; Table 4.3).

1991).

Discussion

The fungal cell wall is a complex structure consisting of chitin, glucan and proteins and this complexity suggests that a large number of proteins must be involved in its biogenesis (Klis 1994; Pardo *et al.*, 2000a). In support of this hypothesis, numerous cell wall proteins have been identified in *S. cerevisiae*, *C. albicans*, and *Trichoderma reesei* (Monteoliva *et al.*, 2002; Lim *et al.*, 2001; Pardo *et al.*, 2000a). Our results with *S. commune* demonstrate the recognition of approximately 10 putative surface antigens (Fig. 3.1A, lane *S. commune*). Unlike previously described cell wall antisera that recognize sugar residues of wall polymers of ascomycetes and basidiomycetes such as *A. nidulans* and *Amanita muscaria* (Behamou *et al.*, 1990; Wycoff and Ayers 1990; Marshall *et al.*, 1997), the ScCW antiserum primarily recognizes cell wall-associated proteins as evidenced by the differing Western and lectin blot profiles (Fig. 3.1). The lectin blotting experiments revealed that some of the proteins recognized by the ScCW antiserum co-migrate with ConA lectin binding activity. However, ConA and the ScCW antiserum recognizes a unique subset of antigens. This clearly shows that the ScCW antiserum recognized proteins and not sugar moieties. The fact that the ScCW antiserum was able to recognize clones expressed in prokaryotic cells strengthens this conclusion (Table 3.1). The cross-reactive properties of the ScCW antiserum indicates its use for studying the cell surface proteins of other closely related fungi. At least some of this binding in the more distant fungi may be due to non-specific or promiscuous interactions and no epitope conservation should be inferred.

Immunochemical screening of a cDNA expression library with the ScCW antiserum led to the identification of 14 clones; 12 of them were further studied. Sequencing information in conjunction with BLASTX searches determined the identity of 9 of them (Table 3.1). Some of the *S. commune* clones reported here encode well characterized cell surface proteins, namely, elongation factor 1- α , heat shock protein 60, ribosomal proteins and a ubiquitin-conjugating enzyme (Table 3.1; Maneu *et al.*, 1996; Sepulveda *et al.*, 1996; Long *et al.*, 2003). Two clones were found to code for ribosomal proteins (Table 3.1). Recently, some of these proteins have been shown to be at the cell surface (Amiri-Eliasi and Fenselau 2001; Delgado *et al.*, 2001; Pitarch *et al.*, 2002).

Consistent with the *C. albicans* work, an *S. commune* ubiquitin-conjugating enzyme has been isolated by screening an expression library with a cell wall antiserum, suggesting the involvement of ubiquitination in cell wall proteins (Sepulveda *et al.*, 1996). Ubiquitin-conjugating enzymes (E2) attach ubiquitin to proteins to target them for degradation or for localization (Jentsch *et al.*, 1990; Jentsch *et al.*, 1991; Hershko 1991). In *C. albicans*, the presence of several proteins in the cell wall that bear ubiquitin-like epitopes have been observed, specifically, in receptor-like molecules (Sepulveda *et al.*, 1996). In addition, clones coding for polyubiquitin have been isolated by screening a cDNA library with antibodies generated against the candidal 37 kDa laminin-binding protein (Sepulveda *et al.*, 1996). Intriguingly, cell surface receptor-like molecules of *C. albicans* such as 58 kDa fibrinogen receptor mannoprotein and the C3d receptor have ubiquitin-like epitopes (Leung *et al.*, 1987; Saxena and Calderone 1990; Cenciarelli *et al.*,

1992; Mori *et al.*, 1992; Paolini and Kinet 1993). It is possible that ubiquitination of some proteins in fungi targets or anchors them to the fungal cell surface. In *C. albicans*, there are two proposed functions of ubiquitination in the cell wall: adhesion and receptor-mediated signalling. At the cell surface, the close association between ubiquitin and different receptors suggest a role for ubiquitination in adhesion (Calderone 1993; Sepulveda *et al.*, 1996). Cell surface receptors are increasingly recognized as multisubunit complexes in which some of the subunits contain the domains used for interaction with ligands whereas others are used for signalling events (Bailey *et al.*, 1995; Sepulveda *et al.*, 1996). In *C. albicans*, the adhesion mediated by ubiquitination appears to be required for its virulence (Sepulveda *et al.*, 1996).

The recovery of an *S. commune* heat shock protein 60 kDa (hsp60) homolog in our study is consistent with the results of co-purification studies, protoplast secretion analysis, indirect immunofluorescence and immunoelectron microscopy of yeast cells (Table 3.1; Mager and Ferreira 1993; Lopez-Ribot and Chaffin 1996; Lopez-Ribot *et al.*, 1996; Eroles *et al.*, 1997; Lussier *et al.*, 1997b; Chaffin *et al.*, 1998; Pardo *et al.*, 1999; Pardo *et al.*, 2000a; Motshwene *et al.*, 2003). Similar results have also been reported for the hsp60 protein of the human respiratory fungal pathogen, *Coccidioides immitis* using antiserum raised in mice against the isolated recombinant protein. Thomas *et al.*, (1997) have immunolocalized hsp60 in the cytoplasm and cell wall of *C. immitis*. Hsp60 was also discovered in the wall of *Histoplasma capsulatum* yeasts by Western blotting of detergent extracted cell wall preparations with anti-hsp60 and immunoelectron microscopy confirmed that hsp60 resided on surface *H. capsulatum* yeasts as discrete

clusters (Long *et al.*, 2003). In addition to hsp60, other proteins from the heat shock protein family have been found at the surface of *N. crassa* as well as other organisms (Chapter 4). For example, hsp70 has been found on the surface of *Histoplasma capsulatum*, *Coxiella burnetti*, *Plasmodium falciparum* merozoites and *Chlamydia trachomatis* elementary bodies (Ardeshir *et al.*, 1987; Danilition *et al.*, 1990; Gomez *et al.*, 1992; Macellaro *et al.*, 1998). Moreover, two hsp homologs, hsp70 and hsp30, have also been identified by mass spectrometry from purified cell walls and intact cells of *N. crassa* (Chapter 4). Members of the hsp70 protein family function as chaperones, playing an important role in folding and translocation of other proteins and this function is also likely required at the cell surface (Pardo *et al.*, 1999; Pitarch *et al.*, 2002).

Several proteins which are desorbed by brief acid treatments from intact *S. cerevisiae* cells are L39 and L40 from the 60S ribosomal subunit and S29-A and S29-B from the 40S ribosomal subunit (Amiri-Eliasi and Fenselau 2001). Immunogold labelling has confirmed that the 60S subunit ribosomal proteins are in the cell wall, as well as in the cytoplasm (Delgado *et al.*, 2001). Our immunochemical protein screen has also detected several ribosomal proteins which appear to be associated with the cell wall of *S. commune* (Table 3.1).

Extracts of the *S. commune* cell wall matrix also contained a elongation factor (Table 3.1). Similarly, Maneu *et al.*, (1996) also reported recovering a cloned expressing *C. albicans* elongation factor-1 beta by screening an expression library with anti-cell wall antibodies. In a genetic screen for cell surface biosynthesis genes in *S. cerevisiae*, translation elongation factor eF-3 mutants were found to be hypersensitive to the mixture

of lytic enzymes in ZymolyaseTM (Lussier *et al.*, 1997b). In *A. thaliana*, a putative elongation factor was found in urea extracts from the cell wall (Chivasa *et al.*, 2002). Cell wall association of elongation factors has also been demonstrated for the bacterial species *Staphylococcus aureas*, *E. coli*, *Neisseria gonorrhoeae*, *Mycobacterium leprae* and the plant *A. thaliana* (Rosenbusch 1976; Jacobson and Porcella *et al.*, 1996; Marques *et al.*, 1998; Chivasa *et al.*, 2002; Valentini *et al.*, 2002; Vytvytska *et al.*, 2002).

A role for translation factors in the cell wall may be more difficult to envision but one possibility is that they may function as protein adhesins (Zhu *et al.*, 1994; Pitarch *et al.*, 2002). In doing so, they might have some chaperone activity, promoting translocation and processing mechanisms of cell wall components inside the wall network (Pitarch *et al.*, 2002).

In *S. cerevisiae*, a multicopy suppressor screen revealed six genes, the overexpression of which allows growth of a *tif51A-1* (translation initiation factor 51A) strain at high temperature (Valentini *et al.*, 2002). three suppressors isolated are simultaneously upstream regulators of the cell wall integrity signalling pathway: *WSC1*, *WSC2* and *WSC3* (Gray *et al.*, 1997; Verna *et al.*, 1997; Jacoby *et al.*, 1998). A fourth suppressor was *PKC1* which encodes a regulator of one of the four mitogen-activated protein-kinase cascades that is involved in cell wall biosynthesis (Posas *et al.*, 1998; Valentini *et al.*, 2002). These data suggest a link between the eIF-5A and cell wall biogenesis and possibly explains the cell wall localization of such a factor in *S. commune* (Valentini *et al.*, 2002).

The proposed cell wall association of other *S. commune* enzymes such as

saccharopine dehydrogenase and xanthine phosphoribosyltransferase (XPRT) are novel findings to date, although bacterial XPRT is periplasmic (Hochstadt-Ozer and Stadtman 1971a, Hochstadt-Ozer and Stadtman 1971b; Liu and Milman 1983; Blemings *et al.*, 1994; Guetsova *et al.*, 1999; Zhu *et al.*, 2000; Kumar *et al.*, 2002).

Several clones encoded proteins which do not resemble anything in the public databases (Table 3.1). It is possible that these clones are derived from novel genes encoding wall proteins unique to *S. commune*. Alternatively, if these are truncated cDNA, obtaining more sequence data for each of these clones might increase the matching score. This is most likely for clone JB-SC-7A (665 bp), but not for the longer clones clones JB-SC-1A (1692 bp) and JB-SC-5A (1201 bp).

The polyclonal ScCW antiserum described here should prove to be a very useful reagent for exploring the architecture of the *S. commune* cell wall. Western blots demonstrated that the ScCW antiserum recognized a small set of proteins. Since immunological recognition does not appear to involve sugar residues, it was also be useful for probing bacterial cDNA expression libraries which recognized genes encoding putative cell surface and novel proteins. Its binding to proteins in other fungal species may also be a useful property in identifying surface proteins which are conserved in related fungal species (Fig. 1.1).

CHAPTER 4

PROTEOMIC AND BIOINFORMATIC APPROACHES TO STUDY THE CELL WALL OF *NEUROSPORA CRASSA*

Introduction

The cell wall is required for the maintenance of the tubular shape of the hyphae of filamentous fungi (Bachman and Bonner 1959; deTerra and Tatum 1963; Reyes and Lahoz 1976; Cabib *et al.*, 1988). Moreover, the cell wall provides physical and osmotic protection, acts as an immobilized protein support and contributes to the cell-cell interactions such as recognition, adhesion and pathogenicity (Pardo *et al.*, 2000a; Lim *et al.*, 2001). The absence of a comparable structure around animal cells has focussed attention on fungal cell walls as a prime target for the development of drugs against fungal pathogens (Pardo *et al.*, 2000a).

While the composition of the cell walls of several yeasts has been well characterized, much less is known about the filamentous fungi such as *Neurospora crassa* (Marshall *et al.*, 1997). The major components of the *N. crassa* cell wall are glucans (β -1,3 and β -1,6) and chitin (Mahadevan and Tatum 1967; Wrathall and Tatum 1974; Cardemil and Pincheira 1979). In addition, alkali and acid extractions of cell wall preparations have been reported to release a proteinaceous component which is thought to comprise about 10 % of the cell wall dry matter (deTerra and Tatum 1963; Mahadevan and Tatum 1965; Warthall and Tatum 1974; Gozalbo *et al.*, 1993). A few *N. crassa* cell wall proteins have been previously identified by classical biochemical and molecular genetic methods including chitin synthases, glucanases, hydrophobins, invertase, trehalase and a copper detoxification protein (Chung and Trevithick 1970; Mahadevan and Mahadkar 1970; Hecker and Sussman 1973a; Sukumaran and Mahadevan 1975; Mehta and Mahadevan 1975; Ruiz-Herrera and Sentandreu 1989; Lauter *et al.*, 1992;

Wessels 1993; Suresh and Subramanyam 1996). The best studied non-covalent, wall-associated protein is the hydrophobic *eas* gene product. This abundant protein has a barrier function which is thought to resist dessication of aerial hyphae and conidiospores (Lauter *et al.*, 1992).

Studies with the yeast *Saccharomyces cerevisiae* suggest that cell wall proteins (CWPs) can be either loosely associated with the cell wall or covalently linked to other wall components in several ways (Kapteyn *et al.*, 1999). Some proteins with an internal repeat structure (Pir-CWPs) are covalently linked to the β -1,3 glucan chains, whereas CWPs that contain glycosyl phosphatidylinositol-derived structure (GPI-CWPs) are linked to the β -1,3 glucan through a β -1,6 glucan sidechain (Pardo *et al.*, 2000a). Recently, a GPI algorithm, which was primarily designed to identify GPI proteins in *S. cerevisiae* and *C. albicans*, predicted the existence of GPI proteins in other fungi such as *Schizosaccharomyces pombe* and *N. crassa* (De Groot *et al.*, 2003). In yeast, the mating factor α -agglutinin is cross-linked to cell wall β -glucans via its GPI anchor (Lu *et al.*, 1995). The covalently linked proteins were released by glucanase and/or chitinase treatments (Pardo *et al.*, 2000a). Some proteins which are not released by β -1,6 glucanase treatment have also been liberated with mild alkali (Pardo *et al.*, 2000a). Besides those proteins which are covalently linked to glucans, some cell wall mannoproteins are connected to other CWPs via disulphide bonds, while the so-called periplasmic proteins are entrapped in the wall and remain soluble (Pardo *et al.*, 2000a). Proteins which are linked to the wall by disulphide bridges have been successfully liberated from the wall matrix with reducing agents (Pardo *et al.*, 2000a).

The growth of proteomics is a direct result of advances made in large-scale nucleotide sequencing of expressed sequence tags and genomic DNA (Graves and Haystead 2002). Without this information, proteins could not be identified even with the improvements made in MS. Protein identification (by MS or Edman sequencing) relies on the presence of some form of database for the given organism (Graves and Haystead 2002). The majority of DNA and protein sequence information has accumulated within the last 5 to 10 years (Graves and Haystead 2002). In 1995, the first complete genome of an organism was sequenced, that of *Haemophilus influenzae* (Fleischmann *et al.*, 1995). The sequencing of the genomes of more than 45 microorganisms, including *N. crassa*, have been completed and that of 170 more is under way.

To catalogue the large set of proteins associated with the *Neurospora* cell wall, we have employed a proteomic approach involving the use of 2-D gel electrophoresis to resolve and isolate individual proteins for sequence analysis (O'Farrell 1975). Due to its speed and sensitivity, mass spectrometry is the method of choice for the large-scale identification of 2-D gel separated proteins. In conjunction with the recently completed sequence of the *Neurospora* genome, mass spectrometry permits unambiguous identification of proteins (Wilkins *et al.*, 1996; Pardo *et al.*, 2000a; Galagan *et al.*, 2003).

In this article, we report on the MS identification of *N. crassa* cell wall proteins which includes several glycolytic enzymes, heat shock proteins, translation factors and homologs of yeast cell wall proteins of unknown function.

Methods and Material

Fungal strains and growth conditions

The *N. crassa* wild type strain (74-OR23-1VA; FGSC 987) was obtained from the Fungal Genetics Stock Center. For mini cultures, 1 ml of spores (1×10^8 spores/ml) were added to a 50 ml tube containing 10 ml of liquid Vogel's minimal media (VMM) (Vogel 1964). For small cultures, spores from one slant tube were resuspended in 5 ml of sterile water and the suspension was used to inoculate a 250 ml media flask containing 50 ml of liquid VMM. For large scale cultures, spores from four 250 ml flasks were resuspended in 200 ml (1×10^7 spores/ml) sterile water and pooled. Fifty ml of the spore suspension was distributed into four 2800 ml flasks containing 1 L of liquid VMM. Cultures were grown with agitation for 18-24 h at 30°C or room temperature. Mycelium was harvested by vacuum filtration, washed 5 times with distilled water, placed in plastic wrap and chilled on ice prior to extraction.

Isolation of cell walls

The *N. crassa* cell wall was isolated by a standard procedure previously described which is known to produce an enriched cell wall protein fraction (Martinez *et al.*, 1991). Essentially, 50 g of fresh mycelium was resuspended in 50 ml of ice cold fungal lysis buffer (FLB containing 10 mM Tris-HCl, pH 7.2, and 1 mM PMSF) and homogenized in a bead beater (6 short, 30 s pulses). The homogenate was centrifuged at 1000 g for 10 min and the pellet was then washed again with FLB and centrifuged. In total, the pellet was washed 5 times with FLB and 10 times with sterile water. The final pellet was resuspended in 100 ml of FLB containing protease inhibitors (10 µg/ml pepstatin, 10 µg/ml leupeptin,

0.05 µg/ml aprotinin, 10 µg/ml TPCK and 0.1 µg/ml PMFS) (Roche). The 100 ml wall suspension was divided into 10 ml aliquots and stored at -20°C until required.

Chemical extractions of cell wall matrix

Aliquots (10 ml) of the purified cell wall mixture were centrifuged at 10000 g for 10 min and the drained cell wall pellets were resuspended in 10 ml of different extraction reagents: 10 mM Tris-HCl, pH 7.2 plus 1 mM PMSF (FLB), 0.5 M sodium hydroxide (NaOH), 1 M hydrochloric acid (HCl), 6 M guanidine hydrochloride (GnHCl) and 100 mM 2-mercaptoethanol plus 8 M urea (ME+U). Each sample was vortexed for 1 min after the addition of glass beads (1 g), incubated at room temperature for 10 min and centrifuged at 10000 g for 15 min and the supernatants were dialyzed (Spectra/Por® MWCO 14000) overnight in 4 L of distilled water (four changes) at 4°C with insignificant loss due to insolubility. The concentration of protein in the supernatant extracts was determined by spectrophotometry (Stoscheck 1990) using an HP 4515 instrument (Hewlett-Packard). Protein samples were lyophilized in a Savant SS2 SpeedVac and stored at -20°C until required. The appropriate amounts of protein (supernatant) were resuspended in 1-D or 2-D sample buffers before 1-D or 2-D SDS-PAGE gel electrophoresis (10% separatory; 4% stacking).

Two-dimensional SDS-PAGE

The cell wall protein extract for 2-D SDS-PAGE was prepared by thawing 1 ml of wall pellet, centrifuging at 16000 g for 5 min and resuspending the wall pellet in 200 µl of 1-D sample buffer. The samples were incubated at room temperature for 15 min and then centrifuged at 16000 g for 15 min. 2-D SDS-PAGE was performed on 10-25 µg

samples as previously described with a Mini-PROTEAN II 2-D cell apparatus (Biorad) according to the manufacturer's instructions (O'Farrell 1975). Isoelectric focusing (IEF) was carried out at 500 V for 10 min and at 750 V for 3.5 h. Following IEF, the gel tubes were extruded and placed on the 4% stacking gels and equilibrated for 10 min in the second dimension sample buffer containing 0.5 M Tris-HCl (pH 6.8), 12.5% v/v glycerol, 10% w/v SDS, 640 mM 2-mercaptoethanol and 0.05% w/v bromophenol blue and electrophoresed as described below. For the second dimension, proteins were separated at 170 V for 1 h through 10% acrylamide separatory gels using the discontinuous buffer system (Laemmli 1970). After electrophoretic separation, gels were silver stained. Briefly, gels were fixed for 30 min in 50% ethanol and 5% acetic acid, washed once for 10 min with 50% ethanol and twice with sterile water. Gels were submerged for 5 min in 0.02% w/v sodium thiosulphate (Sigma), washed twice for 5 min with sterile water, and incubated in 0.1% w/v silver nitrate (Sigma) for 30 min at 4°C. Gels were rinsed for 2 min with water. Gels were developed by submerging them in developer (0.04% v/v formalin and 2% w/v sodium carbonate) once briefly to minimize non-specific staining. New developer was added to the gels until protein spots appeared and the desired intensity had been achieved. Gel were rinsed with water, placed in 5% acetic acid for 5 min and stored in 1% acetic acid at 4°C until required. Gels were scanned on the Biorad Fluor S multimager and analyzed using Biorad's PDQuest™ 7.0. Selected protein spots were cut out and analyzed by mass spectrometry as described below.

Mass spectrometry

Excised gel spots were digested overnight at 37°C with 200 ng of modified

sequencing grade trypsin (Promega, Madison WI) in 50 mM ammonium bicarbonate. The trypsin digests were analyzed by rapid capillary LC-MS/MS (CapLC-MS/MS) using a Waters CapLC liquid chromatograph (Waters, Milford MA) coupled to a Q-TOF 2 hybrid quadrupole time-of-flight mass spectrometer (Micromass/Waters, Manchester, UK) via an electrospray ionization interface. The samples were loaded onto a 0.3 x 5 mm C₁₈ micro pre-column cartridge (Dionex/LC-Packings) for each analysis. Rapid peptide elution was achieved using a linear gradient of 5 to 60% acetonitrile, 0.2% formic acid in 6 min (flow rate of 1 ml/min). The mass spectrometer was operated in data dependent acquisition mode where a survey scan was used to identify suitable precursor ions for subsequent MS/MS analysis. Three precursor ions were selected per survey scan and four MS/MS scans were acquired per product ion (one second per MS/MS scan). MS/MS spectra were obtained only on doubly and triply protonated ions.

Trypsin-released surface proteins and microscopy of intact cells

For the extraction of proteins from intact cells, four mini cultures were grown in 50 ml tubes containing 10 ml of VMM with shaking at 30°C overnight. Mycelia were pelleted at 3000 g for 15 min and gently resuspended in a Tris buffer containing 10 mM Tris-HCl, pH 7.2. To prevent cells from regenerating surface proteins, mycelia were killed by incubating the mini cultures at 60°C for 60 min. Mycelia were thoroughly washed with a 10 mM Tris-HCl, pH 7.2 buffer and pelleted at 3000 g for 15 min. The washing procedure was repeated 14 times. Mycelia from 2 mini cultures were resuspended in 1 ml of a denaturing buffer (2 M urea, 5 mM dithiothreitol, 10 mM Tris-HCl, pH 8.0) and mycelia from the other 2 mini cultures were resuspended in 1 ml of

non-denaturing Tris buffer (10 mM Tris-HCl, pH 8.0). To denature surface proteins, mycelia were incubated for 60 min at 60°C, followed by the addition of 1 ml of 50 mM ammonium bicarbonate or until the final concentration of urea was less than 1 M. Finally, 10 µg of modified sequencing grade trypsin (Promega, Madison WI) were added to only two of the cultures and none were added to the controls. Cultures were incubated at 37°C for 4 h or 24 h. The supernatants, containing wall-released peptides, were obtained after centrifuging the mycelia at 3000 g for 30 min, concentrated by lyophilization and stored at -20°C until required. Supernatants containing tryptic digests were directly analyzed by CapLC-MS/MS. To identify the proteins, databases searches were performed as described below. The integrity of the intact cells was checked by phase-contrast or fluorescence microscopy. In short, 6 h or overnight cultures were treated with trypsin for 4 h or 24 h and observed using phase-contrast optics with a Zeiss microscope. For fluorescence microscopy, mycelia were stained with 5-chloromethylfluorescein diacetate (CMFDA, Molecular Probes), a long-term cell tracking dye, for 15 min at room temperature, centrifuged at 14000 RPM and washed 3 times with Tris buffer containing 10 mM Tris-HCl, pH 7.2. Finally, mycelia were treated with trypsin as previously described and observed using the FITC channel of a Zeiss microscope.

NovozymeTM-released proteins from purified cell walls

A ml of purified cell wall mixture was thawed and aliquots were distributed to as many mini centrifuge tubes as necessary. The tubes were centrifuged at 14000 RPM for 5 min and drained. The cell walls were resuspended in a 99 µl buffer containing 10 mM Tris-HCl and 10X strength protease cocktail mix (Roche Diagnostics, Palo Alto, CA). An

appropriate amount (0.001 to 100 μg) of NovozymeTM 234 (Novo Biolabs, Danbury, CT), in a 1 μl volume, was added to the cell wall mixture. One μl of Tris buffer was added to the control reaction instead of the NovozymeTM. The reactions were incubated for 1 or 5 h at 37°C. Following incubation, the samples were centrifuged at 14000 RPM for at least 30 min or until the cell walls formed a solid pellet. The supernatants containing the released proteins were transferred to new tubes and the protein concentrations were measured by spectrophotometry as previously described. The appropriate amount of release proteins (5-25 μg) were separated by 1-D SDS PAGE previously described. Gels were silver stained, spots were selected and cut, and proteins were identified by mass spectrometry as previously mentioned.

Proteins secreted into the media

Flasks containing 50 ml of VMM were inoculated with 1×10^6 spores and grown at room temperature for 12, 18 or 24 h. Mycelium was harvested by vacuum filtration and the media containing the secreted proteins was collected. The media was dialyzed in a 10 ml Spectra/Por[®] Floalyzer membrane (cut off MW 1000 Da, Spectrum, Laguna Hills, CA) overnight with 4 changes of deionized water. The protein concentrations were determined by spectrophotometry (Stoscheck 1990). Proteins were resuspended (5-15 μg) in 1-D SDS PAGE sample buffer and separated by gel 1-D SDS PAGE. Gels were silver stained, spots were selected and cut, and protein identification was done by mass spectrometry.

Database searching

Database searching was carried out using Mascot™ (MatrixScience, London, UK), a program that correlates mass spectral data with sequence databases. The MS/MS spectra were transformed into peak lists which were then used to search sequence databases such as GenBank (NCBI) and the Whitehead Institute *Neurospora* database (<http://www-genome.wi.mit.edu/annotation/fungi/neurospora/>). The identity of cell wall proteins which matched *Neurospora* entries listed as hypothetical, their identity was successfully deduced in most cases by searching the Munich Information Center *Saccharomyces cerevisiae* database through BlastP (<http://www.mips.biochem.mpg.de/proj/yeast/>). The BLASTP matches were categorized into highly significant (E values $< 10^{-20}$) and moderately significant (E values 10^{-5} to 10^{-19}) hits. The E-values for the matching the hypothetical proteins against the yeast proteins ranged from $6.5\text{E-}17$ to $1.3\text{E-}241$. Weakly (10^{-2} to 10^{-4}) and statistically insignificant (E values $> 10^{-2}$) hits were not obtained. Therefore, the *Neurospora* hypothetical proteins were conjectured to be homologues of yeast proteins and named accordingly (Tables 4.1 and 4.2). The presence of putative cleavable signal peptides or noncleavable signal anchors was predicted using the SignalP V1.1 program (Nielsen *et al.*, 1997). The PROSITE Release 17.42 database was searched using ScanProsite available from the Expasy Molecular Biology Server to identify potential protein domains (<http://us.expasy.org/>).

To determine if the genes encoding these proteins are unique, all proteins identified by mass spectrometry were analyzed by submitting the *N. crassa* protein sequences to the *Neurospora* database in order to check for existence of two or more

closely related proteins. One of these proteins might be targeted towards the cell wall and the other might be targeted to the mitochondria.

In silico identification of putative cell wall proteins

A literature search was performed to discover which gene deletions had been shown to affect the *S. cerevisiae* cell wall directly or indirectly. For example, deletion mutants whose phenotype included sensitivity to cell wall binding dyes, such as calcofluor white, were selected by this first screen. The amino acid sequence were obtained from the Munich Information Center *S. cerevisiae* database (<http://www.mips.biochem.mpg.de/proj/yeast/>). The amino acid sequence of proteins which are directly or indirectly involved in the cell wall integrity of *S. cerevisiae* were searched against the *Neurospora crassa* database by using BLASTP. A total of 180 yeast proteins were used in this search of the *Neurospora* putative cell wall proteome. BLASTP was used because protein to protein searches gave more biologically relevant comparisons because amino acids were being compared instead of nucleotides. The BLASTP matches were divided into groups according to statistical significance values (E values): highly significant (E values $< 10^{-20}$) and moderately significant (E values 10^{-5} to 10^{-19}). Weakly (10^{-2} to 10^{-4}) and not statistically significant (E values $> 10^{-2}$) matches were not included.

Results

Purified cell walls extracted by chemical treatments

Proteins extracted from purified cell walls of *N. crassa* were separated by 1-D gel electrophoresis (Fig. 4.1). This procedure includes extensive washing and low speed

Fig. 4.1 Chemically-extracted proteins from the cell wall of *N. crassa*. The chemical treatments included, 10 mM Tris-HCl, pH 7.2 +1 mM PMSF (FLB), 0.5 M sodium hydroxide (NaOH), 1 M hydrochloric acid (HCl), 6 M guanidine hydrochloride (GnHCl) and 8 M urea+100 mM 2-mercaptoethanol (U+ME).

centrifugation to minimize contamination by cytoplasmic/organelar proteins not normally associated with the cell wall matrix (Lim *et al.*, 2001). Several chemical extractions were employed to determine the type of proteins that could be released from the cell wall matrix (Fig. 4.1). In earlier studies, cell wall proteins were shown to be liberated from the carbohydrate matrix of the *Neurospora* wall by 0.5 M NaOH extraction and 1 M HCl (Wrathall and Tatum 1973). While the NaOH extraction was effective in removing many of the proteins from the wall preparation, alkaline extraction was not used further since the protein profile was very smeared.

Since several cell wall proteins of *C. albicans* and *S. cerevisiae* have been reported to be released by incubation with 1 mM 2-mercaptoethanol (Casanova and Chaffin 1991; Moukadiri *et al.*, 1999), we tested whether the extraction with 8 M urea and 100 mM ME, could effectively release proteins from the *N. crassa* wall. Approximately 18 protein bands were detected on 1-D SDS-PAGE gels with the molecular masses ranging from approximately 10 kDa to 97 kDa (Fig. 4.1, U+ME lane). In contrast, the brief treatments with 0.5 M NaOH, 1 M HCl or 6 M GnHCl at room temperature were less effective in solubilizing proteins from the cell wall preparation (Fig. 4.1). The protein profiles of each chemical extraction had some unique features (Fig. 4.1). At a given molecular mass, some of proteins appear more intense in one treatment in comparison to others, indicative of the selectivity of the chemical used for the extraction. The most effective solution for solubilizing proteins from the *Neurospora* cell wall matrix prior to 2-D gel electrophoresis (IEF gradient pH 3-10) was determined to be a combination of urea plus 2-mercaptoethanol. Due to the limited resolution of some of the

proteins, CapLC-MS/MS was then used to analyse proteins in excised gel fragments because it permits recognition of several proteins present in a single spot that have been separated by chromatography (Fig. 4.2) (Shevchenko *et al.*, 1996). Typically 51 different protein spots were resolved by 2-D PAGE following extraction with the combined denaturant and reducing agent (Fig. 4.2; Table 4.1). From this gel twenty seven protein spots which contained adequate amounts of protein were subjected to CapLC-MS/MS analysis and successfully identified (Fig. 4.2 and Table 4.1, spots 1 to 27). In addition, to identify proteins with a low molecular mass a separate 2-D gel loaded with a higher amount of proteins, extracted with 9.5 M urea and 640 mM 2-mercaptoethanol, was run. Subsequently, spots were cut out and analyzed by mass spectrometry (Table 4.1, spots 28 to 36). Extractions with i) urea (data not shown), ii) guanidine hydrochloride or iii) hydrochloric acid alone solubilized fewer proteins. Three 2-D gels resolving the most abundant proteins extracts yielded a total of 49 proteins which were also excised and subjected to CapLC-MS/MS analysis. These alternative extractions yielded many of the same proteins as the U+ME protocol.

A total of 100 protein spots were analyzed by CapLC-MS/MS, but only 56 of them yielded sequence information (Tables 4.1 and 4.2, chemical treatments). In total, the derived amino acid sequences enabled us to identify 50 distinct cell wall associated proteins (Tables 4.2 and 4.2).

NovozymeTM-released proteins from purified cell walls

In contrast to protein being released by chemical treatments, proteins were released from purified cell walls by mild NovozymeTM treatments (Table 4.2). Many of

Table 4.1 Proteins extracted from purified cell walls by chemical treatments. Summary of cell wall proteins extracted with various chemicals including 9.5 M urea plus 640 mM 2-mercaptoethanol (Refer to Fig. 4.2, spots 1 to 27) and identified by CapLC-MS/MS.

Protein No. ^f	Mass	No. peptide sequences matched ^g	Mascot score	% sequence coverage	Protein name	<i>Neurospora</i> Acc. No.
1	29981	4	213	16.96	Outer mitochondrial membrane protein porin	NCU04304.1
	32178	6	177	19.06	Enoyl coA hydratase ^d	NCU06448.1
2	30267	11	418	36.33	Adenylate kinase	NCU01550.1
3	35496	16	405	34.23	Malate dehydrogenase precursor, mitochondrial ^{b, d}	NCU04899.1
4	29311	12	458	40.84	14-3-3 protein homolog	NCU02806.1
	35496	4	196	12.80	Malate dehydrogenase precursor, mitochondrial ^{b, d}	NCU04899.1
5	35496	13	519	35.42	Malate dehydrogenase precursor, mitochondrial ^{b, d}	NCU04899.1
6	38013	11	368	19.41	NMT1 protein homolog	NCU09345.1
	35496	6	253	19.34	Malate dehydrogenase precursor, mitochondrial ^{b, d}	NCU04899.1
7, 11a	47565	15, 11	544, 519	23.44, 23.29	Enolase ^b	NCU10042.1
8	37272	12	404	26.01	Alcohol dehydrogenase III ^{b, d}	NCU09285.1
9	36270	16	417	25.15	Glyceraldehyde-3-phosphate dehydrogenase ^{a, b, c}	NCU01528.1
10	44774	4	193	11.41	Hypothetical GTP-binding protein ^d	NCU02044.1
11	51956	7	289	14.29	Citrate synthase, mitochondrial	NCU01692.1
12	33170	4	120	10.37	Galactose-induced of aldo/keto reductase family ^d	NCU04510.1
13	38013	17	425	29.71	NMT1 protein homolog	NCU09345.1
14	47565	16	687	31.96	Enolase ^b	NCU10042.1
15, 12a	55602	16, 5	518, 106	25.00, 11.92	ATP synthase beta chain, mitochondrial precursor ^{a, b}	NCU05430.1
16	29311	4	104	15.02	14-3-3 protein homolog	NCU02806.1
17	31533	4	148	11.03	Predicted protein ^e	NCU08330.1
18	54799	4	92	6.93	Protein disulfide isomerase precursor ^d	NCU09223.1

Protein No. ^f	Mass	No. peptide sequences matched ^g	Mascot score	% sequence coverage	Protein name	<i>Neurospora</i> Acc. No.
19	31481	7	194	15.17	Ribosomal protein p40 homolog A ^d	NCU03393.1
	42905	3	122	9.60	Vacuolar protease A precursor	NCU02273.1
20, 14a	42215	6, 9	246,	17.06, 24.67	Actin	NCU04173.1
	38013	5	363	12.35	NMT1 protein homolog	NCU09345.1
			191			
21	44596	5	221	14.68	ketol-acid reductoisomerase precursor	NCU03608.1
22	39864	21	564	31.77	Fructose biphosphate aldolase ^d	NCU07807.1
23	25581	8	200	31.90	Heat shock protein 30 kDa ^{a, b, d}	NCU07232.1
	28787	7	146	16.97	Related to sorbitol utilization protein ^d	NCU09041.1
24	27217	3	133	11.69	Triose phosphate isomerase ^d	NCU07550.1
25	56292	5	146	9.49	Related to enoyl CoA hydratase	NCU03777.1
	61024	5	102	8.61	UTP glucose-1-phosphate uridylyltransferase ^d	NCU02797.1
26	35095	7	280	22.78	40S small subunit ribosomal protein ^d	NCU05810.1
27	38013	29	517	28.82	NMT1 protein homolog	NCU09345.1
	35206	14	473	26.54	Transaldolase ^d	NCU02136.1
28 ^h	32334	6	145	14.93	Snz-type pyridoxine vitamin B6 biosynthetic protein SNZ1	NCU06550.1
	31472	3	121	9.12	Putative monooxygenase	NCU08549.1
	36841	2	87	4.65	Thiazole biosynthetic enzyme, mitochondrial precursor	NCU06110.1
29 ^h	37419	5	158	13.59	Alcohol dehydrogenase	NCU01754.1
	39258	3	154	9.75	Putative disulfide isomerase ERP38	NCU03739.1
	38773	3	115	10.85	Translation initiation factor 3 (eif3)	NCU03876.1
	37465	1	62	2.49	Ribosomal protein L4	NCU03757.1
30 ^h	28791	2	93	9.12	40S ribosomal protein S3	NCU00489.1
31 ^h	32029	4	83	14.53	Related to 2, 5-diketo-D-gluconic acid reductase	NCU01233.1
32 ^h	22059	1	53	7.00	Predicted protein	NCU00635.1
33 ^h	23033	6	184	28.63	ATP synthase oligomycin sensitivity conferral protein	NCU01606.1
34 ^h	19353	5	125	23.69	H ⁺ -transporting two-sector ATPase	NCU00636.1
35 ^h	24458	2	57	11.92	NADH-Ubiquinone oxidoreductase 21 kDa	NCU05221.1
36 ^h	22859	3	40	14.85	40S ribosomal protein S7	NCU00258.1
1a	86259	12	514	17.82	Homocysteine methyltransferase ^e	NCU06512.1
4a	64848	19	637	26.71	Hypothetical heat shock protein 70 kDa ^d	NCU02075.1
5a	70510	10	287	14.55	Heat shock protein 70 kDa	NCU09602.1

Protein No. ^f	Mass	No. peptide sequences matched ^g	Mascot score	% sequence coverage	Protein name	<i>Neurospora</i> Acc. No.
6a	59485	13	609	24.14	ATP synthase alpha chain, mitochondrial precursor	NCU02514.1
7a	61360	6	249	9.27	Glucose-6-phosphate isomerase ^c	NCU07281.1
9a	52092	12	408	18.67	Elongation factor 1-alpha ^b	NCU02003.1
10a	45029	7	247	21.29	Phosphoglycerate kinase	NCU07914.1
13a	48823	11	502	24.01	NADP-specific glutamate dehydrogenase	NCU01195.1
15a	47937	3	107	6.82	Eukaryotic initiation factor 4A	NCU07420.1

^a Proteins also identified in 100 mM 2-mercaptoethanol plus 8 M urea extracts.

^b Proteins identified in 6 M guanidine hydrochloride extracts.

^c Protein identified in 8 M urea extracts.

^d Identity of hypothetical proteins by BlastP searches against NCBI and yeast databases (E-values range 6.5E-17 to 5.8E-175).

^e Predicted protein is not similar to any protein in the yeast and NCBI databases.

^f Spots 1 to 27 refer to proteins found on Fig. 4.2 Spots 1a to 15a are of cell wall proteins purified by 2D gel electrophoresis (see Methods and Materials, purified cell wall proteins).

^g This indicates the number of peptide sequences that were matched between the unknown protein and the identified protein.

^h Proteins extracted by 9.5 M urea plus 640 mM 2-mercaptoethanol. A separate 2D gel was run to identify these low molecular weight proteins.

Fig. 4.2 Two-dimensional silver-stained gel of proteins found in the cell wall.

Neurospora crassa walls were extracted with 1-D sample buffer containing 9.5 M urea and 5% v/v 2-mercaptoethanol. Example proteins were labelled as follows: *OMMP*, outer mitochondrial membrane porin; *ENO*, enolase; *GADPH*, Glyceraldehyde-3-phosphate dehydrogenase; *ASB*, ATP synthase beta chain; *PP*, predicted protein; *PDI*, protein disulfide isomerase and *NMT1*, NMT1 protein homolog.

the proteins released by Novozyme™ were also released by chemical treatments from purified walls. An example of such a protein is enolase. Furthermore, there were many proteins identified in the Novozyme™-extracts that were also identified in the trypsin-extracts. Categories to which the identified proteins belong to include glycolytic enzymes, heat shock proteins, mitochondrial proteins and proteins with a diverse variety of functions. Examples of the latter category of protein include phosphoglucosmutase 2, methionine synthase and NMT1 (Table 4.2).

Surface proteins released by trypsin from intact cells

To address the issue of possible contamination in the purified cell wall fraction, extractions of proteins with mild trypsin treatments were performed on intact cells. Fluorescence and phase-contrast microscopy was used to check the integrity of cells after trypsin treatments (Fig. 4.3). No differences were observed between trypsin-treated and untreated samples. The 6 h old mycelia did not show any sign of cellular lysis after 4 h or 24 h trypsin treatments. Mycelia of trypsin-treated samples were composed of filamentous tubes similar to those of untreated mycelia. Viability tests were performed by testing the growth of trypsin-treated and untreated hyphae in liquid media (data not shown). The untreated mycelia grew more vigorously than the trypsin-treated ones. A clear distinction between the two samples was the fact that trypsin-treated mycelia did not form growth aggregates as did the untreated mycelia.

Many proteins were released from the cell surface of intact hyphae by the action of trypsin (Table 4.2). In total, 39 of the most abundant proteins were identified in the 4 h (containing 2 M urea, 5 mM dithiothreitol, 50 mM Tris-HCl, pH 8.0) and 24 h

Table 4.2 Proteins released from purified walls and intact cells by chemical and enzymatic treatments. Proteins were released from purified cell walls with various treatments including 9.5 M plus 640 mM 2-mercaptoethanol and 6 M guanidine hydrochloride (alone). Proteins were also released from intact cells with 10 µg of trypsin in denaturing (4 h) and nondenaturing conditions (24 h).

Acc. No.		Purified walls + Chemical treatments ^a	Purified ^b wall + Novozyme	Intact cells - Trypsin (4h/24h)	Intact ^d cells + Trypsin (4 h)	Intact ^e cells + Trypsin (24 h)	Secreted ^f proteins into media
Known cell wall proteins							
NCU03685.1	α-1,4 glucanase						+ ^h
NCU07523.1	β-1,3 glucanase						+ ^{i,j}
NCU08909.1	Gas1 (GPI-anchored surface glycoprotein)						+ ^j
NCU06781.1	Gas3 precursor (GPI-wall anchored protein)						+ ⁱ
NCU09175.1	Soluble cell wall protein						+ ^j
NCU08936.1	ECM33 homolog involved in cell wall biogenesis						+ ^j
Sugar metabolism and fermentation enzymes							
NCU01754.1	Alcohol dehydrogenase	+					
NCU09285.1	Alcohol dehydrogenase	+					
NCU10042.1	Enolase	+	+ ^{f,g}		+	+	
NCU07807.1	Fructose biphosphate aldolase	+	+ ^{f,g}		+		
NCU02252.1	Phosphoglyceromutase				+	+	
NCU07914.1	Phosphoglycerate kinase	+	+ ^{f,g}			+	
NCU01528.1	Glyceraldehyde-3-phosphate dehydrogenase	+			+	+	
NCU07550.1	Triose phosphate isomerase	+	+ ^{f,g}	+ ^b	+		

Acc. No.		Purified walls + Chemical treatments ^a	Purified ^k wall + Novozyme	Intact cells - Trypsin (4h/24h)	Intact ^d cells + Trypsin (4 h)	Intact ^e cells + Trypsin (24 h)	Secreted ^f proteins into media
NCU04510.1	Galactose-induced of aldo/keto reductase family	+					
NCU09041.1	Related to sorbitol utilization protein	+					
NCU02797.1	UTP glucose-1-phosphate uridylyltransferase	+					
NCU07281.1	Glucose-6-phosphate isomerase	+ ^{f, g}	+				
NCU01328.1	Transketolase					+	
NCU03100.1	6-phosphogluconate dehydrogenase		+ ^f		+	+	
NCU03982.1	78 kDa glucose-regulated protein homolog precursor					+	
NCU02193.1	Pyruvate decarboxylase				+		
NCU02136.1	Transaldolase	+	+ ^{f, g}	+ ^b			+ ^h
Heat shock proteins							
NCU04142.1	Heat shock protein 80 kDa				+		
NCU08693.1	Heat shock protein 70 kDa					+	
NCU02075.1	Heat shock protein 70 kDa	+	+			+	
NCU09602.1	Heat shock protein 70 kDa	+					
NCU07232.1	Heat shock protein 30 kDa ^{b)}	+					
Mitochondrial proteins							
NCU04304.1	Outer mitochondrial membrane protein porin ⁸⁾	+					
NCU01692.1	Citrate synthase, mitochondrial	+				+	
NCU05430.1	ATP synthase beta chain, mitochondrial precursor	+			+	+	
NCU02514.1	ATP synthase alpha chain, mitochondrial precursor	+			+	+	

Acc. No.		Purified walls + Chemical treatments ^a	Purified ^k wall + Novozyme	Intact cells - Trypsin (4h/24h)	Intact ^d cells + Trypsin (4 h)	Intact ^e cells + Trypsin (24 h)	Secreted ^f proteins into media
NCU07659.1	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial precursor					+	
NCU01550.1	Adenylate kinase	+					
NCU03559.1	Ubiquinol cytochrome c reductase complex core protein 2					+	
NCU04899.1	Malate dehydrogenase precursor, mitochondrial	+	+				+ ^h
Other functions							
NCU03486.1	Predicted protein			+ ^{b, c}			
NCU02411.1	Putative protein			+ ^{b, c}			
NCU06031.1	Putative thiol antioxidant protein			+ ^b			
NCU06603.1	Putative intracellular protease/amidase			+ ^b			
NCU09057.1	Soluble cell wall protein awa1p			+ ^b			
NCU02806.1	14-3-3 family, chaperone	+			+		
NCU03300.1	14-3-3-like protein					+	
NCU10058.1	Phosphoglucomutase 2		+ ^g				
NCU02273.1	Vacuolar protease A precursor	+					
NCU09223.1	Protein disulfide-isomerase precursor	+					
NCU02003.1	Elongation factor 1-alpha	+			+	+	
NCU03826.1	Elongation factor 1-gamma					+	
NCU07700.1	Elongation factor 2				+		
NCU07922.1	Elongation factor 3b				+	+	
NCU01021.1	Translation initiation factor 3	+				+	

Acc. No.		Purified walls + Chemical treatments ^a	Purified ^k wall + Novozyme	Intact cells - Trypsin (4h/24h)	Intact ^d cells + Trypsin (4 h)	Intact ^e cells + Trypsin (24 h)	Secreted ^f proteins into media
NCU06035.1	Translation elongation factor eEF-1 beta chain				+	+	
NCU06512.1	Methionine synthase (homocysteine methyl transferase)	+	+ ^{f, g}		+	+	+ ^h
NCU06110.1	Thiazole biosynthetic enzyme	+			+	+	
NCU09345.1	NMT1 protein homolog	+	+ ^{f, g}		+	+	
NCU06550.1	Snz-type pyridoxine vitamin B6 biosynthetic protein	+				+	
NCU04173.1	Actin	+	+ ^g	+ ^b	+	+	
NCU03608.1	ketol-acid reductoisomerase precursor	+					
NCU01439.1	Phosphoglycerate dehydrogenase				+	+	
NCU03777.1	Related to enoyl CoA hydratase	+					
NCU06448.1	Enoyl CoA hydratase	+					
NCU01680.1	Plasma membrane ATPase					+	
NCU09477.1	ADP, ATP carrier protein (translocase)				+		
NCU08549.1	Putative monooxygenase	+					
NCU03739.1	Putative disulfide isomerase ERP38	+					
NCU01233.1	Related to 2, 5-diketo-D-gluconic acid reductase	+					
NCU00635.1	Predicted protein	+					
NCU01606.1	ATP synthase oligomycin sensitivity conferral protein	+					
NCU00636.1	H ⁺ -transporting two-sector ATPase	+					
NCU05221.1	NADH-Ubiquinone oxidoreductase 21 kDa	+					

Acc. No.		Purified walls + Chemical treatments ^a	Purified ^b wall + Novozyme	Intact cells - Trypsin (4h/24h)	Intact ^d cells + Trypsin (4 h)	Intact ^e cells + Trypsin (24 h)	Secreted ^f proteins into media
NCU01195.1	NADP-specific glutamate dehydrogenase	+			+		
NCU07420.1	Eukaryotic initiation factor 4A	+				+	
NCU10051.1	Flavohemoglobin				+		
NCU07307.1	Fatty acid synthetase					+	
NCU08330.1	Predicted protein	+					+ ^h
NCU05137.1	Hypothetical protein				+		+ ^{i,j}
NCU02044.1	Hypothetical GTP-binding protein	+					
NCU03757.1	Ribosomal protein L4	+			+	+	
NCU03806.1	Ribosomal protein L27				+		
NCU03393.1	Ribosomal protein p40 homolog A	+			+		
NCU00258.1	40S ribosomal protein S7	+			+	+	
NCU08344.1	60S ribosomal protein				+		
NCU06843.1	60S ribosomal protein L3					+	
NCU04331.1	60S ribosomal protein L5					+	
NCU01317.1	60S ribosomal protein L12					+	
NCU08627.1	40S ribosomal protein S21				+		
NCU06431.1	40S ribosomal protein S22					+	
NCU05599.1	40S ribosomal protein S28					+	
NCU00971.1	40S ribosomal protein S15					+	
NCU00489.1	40S ribosomal protein S3	+				+	
NCU05810.1	40S small subunit ribosomal protein	+					
NCU03897.1	Related to SCP160 protein					+	

Acc. No.		Purified walls + Chemical treatments ^a	Purified ^k wall + Novozyme	Intact cells - Trypsin (4h/24h)	Intact ^d cells + Trypsin (4 h)	Intact ^e cells + Trypsin (24 h)	Secreted ^f proteins into media
NCU02657.1	Methionine adenosyltransferase				+	+	
NCU00018.1	Transitional endoplasmic reticulum ATPase				+		
NCU05290.1	Orotate phosphoribosyltransferase				+		
NCU01985.1	Sulfate adenylyltransferase				+		
NCU06666.1	Myc-inositol 1-phosphate synthase				+		
NCU07697.1	Isocitrate dehydrogenase				+		
NCU06783.1	Probable ATP citrate lyase subunit 2				+		
NCU08332.1	Woronin body major protein precursor				+		
NCU00355.1	Catalase						+ ^{i,j}
NCU00798.1	Predicted protein						+ ^{i,j}
NCU09788.1	Putative large secreted protein						+ ^j
NCU09133.1	Predicted protein						+ ⁱ

^a Proteins released from purified cell walls by 9.5 M urea plus 640 mM 2-mercaptoethanol, 8 M urea plus 100 mM 2-mercaptoethanol, 8 M urea and 6 M guanidine hydrochloride and boiling in SDS-PAGE sample buffer (electroelution purified proteins).

^b Proteins released from the cell surface after incubation of intact cells in 2 M urea at 37°C for 4 h without trypsin. The Mascot™ score varied from 20 to 181.

^c Proteins released from the cell surface after incubation of intact cells in 0 M urea at 37°C for 24 h without trypsin.

^d Proteins released by 10 µg of trypsin under denaturing conditions (2 M urea) for 4 h at 37°C.

^e Proteins released by 10 µg of trypsin under nondenaturing conditions (0 M urea) for 24 h at 37°C.

^f Proteins released from purified cells walls by 0.001 µg/µl of novozym 234.

^g Proteins released from purified cell walls by 1 µg/µl of novozym 234.

^h Proteins identified in Vogel's minimal medium after 12 h of growth.

ⁱ Proteins identified in Vogel's minimal medium after 18 h of growth.

^j Proteins identified in Vogel's minimal medium after 24 h of growth.

^k Mascot™ score for the proteins identified in novozym samples varied from 40 to 362. The number of peptides sequenced per protein was 1 to 15.

^l Mascot™ score for the proteins identified in the growth media samples varied from 38 to 346. The number of peptides sequenced per protein was 1 to 8.

Table 4.3 Comparison of wall proteins between species. List of all wall proteins identified in *Neurospora crassa* by mass spectrometry in comparison to wall proteins of other organisms reported in the literature. Evidence for cell wall localization in other species comes from immunofluorescence and immunogold transmission electron microscopy, secretion by protoplasts, antibodies recognition of cell surface antigens, cell surface biotinylation experiments and chemical extractions from purified walls and/or intact cells. The proteins have been classified according to their known biological function.

Acc. No.	<i>Neurospora crassa</i>	<i>Saccharomyces cerevisiae</i>	<i>Candida albicans</i>	<i>Trichoderma reesei</i>	<i>Arabidopsis thaliana</i>	References
Known cell wall proteins						
NCU03685.1	α -1,4 glucanase	+				Pardo <i>et al.</i> , 2000a
NCU07523.1	β -1,3 glucanase	+	+			Pardo <i>et al.</i> , 2000a Pitarch <i>et al.</i> , 2002
NCU08909.1	Gas1 (Glycophospholipid-anchored surface glycoprotein)	+	+			Pardo <i>et al.</i> , 2000a Pitarch <i>et al.</i> , 2002
NCU06781.1	Gas3 precursor (GPI wall anchored protein)	+	+			Pardo <i>et al.</i> , 2000a Pitarch <i>et al.</i> , 2002
NCU09175.1	Soluble cell wall protein	+				Giaever <i>et al.</i> , 2002
NCU08936.1	ECM33 homolog involved in cell wall biogenesis	+				Giaever <i>et al.</i> , 2002
Sugar metabolism and fermentation enzymes						
NCU09285.1	Alcohol dehydrogenase	+	+			Pardo <i>et al.</i> , 2000a Klotz <i>et al.</i> , 2001 Pitarch <i>et al.</i> , 2001 Chaffin <i>et al.</i> , 1998 Pitarch <i>et al.</i> , 2002

Acc. No.	<i>Neurospora crassa</i>	<i>Saccharomyces cerevisiae</i>	<i>Candida albicans</i>	<i>Trichoderma reesei</i>	<i>Arabidopsis thaliana</i>	References
NCU10042.1	Enolase	+	+	+	+	Pardo <i>et al.</i> , 2000a Lim <i>et al.</i> , 2001 Eroles <i>et al.</i> , 1997 Martinez <i>et al.</i> , 1998 Edwards <i>et al.</i> , 1999 Pitarch <i>et al.</i> , 2002 Pardo <i>et al.</i> , 1999 Chivasa <i>et al.</i> , 2002 Pitarch <i>et al.</i> , 1999 Angiolella <i>et al.</i> , 1996
NCU07807.1	Fructose biphosphate aldolase	+	+			Pardo <i>et al.</i> , 2000a Pitarch <i>et al.</i> , 2001 Chaffin <i>et al.</i> , 1998 Martinez <i>et al.</i> , 1998 Pitarch <i>et al.</i> , 2002 Pardo <i>et al.</i> , 1999
NCU02252.1	Phosphoglycerate mutase	+	+			Pardo <i>et al.</i> , 2000a Pitarch <i>et al.</i> , 2002 Pardo <i>et al.</i> , 2000b
NCU07914.1	Phosphoglycerate kinase	+	+			Pardo <i>et al.</i> , 2000a Pitarch <i>et al.</i> , 2001 Martinez <i>et al.</i> , 1998 Pitarch <i>et al.</i> , 2002 Alloush <i>et al.</i> , 1997
NCU01528.1	Glyceraldehyde-3-phosphate dehydrogenase	+	+	+	+	Pardo <i>et al.</i> , 2000a Lim <i>et al.</i> , 2001 Chaffin <i>et al.</i> , 1998 Martinez <i>et al.</i> , 1998 Pitarch <i>et al.</i> , 2002 Pardo <i>et al.</i> , 1999 Chivasa <i>et al.</i> , 2002 Gil-Navarro <i>et al.</i> , 1997 Gozalbo <i>et al.</i> , 1998 Pitarch <i>et al.</i> , 1999 Delgado <i>et al.</i> , 2001
NCU07550.1	Triose phosphate isomerase	+	+		+	Pardo <i>et al.</i> , 2000a Pitarch <i>et al.</i> , 2001 Pitarch <i>et al.</i> , 2002 Robertson <i>et al.</i> , 1997
NCU04510.1	Galactose-induced of aldo/keto reductase family					
NCU09041.1	Related to sorbitol utilization protein					
NCU02797.1	UTP glucose-1-phosphate uridylyltransferase	P ^a				Daran <i>et al.</i> , 1997
NCU07281.1	Glucose-6-phosphate isomerase	P ^a				Dickinson 1991
NCU01328.1	Transketolase					
NCU03100.1	6-phospho gluconate dehydrogenase					
NCU03982.1	78 kDa glucose-regulated protein homolog precursor					

Acc. No.	<i>Neurospora crassa</i>	<i>Saccharomyces cerevisiae</i>	<i>Candida albicans</i>	<i>Trichoderma reesei</i>	<i>Arabidopsis thaliana</i>	References
NCU02193.1	Pyruvate decarboxylase		+			Pitarch <i>et al.</i> , 2002
NCU02136.1	Transaldolase			+		Lim <i>et al.</i> , 2001
Heat shock proteins						
NCU04142.1	Heat shock protein 80 kDa					
NCU08693.1	Heat shock protein 70 kDa	+	+			Pardo <i>et al.</i> , 2000a Lopez-Ribot and Chaffin 1996 Eroles <i>et al.</i> , 1997 Chaffin <i>et al.</i> , 1998 Lopez-Ribot <i>et al.</i> , 1996 Martinez <i>et al.</i> , 1998 Pitarch <i>et al.</i> , 2002 Pardo <i>et al.</i> , 1999 Pitarch <i>et al.</i> , 1999
NCU02075.1	Heat shock protein 70 kDa	+	+			Pardo <i>et al.</i> , 2000a Lopez-Ribot and Chaffin 1996 Eroles <i>et al.</i> , 1997 Chaffin <i>et al.</i> , 1998 Lopez-Ribot <i>et al.</i> , 1996 Martinez <i>et al.</i> , 1998 Pitarch <i>et al.</i> , 2002 Pardo <i>et al.</i> , 1999 Pitarch <i>et al.</i> , 1999
NCU09602.1	Heat shock protein 70 kDa	+	+			Pardo <i>et al.</i> , 2000a Lopez-Ribot and Chaffin 1996 Eroles <i>et al.</i> , 1997 Chaffin <i>et al.</i> , 1998 Lopez-Ribot <i>et al.</i> , 1996 Martinez <i>et al.</i> , 1998 Pitarch <i>et al.</i> , 1999
NCU07232.1	Heat shock protein 30 kDa ^{b)}	p ^{b, c}				Plesofsky-Vig and Brambl 1998 Piper <i>et al.</i> , 1998 Wu <i>et al.</i> , 2000
Mitochondrial proteins						
NCU04304.1	Outer mitochondrial membrane protein porin ^{g)}			+		Lim <i>et al.</i> , 2001 Bathori <i>et al.</i> , 1999
NCU01692.1	Citrate synthase, mitochondrial				+	Chivasa <i>et al.</i> , 2002 lung <i>et al.</i> , 1999
NCU05430.1	ATP synthase beta chain, mitochondrial precursor		+			Pitarch <i>et al.</i> , 2002
NCU02514.1	ATP synthase alpha chain, mitochondrial precursor		+			Pitarch <i>et al.</i> , 2002

Acc. No.	<i>Neurospora crassa</i>	<i>Saccharomyces cerevisiae</i>	<i>Candida albicans</i>	<i>Trichoderma reesei</i>	<i>Arabidopsis thaliana</i>	References
NCU07659.1	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial precursor					
NCU01550.1	Adenylate kinase	P ^M			+ ^e	Chivasa <i>et al.</i> , 2002 Bandlow <i>et al.</i> , 1988 Anderson <i>et al.</i> , 2001
NCU03559.1	Ubiquinol cytochrome c reductase complex core protein 2					
NCU04899.1	Malate dehydrogenase precursor, mitochondrial					
Other functions						
NCU02806.1	14-3-3 family, chaperone	+	+			Pardo <i>et al.</i> , 2000a Pitarch <i>et al.</i> , 2002
NCU03300.1	14-3-3-like protein	+	+			Pardo <i>et al.</i> , 2000a Pitarch <i>et al.</i> , 2002
NCU02273.1	Vacuolar protease A precursor	P ^a		+		Lim <i>et al.</i> , 2001 Packeiser <i>et al.</i> , 1999
NCU09223.1	Protein disulfide-isomerase precursor		+	+	+	Lim <i>et al.</i> , 2001 Pitarch <i>et al.</i> , 2002 Chivasa <i>et al.</i> , 2002
NCU02003.1	Elongation factor 1-alpha	P ^a	+	+	+ ^d	Lim <i>et al.</i> , 2001 Pitarch <i>et al.</i> , 2002 Chivasa <i>et al.</i> , 2002 Lussier <i>et al.</i> , 1997b Valentini <i>et al.</i> , 2002
NCU03826.1	Elongation factor 1-gamma					
NCU07700.1	Elongation factor 2		+			Pitarch <i>et al.</i> , 2002
NCU07922.1	Elongation factor 3b		+			Pitarch <i>et al.</i> , 2002
NCU01021.1	Translation initiation factor 3					
NCU06035.1	Translation elongation factor eEF-1 beta chain			+		Lim <i>et al.</i> , 2001
NCU06512.1	Methionine synthase		+			Pitarch <i>et al.</i> , 2001 Pardo <i>et al.</i> , 2000b

Acc. No.	<i>Neurospora crassa</i>	<i>Saccharomyces cerevisiae</i>	<i>Candida albicans</i>	<i>Trichoderma reesei</i>	<i>Arabidopsis thaliana</i>	References
NCU06110.1	Thiazole biosynthetic enzyme					
NCU09345.1	NMT1 protein homolog					
NCU06550.1	Snz-type pyridoxine vitamin B6 biosynthetic protein					
NCU04173.1	Actin	P ^a				Li <i>et al.</i> , 2002 Mulholland <i>et al.</i> , 1997
NCU03608.1	ketol-acid reductoisomerase precursor					
NCU01439.1	Phosphoglycerate dehydrogenase					
NCU03777.1	Related to enoyl CoA hydratase					
NCU06448.1	Enoyl CoA hydratase					
NCU01680.1	Plasma membrane ATPase		+			Pitarch <i>et al.</i> , 2002
NCU09477.1	ADP, ATP carrier protein (translocase)		+			Pitarch <i>et al.</i> , 2002
NCU01195.1	NADP-specific glutamate dehydrogenase	P ^a				Lussier <i>et al.</i> , 1997b
NCU07420.1	Eukaryotic initiation factor 4A	P ^a				Valentini <i>et al.</i> , 2002
NCU10051.1	Flavohemoglobin					
NCU07307.1	Fatty acid synthetase					
NCU08330.1	Predicted protein					
NCU05137.1	Hypothetical protein					
NCU02044.1	Hypothetical GTP-binding protein					
NCU03757.1	Ribosomal protein L4					
NCU03806.1	Ribosomal protein L27					

Acc. No.	<i>Neurospora crassa</i>	<i>Saccharomyces cerevisiae</i>	<i>Candida albicans</i>	<i>Trichoderma reesei</i>	<i>Arabidopsis thaliana</i>	References
NCU03393.1	Ribosomal protein p40 homolog A	+				Amiri-Eliasi and Fenselau 2001 Boguszewska <i>et al.</i> , 2002
NCU00258.1	40S ribosomal protein S7	+				Amiri-Eliasi and Fenselau 2001 Boguszewska <i>et al.</i> , 2002
NCU08344.1	60S ribosomal protein					
NCU06843.1	60S ribosomal protein L3					
NCU04331.1	60S ribosomal protein L5					
NCU01317.1	60S ribosomal protein L12					
NCU08627.1	40S ribosomal protein S21	+				Amiri-Eliasi and Fenselau 2001 Boguszewska <i>et al.</i> , 2002
NCU06431.1	40S ribosomal protein S22	+				Amiri-Eliasi and Fenselau 2001 Boguszewska <i>et al.</i> , 2002
NCU05599.1	40S ribosomal protein S28	+				Amiri-Eliasi and Fenselau 2001 Boguszewska <i>et al.</i> , 2002
NCU00971.1	40S ribosomal protein S15	+				Amiri-Eliasi and Fenselau 2001 Boguszewska <i>et al.</i> , 2002
NCU00489.1	40S ribosomal protein S3	+				Amiri-Eliasi and Fenselau 2001 Boguszewska <i>et al.</i> , 2002
NCU05810.1	40S small subunit ribosomal protein	+				Amiri-Eliasi and Fenselau 2001 Boguszewska <i>et al.</i> , 2002
NCU03897.1	Related to SCP160 protein					
NCU02657.1	Methionine adenosyltransferase					
NCU00018.1	Transitional endoplasmic reticulum ATPase			+		Pitarch <i>et al.</i> , 2002
NCU05290.1	Orotate phosphoribosyltransferase					
NCU01985.1	Sulfate adenylyltransferase					

Acc. No.	<i>Neurospora crassa</i>	<i>Saccharomyces cerevisiae</i>	<i>Candida albicans</i>	<i>Trichoderma reesei</i>	<i>Arabidopsis thaliana</i>	References
NCU06666.1	Myc-inositol 1-phosphate synthase		+			Pitarch <i>et al.</i> , 2002
NCU07697.1	Isocitrate dehydrogenase					
NCU06783.1	Probable ATP citrate lyase subunit 2		+			Pitarch <i>et al.</i> , 2002
NCU08332.1	Woronin body major protein precursor			+		Lim <i>et al.</i> , 2001

+, presence of a cell wall protein in a given organism.

P, probable wall localization due to its involvement in the wall integrity pathway or wall biogenesis directly or indirectly.

M, protein has been localized to the mitochondria, but there are several examples of dual mitochondrial/cell wall localizations.

^a Proteins that have not been localized to the wall, but gene mutation(s) had an affect on the wall deduced from cell morphology studies, wall component studies, gene expression experiments or chemical wall sensitivity assays.

^b Heat shock protein 70 kDa has been found to interact with heat shock protein 30 kDa.

^c Heat shock protein 30 kDa has been found on cell surfaces, namely, plasma membranes (Piper *et al.*, 1997).

^d Putative elongation factor found in the wall of *Arabidopsis*.

^e Putative protein kinase found in the wall of *Arabidopsis*.

^f Elongation factor-1 beta was found by screening an expression library with wall antibodies.

^g Porin has been localized on the outer face of the plasma membrane (Allouh *et al.*, 1997).

Fig 4.3 Fluorescence and phase-contrast microscopy of trypsin-treated intact 6 h old mycelia. -Trypsin, indicates the absence of trypsin-treatments for 4 or 24 h. + Trypsin, indicates mycelia treated with 10 μ g of trypsin for either 4 h or 24 h. The top four panel are of 5-chloromethylfluorescein diacetate labelled mycelia with and without trypsin treatments. The bottom two panels are phase-contrast micrographs of trypsin-treated mycelia.

- Trypsin, 4 h

- Trypsin, 24 h

+ Trypsin, 4 h

+ Trypsin, 24 h

+ Trypsin, 4 h

+Trypsin, 24 h

trypsin-treated (containing 10 mM Tris-HCl, pH 8.0) samples. Of these 39 proteins, 37 were liberated by 4 h of trypsin digestion. Moreover, only 2 protein, namely, triose phosphate isomerase and actin, were also found in the untreated samples, indicating a loose association with the surface. In contrast, 39 proteins were identified in the 24 h samples and these proteins were all released by the trypsin since no equivalent proteins were found in the untreated samples. In general, proteins identified in the trypsin extracts were in the same functional categories, namely, glycolytic enzymes, heat shock proteins and mitochondrial proteins, as those found in chemical extracts (Tables 4.2 and 4.3). There were proteins that were found in the chemical, 4 h and 24 trypsin extracts such as enolase. In contrast, some proteins were only found in one or two of the extracts. For example, citrate synthase was found in the chemical and 24 h trypsin extracts, but not in the 4 h trypsin extract.

Proteins secreted into the media

Proteins that are either secreted or otherwise released from hyphae into the growth media were identified by mass spectrometry (Table 4.2). Several of these proteins were distinct from those identified in the chemical and enzymatic-extracts from purified cell wall and intact cells, respectively. Homologs of six of these proteins have been shown to be wall proteins, namely, α -1,4 glucanase, β -1,3 glucanase, Gas1, Gas3, soluble wall protein and ECM33 in other organisms (Tables 4.2 and 4.3; Pitarch *et al.*, 2002). In contrast, transaldolase, a glycolytic enzyme, and malate dehydrogenase, a mitochondrial protein, were also found in the secreted media. Malate dehydrogenase was also identified in chemical and NovozymeTM extracts, but not in trypsin-treated samples from intact

cells. Moreover, a number of hypothetical and predicted proteins were also identified. Methionine synthase is an example of a protein that was identified in the secreted media, in trypsinized surface proteins and was liberated from cell wall extracts by Novozyme™ and chemical treatments.

CapLC-MS/MS sequencing, database matches and protein identification

Proteins in each of the excised gel fragments were digested with trypsin, as previously described, and then submitted to CapLC-MS/MS. The numbered proteins identified from the 2-D gel shown in Fig. 4.2, the number of matched peptides, the Mascot™ scores, percentage of sequence coverage and the identified proteins, are listed in Table 4.1. Proteins which were solubilized by one or more of the alternative extraction methods are also identified. In addition, fifteen proteins which were purified from a preparative 1-D gel by electroelution, were resolved on a second 2-D gel. The most prominent protein spots from each of these gels were then subjected to CapLC-MS/MS analysis and yielded sequence information for multiple tryptic peptides (Table 4.1, spots 1a to 15a).

Generally, the number of matched peptide sequences per protein ranged from 3 to 21 (Table 4.1). A large number of proteins had more than 6 matching peptide sequences, indicative of good protein identifications. For example, 21 peptide sequences obtained from protein spot 22 were matched to fructose biphosphate aldolase with a mass of 39 kDa (Table 4.1). The masses of the identified proteins have also been tabulated (Table 4.1). This result, in combination with high Mascot™ score values, consistently indicated good protein identifications.

Protein spot 1a, with a mass of 86 kDa, was identified as homocysteine methyltransferase (Table 4.1). The resulting tryptic peptides subjected to CapLC-MS/MS gave 12 unique peptide sequences (Fig. 4.4). For example, the doubly charged precursor ion 794.42 $[M+2H]^{2+}$, for a peptide with a molecular mass of 1587.84 Da, gave a full sequence of NGIYVIQVDEPALR (Fig. 4.4 peptide j). The clean MS/MS spectrum for this peptide, as indicated by well separated y-ions, is representative of MS/MS spectra obtained for this study (Fig. 4.5).

Identification of putative wall proteins by an *in silico* approach

In the search for cell wall proteins we took advantage of molecular sequence data from *S. cerevisiae*. Amino acid sequencing from *S. cerevisiae* proteins known, to affect the cell wall directly or indirectly, were searched against the *Neurospora* database (Table 4.4). In view of the complexity of the wall, the number of genes directly or indirectly involved in the cell wall synthesis and elaboration was expected to be large. From 107 searches, 93 of them (87%) gave significant matches with E-values less than E-07. Proteins involved in the cell wall biosynthesis secretory pathway, bipolar budding, signal transduction, metabolism, mitochondrial function, and others, were represented in the searches. Deletions in genes encoding for these types of proteins have been shown to affect the cell wall directly or indirectly (Lussier *et al.*, 1997b). In many of the *S. cerevisiae* strains possessing a deletion in the genes, encoding all of these types of proteins, made the strains sensitive to calcofluor and zymolyase.

Functional categorization of identified wall proteins

This proteomic approach has identified well characterized cell wall proteins,

Table 4.4 *Saccharomyces cerevisiae* cell wall protein homologs found in *Neurospora crassa*. Known *S. cerevisiae* cell wall proteins were searched against the *Neurospora* database to identify possible cell wall protein homologs. The matching *Neurospora crassa* proteins are shown on the left side. The name of the *S. cerevisiae* proteins that were used for the search are shown on the right side along with the known function and phenotype of deletion mutants. The BLASTP algorithm was used for all searches.

Matching <i>Neurospora crassa</i> protein	Identity (%)	Similarity (%)	E-value	<i>Saccharomyces cerevisiae</i> protein	Known Function(s)	Mutant Phenotype	References
NCU01266.1 ^a Hypothetical protein L=1151	31	49	4E-96	YPL268w PLC1 1-phosphatidylinositol -4,5-bisphosphate phosphodiesterase	Production of the second messenger molecules diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) is mediated by activated phosphatidylinositol-specific phospholipase C enzymes	S ^{CW} , S ^T , S ^{HO}	Lussier <i>et al.</i> , 1997b Flick and Thorner 1993
NCU00812.1 ^a Hypothetical protein L=273	35	59	5E-42	YGR195w SKI6 3'→5' exoribonuclease required for 3' end formation of 5.8S rRNA	Involved in cell surface biosynthesis and architecture	S ^{CW} , 1 M:G, S ^C , S ^P , S ^H , abnormal cell morphology	Lussier <i>et al.</i> , 1997b
NCU06836.1 ^a Acetyl-coenzyme A synthetase L=631	61	75	0	YAL054c ACS1 acetyl-CoA synthetase	Catalyzes the synthesis of acetyl-CoA out of acetate and ATP, involved in cell wall biogenesis and architecture	S ^{CW} , S ^Z	Lussier <i>et al.</i> , 1997b
NCU06541.1 ^a Hypothetical protein L=436	43	61	E-106	YDR483w KRE2 alpha-1,2-mannosyl transferase	Directly involved in cell surface assembly by adding third mannose onto O-linked carbohydrate chains	S ^{CW} , S ^Z , S ^P	Lussier <i>et al.</i> , 1997b
NCU04314.1 ^a Predicted protein L=191	34	47	0	YGR008c STF2 ATPase stabilizing factor	Facilitates binding of inhibitor and 9 kDa protein to F1-ATPase Involved in cell surface biosynthesis and architecture	S ^{CW} , 1 M:G	Lussier <i>et al.</i> , 1997b
NCU03773.1 ^a Hypothetical protein L=464	25	41	0	YLR353w BUD8 budding protein	Required for bipolar budding, involved in cell wall biogenesis and architecture	S ^{CW} , S ^Z , R ^C , 1 GlcNac	Lussier <i>et al.</i> , 1997b

Matching <i>Neurospora crassa</i> protein	Identity (%)	Similarity (%)	E-value	<i>Saccharomyces cerevisiae</i> protein	Known Function(s)	Mutant Phenotype	References
NCU04439.1 ^a Hypothetical protein L=823	52	68	0	YJL033w HCA4 U14 snoRNA rRNA	Suppresses the U14 snoRNA rRNA processing function Involved in cell wall biogenesis and architecture	S ^{CW} , S ^Z , ↓ M:G	Lussier <i>et al.</i> , 1997b
NCU06981.1 ^a Hypothetical protein L=404	34	51	0	YHL028w WSC4 Cell wall integrity and stress response component 4	Similarity to equine herpesvirus glycoprotein	Viable	Verna <i>et al.</i> , 1997
NCU05686.1 ^a Hypothetical protein L=477	49	67	1E-94	YEL040w UTR2 cell wall protein	Weak similarity to <i>Bacillus</i> β-1,3 and 1,4 glucanase	Viable	Smith <i>et al.</i> , 1996
NCU09324.1 ^a Chitin synthase 4 L=1195	53	68	0	YBR023c CHS3 chitin synthase III	Required in chitin ring formation and lateral cell wall chitin synthesis	S ^{CW} , ↓ chitin R ^{KT}	Ziman <i>et al.</i> , 1998 Trilla <i>et al.</i> , 1999
NCU03460.1 ^a Hypothetical protein L=359	42	63	5E-39	YMR101c SRT1 cis-prenyltransferase homologue	Protein amino acid glycosylation.	viable	Sato <i>et al.</i> , 1999
NCU03460.1 ^a Hypothetical protein L=359	49	72	3E-43	YBR002c RER2 cis-prenyltransferase, a key enzyme in dolichol synthesis	Key enzyme in dolichol synthesis	S ^H , defects in N and O glycosylation	Sato <i>et al.</i> , 1999
NCU01929.1 ^a Hypothetical protein L=605	27	44	7E-51	YKL025c PAN3 component of the PAB1P-dependent poly(A) ribonuclease	Involved in cell wall biogenesis and architecture	R ^{CW}	Lussier <i>et al.</i> , 1997b
NCU05720.1 ^a Hypothetical protein L=358	44	64	1E-72	YHR142w CHS7 control of protein export from the ER (like chitin synthase III)	Chs7p controls protein export from the endoplasmic reticulum specifically engaged in the regulation of chitin synthesis	↓ mating efficiency	Trilla <i>et al.</i> , 1999
NCU00261.1 ^a Hypothetical protein L=568	59	76	0	YBL039c URA7 CTP synthase I	Catalyzes last step in pyrimidine biosynthesis pathway	S ^{CW} , ↓ M:G	Lussier <i>et al.</i> , 1997b
NCU09419.1 ^b Hypothetical protein L=561	25	40	4E-17	YDR402c DIT2 cytochrome P450 56	Directly involved in cell surface assembly involved in pathway for biosynthesis of dihydroxytyrosine in the outer layer of the spore wall	S ^{CW} , ↓ M:G, S ^Z , S ^T , lack the outermost layer of the spore wall	Lussier <i>et al.</i> , 1997b
NCU01657.1 ^a Probable oxidase assembly protein L=279	57	72	3E-50	YPL132w COX11 cytochrome-c oxidase assembly protein	Involved in synthesis of formyl group of position 8 of heme A	S ^{CW}	Lussier <i>et al.</i> , 1997b

Matching <i>Neurospora crassa</i> protein	Identity (%)	Similarity (%)	E-value	<i>Saccharomyces cerevisiae</i> protein	Known Function(s)	Mutant Phenotype	References
NCU04242.1 ^a Hypothetical protein Probable nonsense-mediated mRNA decay protein L=1093	34	52	1E-59	YER176w ECM32 DNA dependent ATPase/DNA helicase B	Involved in the unwinding of the DNA double helix during replication, involved in cell wall biogenesis and architecture	S ^{CW} , S ^H , S ^P , ↑ M:G	Lussier <i>et al.</i> , 1997b
NCU08730.1 ^a Hypothetical protein L=525	54	70	E-123	YMR224c MRE11 DNA repair and meiotic recombination protein	Required for double-strand DNA break repair and meiotic recombination	S ^{CW} , S ^{AT} , S ^P , ↑ M:G, ↓ GlcNac	Lussier <i>et al.</i> , 1997b
NCU00727.1 Hypothetical protein L=410	29	48	4E-26	YNR003c RPC34 DNA-directed RNA polymerase III, 34 KD subunit	Involved in cell wall biogenesis and architecture	S ^{CW} , C ^H , ↓ M:G	Lussier <i>et al.</i> , 1997b
NCU03657.1 ^a Related to ER glucosidase I L=834	38	56	E-149	YGL027c CWH41 ER glucosidase I	Involved in cell surface biosynthesis and architecture	R ^{AT} , S ^{CW} , ↓ β-1,6 glucan	Jiang <i>et al.</i> , 1996 Lussier <i>et al.</i> , 1997b
NCU01753.1 ^a Hypothetical protein L=581	32	48	2E-59	YLR299w ECM38 gamma-glutamyltransferase, involved in glutathione synthesis	Involved in glutathione synthesis, involved in cell wall biogenesis and architecture	R ^{CW}	Lussier <i>et al.</i> , 1997b
NCU00668.1 ^a Hypothetical protein L=1251	37	57	E-138	YLR371w ROM2 GDP/GTP exchange factor for RHO1P	Involved in cell wall biogenesis and architecture, involved in regulation of beta-1,3-glucan synthesis	S ^{CW} , S ^C , ↑ percentage of small budded cells, no mating projections	Lussier <i>et al.</i> , 1997b Manning <i>et al.</i> , 1997
NCU08909.1 ^a Hypothetical protein L=542	45	60	E-123	YMR307w GAS1 glycophospholipid-anchored surface glycoprotein	Plays role in the retention of β-1,3-glucan and glycosylated proteins	S ^{CW} , R ^Z , S ^N , ↑ chitin, ↑ wall proteins are β-1,6 linked cell wall glucan levels, ↓ β-1,3 glucan	Popolo <i>et al.</i> , 1997
NCU01207.1 ^a Vacuolar ATP synthase catalytic subunit L=607	75	86	0	YDL184w-b TFP1 H+-ATPase V1 domain 69 KD catalytic subunit, vacuolar	Involved in cell wall biogenesis and architecture	S ^{CW}	Lussier <i>et al.</i> , 1997b
NCU08693.1 ^a Hypothetical protein L=516	65	84	0	YEL030w ECM10 heat shock protein of HSP70 family	Involved in cell wall biogenesis and architecture and protein folding	S ^{CW} , S ^H , ↑ M:G	Lussier <i>et al.</i> , 1997b
NCU08180.1 ^a Hypothetical protein L=662	45	62	E-120	YDL194w SNF3 high-affinity glucose transporter/regulatory protein	Involved in cell wall biogenesis and architecture, acts as a glucose receptor	S ^{CW} , S ^Z , S ^H , S ^P , S ^C	Lussier <i>et al.</i> , 1997b Neigeborn and Carlson 1984

Matching <i>Neurospora crassa</i> protein	Identity (%)	Similarity (%)	E-value	<i>Saccharomyces cerevisiae</i> protein	Known Function(s)	Mutant Phenotype	References
NCU08828.1 ^a Hypothetical protein L=894	48	63	0	YKR009c FOX2 hydratase-dehydrogenase-epimerase, peroxisomal	Involved in cell wall biogenesis and architecture	S ^{CW} , S ^H , ↑ M:G	Lussier <i>et al.</i> , 1997b
NCU06201.1 ^a Hypothetical protein L=121	48	71	6E-25	YBL001c ECM15	Involved in cell wall biogenesis and architecture	S ^{CW} , S ^H	Lussier <i>et al.</i> , 1997b
NCU05721.1 ^a Hypothetical protein L=995	32	59	0	YBL101c ECM21	Involved in cell wall biogenesis and architecture	S ^{CW} , ↓ GlcNac	Lussier <i>et al.</i> , 1997b
NCU03007.1 ^b Hypothetical protein L=392	24	41	6E-17	YBR065c ECM2	Involved in cell wall biogenesis and architecture	S ^{CW} , S ^Z , S ^H , S ^P , S ^{AT} , S ^C , abnormal morphology	Lussier <i>et al.</i> , 1997b
NCU08936.1 ^a Hypothetical protein L=411	31	53	2E-50	YBR078w ECM33	Involved in cell wall biogenesis and architecture	S ^{CW} , S ^Z , S ^H , S ^{AT} , ↑ M:G	Lussier <i>et al.</i> , 1997b
NCU10048.1 ^a Hypothetical protein L=309	37	59	2E-47	YBR176w ECM31	Involved in cell wall biogenesis and architecture	S ^{CW} , S ^H , S ^P , ↑ M:G	Lussier <i>et al.</i> , 1997b
NCU06332.1 ^a Hypothetical protein L=533	23	40	2E-22	YDR125c ECM18	Involved in cell wall biogenesis and architecture	S ^{CW}	Lussier <i>et al.</i> , 1997b
NCU00770.1 ^a Hypothetical protein L=1845	26	45	E-145	YHL030w ECM29	Involved in cell wall biogenesis and architecture	S ^{CW} , S ^Z , S ^H	Lussier <i>et al.</i> , 1997b
NCU01464.1 ^a Hypothetical protein L=536	40	58	7E-78	YHR132c ECM14	Involved in cell wall biogenesis and architecture	S ^{CW} , S ^H , S ^P , ↑ M:G	Lussier <i>et al.</i> , 1997b
NCU04826.1 ^a Hypothetical protein L=1422	23	39	0	YJL201w ECM25	Involved in cell wall biogenesis and architecture	S ^{CW} , no mating	Lussier <i>et al.</i> , 1997b Jaquet and Jauniaux 1999
NCU02826.1 ^b Hypothetical protein L=898	48	79	1E-10	YJR106w ECM27	Involved in cell wall biogenesis and architecture	S ^{CW} , S ^P	Lussier <i>et al.</i> , 1997b
NCU05238.1 ^a Hypothetical protein L=1520	51	68	0	YJR137c ECM17	Involved in cell wall biogenesis and architecture	S ^{CW} , S ^Z , S ^H	Lussier <i>et al.</i> , 1997b
NCU04368.1 ^a Hypothetical protein L=346	50	61	2E-90	YKR076w ECM4	Involved in cell wall biogenesis and architecture	S ^{CW} , ↑ GlcNac	Lussier <i>et al.</i> , 1997b

Matching <i>Neurospora crassa</i> protein	Identity (%)	Similarity (%)	E-value	<i>Saccharomyces cerevisiae</i> protein	Known Function(s)	Mutant Phenotype	References
NCU04616.1 ^a Predicted protein L=624	27	45	0	YLR436c ECM30	Involved in cell wall biogenesis and architecture	S ^{CW} , S ^P	Lussier <i>et al.</i> , 1997b
NCU01238.1 ^a Hypothetical protein L=1736	39	60	3E-27	YMR176w ECM5	Involved in cell wall biogenesis and architecture	S ^{CW} , S ^H , S ^C , † M:G, † GlcNac, abnormal morphology	Lussier <i>et al.</i> , 1997b
NCU07472.1 ^a Hypothetical protein L=576	33	48	1E-62	YNR030w ECM39	Involved in cell wall biogenesis and architecture	R ^{CW} , S ^P	Lussier <i>et al.</i> , 1997b
NCU00589.1 ^a Hypothetical protein L=579	17	40	0	YOR092w ECM3	Involved in cell wall biogenesis and architecture	S ^{CW} ,	Lussier <i>et al.</i> , 1997b
NCU06425.1 ^a Predicted protein L=310	27	40	0	YBL043w ECM13	Involved in cell wall structure of biosynthesis	S ^{CW} , S ^Z , S ^P , S ^C , † GlcNac	Lussier <i>et al.</i> , 1997b
NCU04160.1 ^a Hypothetical protein L=360	24	39	0	YOL007c CSI2	Appears component of the chitin synthase 3 complex	Delocalized chitin deposition	
NCU01206.1 ^a Hypothetical protein L=1313	41	54	4E-78	YHR119w SET1 involved in chromatin-mediated gene regulation	Involved in diverse aspects of cell morphology	Reduced mannoprotein layer, abnormal growth and sporulation defects	Nislow <i>et al.</i> , 1997
NCU07170.1 ^b Hypothetical protein L=263	30	52	3E-19	YIR030c DCG1	Involved in cell wall biogenesis and architecture, involved in nitrogen-catabolite metabolism	S ^{CW} , S ^Z , S ^C , † GlcNac	Lussier <i>et al.</i> , 1997b
NCU03137.1 ^a Hypothetical protein L=872	40	57	E-129	YMR165c SMP2	Involved in plasmid maintenance, respiration and cell proliferation, Involved in cell wall biogenesis and architecture	S ^{CW} , S ^{K7} , † M:G, † GlcNac	Irie <i>et al.</i> , 1993 Lussier <i>et al.</i> , 1997b
NCU06003.1 ^a Hypothetical protein L=364	72	84	E-151	YDL055c PSA1 mannose-1-phosphate guanylttransferase	Required for bud-site selection and cytokinesis	Pleiotropic cell wall integrity defects, requires osmotic stabilizers	Warit <i>et al.</i> , 1998
NCU02282.1 ^b Hypothetical protein L=469	36	50	3E-18	YDR072c IPT1 mannosyl diphosphorylinositol ceramide synthase	Necessary for synthesis of mannose-(inositol-P) 2-ceramide	Accumulates precursor mannose-inositol-ceramide	Dickson <i>et al.</i> , 1997
NCU06541.1 ^a Hypothetical protein L=436	30	51	1E-63	YPL053c KTR6 mannosylphosphate transferase	Required for N-glycosylation, involved in the assembling mannose-containing wall proteins	S ^{CW} , S ^H , † M:G	Lussier <i>et al.</i> , 1997b

Matching <i>Neurospora crassa</i> protein	Identity (%)	Similarity (%)	E-value	<i>Saccharomyces cerevisiae</i> protein	Known Function(s)	Mutant Phenotype	References
NCU04454.1 ^a Hypothetical protein L=599	31	48	2E-71	YNL219c ALG9 mannosyltransferase	Involved in protein glycosylation, involved in cell wall biogenesis and architecture	R ^{CW} , R ^{KT} , S ^C	Lussier <i>et al.</i> , 1997b
NCU03071.1 ^a Hypothetical protein L=1367	41	59	E-128	YNR031c SSK2 MAP kinase kinase kinase	High-osmolarity signal transduction pathway, involved in cell wall biogenesis and architecture	R ^C , R ^{CW}	Lussier <i>et al.</i> , 1997b
NCU09757.1 ^a Hypothetical protein L=534	50	63	E-136	YPL175w SPT14 N-acetylglucosaminyltransferase	Required for GPI anchor synthesis	S ^{CW} , defective synthesis of GlcNAc-phosphatidylinositol, loss of GPI-anchored proteins, cell wall proteins, secretion of cell wall precursors into the medium	Lussier <i>et al.</i> , 1997b
NCU01195.1 ^a NADP-specific glutamate dehydrogenase L=454	64	75	E-160	YAL062w GDH3 NADP-glutamate dehydrogenase	Involved in cell wall biogenesis and architecture	S ^{CW} , production colonies with pseudohyphal cells	Lussier <i>et al.</i> , 1997b
NCU07542.1 ^a Hypothetical protein L=408	34	51	5E-53	YEL037c RAD23 nucleotide excision repair protein (ubiquitin-like protein)	Involved in cell wall biogenesis, architecture and UV excision repair	S ^{CW} S ^C , ↓ M:G	Lussier <i>et al.</i> , 1997b
NCU01271.1 ^a Hypothetical protein L=484	44	62	E-110	YKL184w SPE1 ornithine decarboxylase	Polyamine biosynthetic pathway, involved in cell wall biogenesis and architecture	S ^{CW} , S ^Z , ↑ M:G, thicker cell wall	Lussier <i>et al.</i> , 1997b
NCU01141.1 ^a Probable PEL1 L=499	42	63	E-109	YCL004w PGS1 phosphatidylglycerophosphate synthase	Converts CDP-diacylglycerol and glycerol-3-phosphate to CMP and phosphatidylglycerophosphate	S ^{CW} , S ^Z , S ^P , S ^C	Lussier <i>et al.</i> , 1997b Chang <i>et al.</i> , 1998
NCU03298.1 ^a Hypothetical protein L=1181	34	50	E-146	YOR109w INP53 phosphatidylinositol phosphate phosphatase	Control of cellular organization (cell wall)	Thick cell wall vacuole	Stoltz <i>et al.</i> , 1998
NCU04047.1 ^a Predicted protein L=471	21	38	0	YNL076w MKS1 pleiotropic regulatory factor	Involved in transcriptional regulation of genes under cAMP control, involved in cell wall biogenesis and architecture	S ^{CW} , S ^{KT} , R ^C , ↑ M:G, ↑ GlcNAc	Lussier <i>et al.</i> , 1997b

Matching <i>Neurospora crassa</i> protein	Identity (%)	Similarity (%)	E-value	<i>Saccharomyces cerevisiae</i> protein	Known Function(s)	Mutant Phenotype	References
NCU06419.1 ^a Hypothetical protein L=587	53	69	2E-89	YPL140c MKK2 protein kinase of the map kinase kinase (MEK) family	Involved cell wall integrity and morphogenesis pathways	S ^{HO}	Lussier <i>et al.</i> , 1997b
NCU06630.1 ^a Serine/threonine protein phosphatase PP2A L=310	78	88	E-138	YDL188c PPH22 protein ser/thr phosphatase PP2A-2	Involved in cell wall biogenesis and architecture, cell morphogenesis, cell cycle and carbohydrate metabolism	R ^{CW}	Lussier <i>et al.</i> , 1997b
NCU09322.1 ^a Hypothetical protein L=765	44	59	2E-76	YBL061c SKT5 protoplast regeneration and killer toxin resistance protein	Required for chitin synthase III activity	R ^{CW} , † chitin, defect in mating	Lussier <i>et al.</i> , 1997b
NCU03808.1 ^a Hypothetical protein L=1258	41	57	0	YMR128w ECM16 putative DEAH-box RNA helicase	Involved in cell wall biogenesis and architecture	S ^{CW} , S ^H , S ^P , S ^Z , † GlcNac	Lussier <i>et al.</i> , 1997b
NCU06727.1 ^a Spermidine synthase L=291	78	87	E-132	YPR069c SPE3 putrescine aminopropyl transferase (spermidine synthase)	Involved in cell surface polyamine biosynthesis	S ^{CW}	Lussier <i>et al.</i> , 1997b
NCU02635.1 ^a Hypothetical protein L=373	59	73	5E-98	YPL050c MNN9	Required for the addition of the long alpha 1,6-mannose backbone of the complex MANNAN	S ^{HO} , S ^{CW} , defect in protein glycosylation	Lussier <i>et al.</i> , 1997b
NCU03718.1 ^b Hypothetical protein L=282	36	55	1E-10	YGR036c CAX4	Required for full levels of dolichol- linked oligosaccharides in the endoplasmic reticulum, involved in cell wall biogenesis and architecture	S ^{CW} , † -N- glycosylation of secretory proteins	Lussier <i>et al.</i> , 1997b
NCU00193.1 ^a Hypothetical protein L=664	27	42	6E-46	YGL142c GPI10 required for glycosyl- phosphatidylinositol synthesis	Alpha 1,2 mannosyltransferase utilized which adds a third mannose onto the GPI core structure	Cannot attach a mannose molecule to the GPI core- structure	Sutterlin <i>et al.</i> , 1998
NCU02255.1 ^a Hypothetical protein L=855	38	59	2E-73	YGR216c GPI1 required for N-acetylglucosaminyl phosphatidylinositol synthesis	Involved in the first step of GPI anchor synthesis	S ^{CW} , in the synthesis of N- acetyl glucosaminyl phosphatidylinosito l, multiple budded cells	Lussier <i>et al.</i> , 1997b

Matching <i>Neurospora crassa</i> protein	Identity (%)	Similarity (%)	E-value	<i>Saccharomyces cerevisiae</i> protein	Known Function(s)	Mutant Phenotype	References
NCU03278.1 ^b Hypothetical protein L=411	24	39	4E-15	YDR414c ERD1 required for retention of luminal ER proteins	Affects glycoprotein processing in the golgi apparatus, involved in cell wall biogenesis and architecture	S ^{CW} , defect in the Golgi-dependent modification of glyco- proteins	Lussier <i>et al.</i> , 1997b
NCU05750.1 ^a Hypothetical protein L=327	28	43	3E-28	YMR234w RNH1 ribonuclease H	Involved in cell wall biogenesis and architecture	S ^{CW} , S ^H , S ^P , S ^C , ↑ M:G	Lussier <i>et al.</i> , 1997b
NCU04047.1 ^b Predicted protein L=471	33	53	1E-16	YLR390w-a CCW14 Secretory Stress Response protein 1	Putative GPI anchor attachment site at aa 220	S ^{CW} , S ^Z	Winzeler <i>et al.</i> , 1999
NCU06179.1 ^a Hypothetical protein L=497	36	52	5E-58	YJL165c HAL5 ser/thr protein kinase	Involved in salt tolerance and pH sensitivity, involved in cell wall biogenesis and architecture	S ^{CW} , S ^H , ↑ M:G	Lussier <i>et al.</i> , 1997b
NCU00772.1 ^a Hypothetical protein L=809	50	68	5E-74	YHR102w KIC1 ser/thr protein kinase that interacts with CDC31P	Required for cell integrity and morphogenesis, involved in cell wall biogenesis and architecture	S ^{CW} , defect in cell separation	Lussier <i>et al.</i> , 1997b
NCU04373.1 ^a Hypothetical protein L=1167	23	39	0	YMR317w YMR317w similarity to mucins, glucan 1,4-alpha-glucosidase and exo-alpha sialidase	Control of cellular organization such as the cell wall	Viable	Ashrafi <i>et al.</i> , 1998
NCU02356.1 ^a White collar 1 protein L=1262	35	59	0	YPL021w ECM23 similarity to SRD1P	Involved in cell wall biogenesis and architecture	S ^{CW} , ↑ M:G	Lussier <i>et al.</i> , 1997b
NCU09871.1 ^a Hypothetical protein L=1051	39	59	6E-28	YOR257w CDC31 spindle pole body component, centrin	Required for cell integrity and morphogenesis	Lysis and morphological defects	Lussier <i>et al.</i> , 1997b
NCU01418.1 ^b Hypothetical protein L=142	39	54	3E-11	YER150w SPI1 Stationary Phase Induced	Strong similarity to putative cell surface glycoprotein Sed1p	Viable	Lussier <i>et al.</i> , 1997b
NCU07277.1 ^a Hypothetical protein L=734	32	49	0	YLR110c CCW12	Strong similarity to FLO1P involved in flocculation	Viable	Lussier <i>et al.</i> , 1997b
NCU08909.1 ^a Hypothetical protein L=542	36	55	2E-96	YLR343w YLR343w	Strong similarity to GAS1P and <i>C.albicans</i> pH responsive protein	Viable	Yeast. 1997 Dec;13(15):1 477-89.

Matching <i>Neurospora crassa</i> protein	Identity (%)	Similarity (%)	E-value	<i>Saccharomyces cerevisiae</i> protein	Known Function(s)	Mutant Phenotype	References
NCU04516.1 ^a Predicted protein L=160	28	50	0	YIL154c (IMP2) sugar utilization regulatory protein	Regulation of carbon compound and sugar utilization, involved in cell wall biogenesis and architecture	S ^{CW} , S ^Z , S ^C , S ^H , S ^{KT}	Lussier <i>et al.</i> , 1997b
NCU00609.1 ^a Hypothetical protein L=372	36	52	2E-63	YJR075w HOC1 suppressor of PKC1	Cell wall metabolism, budding, cell polarity and filament formation	S ^{CW} , S ^H	Genetics. 1997 Mar;145(3):6 37-45.
NCU04963.1 ^a Hypothetical protein L=523	43	60	E-108	YDL138w RGT2 suppressor of SNF3 mutant	Involved in regulation of glucose transport, involved in cell wall biogenesis and architecture	S ^{CW} , ↓ M:G, ↑ GlcNac	Lussier <i>et al.</i> , 1997b
NCU03787.1 ^b Hypothetical protein L=866	23	40	9E-14	YBR123c TFC1 TFIIIC (transcription initiation factor) subunit, 95 kD	RNA transcription, involved in cell wall biogenesis and architecture	S ^{CW} , S ^Z , ↓ M:G	Lussier <i>et al.</i> , 1997b
NCU07587.1 ^a Hypothetical protein L=958	28	49	6E-53	YLR182w SWI6 transcription factor	Involved in controlling genes involved in cell wall biogenesis and architecture	S ^{CW} , S ^{SDS} , cells have elongated morphology	Lussier <i>et al.</i> , 1997b
NCU07922.1 ^a Hypothetical protein L=1056	62	76	0	YLR249w YEF3 translation elongation factor eEF3	During protein synthesis it binds to the 80S, 60S and 40S ribosomal particles, involved in cell wall biogenesis and architecture	S ^{CW} , S ^Z , ↓ GlcNac	Lussier <i>et al.</i> , 1997b
NCU06103.1 ^a Hypothetical protein L=1223	43	62	E-140	YOL023w IFM1 translation initiation factor 2, mitochondrial	Involved in the hydrolysis of GTP during the formation of the 70S ribosomal complex, involved in cell wall biogenesis and architecture	S ^{CW} , S ^Z , S ^P	Lussier <i>et al.</i> , 1997b
NCU02109.1 ^a Hypothetical protein L=487	47	65	E-118	YDL103c QRI1 UDP-N-acetylglucosamine pyrophosphorylase	Chitin biosynthesis	Lethal	Mio <i>et al.</i> , 1998
NCU06268.1 ^a Hypothetical protein L=870	26	47	8E-54	YPL045w VPS16 vacuolar sorting protein	Required for vacuolar protein sorting and vacuolar biogenesis and stability	S ^{CW} , vacuolar protein targeting	Wada <i>et al.</i> , 1992
NCU08024.1 ^a Hypothetical protein L=416	54	65	4E-93	YML115c VAN1 vanadate resistance protein	Involved in regulation of the phosphorylation of a number of proteins, involved in cell wall biogenesis and architecture	S ^{CW} , S ^Z , S ^H , R ^V , ↓ M:G, defects in protein glycosylation	Lussier <i>et al.</i> , 1997b

Matching <i>Neurospora crassa</i> protein	Identity (%)	Similarity (%)	E-value	<i>Saccharomyces cerevisiae</i> protein	Known Function(s)	Mutant Phenotype	References
NCU02349.1 ^a Hypothetical protein L=1500	13	38	3E-32	YOR336W KRE	Required for beta-1,6-glucan biosynthesis	No β -1,6 glucan, aberrant morphology	Brown and Bussey 1993
NCU09117.1 ^a Hypothetical protein L=375	48	65	9E-67	YGR189C CRH1	Cell wall protein, family of putative glycosidases	R ^K	Rodriguez-Pena <i>et al.</i> , 2000
NCU06381.1 ^b Hypothetical protein L=406	28	44	4E-19	YGR282C BGL2 cell wall endo-beta-1,3 glucanase	Successive hydrolysis of beta-D-glucose units from the non-reducing ends of 1,3-beta-D-glucans, releasing alpha-glucose	No obvious phenotype	Cappellaro <i>et al.</i> , 1998
NCU09326.1 ^a Hypothetical protein L=624	35	54	2E-40	YGL028 SCW11	Soluble cell wall protein, similarity to glucanase	Clumpy growth	Cappellaro <i>et al.</i> , 1998
NCU07435.1 ^a Hypothetical protein L=454	34	50	1E-59	YLR330W CHS5 chitin synthesis protein	Involved in cell wall biogenesis and also required for mating	R ^{CW}	Santos <i>et al.</i> , 1997
NCU04500.1 Hypothetical protein L=440	39	54	8E-53	YLR286C CTS1 endochitinase	Hydrolysis of the beta-1,4 linkages of N-acetyl-D-glucosamine polymers of chitin	Viable	King and Butler 1998
NCU05680.1 ^a Hypothetical protein L=503	39	54	7E-97	YNL029C KTR5 mannosyltransferase	Involved in assembling mannose-containing molecules of the cell wall	S ^{CW}	Lussier <i>et al.</i> , 1997a
NCU06508.1 ^a Hypothetical protein L=1122	38	56	3E-87	YLL031C GPI13 phosphoryltransferase that adds phosphoethanolamine to the third mannose residue of the GPI anchor precursor	Involved in glycosyl phosphatidylinositol biosynthesis	Lethal	Hong <i>et al.</i> , 1999
NCU07259.1 ^a Hypothetical protein L=401	67	79	E-111	YDR331W GPI8 (putative) transamidase involved in GPI anchor attachment	Essential for GPI anchor attachment	Deficient in addition of GPIs to proteins	Meyer <i>et al.</i> , 2000
NCU04170.1 ^a Hypothetical protein L=2076	33	47	0	YOR008C SLG1 involved in cell wall integrity and stress response	Required for cell wall integrity and for stress response	S ^{CW} , S ^H , S ^P , S ^C , ↓ M:G	Lussier <i>et al.</i> , 1997b Verna <i>et al.</i> , 1997
NCU09332.1 ^a Hypothetical protein L=744	43	60	E-172	YJR143C PMT4 dolichyl phosphate-D-mannose:protein O-D-mannosyl transferase	Transfers mannose from DOL-P-mannose to ser or thr residues on proteins	↓ Glycosylation	Immervoll <i>et al.</i> , 1995

Matching <i>Neurospora</i> <i>crassa</i> protein	Identity (%)	Similarity (%)	E-value	<i>Saccharomyces</i> <i>cerevisiae</i> protein	Known Function(s)	Mutant Phenotype	References
NCU05273.1 ^a Hypothetical protein L=644	28	47	2E-47	YDR484W SAC2 involved in localization of actin and chitin	Vesicular transport	Missorting of proteins	Lussier <i>et al.</i> , 1997b
NCU03503.1 ^a Hypothetical protein L=471	43	59	2E-84	YGL065C ALG2 glycosyltransferase	Involved in N- glycosylation	Cell lysis, distorted cell walls	Jackson <i>et al.</i> , 1993

^aHighly significant BLASTP matches (refer to Methods and Materials).

^bModerately significant BLASTP matches.

L, length of the protein

S^W, sensitivity to calcofluor white

S^T, sensitivity to temperature

S^C, sensitivity to caffeine

S^Z, sensitivity to zymolyase

S^H, sensitivity to hygromycin B

S^N, sensitivity to nikkomycin Z

S^P, sensitivity to papulacandin

S^{HO}, sensitivity to hyperosmotic conditions

S^{SDS}, sensitivity to sodium dodecyl sulfate

R^C, resistance to caffeine

R^W, resistance to calcofluor white

R^{KT}, resistant to the *K. lactis* killer toxin

R^Z, resistant to zymolyase

R^V, resistance to vanadate

R^K, resistance to ketoconazole

↑M:G, increased mannose to glucose ratio of walls

↓G:M, decrease mannose to glucose ratio of walls

↓GlcNac, decreased level of N-acetylglucosamine relative to glucose and mannose in cell wall

Fig. 4.4 Complete peptide sequence information obtained for protein spot 1a that matched the protein sequence of homocysteine methyltransferase. All peptide sequences obtained by CapLC-MS/MS. Match between peptides and the protein sequence of homocysteine methyltransferase (NCU06512.1). Matching peptides are shown in bold print and underlined. MascotTM scores are tabulated on Table 4.1.

(a)
 1 MQSSVLGFP RMGVLRDLKK ANEAYWADKI SQEALLAEGK RLRLAHWKIQ KDAGVDIIPS
 (b) (c)
 61 NDFAHYDHVL DHIQLFNAVP ERYTSQKLSP LDEYFAMGRG HQKGGVDVPA LEMVKWFDSN
 (d)
 121 YHYVKPTLQD NQTFSLAKDP KPVREFLEAK EAGFQTRPVL VGPVSFLALG KADRGSSVDP
 (e)
 181 ITLLDKLVPV YVELLKQLKA AGAESVQIDE PVLVFDLRPE VKAAFKPAYE AIAAAGDAVP
 (f)
 241 KVVVATYFGD IVHNFDVLPA FSGAAGLHVD LVRNPEQLEP VLKQLGPNQI LSAGVVDGRN
 (g)
 301 IWKNDFAKSL EILQTAVKAL GSERVIVATS SSLIHTPHTL ASEKKLPSDV YEWFSFAVEK
 (h)
 361 VKEVATLAKA VTEPEAVKAE LEANAAAIKA RTDSKRTNDP AVKERQAQVT PEQHNKAPF
 (i)
 421 NTRYAEQKKH LSLPLFPTTT IGSFPQTSEI RVQRNKFTKG EISAEYERF IEKEIELAVK
 481 IQDELDLDVY VHGEPERNDM VQYFGERLNG YVFTTHAWVQ SYGSRCVRPP IIVGDISRPA
 541 PMTVKESKYA ASISKKPMKG MLTG PVTCLR WSFPRVDVHQ SVQCQQLALA LRDEVVDLEK
 (j)
 601 NGIYVIQVDE PALREGLPLR KGQEREAYLK WAVDSFKLAT AGVENSTQIH SHFCYSEFQD
 (k) (l)
 661 FFHAIAALDA DVLSIENSKS DAKLLKVFID EEYPRHIGPG VYDIHSPRV TLEEFKQRIE
 721 EMLAYLKPEQ LWINPDCGLK TRKWDEVKGA LSHMVEAAKY FREKYANKA

Fig. 4.5 Sequencing of doubly charged peptide by tandem mass spectrometry. MS/MS fragmentation pattern of a doubly charged ion at m/z 794.42 corresponding to a peptide of molecular mass 1587.84 Da. The sequence tag originates from homocysteine methyltransferase (NCU06512.1). The y ions were used for sequencing as indicated.

namely, GPI anchored proteins (Tables 4.2 and 4.3). Gas1 and Gas3 (GPI proteins) were identified in the media of cultures. As expected, glucanases, suspected of being involved in wall expansion, were also detected. These proteins were not identified in other extracts and an explanation for this is mentioned in the discussion section. Mitochondrial , glycolytic enzymes and proteins with other functions were found in these secretion samples. These include several predicted and hypothetical proteins.

In *N. crassa*, glycolytic and fermentation enzymes were identified in trypsin extracts from intact cells and in secretion samples, Novozyme™ and chemical extracts from the purified cell walls (Tables 4.2 and 4.3). Examples of wall associated glycolytic proteins include enolase, fructose biphosphate aldolase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase. Alcohol dehydrogenase was only identified in chemical extracts.

Heat shock proteins (hsps) were also among the proteins released, from the purified *N. crassa* cell wall matrix and from intact cells, by chemical and enzymatic treatments, respectively (Tables 4.1, 4.2 and 4.3). Specifically, heat shock proteins were released by trypsin, Novozyme™ and chemical treatments, but these proteins were not found in secretion samples. Protein spot 4a yielded 19 sequenced peptides (26.71% sequence coverage), which, when matched against databases, identified heat shock protein 70 kDa.

From the *N. crassa* data, we also identified several proteins only in purified wall extracts with a strong similarity to organelle proteins: a vacuolar protease, and protein disulfide isomerase (Table 4.1). In addition, a number of proteins with strong similarity to

mitochondrial components were discovered in purified wall and trypsin extracts including: citrate synthase and the ATP synthase beta chain. Other mitochondrial proteins were found in chemical, NovozymeTM and secretion extracts. An example of such a protein is malate dehydrogenase, a protein only found in chemical extracts from purified walls (Table 4.2).

Some proteins were identified in multiple spots, often co-migrating with other proteins. For example, malate dehydrogenase was detected in spots 3 and 4 (Fig. 4.2 and Table 4.1). Peptides derived from the enzyme enolase were detected following tryptic digestion of protein spots 7 and 14. These protein spots have a considerable charge separation which may be attributable to a post-translational modification such as phosphorylation. An example of a protein with apparent isoforms which differ by charge and by molecular mass was also detected. Peptides derived from NMT1 were detected in spots 6, 13, 20 and 27 from the 9.5 M urea, 5% ME solubilization and spot 14a from the 1% SDS, 5% ME solubilization. Spots 6 and 13 have comparable molecular mass but a large IEF separation. In contrast, proteins in spot 20 have a comparable charge to spot 13 but an estimated molecular weight of approximately 55 kDa while proteins in spot 13 are estimated to be about 38 kDa which corresponds to the predicted molecular mass of the NMT1 protein.

Discussion

The complexity of the fungal cell wall suggests that numerous proteins are involved in its biogenesis and maintenance (Pardo *et al.*, 2000a). To characterize this proteinaceous component, *N. crassa* cell walls were isolated and purified using a method

which has been shown to result in highly enriched cell wall material from other fungi (Martinez *et al.*, 1991). While these methods cannot completely exclude co-purification of proteins from membrane-bound organelles or the cytoplasm, no significant contamination problems have been reported (Lim *et al.*, 2001).

The nature of the interaction between wall-associated proteins and glucan/chitin matrix was explored by testing the ability of several different chemical extractions to solubilize proteins (Fig. 4.1). High concentrations of denaturants such as urea or GnHCl successfully released a limited set of proteins compared to the FLB (10 mM Tris buffer) alone (Fig. 4.1, FLB control lane). Reducing agents such as ME have been shown to release non-covalently linked and disulphide-linked proteins from *S. cerevisiae* and *C. albicans* walls (Casanova and Chaffin 1991; Edwards *et al.*, 1999; Moukadiri *et al.*, 1999). In contrast, ME without a denaturant was not effective in the solubilization of *Neurospora* cell walls (data not shown). The combination of 9.5 M Urea plus 640 mM ME proved to be very effective in releasing more than 50 *Neurospora* proteins including several which are not apparent following any other treatment (Fig. 4.1). These results suggest that some proteins are loosely associated with the cell wall and are readily released by denaturation alone, whereas some proteins are only released by reduction of exposed disulphide bonds following denaturation (Fig. 4.1). This is consistent with the observation that over 70% of the radioactivity of amino acids in *C. albicans* walls is released by denaturation with SDS alone (Pitarch *et al.*, 2002).

Wrathall and Tatum (1973) originally reported treating *N. crassa* cell walls with mild (0.5 M NaOH) alkali to obtain an enriched fraction of proteins directly linked to β -

1,6 glucan through their O-glycosyl side chains or by other alkali-sensitive linkages (Pitarch *et al.*, 2002). NaOH-extractable proteins such as members of the Pir family of proteins (*CW5*, *CCW6*, *CCW7*, *CCW8*) have also been reported from *S. cerevisiae* (Mrsa and Tanner 1999). When the genes which encode the Pir protein family were disrupted, yeast cells grew more slowly, formed large irregular-shaped cells and showed pronounced susceptibility to cell wall synthesis inhibitors like Calcofluor (Mrsa and Tanner 1999). However, our experiments indicate that HCl and NaOH are less useful for extraction of cell-wall associated proteins, since treatment with a strong acid or a strong base resulted in ineffective protein release or a smeared protein pattern, respectively (Fig. 4.1). The smearing and presence of higher molecular mass protein bands in the alkali-treated lanes may be the result of the formation of SDS-resistant aggregates or the result of non-specific cross-linkages (manuscript in preparation). Due to this smearing the NaOH-treated proteins were not suitable for downstream MS analysis.

The subset of *N. crassa*, cell wall proteins released by U+ ME treatment were resolved by 2-D SDS PAGE (Fig. 4.2 and Table 4.3). Although the resolution of the proteins was hampered somewhat by a number of factors including: heterogeneity, low abundance, low solubility, hydrophobicity and probable linkages with glucan and/or chitin, a total of 51 proteins were picked for sequencing by CapLC-MS/MS (Pitarch *et al.*, 2002). The availability of the complete genome of *N. crassa* facilitated identification of most of the proteins released from the fungal cell wall. We found a number of proteins such as known wall proteins, glycolytic enzymes, organellar proteins and heat shock proteins (Table 4.3). Our results corroborate several very recent reports of the cell wall

co-purification and co-localization of homologues of many of these proteins in yeasts, other filamentous fungi, bacteria and plants.

To address the issue of possible contamination of the purified cell wall fraction by cytoplasmic proteins, we examined the surface protein content by trypsin treatments of intact cells. Fluorescence and phase-contrast microscopy of 6 h or overnight cultures did not show any release of cellular components using our digesting conditions adapted from the procedure recommended by Promega (Fig. 4.3). This demonstrates that the peptides identified in the trypsin extracts were located at the surface and are unlikely contaminants (Table 4.2). Interestingly, the proteins identified in the trypsin extracts were in the same functional categories as those found in chemical extracts (Table 4.2). Glycolytic enzymes, heat shock proteins, mitochondrial proteins and translation factors were common to both trypsin release and chemical solubilization.

A total of 100 proteins solubilized by various extraction regimens and resolved by 2-D gel electrophoresis gels were processed for CapLC-MS/MS and amino acid sequences were obtained for 56 of them (Tables 4.1 and 4.2). In general, the proteins were relatively well resolved by 2-D gel electrophoresis although multiple co-migrating proteins were still identifiable even when present in a single spot. In contrast to the proteins released from the purified walls, 78 proteins were identified by CapLC-MS/MS after trypsin treatments of intact cells (Table 4.2). Moreover, over 100 putative cell wall proteins were identified by comparing the sequences of known cell wall proteins of *S. cerevisiae*, including metabolic and mitochondrial proteins, against the *Neurospora* database (Table 4.4).

The majority of the proteins reported here had more than 6 matching peptide sequences, increasing the confidence of the identification (Table 4.1). For example, 21 peptide sequences obtained from protein spot 22 were matched to fructose biphosphate aldolase with a predicted mass of 39 kDa (Table 4.1).

Several of the proteins identified in this work are clearly homologs of well-known cell wall proteins or enzymes involved in cell wall construction (Table 4.2). Several of the proteins which are secreted by *N. crassa* (MW 23 kDa -231 kDa) are homologs of proteins secreted by protoplasts of *S. cerevisiae* and have also been extracted from isolated cell walls (Pardo *et al.*, 1999; Pitarch *et al.*, 2002). In yeasts, they are produced as mannoproteins are covalently linked through a GPI remnant to β -1,6 glucan, which is linked, in turn, to β -1,3 glucan and/or chitin (Kapteyn *et al.*, 1996; Terashima *et al.*, 2003). It has been speculated that the GPI anchorage of some proteins to the membrane represents an intermediate stage after which cross-linking to the cell wall is finally achieved (Terashima *et al.*, 2003). However, Pardo *et al.*, (2000a) have suggested that all GPI-CWPs are constitutively targeted to the cell wall without forming an intermediate membrane-bound pool.

Gas1 is an example of a GPI protein, which we found to be secreted by *N. crassa* (Table 4.2). In *S. cerevisiae*, Gas1 has been reported to be covalently linked to the cell wall from where it can be extracted by glucanase treatment (De Sampaio *et al.*, 1999). Similarly, in *C. albicans* the Gas1 homolog has been proposed to be retained in the cell walls by non-covalent and covalent linkages (Pitarch *et al.*, 2002). A catalytic activity has been suggested lately for Gas1. This is β -1,3 glycosyl transferase that catalyzes the

formation of a β -1,3 glycosidic linkages between two β -1,3 glucan chains, thus being involved in cell wall polymer cross linking (Ram *et al.*, 1994; Popolo and Vai 1999). Our results suggest the *N. crassa* Gas1 homolog is a secreted protein. If it is also a component of the cell wall it is unlikely to be particularly abundant since it was not detectable in chemically solubilized or trypsin released protein sets. A genome-wide bioinformatic study identified 97 putative GPI proteins in *N. crassa* (De Groot *et al.*, 2003). The existence of putative GPI proteins in *N. crassa* indicates a possible involvement of a GPI anchor in cell wall protein attachment (De Groot *et al.*, 2003). The direct attachment of Gas1 and other GPI proteins to the *N. crassa* wall remains to be explored.

Glucanases were other proteins we found to be secreted by *N. crassa* as well as by yeast protoplasts (Table 4.2; Pardo *et al.*, 1999). This protein may play a role in cell wall expansion during growth, mating and spore release (Pardo *et al.*, 1999). Moreover, β -1,3 glucanase has been implicated in cell wall β -glucan assembly (Pardo *et al.*, 1999).

A number of glycolytic and fermentative enzymes including enolase, glyceraldehyde-3-phosphate dehydrogenase, fructose biphosphate aldolase, phosphoglycerate kinase, triose phosphate isomerase, alcohol dehydrogenase were extracted from the wall matrix of *N. crassa* (Table 4.2). This is consistent with the results of recent co-purification studies reported in the literature for yeasts and other fungi (Table 4.3) (Angiolella *et al.*, 1996; Lopez-Ribot and Chaffin 1996; Chaffin *et al.*, 1998; Pardo *et al.*, 1999; Eroles *et al.*, 1997; Pardo *et al.*, 2000a; Klotz *et al.*, 2001; Pitarch *et al.*, 2001). For many of these proteins, immunofluorescence and immunoelectron microscopy have been used to confirm localization to the bacterial or yeast cell walls (Casanova and

Chaffin 1991). For example, 3-phosphoglycerate kinase has been localized to the wall of *C. albicans* by transmission electron microscopy (Alloush *et al.*, 1997). Alcohol dehydrogenase has been found in the wall by immunofluorescence microscopy in *C. albicans* (Klotz *et al.*, 2001).

Enolase is an example of a known glucan-associated, integral component of the cell wall of *C. albicans* and *S. cerevisiae* which we have detected in *N. crassa* cell wall extracts (Tables 4.1 and 4.2). It has also been found in to be extractable by urea + the reducing agent dithiothreitol, from the cell wall of the plant *A. thaliana* (Angiolella *et al.*, 1996; Chivasa *et al.*, 2002). Immuno-electron microscopy has confirmed that this enzyme is present in abundance within the inner wall layers of intact yeast cells and it has been shown to be one of the proteins secreted by regenerating protoplasts of *S. cerevisiae* (Angiolella *et al.*, 1996; Pardo *et al.*, 1999). The absence of a conventional N-terminal signal sequence in this enzyme suggests a nonclassical export mechanism being involved in their targeting to the cell surface (Cleves *et al.*, 1996).

Similarly, the discovery of GAPDH in *N. crassa* cell wall extracts supports the results of similar studies with yeasts which have also recently been confirmed by indirect immunofluorescence microscopy of *C. albicans* and by the analysis of proteins which are secreted by regenerating *S. cerevisiae* protoplasts (Angiolella *et al.*, 1996; Gil-Navarro *et al.*, 1997). GAPDH has also been found in the cell wall of *A. thaliana*, the yeast *Kluyveromyces marxianus* and the parasite *Schistosoma mansoni* (Goudot-Crozel *et al.*, 1989; Shoemaker *et al.*, 1992; Lee *et al.*, 1995; Robertson *et al.*, 1997; Chivasa *et al.*, 2002). In streptococcal bacteria, both enolase and GAPDH have been found at the

surface, where they show transferrin-binding properties (Pancholi and Fischetti 1992; Gase *et al.*, 1996; Winram and Lottenberg 1996; Modun and Williams 1999).

Although a growing number of studies have confirmed the presence of these enzymes in the walls of fungi and other organisms, their function outside of the plasma membrane remains a mystery (Pardo *et al.*, 2000a). It has been proposed that many of these wall-associated proteins can be categorized as ‘moonlighting proteins’, having multiple functions (Jeffery 1999; Lussier *et al.*, 1997b). For example, phosphoglucose isomerase, a cytosolic enzyme that catalyzes the second step in the glycolytic pathway, is also secreted by mammalian cells and has at least four other unique roles: as neuroleukin, as the autocrine motility factor and as mediator of differentiation and maturation of cells in human myeloid leukemia (Pardo *et al.*, 2000a). It is plausible that some of these glycolytic enzymes at the cell surface of *N. crassa* and other fungi could have alternative functions involving formation of the glucan/chitin matrix.

One of the evolutionarily conserved characteristics of living cells is the ability to respond to a sudden change in temperature by producing a set of proteins referred to as heat shock proteins (hsps) (Chaffin *et al.*, 1998). Several families of hsps have been identified and the nomenclature reflects their molecular mass (i.e., the hsp70 family). Recent research has shown that hsps are involved in all major growth-related processes, such as cell division, DNA synthesis, transcription, translation, protein folding and transport, membrane translocation (Mager and Ferreira 1993). Members of the Hsp70 protein family function as chaperones, playing an important role in folding and translocation of other proteins (Pardo *et al.*, 1999; Pitarch *et al.*, 2000). Their main

function is assumed to be the protection and damage repair of proteins following heat shock (Chaffin *et al.*, 1998). Recently, members of the heat shock family have been found to play key roles in fundamental immunological phenomena (Srivastava *et al.*, 2002).

In mammalian cells, members of the heat shock protein family interact with peptides and antigen presenting cells (Srivastava *et al.*, 2002). Heat shock proteins also interact with antigen presenting cells through CD91 and other receptors, eliciting a cascade of events including re-presentation of heat shock protein-chaperoned peptides by MHC, and translocation of NF kappa B into the nucleus (Srivastava *et al.*, 2002). Furthermore, heat shock proteins are involved in the activation of antigen presenting cells, indirect presentation (cross-priming), and chaperoning of peptides during antigen presentation (Srivastava *et al.*, 2002). This is an example of the external roles that heat shock proteins are involved in.

In our studies, two hsp homologs, hsp70 and hsp30 were recovered by extraction from purified *N. crassa* cell walls (Table 4.3). In *Neurospora*, hsp30 associates with both hsp70 and hsp88 and may assist in protein folding (Piper *et al.*, 1997; Plesofsky-Vig and Brambl 1998). An association between hsps and yeast cell walls has been reported previously based on co-purification studies, protoplast secretion, indirect immunofluorescence and immunoelectron microscopy (Matthews and Burnie 1989; Mager and Ferreira 1993; Lopez-Ribot *et al.*, 1996; Lopez-Ribot and Chaffin 1996; Eroles *et al.*, 1997; Lussier *et al.*, 1997b; Chaffin *et al.*, 1998; Pardo *et al.*, 1999; Pardo *et al.*, 2000a; Motshwene *et al.*, 2003). Biotinylation experiments with intact yeast cells have also established that hsp70 is external to the plasma membrane (Chaffin *et al.*,

1998). Hsp70 has also been found on the surface of *Histoplasma capsulatum*, *Coxiella burnetti*, *Plasmodium falciparum* merozoites and *Chlamydia trachomatis* elementary bodies (Ardeshir *et al.*, 1987; Danilition *et al.*, 1990; Gomez *et al.*, 1992; Macellaro *et al.*, 1998).

It has been proposed that these chaperone functions are required during biosynthesis, secretion and assembly of the cell wall. Since we have found both hsp70 and hsp30 associated with the cell wall matrix, they may function together in a complex outside of the cell as well as within it.

In *N. crassa*, we also recovered a number of proteins from the cell wall which are normally within membrane-bound organelles including a vacuolar protease, and an enzyme from the endoplasmic reticulum, protein disulfide isomerase (Table 4.3). Some support for the apparent targeting of vacuolar proteases to the cell surface is provided by a report on the consequences of over-expression of the *S. cerevisiae* Pep4p, vacuolar protease (Suresh and Subramanyam 1996; Lim *et al.*, 2001). When expressed at elevated levels from a multicopy plasmid, a large proportion of enzyme was directed to the yeast cell surface instead of the vacuole (Eroles *et al.*, 1997).

Protein disulfide isomerase (PDI) is an enzyme which catalyzes the isomerization and formation of disulphide bonds of secretory proteins within the lumen of the endoplasmic reticulum (ER). An essential enzyme in yeast cells, PDI has also been found associated with the cell wall matrix of the fungus *T. reesei*, the protist *Giardia lamblia* and the plant *A. thaliana* (Lim *et al.*, 2001; Reiner *et al.*, 2001; Chivasa *et al.*, 2002). The gene encoding PDI from *T. reesei* contains a predicted N-terminal signal sequence and a

consensus ER retention signal at the C-terminus which are thought to localize this enzyme to the endoplasmic reticulum (Saloheimo *et al.*, 1999; Lim *et al.*, 2001). The putative *N. crassa* PDI also possesses an N-terminal signal sequence as numerically predicted by SignalP (Neilsen *et al.*, 1997). Currently, no pathway for export of this enzyme is known in any organism. A plausible function which has been suggested for PDI in the cell wall may be protein externalization and maturation of the cell surface, by cross-linking cell wall proteins to the carbohydrate matrix via its transglutaminase activity (LaMantia *et al.*, 1991; Knodler *et al.*, 1999; Reiner *et al.*, 2001; Pitarch *et al.*, 2002).

The mitochondrial proteins liberated from the *Neurospora* cell wall matrix include: an outer mitochondrial membrane porin, malate dehydrogenase, citrate synthase and the ATP synthase beta chain (Table 4.1). Possible functions for these proteins are less apparent. However, this is also concordant with the reports of several yeast mitochondrial proteins, Uth1p and Sun4p being found in the *S. cerevisiae* cell wall (Rothman *et al.*, 1986; Velours *et al.*, 2002). In addition, Uth1p and Sun4p were found to have a mitochondrial localization (Velours *et al.*, 2002). Gas1 and Pir1 are other examples of dual cell wall/intracellular localization (Msra and Tanner 1999; De Sampaio *et al.*, 1999). It is possible that some fraction of a protein is targeted to mitochondria while the remainder is sent to the cell wall by some mechanism such as ubiquitination. Alternatively, the dual localization may be explained by assuming the presence of two distinct but closely related proteins; one that is found in the mitochondria and the other as a component of the cell wall. A bioinformatic approach was used to explore the latter

possibility (Table 4.5). All of the *N. crassa* proteins identified by mass spectrometry were used to search the *Neurospora* database with the aim of finding paralogous proteins. For example, following a database search, citrate synthase was found to have a closely related hypothetical protein (NCU02482.1, similarity 70%, identity 55%). It can be envisioned that one citrate synthase is targeted to mitochondria while a homologous protein is targeted to the wall. Further experiments are necessary to determine which of the two possibilities explain the dual localization. It is conceivable that both mechanisms are operative.

Alternative splicing and dual localization may be mechanisms that increase the diversity of proteins in fungi since the creation of new genes through duplications is nonexistent. RIP has had a powerful impact in suppressing the creation of new genes or partial genes through genomic duplications in *N. crassa* (Galagan *et al.*, 2003). RIP is a process that efficiently detects and mutates both copies of a sequence duplication (Galagan *et al.*, 2003). Thus, *N. crassa* has a low proportion (10%) of genes in multigene families. It has been proposed that the few paralogous genes duplicated and diverged before the emergence of RIP and since that point evolution of new genes has been arrested (Galagan *et al.*, 2003). To overcome these evolutionary constraints, *N. crassa* may use alternative splicing and dual localization as mechanisms to increase protein diversity, but the relative impact of each mechanism is currently unknown. In contrast to fungi, alternative splicing and the creation of genes families are more frequent mechanisms that increase protein diversity in animals and plants, respectively (Galagan *et al.*, 2003).

Table 4.5 Identification of paralogous proteins of detected *N. crassa* cell wall associated proteins. The proteins on the right column were identified by mass spectrometry in chemical and enzymatic extracts. Database searches using BLASTP were performed to identify closely related proteins that may have different cellular locations.

Matching <i>Neurospora crassa</i> protein	Identity (%)	Similarity (%)	E-value	<i>Neurospora crassa</i> protein
NCU06303.1 Hypothetical protein ^a	39%	55%	3E-64	NCU09285.1 Alcohol dehydrogenase
NCU07042.1 Hypothetical protein ^a	33%	47%	1E-31	
NCU01870.1 Hypothetical protein ^a	52%	65%	E-120	NCU10042.1 Enolase
NCU04399.1 Hypothetical protein ^b	28%	41%	6E-17	NCU07550.1 Triose phosphate isomerase
NCU01906.1 Hypothetical protein ^a	42%	58%	5E-56	NCU04510.1 Galactose-induced of aldo/keto reductase family
NCU04923.1 Hypothetical protein ^a	39%	53%	3E-46	
NCU03803.1 Hypothetical protein ^a	46%	63%	1E-64	NCU09041.1 Related to sorbitol utilization protein
NCU03893.1 Hypothetical protein ^a	39%	59%	7E-45	
NCU00837.1 Hypothetical protein ^a	35%	51%	1E-65	NCU03100.1 6-phosphogluconate dehydrogenase
NCU02397.1 Hypothetical protein ^a	31%	47%	3E-69	NCU02193.1 Pyruvate decarboxylase
NCU09602.1 Heat shock protein 70 kDa ^a	43%	60%	E-109	NCU08693.1 Heat shock protein 70 kDa
NCU02075.1 Heat shock protein 70 kDa ^a	39%	55%	5E-87	
NCU09364.1 30 kDa Heat shock protein ^a	35%	46%	2E-33	NCU07232.1 Heat shock protein 30 kDa
NCU09420.1 Hypothetical protein ^a	30%	43%	2E-22	
NCU02482.1 Hypothetical protein ^a	55%	70%	E-144	NCU01692.1 Citrate synthase, mitochondrial
NCU02438.1 Hypothetical protein ^a	27%	43%	7E-33	NCU07659.1 Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial precursor
NCU03216.1 Hypothetical protein ^a	30%	48%	7E-30	NCU01550.1 Adenylate kinase
NCU06211.1 Hypothetical protein ^a	57%	71%	4E-96	NCU04899.1 Malate dehydrogenase precursor, mitochondrial
NCU03300.1 14-3-3-like protein ^a	78%	89%	E-100	NCU02806.1 14-3-3 family, chaperone
NCU04269.1 Related to pepsin precursor ^a	29%	46%	1E-26	NCU02273.1 Vacuolar protease A precursor
NCU03168.1 Hypothetical protein ^a	25%	42%	7E-24	
NCU08739.1 Hypothetical protein ^a	27%	44%	2E-23	
NCU03739.1 Putative disulfide isomerase ^a	48%	64%	7E-21	NCU09223.1 Protein disulfide-isomerase precursor
NCU03981.1 Hypothetical protein ^a	37%	53%	1E-74	NCU02003.1 Elongation factor 1-alpha
NCU04790.1 Hypothetical protein ^a	35%	54%		
NCU02572.1 Hypothetical protein ^a	37%	55%	E-165	NCU07700.1 Elongation factor 2
NCU06278.1 Hypothetical protein ^a	37%	55%	1E-72	

Matching <i>Neurospora crassa</i> protein	Identity (%)	Similarity (%)	E-value	<i>Neurospora crassa</i> protein
NCU09903.1 Hypothetical protein ^a	38%	54%	E-177	Elongation factor 3b
NCU04051.1 Hypothetical protein ^a	32%	45%	5E-42	
NCU03989.1 Hypothetical protein ^a	31%	44%	2E-29	NCU09477.1 ADP, ATP carrier protein (translocase)
NCU01234.1 Probable translation initiation factor eIF-4A ^a	67%	84%	E-150	NCU07420.1 Eukaryotic initiation factor 4A
NCU06149.1 Hypothetical protein ^a	40%	58%	4E-74	
NCU02096.1 Hypothetical protein ^a	41%	57%	5E-83	NCU10051.1 Flavohemoglobin
NCU01809.1 Hypothetical protein ^a	37%	55%	3E-94	NCU00018.1 Transitional endoplasmic reticulum ATPase
NCU06960.1 Hypothetical protein ^a	38%	52%	5E-24	NCU06448.1 Enoyl CoA hydratase
NCU09058.1 Hypothetical protein ^a	31%	44%	6E-24	
NCU09896.1 Hypothetical protein ^a	39%	56%	1E-28	NCU01985.1 Sulfate adenylyltransferase
NCU00775.1 Hypothetical protein ^a	46%	67%	5E-82	NCU07697.1 Isocitrate dehydrogenase
NCU02954.1 Hypothetical protein ^a	36%	53%	4E-46	

^a Highly significant BLASTP matches (refer to Methods and Materials).

^b Moderately significant BLASTP matches.

The *N. crassa* cell wall matrix also contained a diverse array of other proteins including protein 14-3-3, an elongation factor, ribosomal proteins, homocysteine methyltransferase, actin and several novel proteins (Table 4.3). Some proteins of similar function have also recently been shown to be genuine wall proteins in other organisms (Table 4.3). For example, translation elongation factor-1 beta was found in *C. albicans* by screening an expression library with wall antibodies as probes (Maneu *et al.*, 1996). Furthermore, a putative elongation factor was found in urea extracts from the *A. thaliana* cell wall (Chivasa *et al.*, 2002). In a genetic screen for cell surface biosynthesis genes in *S. cerevisiae*, translation elongation factor *eF-3* mutants were found to be zymolyase hypersensitive (Lussier *et al.*, 1997b). It has been suggested that translation initiation factors are involved in a signaling pathway for cell wall integrity and related processes (Valentini *et al.*, 2002). Cell wall association of elongation factors, which have been proposed to be cell surface adhesion proteins, have also been shown for the bacterial species *Staphylococcus aureus*, *E. coli*, *Neisseria gonorrhoeae* and *Mycobacterium leprae* (Jacobson and Rosenbusch 1976; Porcella *et al.*, 1996; Marques *et al.*, 1998; Vytvytska *et al.*, 2002).

A role for translation factors in the cell wall may be more difficult to envision but one possibility is that they may function as protein adhesins (Zhu *et al.*, 1994; Pitarch *et al.*, 2002). In doing so, they might have some chaperone activity, promoting translocation and processing mechanisms of cell wall components inside the wall network (Pitarch *et al.*, 2002).

Several proteins which are desorbed by brief acid treatments from intact *S.*

cerevisiae cells are L39 and L40 from the 60S ribosomal subunit and S29-A and S29-B from the 40S ribosomal subunit (Amiri-Eliasi and Fenselau 2001). Immunogold labelling has confirmed that the 60S subunit ribosomal proteins are definitely in the cell wall, as well as the cytoplasm (Delgado *et al.*, 2001). Our proteomics survey has also detected several ribosomal proteins which appear to be associated with the cell wall of *N. crassa* (Table 4.3).

In addition to the readily identifiable proteins, several novel proteins of unknown function were extracted from the *N. crassa* cell wall (Table 4.2). One of them shows a strong similarity to a hypothetical GTP-binding protein (NCU02044.1) of *S. cerevisiae*, which implies a possible involvement in the cell wall integrity pathway. PROSITE searches indicate that an ATP/GTP-binding site motif A is located from amino acids 27 to 34 (Fry *et al.*, 1986; Dever *et al.*, 1987).

In *S. cerevisiae*, wall remodelling during stress is monitored and controlled by a GTPase, Rho1 (Philip and Levin 2001). This signalling pathway maintains cell integrity by activating the transcription of several genes directly involved in wall biogenesis. Two essential functions have been identified for Rho1. First, it serves as an integral regulatory subunit of the β -1,3 glucan synthase (GS) complex that stimulates GS activity in a GTP-dependent manner (Philip and Levin 2001). A pair of closely related yeast genes, *FKS1* and *FKS2*, encode alternative catalytic subunits of the GS complex that are the presumed targets of Rho1 activity (Philip and Levin 2001). A second essential function of Rho1 is to bind and activate protein kinase C, which is encoded by *PKC1* (Philip and Levin

2001). Loss of the *PKC1* function results in cell lysis defect that is attributable to a deficiency in cell wall construction (Philip and Levin 2001).

A novel protein, has no similarity to other proteins in the public databases. Analysis using the ScanProsite software tools indicates that this protein possesses a helix-loop-helix domain similar to those found in bacterial wall peptidoglycan hydrolases (Garrell and Campuzano 1991; Ghuysen *et al.*, 1994). The *Streptomyces albus* G Zn DD-peptidase is a bipartite protein of known three-dimensional structure (Ghuysen *et al.*, 1994). Its noncatalytic N-terminal module possesses, exposed at the surface, an elongated crevice which is defined by a helix-loop-helix domain connected to a heptapeptide element whose design is compatible with its being a substrate recognition and binding site (Ghuysen *et al.*, 1994). Mutagenesis of these ORFs may help discern their function.

N. crassa cell wall proteins previously identified by standard biochemical methods include chitin synthases, glucanases, hydrophobins, invertase, trehalase and a copper detoxification protein (Chung and Trevithick 1970; Mahadevan and Mahadkar 1970; Hecker and Sussman 1973a; Sukumaran and Mahadevan 1975; Mehta and Mahadevan 1975; Ruiz-Herrera and Sentandreu 1989; Lauter *et al.*, 1992; Wessels 1993; Suresh and Subramanyam 1996). However, none of these proteins were identified by proteomics methods for several possible reasons. The most likely reason is that these proteins are found in lower abundance and are typically masked by the more abundant ones, such as those listed Table 4.1. Similarly, other yeast proteomic studies did not identify endoglucanases, glucan synthases and chitin synthases (Pardo *et al.*, 2000a). In addition, hydrophobins were not identified because they resist standard extraction

procedures, partly due to their hydrophobic properties (Wessels 1993). To obtain hydrophobins, SDS insoluble cell wall material must be extensively washed and extracted with trifluoroacetic acid (Wessels 1993).

In addition to proteins identified by mass spectrometry, a bioinformatic search, has also identified many probable wall-associated proteins or wall proteins in the *Neurospora* proteome (Table 4.4). Many of the *S. cerevisiae* genes used in my search of the *Neurospora* database, catalogued in databases and literature, were originally identified by genetic screens which involved the use of enzymes and chemicals known to attack the wall (Lussier *et al.*, 1997b). Yeast deletion mutants that were resistant or sensitive to calcofluor white, zymolyase (β -1,3 glucanase), hygromycin B, papulacandin B and caffeine, were selected for the *N. crassa* database searches (Table 4.4, right side; Parsons *et al.*, 1988; Ballou *et al.*, 1991). For example, sensitivity of yeast cells to zymolyase (β -1,3 glucanase) was used to detect changes in cell wall composition. Possible explanations of a greater or diminished accessibility of the glucanase towards the cell wall β -1,3 glucan include i) incomplete N- and O-linked polysaccharides; ii) defect in incorporation of cell wall proteins and iii) diminished levels of branched β -1,3 glucan polymers (Lussier *et al.*, 1997b). Papulacandin B is thought to be an inhibitor of β -1,3 glucan synthesis that it may directly hinder some components of the β -1,3 glucan synthase complex or inhibit incorporation of β -1,3 glucans into the molecular organization of the extracellular matrix (Ram *et al.*, 1994). Several mutants involved in growth control and in the PKC1-MPK1 signal transduction pathway showed increased sensitivity to caffeine. This phenotype is loosely indicative of a defect in regulation/signal transduction. Caffeine sensitivity is a

common phenotype among the calcofluor white sensitive or resistant strain collections (Table 4.4). A large number of positive hits revealed significant similarity between putative *Neurospora* gene products and yeast proteins (Table 4.4).

The unexpected association of some known genes with cell wall phenotypes emphasizes the value of genome-wide screens to define function and to examine global aspects of regulation (Lussier *et al.*, 1997b; Table 4.4). However, the effects of some of these gene knock-outs in yeast mutants are likely indirect. For example, a cell that transcribes or translates incorrectly because of enzymatic machinery problems may produce defective proteins and ultimately the cell wall and other cellular organelles will have a modified compositions (Lussier *et al.*, 1997b). Another possible example of global regulatory response is illustrated by mitochondrial defects that appear to perturb the yeast cell surface (Lussier *et al.*, 1997b; Table 4.4). This data will be most useful when it is supported by subcellular localization and functional assays.

The wide-range of protein categories obtained bioinformatically re-emphasizes that cell surface synthesis and its integration with cellular growth and division is complex, with regulation of the individual polymers and likely some global overall sensing and control (Lussier *et al.*, 1997b; Table 4.4). In addition there is much physiological evidence that environmental such as nutrient and carbon source, temperature and the medium osmoticum affect the composition of the cell wall (Klis 1994). Recently several major signal transduction cascades have been found to regulate the cell surface. Known components include the protein kinase C cascade, the osmotic sensing HOG pathway, a two component regulatory system with the *SKN7* transcription

factor as a receiver module, and the calcium-modulated protein phosphatase, calcineurin, has been implicated in β -glucan synthesis (Garret-Engle *et al.*, 1995; Igual *et al.*, 1996). Other *N. crassa* protein kinase and phosphatase homologs, identified by bioinformatic searches, have been shown to have cell wall effects in *S. cerevisiae* (Table 4.4). It is likely that these different pathways are coordinated and that cell surface biosynthesis and assembly is controlled at many levels, from transcriptional regulation to the cell wall itself (Lussier *et al.*, 1997b).

In conclusion, proteomic and bioinformatic methods have permitted the identification of a large number of putative cell wall and cell wall-affective proteins of *N. crassa* (Tables 4.1, 4.2, 4.3 and 4.4). The extraction of proteins with trypsin from intact hyphae and with denaturants with a reducing agent, from purified cell walls, have revealed more than 50 proteins which have a strong association with the cell wall (Table 4.3). Additionally, the analysis of proteins in the growth media shed light on the composition of the cell wall and secretion. The use of 2-D electrophoresis and mass spectrometric techniques in concert with the *Neurospora* genome project, has contributed to the identification of many known proteins which are thought to play a role in cell wall structure or biogenesis. These include known wall proteins, glycolytic enzymes, heat shock proteins and organelle-related proteins (Table 4.3). Many of these associations with the cell wall are in agreement with recent findings from work with other organisms and a number of these observations have been corroborated by immuno-labeling experiments. Furthermore, in a complementary to proteomic and immunological studies, computational methods were able to identify a large number of *S. cerevisiae* homologs in *N. crassa* that

are known to affect the composition of the wall. The major challenges are to determine how these proteins are externalized and what functions they may be involved in.

SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

Summary, conclusions and future directions

The fungal cell wall has a complex, laminate structure composed primarily of polymeric, mixed glucans and chitin, as well as a minor fraction of proteins and glycoproteins. In *N. crassa*, immuno-gold labelling confirmed that the NcCW antiserum binds primarily to the cell envelope and cleavage with endo-proteinases and chemical reagents confirmed that the reacting antigens are proteinaceous. Western blot analysis revealed that five prominent proteins and more than 20 minor proteins are recognized by the NcCW antiserum and several of the most immuno-reactive antigens were resolvable as multiple charge variants by two-dimensional gel electrophoresis and Western blotting, suggestive of multiple, post-transcriptional modifications. Western blot analysis, of both fungi, revealed that a subset of these antigens were also recognized by lectin conjugates which bind to mannosyl, glucosyl and galactose residues. A comparison of these multi-species profiles of NcCW antiserum binding and lectin-binding supports the conclusion that this antiserum primarily recognizes polypeptides and demonstrates its potential utility for inter-species comparisons. Extractions of the *N. crassa* cell wall fraction with detergents, urea, sodium hydroxide, hydrochloric acid and trifluoromethanesulfonic acid suggest that several of the antigens are covalently linked to the cell wall through an O-glycosyl linkage. The NcCW antiserum was utilized to identified 6 cDNA clones encoding putative cell wall-associated proteins from an expression library. A similar approach using a ScCW antiserum resulted in the discovery of 14 immunoreactive *S. commune* clones, 12 which were identified through DNA sequence analysis, after expression in prokaryotic cells and provides more evidence that the ScCW antiserum

mainly recognized proteins. Genes encoding well documented cell wall proteins were identified including heat shock protein 60 kDa, elongation factor 1- α , and ubiquitin conjugating enzyme. Several novel genes, whose sequence is not statistically similar to others in the public databases, that might encode cell surface proteins were also found in our screen.

Complementary approaches, namely, bioinformatic and proteomic-based methods, were also employed to further identify the proteins associated with the cell wall of *N. crassa*. To characterize the surface proteins intact hyphae were treated with trypsin and tryptic fragments were compared to proteins released by chemical treatments, including, hydrochloric acid, sodium hydroxide, guanidine hydrochloride, and urea plus 2-mercaptoethanol, from purified walls. Proteins extracted with i) urea plus 2-mercaptoethanol were separated by two-dimensional polyacrylamide gel electrophoresis (2-D SDS PAGE), whereas, ii) the trypsin extracts were directly examined by on-line liquid chromatography tandem mass spectrometry (LC-MS/MS). In the previous case, 100 of the most prominent protein spots were cut out following gel electrophoretic separation, digested with trypsin and examined by LC-MS/MS. Amino acid sequences were obtained for 56 of the chemically extracted proteins separated by 2-D SDS PAGE. From the derived sequences, more than 50 proteins were identified by MascotTM searches against the NCBI and Whitehead Institute *Neurospora* protein databases. Furthermore, proteins secreted into the growth media and proteins released by NovozymeTM treatments from purified cell walls were also identified by mass spectrometry. In contrast to chemical and enzymatic extractions of proteins from purified wall, 78 proteins from

intact cells were released by mild trypsin treatments and identified by protein sequencing. The proteins identified in the datasets included homologues of known yeast cell wall proteins, glycolytic enzymes, heat shock proteins and translation factors. Examples of identified proteins include: enolase, glyceraldehyde-3-phosphate dehydrogenase, heat shock 70 kDa protein, elongation factor 1-alpha, outer mitochondrial membrane porin, alcohol dehydrogenase and vacuolar protease A. Moreover, this approach identified several novel proteins which may be involved in cell wall biogenesis. In general, all methods used in this study, in a complementary way, led us to better understand the architecture of cell walls, in particular, the cell wall protein component.

There are unresolved issues that should be addressed by future research studies. The cell wall is a dynamic organelle and changes of the protein composition should be examined under different growth conditions, following stress such as osmotic shock and during the cell cycle. The cell wall-associated proteins identified here only represent a small component of a larger set of proteins involved in cell wall biogenesis. The identification and characterization of signal transduction components involved in cell wall maintenance and biogenesis requires close attention. Intriguingly, many of the wall associated proteins do not possess N-terminal signal peptides. It would of great interest of future research to elucidate the nonclassical protein secretion mechanism in *N. crassa*.

Most of the examples of protein translocation across a membrane (such as the import of classical secretory proteins into the endoplasmic reticulum, import of proteins into mitochondria and peroxisomes, as well as protein import into and export from the nucleus), are understood in great detail. In striking contrast, the phenomenon of

unconventional protein secretion (also known as nonclassical protein export or ER/Golgi-independent protein secretion) from eukaryotic cells was discovered more than 10 years ago and yet the molecular mechanism and the molecular identity of machinery components that mediate this process remain elusive (Nickel 2003). This problem appears to be even more complex as several lines of evidence indicate that various kinds of mechanistically distinct nonclassical export routes may exist, all of which can be explored in *N. crassa* (Fig. 4.6). To study the mechanisms of protein transport in filamentous fungi, *N. crassa* cultures may be treated with chemical agents that interfere with this process. Dithiothreitol and brefeldin A have been shown to inhibit protein secretion in the filamentous fungus *T. reesei* (Pakula *et al.*, 2003). In *N. crassa*, the effects of the drugs on transport can be characterized by Western blots, Northern blots and MS to see whether any of the identified genes and gene products are repressed and/or not transported to the surface, respectively.

By a combination of immunological, proteomic and bioinformatic methods, we identified approximately 230 distinct surface related proteins. Using an immunological approach we were able to identify 18 and by various chemical and enzymatic extractions followed by 2D SDS PAGE plus MS, were identified a further 107 distinct proteins. Moreover, bioinformatic searches permitted the discovery of 105 proteins known to be directly or indirectly involved in cell wall biogenesis.

This is the first comprehensive proteomic study of the cell wall of *N. crassa* and it is a powerful tool that has significantly contributed to the understanding of cell wall

structure. These exciting data will lead to further studies on wall growth, cell wall stress and cell stability.

Fig. 4.6 Cargo proteins and potential export routes of unconventional protein secretion (Adapted from Nickel 2003). At least four distinct types of nonclassical export can be distinguished in mammalian cells. I) The first postulated pathway of unconventional protein secretion involves the formation of exosomes, vesicles that form on the outer surface of the cell in a process known as membrane blebbing. Exosomes are labile structures that release their contents into the extracellular space. It has been suggested that this pathway may be used by the galectins, a class of animal lectins secreted by immune cells. II) FGF-1 and FGF-2 probably reach the extracellular space by direct translocation across the plasma membrane, but they apparently use distinct transport systems. III) For IL-1 β , export involves import into intracellular vesicles, which are probably endosomal subcompartments. IV) The *Leishmania* cell surface protein HASPB also translocates directly across the plasma membrane and requires that the protein is membrane-anchored through dual acylation at the N-terminus. Therefore, a flip-flop mechanism is required to locate the protein in the outer leaflet of the plasma membrane.

Appendix A

Nucleotide sequences of the clones isolation by screening an *S. commune* library with a

antiserum.

A) JB-SC-1A

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1      GCACGAGTTC CATTCTCCCA TCTCCCTTCT TGAGCCTTCG TACCTTCAAC GACTCCCTAT
61     CTGTTCACGT CTGTCTCTTC GACTTGCACA TCTACCCACT TCATTCTTTG CAATGGCCCG
121    CCTTACGTTT ACCTTTGTTG CTGCCTCCTT GCGCTGTGCC ATGCTCGCTG CCGCCTCGCC
181    GATGCCTGCG CCCCGCCTGA ACGCGGTCCC GCGTGACGCG GCGAAGCTCG CCGTTCGACG
241    GAAGACGGGC AACTACATCG CGTTCGATGC CGCCGGCAAG CACCTTGGCT TCATTGAGCC
301    CAGCGCCTCG CTCGGGCGCC GCGACGGCTC GTGCGCTGCG CTCAGCTCGG ATGATGTCCA
361    GAAGCTTCCG GGCTGGGACA AGCTCGTCTC CCAGGCCAAC GACAACTGGG GCGACCACAA
421    GCGCAAGATC GTCACCAACG ACTCGGACTA CCCTGACAGG CCCGCGTCGA TCTGCGCCAT
481    GGACGCTGGC GACATCGCCA TCGACGGTGA CCCGGAGTGC ACGACCTCCA CGCAGACTCT
541    GTCCACTGGC GTGAAGGACA CGACCGGTAT CGCCACCGTG TCCCAGGTCA CTGGCACGAG
601    CTTCTCCTCG TCGCAGACCG TCACTCAGGA GTCCTCCATC TCAGTCGGCG AGACCGTTGA
661    TGTGAAGGTC GGCATTCCGG AGGTCGCAGA CGTCACGTCT TCGACGTCCC TCACCGCGAC
721    CTTACGAAC AGCCTGTCCA AGACCACCAC CTCGACGTCC AACCAGCAGA CCACGCAGAC
781    GGTCGCGATC AACATCCCCA AGGACTCGAC GTGCAACATC ACCTACAAGC AGACGTCTTG
841    CTCCACGCAC GGCTCCGGCC AGGTCCCCTT CACCGCGACC GGCTGGGTGT GGTTCGAGTA
901    CGACTCGAAG ACGGAGGGCC ACTACAAGTG GCGCTCAAC ATGGACAACC TCCTCTCCGA
961    CGACGAGCGC TCGTCGTACA TGAAGTTCGA CTCCGTCTGC AGCACCGACA CCAAGGGCGA
1021   CTACCAGGCC GACTGCTAGA GGACCGGACG TCTTCGCTCC TCACGACACT AGGGACGACC
1081   ACGACATCAT GAAGGGACAA TGGGATTTAT TTATCGGTCA ATCCTTGCGG GCTTGTACTC
1141   TACATTCAAT AATTATTTAT CACCGTGTTC GACGCCTGGT GTTCACGCTA AACAAAAAAA
1201   AAAAAAAAAA A

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B) JB-SC-2A

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1      GGCACGAGCC TCCCTCCATC CGCTCCGCAC CCCTTCCCTC GCCCGCCCAA CACTCTCCAC
61     TCACTCCGCC CCCAAAATGT CCGACGATGA GCAGCACAAC ACTCTTTCGA GGAGGCCAAC
121    GCTGGGGCGT CCCTCACGTT CCCCATGCAG TGCTCGGCGC TGAGGAAGAA CGGGCACGTC
181    GTCATCAAGG GCCGCCCCTG CAAGATCGTC GAGATGTCCA CCTCGAAGAC GGGCAAGCAC
241    GGTCACGCCA AGGTCCACCT TGTCGCCATC GACATCTTCA CCGGCAAGAA GCTTGAGGAT
301    ATCTCCCCGT CCACCCACAA CATGGATGTG CCGCACGTCA AGAGGGACGA ATACCAGCTC
361    GTCAACATCG ACGAGGGATT CCTCAACCTG ATGACGTCCG ACGGTACCCC CAAGGACGAT
421    GTGAAGGTCC CCGAGGGTGA GCTCGGTCAG GAGATCGAGG CTGCCTTTGA GGACGGCAAG
481    GACGTTCTGG TCACCATCGT TAGCTCGATG GGTGAGGAGC AGGCGATCTC CTGGAAGGAG
541    GCCCCCAAGG GTGCCGGTGC TTAGAGTGGC CCGCCCCCTT CTCCGCCCTT GTACATGGAT
601    GTGTGTGGGG AGATACGCGT GAAGGCTCGC CTCGTTACCG TCGGAAGACA CGTTTCGTTA
661    CTGTCGGCAG ACACGTTTCG TCACCGTTGG AAGACACGTC ACGACCGCCC TGGGCAGGCT
721    TGTATCGCGG CACTGCGGCC TTCGTTTTTC GTGTACTCTC GTGTTGTTCA CTGTGGAAGC
781    AGATGTATGT ATCGTGTAAAT GTATCCGCT GCCGCCCAA TGAGTGTATG TGCGCGTGTA
841    CAGAGAAAAA AAAAAAAAAA AAA

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C) JB-SC-2B

1	GGCACGAGGG	TCATCCAGGC	CGCCATCAAG	GGCAAGACGA	ACGTCGTGAC	GACCTCCTAC
61	GTCTCCCCCG	CTATCCGCGC	GCTCGAGCCC	GAGATCAAGG	CCGCCGGCAT	CGTCGTCTATG
121	AACGAGATCG	GGCTCGACCC	CGGCATCGAC	CACCTGTACG	CGGTGAAGAC	GATCAGCGAG
181	GTGCACGCAA	AGGGCGGCAA	GATCAAGAAG	TTCCTCTCCT	ACTGCGGCGG	CCTGCCCGCG
241	CCTGAGTGCT	GCGGGAATCC	GCTCGGGTAC	AAGTTCTCGT	GGTCGTGCGG	GGCGTGCTGC
301	TCGCGCTCCT	GAACAACGCG	GCGTACATCG	ACGGCGGGAA	GAAGGTGGAG	GTGTGCGGGA
361	AGGACTTGAT	GGGCCACGCG	AAGCCGTATT	ACATCTCGCC	GGCGTACGCG	TATGTGGCGT
421	ACCCGAACCG	CGACTCGACG	CCGTTAGGG	AGTTCTATGG	GATCCCGGAG	GCGGAGACGT
481	GCGTCCGCGG	GACGCTGCGG	TATCAGGGCT	TCCCGGAGTT	CGTGAAGGCG	CTGGTGCGAG
541	CTGGGTGGCT	TGACCAGGGC	GAGAAGGACT	GGCTCAAGGA	GGGCTTGACC	TGGGCGGAGA
601	TATATGCGAA	GGCTTTGGGA	ACGGCCGACG	CGAACGAGGC	GACCCTCGTG	GCCAAGGTCA
661	AGGAGCTCTG	CCAGTTCCCC	GACGAATCCG	AGGCGACGCG	CATCATCTCC	GGCATGCGCT
721	GGGTGCGCCT	CTTCTCGAGC	GAGCCCGCGC	CTATCCGCGC	CTCCAACCTC	CTCGACACGC
781	TCTGCGTCTC	GCCTCGAGAA	GCTCATGGCT	TACGAGCAGG	GCGAGCGCGA	CCTCGTCATC
841	CTCCAGCACA	AGTTCGTCTG	CGAGTGGGCG	GACGGGAAGG	AGGACACGCT	CACGTGACG
901	CTCGAGGCGT	ACGGGAGCCC	GATCGGGTAC	TCGGCGATGG	CGCGCACCCT	CGGTGTGCCC
961	TGTGGGATTG	CGGTGCAGTT	GGTGCTGGAC	GGTGTGATCA	ACACGCCCTG	TGTCATTGTG
1021	CCCTACACGC	CGGAGATTTG	CGACCCATATC	CGGGAGGTGT	TGGAGAAGGA	GGGCTTGGGG
1081	CTGATTGAGC	GGGTGCTGTA	GGTGTATTTG	GATATAGACG	ATCGGAACGG	TGAGAGGGTA
1141	GGCAGCTTTG	TATCAACTCT	GTAAAGGCGC	TGTATCTCTA	GAGTACGGCC	CCTCCAAAAT
1201	TGATGTATTT	TAGACGAAAA	AAAAAAAAAA	AAAAAAA		

D) JB-SC-3A

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121	TATCGGTGGA	GGCGGTTTCT	TCCCCGCTCG	TGTCTTGCGC	ACCTTCATCC	GCGCGCAAGG
181	ATCGCACAA	GCCATCCAAA	TACAAGCTAT	TGGCCTCTCC	CTCTACGAAC	CCGTTGAGGG
241	CACCACCGCG	CAGCAGATCG	GCACAGAGGT	CATCCGCACA	CAATGGCTGG	AAGACGCTGG
301	AAAGAACCTG	GTCGGCAAGA	AGATCCTCAT	CATCGACGAG	GTTGACGATA	CCCGCAAGAC
361	CCTCCAATAC	GCCGTCTCCG	AGCTCAAAAA	GGACATCGCA	GCCGAGCTCG	TCACCCGCCC
421	CGAGCACGAG	GCCGCCGTCC	TCGCCACGCA	GACCGCCTTC	GGCATCTTCG	TCGTGCATAA
481	CAAGAAGAAG	GCGAAGATCG	GCGAGCTCAC	GGACGACGTG	AAGTACTTCC	CCGGGATGGA
541	GGTGGACGAT	GTGTGGTTGG	ATTATCCGTG	GGAGGTGCTG	GACATCGAGG	AGCACGACCG
601	TCTCGTGGCG	GCGGACAAGG	CGCGTAGGAA	GGCGCGTGCG	GGGCAGCAGG	CGTCTTCATA
661	ACGTACGGGA	TGTCGCATTG	CGTCAACATC	GTGAGGGCAT	GAGCCGTCGC	TCCTCCACTG
721	AATTCCAGGG	CTCACGCGTC	GGACTGCAAC	AGGTCTCCTG	CCCTTCTCCA	AACCTCCAGC
781	GAATCGCCCC	AGACGCGCGC	ACGAGCCTAT	CATTGCGTCC	TGTGATATGC	GAAGCAGAGA
841	ACAATGGCAT	CGACATGTCT	CCAGGTTGTA	GTACAAAATT	GAGTACCCAG	ATGTACCTTC
901	TCTATCTATC	GATTATTATT	TCAAAAAAAAA	AAAAAAAAAA	AA	

E) JB-SC-3B

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121	TCCGCAAGAT	CAAGCTACGG	GTGGACGAGG	TTCAGGGCAA	GAAGTGCCTT	ACCAACTTCC
181	ACGGCATGGA	CTTCACGTCC	GACAAGCTCC	GTTCCCTTGT	CCGCAAGTGG	CAAACCCCTCA
241	TTGAGGCCCA	CGTCGACGTC	AAGACGACTG	ACGGCTACCT	CCTCCGCCTC	TTCGCCATCG
301	GCTTCACGAA	GCGCAGACCG	ACGCAGGTTT	GCAAGACGAC	CTACGCCCAG	TCTTCGCAGG
361	TGCGGGAGAT	CCGGAAGAAG	ATGTTCCGAG	TCATGACGCG	CGAGGCGACG	AGCTGCGACC
421	TCAAGGAGCT	CGTGCAGAA	TTCGTTCCGG	AGGCGATCGG	TCGCGAGATC	GGAAGGCGTC
481	GCGGAGCATC	TACCCCTTCC	AAAACGTCTA	CGTGCGGAAG	GCCAAGATCC	TGAAGGCGCC
541	CAAGTTCGAC	GTCTCCAAGC	TCATGGAGCT	GCACGGCGAG	TCGACGGACG	AGACTGGCAC
601	GAAGATCGCG	AAGGACTTCA	AGGAGCCGGA	GATC		

F) JB-SC-4A

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1      GCACGAGCCG AGAAGAAGAT CTCGCTCCTG CAGGACATCC TCCCGTCGTT GGAGACCGCT
61     GTCCAGGCCC GCCGTCCGCT CGTCATCATC GCGGAGGACG TTGACGGCGA GGCGCTCGCT
121    GCGCTCATCC TCAACAAGCT CCGCGGCCAG CTCCAGGTCG CCGCCGTCAA GGCCCCGGGC
181    TTCGGTGACA ACCGCAAGTC CATCCTCGGC GATATTGCCA TCCTCACCGG CGGTACCGTC
241    TTCACGGACG AGCTCGACAT CAAGCTCGAG AAGGCGACCG CGGACCTCCT CGGCTCGACC
301    GGCTCCATCA CGATCACCAA GGAGGACACC ATTGTGCTCA ACGGCGAGGG CTCGAAGGAC
361    AACATCCAGG CCCGTTGCGA GCAGATCCGC GCGCTCATTG ACGACCCGAC GACGAGTGAC
421    TACGACCGCA CGAAGCTCCA GGAGCGCCTC GCGAAGCTCC AGCGGTGGTG TCGCTGTCAT
481    CAAGGTCGGC GGCTCATCCG AGGTCGAGGT CGGCGAGAAG AAGGACCGCT ACGACGACGC
541    GCTCAACGCT ACCCGCGCCG CTGTCGAGGA GGGCATCCTC CCCGGCGGCG GTGTCGCGCT
601    CCTGAAGGCG TCCCTCATGC TCGCGACCAA CTCCCAGGCC GCGTCTTCCA CCTCCGCGCC
661    CGTCTCGCCC GACGCGAAGC CGATACCGAC CGCCAAC TTC GACCAGGAGC TCGGCGTCGC
721    GATCATCCGC CGCGCGCTCA CGAACCCCTC GCGCGCGATC CTCTCCAACG CGGGCGAGGA
781    GGCGAGCGTG ATCGTCGGCA CGCTGCTCAA GCAGTACGGC GGCGCGGACA AGTTCGCGTG
841    GGGCTACGAC GCCGCGAAGG GCGAGTACGT GGACATGATC AAGGCGGGGA TCGTCGACCC
901    GCTGAAGGTG GTGCGCACGG CGCTCGTGGA TCGGTCGGGC GTGGCGTCGC TGTTGACGAC
961    GAGCGAGTGC TCCGTGGTGG ATGCGCCGGA GGAGGACAAG CCGGCGGGTG GGATGGGCGG
1021   CATGGGGGGT ATGGGAGGCA TGGGTGGCAT GGGCGGCTTC TAAGCTAGCT GTGGCGCCTC
1081   TGATTAACCT TTTCTCCCTC CCTCCCTCTC CCTCACTGCC TTCACCTACT GGGTCCGCTA
1141   TCCGGTCCCT TTGCGCATGA GCATCGGACA CGCCTCATCG CTGACATGTA CACCATGTCT
1201   TTCCTCATCG CTGATCGTTG GTCTCTTCAG GGTTCATGAT TATTGACGCA CTCACCAAGT
1261   CGCATCCGTC GCGCTAACCG CATGTCGCAC TCAACGCGCT GCCGCGCTCC TCACCACCAA
1321   GTACAATACT CGTATGTTAC TAGHTTTGAC ACTGCTATTC TAGTCTACGT ACCATAGTCB
1381   CCACGAATGC AAGCCGCTAC TCCGTAAAAA AAAAAAAAAA AAAAAAA

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G) JB-SC-5A

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121    CCTTTCACAA TCTTTTGCCT GCATTTGACG AGCAATACCA TCAGGAAACG AGCCTCTTGT
181    CGCGCTGTGT GGAGAGAGGC ACTATGTCGG ACCATGGTGG AGAATGATAT CTTTGGACCG
241    ACATTGACAC AAGCACACAT GTCTATCGAA GAACTCCGTT GTGCTGCCCT GCGCCCTCAG
301    CTCTTCCTGA ACAGCATTA AAGCGATTCA TCTGACGGTC CTGACTACGG ATGGCCTGAT
361    TCCACCCGCG AAATCGTGCT GGAGACCGCT TGGAATTTCA CCCAGTGGGA GGATCCTGTA
421    AAGGGGCTTC ATCTGATTCA GGGAGGCCGA TATCTCCTTC ATGGACACTC ACCTTTCCCT
481    CTGTATCTAC GACATGGAGG ATCCCTTGAA CGACGTGGAA AGGCTCGAAC CCATTTTCTC
541    GTTGGACAAA CACGGCAACA GAGCGGACGA CGATCTGAGG CCGTGGCTAC CTTTCGGAAG
601    CACCCTTGGA AAATATGCAT GTGTTGGAGA CGAGATCATT CTCTTCTTCT CCTCTCGTCC
661    CAATCATATG ACGAACTCGC CAGCATCCGC TTCTGGCGTA CAAAATCCGA GGCTTTCCCT
721    ATGAAGTCAC GGGCGAAAAG CTGCGCCACT GACCGTCGAC TGGCTTTCGT TGGCCATGGG
781    TCGGAGATCA TTGGCGTATG GGACTACGAG CATAATCAGG TCGCGCTGAT GAAGGTCTCG
841    GACCGATATG GCCTTGACTG CCTCAATGGG TGCGTCTTGA CGCATAATAG CATCATCTTC
901    AAGAACTCTC GAGGCTTTTA TACATACGCT ATTCCGCCTT TCGCGCCGGC GACGATGCCG
961    CCGCTTCCTT TGAAGCATGA AGTGATGCAC TCGATCGAGA GCGGTTGGAT TGGGCTGCGC
1021   TTTCTCTACT CAACAAATCA GCTGGCGCCA CTTTCCTCGA CCTCCATTAC CTTGCGCAGC
1081   ACAGCTACCT CGACAGGTAC TGCATCGAAT CCGTGTGCGC AAACGATTTT GCCAGCTTGC
1141   TCGTGCCAGC GCCGACCTCC GAGAACCTGA ATCTTGATC CGGACAGCGG TTACGAGATG
1201   CAGTGCTGCG ATGTGCGAAC GCCCCGTGCC TCGCTCGGTA GCGTGTTTAT CCTGGGGCAT
1261   CGAACAGGAT GGATCGATCC CGACATCGAC GCGCAACTCG GGAGGCGCGA GACTTATTTT
1321   GAGCGTTTGG GCATCCCGTA CGAGGAGTGG AACGAGGAGG ATTATACCGA GGAGATGGAC
1381   GAAGCGATTG GGCGCGAGAA CGACCAGGCG GGCGTGTTTA TCCACGTCGA GCACAGAAAC
1441   TCGGCACGAG ATGAGGACGG TCAGTAGCAG ATCTCCGTTG TTGAGCTGCT GGACGCGCAG
1501   AATTCCGCAT GGGGGCAGCG TGAATTGCGT TTCTGCGGCT TCTGCCCCGC GTCTGGTCTG
1561   CTTTGCGCCA TCGTGGAAGA GGGTGATGAG CCTCCCCGAT TGCTTGTTTT CGATTATCTA
1621   CCCGCTTCCA TGTAGACTCA ATATGCCCCC CATATTTTGC ACTCTGGCGC ACTTTAAAAA
1681   AAAAAAAAAA AA

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H) JB-SC-5B

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121    AGAGGGCTCT CGCAGGACGT GTTCATCCCC CCGGACGACG CCACTTGGCC GACGTTCAAC
181    GCTACAGCTG GCGATAGCCC GCACCCGTCA CCACCGCAGC AGGACGCACT CATGATCCTA
241    CTCCCTCTGC TCATCGTGCT CTCAACCTTC CTCTTCCTAC TCCTTGATAT CCTCATATGC
301    GTAGTGCTCG TCCGCCGTG CAGAGGCATA GCCCTGCGAG ACTCCGATGG TCCCGTGGAC
361    ATGAGTCGCG AGGAGCTCAT CGATGGGGAA GGCGGCTTTG AGGGCGTAGA GTCCCGCTGG
421    CTGGAGACCG TACCCGAGCA CGTACAGCGA GATTACAGGC GGGCGAAAGA GTACCAGCTC
481    GACTACCCGC CCAATTCCTT CCCGACAGAT ATTACTCTCT CGCAGTTCCT ATCTATTCAG
541    GAGAAGGGCG TGTCGGCCTG GTGCTTTGAG CCCGACTTCG AGACGGTTAA CAACCTTCTT
601    GTGCAAGCGC GCACAGAGAT AACATTCTTT CCCGACCCGG CGTCGGCTTC CTGCGTTCAA
661    AGCAACCTCC CCCTTCCGAA ACTCAACGAG GTCTACTACT GGAAGTCAA GATGTTTCGAC
721    CTACCCATCA CTACCAACGT CAGCGTCGGC CTCGMCACCA AGCCATACCC TCAGTTCAGC
781    TTGCCCCGCA TGAACCGCTA CTCTGTCGCA TACCACTCCA ACGGTGACAT ATCATATAAC
841    TACCCCTTCA MCGCGTCGTC CTTCCGACCC GGACTCAAGG AAGGCGATGT GCTTGGCGTT
901    GGCTATAGAC CTCGGTCGGG CACGTCTTCT TCACACGGAC GGGCGAAAAA AAAAAA

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I) JB-SC-6A

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121    GTACGTCTGG GCGGTGCCCG ACGAGCGCAA CATCTTGACC TGGAACCTTA TCATCCGCGG
181    TCCGCCTGAC TCGCCGTACG ATGGCGGAGA GTACCACGGG CAGTTGCTAT TCCCCGCCGA
241    GTACCCGTTC AAGCCGCCGG GCATCAAGAT GCTCACGCCG TCGGGGCGCT TCTACCCAGA
301    CAAGAAGATC TGCTTCTCCA TGTCGGACTT TCACCCTGGG AGCTGGAATC CTGCATGGAG
361    CGTCCAGACG ATTCTCACCG GCCTCCTCTC CTTTCATGCTC TCCGACGAGA TTACGACCGG
421    CTCCGTTAAC TCGTCACCCG CACACAAGCG CGGCTTCGCT GCGCAGAGCC ATAACCTGGA
481    TATCCAGCAG GCGCGGTTTA GAGAGGCGTT TCCGGAGTAC GCCACCCCGG AGATGCGCGA
541    CCTTCCGAAT ATGGGCGAGA AGGAGCGCGG CCGGGCGGAT AAGCCGCCTA CGCCTCCGCC
601    CGCTCCATCA CGACCTGCCT CTTCTACCGG AGCGAACGCA ACCTCATCTG CAGCCCAAGC
661    CGCCAACGCA GCCCAGCCCG GAAACGGCGC ACAACCAGCA CCCGCCCCAG GTGCGTGGGG
721    CGCGGCGCTG TGGGAGAAAGT GGCGGTGGGG GGTGCTGATC GTGCTCGCGG TGCTGGTGTG
781    GAGGATGTCT GGGCCGTAGT GGGGAGGCGA TCGAGGCGAC GTGGGCGGGA GGCATGGAG
841    GCGACGTGGG TGGGTGGGTG CGTGTCTTGG TGCTGGGGTC TCGTAGGTAG TATGGTGGGA
901    GGGCTAAGAA GAGAAGGGAT GCCAGGCGAT GGGCAGAGGG GTTGGATGGG TTGGGCAGGG
961    GAGGGGAGGG TGGAGTCGTC GGATGAAGGA ATGCAGGGCG CTAACGGGGC ACCAGACATG
1021   GCCTGCTTCG TCGCGTAGAA TGGCAGCGGT ACGCCCTCTG GGGGAAACTA AGGATCTTGC
1081   AGAGATCTGC TAGACGCTTA GTGGGCGTAC TTTGGAAGCC TAGTGGGTGT ACTTGACTTG
1141   GAATGGCTGC CGACGTCTTA GTCGGCGGCT TGGACGATGG TGATTCTATC TAATGTTTTT
1201   GGCACGCTT TGTATATCGT ATCATACCTC AAGAAACCTT CTCAATCTTT CACAAAAAAA
1261   AAAAAAAAAA AAA

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J) JB-SC-6B

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121    GGCTACGTCC GCGGCGTGGT GAAAGAGATC ATTACGACT CTGGCCGTGG CGCGCCCTC
181    GCCGCGCTCG TCTTCCGGGA CCCGTACCGC TACAAGCTCC GCAAGGAGAC CTTTCATTGCG
241    ACCGAGGGCC TCCACACCGG CGCGTTCGTC TACTGCGGCA AGAAGGCGCA GCTCGCAGTT
301    GGCAACGTTA TCCCGTCTGG CCAGCTCCCT GAGGGTACGA TCATCTGCAA CGTCGAGGAG
361    AAGGTGCGCG ACCGCGGTGC GCTCGCCCGC ACTTCCGGCA ACTACGCGAC CGTCATTGGC
421    CACTCGCCGG ACGAGAACAA GACGCGCATT CGTCTGCCCT CCGGCTCGAA GAAGACCGTC
481    TCTGGCTCTG CCCGTGCGAC CGTCGGCATT GTCGCCGGTG GTGGCCGTAT CGACAAGCCT
541    TTGCTCAAGG CTGGCCGTGC GTACTACAAG TTCAAGGCCA AGCGTTACAA CTGGCCCCGT
601    ACTCGTGGTG TTGCTATGAA CCCCGTTGAC CATCTCACG GTGGTGGTAA CCACCAGCAT
661    ATCGGTAAGG CGTCCACGAT CGCCCGCTCC GCTGTTCTTG GCCAGAAAGT CGGTCTCATC
721    GCCGCTCGCC GGACGGGTCT CCTCCGCGGT ACCCAGAAGG TCAAGGAGGT TTAAGGGGCT
781    TGCGGCTGCG CCTACCTTTC ACCTCGACTT TCTATGTACG ACCCCACCCT ACAAACATG
841    TACCGCGTAT GCTATGTATG CTATCGACCC ATGCAGTTTC ATATGCCCA TCAAAAAAAA
901    AAAAAAAAAA A

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K) JB-SC-7A

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61     GTCGTCCCTT CCGCATCCAT CCCGACGAGA CGAACACCCC GATGCCTGTG CCCTACCCGG
121    ACCCGACTCG GGAGATCGCA AGCAGCCACT TCTATCTCAG CCATGCTGTT GCCGATATCC
181    AGAAGGAGAT TATCCTCAAG CTTTATGCCC CCAAGACGCC CCCGCGCTCC GCGCTGAGC
241    TGATCGGGAG CGCGAAGGCG ATTCTAGACA AGATGGAGAC TATACTTCCT CAGTCGTTCC
301    GTGATTATAT GGGTGGTACG GAGAATTGGC CCGTTGAGCG CAGGCGTGAT CTCGTGAAGA
361    GCATCTCGAC GGACATTGGA TTGACGCTCT TGAAGTGGGC CATCGCCAAG ATCTTCCTCC
421    TTCGCGCCCT TGTCGCGAGT AGCACGACAG GTTACAAGGA CAAGGTTCCG GCCCTGCAGC
481    CTGCCGTCTG CACCGCGCAC AACATCATTG TCGTGATCA CCAACTTATC AATCAGCCCG
541    ATATCTCGTT CTTACCTCA CCCCTCCCGT TGCAGACGGC GGCTATGGTG TTGCTCTATG
601    GCTGGATGTC CCGACGCGTT GAGGCTGTCA CTCGTGAAGT TCGGTTGGAG GATGTTACCA
661    TGGCC

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L) JB-SC-7B

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121    GGCCATCTCA TCTACAAGTG CGGTGGTATC GACAAGCGTA CGATCGAGAA GTTCGAGAAG
181    GAGGCCGCTG AGCTTGGCAA GGGTTCCTTC AAGTATGCGT GGGTGCTTGA CAAGCTCAAG
241    GCTGAGCGTG AGCKGGTATC ACCATTGATA TCGCCCTCTG GAAGTTCGAG ACCCCGAAGT
301    ACATGGTCAC CGTCATCGAC GCCCCGGGTC ACCGTGACTT CATCAAGAAC ATGATCACCG
361    GTACCTCCCA GGCTGACTGC GCTATCCTCA TCATCGCCGG TGGCACTGGT GAATTCGAGG
421    CTGGTATCTC CAAGGATGGC CAGACCCGCG AGCACGCTCT CCTTGCTTC ACCCTCGGTG
481    TCCGTCAGCT CATCGTCGCC GTCAACAAGA TGGACACGAC CAAGRGGAGC GAGGACCGTT
541    TCAACGAAAT CGTCAAGGAG ACCTCCACCT TCATCAAGAA GGTCGGCTAC AACCCGAAGA
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661    ACATGCCGTG GTACAAGGGC TGGACCAAGG AGACCAAGGC TGGTGTCGTC AAGGGCAAGA
721    CCCTCCTCGA TGCCATCGAC GCCATCGAGC CCCCCGTTTC TCCCTCCGAC AAGCCCCTCC
781    GTCTCCCCCT CCAGGATGTC TACAAGATCG GTGGTATTGG GACGGTGCCC GTCGGCCGTG
841    TCGAGACCGG TATCATCAAG GCCGGCATGG TCGTCACCTT CGCCCCCTCC AACGTCACCA
901    CTGAGGTCAA GTCCGTCGAG ATGCACCACG AGCAGCTCGC TGAGGGCAAG CCGGGTGACA
961    ACGTCGGGTT CAACGTCAAG AACGTCTCCG TCAAGGACAT TCGCCGTGGC AACGTCGCGT
1021   CTGACTCCAA GAACGACCCC GCGAAGGAGG CGGCGTCCTT CAACGCCAG GTCATCGTTC
1081   TCAACCACCC CGGTCAGATC GCGCTGGTA CGCACCCGTT CTCGATTGCC ACACCGCGCA
1141   CATTGCCTGC AAGTTCGCCG AGCTGCTCGA GAAGATCGAT CGCCGGACGG GTAAGTCGCT
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1261   CAAGCCGATG TGCCTTGAGT CCTACAACGA GTACCCGCCC CTTGGTCGTT TCGCTGTCCG
1321   CGACATGAGG CAGACGGTTG CCGTCGGCAT CATCAAGTCG GTCGACAAGA CTGACAAGTC
1381   CGGTGGCAAG GTCACCAAGT CCGCCGAGAA GGCCGCCAAG AAGAAGTAAA TGTAGATGTA
1441   CATATGTATT TTCTCATTCC GTTTCCTTCC TCTTGTTGTT GTTTCCTGTT TCCTCTCGTG
1501   CTCGCTCGCA TCGCATACAG CCATTGTTGT CACCACTATA ACTTCACGCA TTCTGTATTT
1561   CATGCCAGGC GACGGGGTCT TGTCTGCCA GGCCTGTCGC TTGTTGTAAC TCTAATGAAT
1621   WGTACGAGT AGTGGACGAA CGACGATGTA AAAAAAAAAA AAAAAAA

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