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**Cloning and characterization of *sbr* :
a new colonial mutant of *Neurospora crassa*.**

Katrina D. Campsall, B. Sc. (Hons.)

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of
Master of Science.

Ottawa-Carleton Institute of Biology
Carleton University
Ottawa, Ontario
Canada

May, 1998

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Research acceptance of the thesis

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Thesis Supervisor

Chairman, Department of Biology

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Date

ABSTRACT

Sbr (small brown) is a colonial mutant of *Neurospora crassa*, which was recovered in a screen of transformants harbouring ectopic integrations of a hygromycin phosphotransferase (*hph*) gene construct. Insertional inactivation by a single copy of this drug resistance marker appears to be responsible for the mutant morphology.

The *sbr* strain forms slow-growing compact colonies, which become dark brown with age. The density of the colony is likely attributed to frequent lateral and aerial branching. Instead of tubular hyphae, *sbr* grows by forming large spherical buds. Immunofluorescence microscopy with an anti-tubulin, monoclonal antibody revealed a scattered arrangement of microtubules. As well, conidiation and sexual spore germination are defective.

The *sbr* genomic DNA flanking the construct and the corresponding wild-type cDNA (incomplete) has been cloned and sequenced. The putative SBR protein encodes poly-glutamine and cysteine-rich regions and a helix-turn-helix DNA binding motif suggesting a regulatory function.

Dedicated to

Mom
&
Dad

(who may not understand what is written on these pages,

but

likely understand the entire work better than most.)

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LIST OF ABBREVIATIONS

aBSA	acetylated bovine serum albumin
amp	ampicillin
APS	ammonium persulfate
ATP	adenosine triphosphate
bp	base pair
cpm	counts per minute
DAPI	4', 6-diamidino-2-phenylindole
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddnTP	dideoxynucleotide triphosphate
dGTP	deoxyguanosine triphosphate
dH ₂ O	distilled water
DMSO	dimethyl sulfoxide
dnTP	dinucleotide triphosphate
dTTP	deoxythymidine triphosphate
EDTA	N,N-ethylenediaminetetracetic acid
EGTA	[ethylenebis(oxyethylenenitrilo)]- tetraacetic acid
FGSC	Fungal Genetics Stock Center
HRP	horse radish peroxidase
IPTG	isopropyl thio beta-D-galactosidase
kb	kilobase pair
kD	kilodalton
mA	milliamps
MCS	multiple cloning site
MW	molecular weight
ng	nanogram (10 ⁻⁹ grams)
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCI	phenol:chloroform:isoamyl alcohol (25:24:1 v/v)
PEG	polyethylene glycol
pfu	plaque forming unit
pH	-log ₁₀ [H ⁺]
rpm	revolutions per minute
RT	room temperature
SEM	scanning electron microscopy
SDS	sodium dodecyl sulfate
TBS	Tris buffered saline
TE	10 mM Tris, 1 mM EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	Tris(hydroxymethyl)aminomethane

ul	microlitre (10^{-6} litres)
uv	ultraviolet
v	volts
W	watts
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactosidase

I. INTRODUCTION

The model organism

In 1927, an enchanting red-bread mold attracted the attention of Shear and Dodge (1927) who published both the first detailed characterizations and the name of the organism, *Neurospora crassa*. In 1941, Beadle and Tatum published a report on the first mutants with biochemically defined nutritional requirements (Beadle and Tatum, 1941). Since this revolutionary discovery, the use of *N. crassa* as a model organism has led to many discoveries in the areas of molecular biology, classical genetics, recombination and cell biology (see review by Perkins, 1992).

Neurospora crassa is classified as an eukaryotic, filamentous fungus belonging to the class Ascomycetes. In nature, it can be found in soils of warm climates especially in areas of sugar cane cultivation. As a model organism, it is favored because it is easily manipulated using standard microbiological techniques, has simple nutritional requirements and a relatively small genome. Growth and maintenance of *N. crassa* cultures is as simple as handling yeast or bacteria.

Studies of the molecular biology of *N. crassa* have revealed a haploid genome of 4.7×10^7 bp (Orbach *et al.*, 1988) which is nearly twice the size of the yeast genome. The DNA is arranged into 7 linkage groups or chromosomes that are predominantly comprised of unique-sequences with only 8% repetitive-

sequences (Krumlauf and Marzluf, 1980). Over 800 genes have been identified by phenotype and/or map location (Radford and Parish, 1997), many genomic and cDNA libraries have been created, hundreds of morphological and biochemical mutants are available for study (Perkins *et al.*, 1982), and a *Neurospora* Genome Project is currently underway at the University of New Mexico.

Macroscopically, *N. crassa* has a life cycle that encompasses both a vegetative (asexual) stage and a sexual stage (Figure 1). A vegetative culture is formed from a network of branched hyphae (mycelium) each approximately 5 μ m in diameter. The tubular hyphae are surrounded by a cell wall composed of a protein layer sandwiched between an outer amorphous network of glycoproteins and glucans and an inner layer of chitin microfibrils (Trinci, 1978). The hyphal filament is subdivided by porous crosswalls called septa that allow cellular material, including nuclei, to migrate along its length.

The asexual life cycle has distinct stages of development resulting in the production of aerially disseminated multinucleate macroconidial spores (see review by Springer and Yanofsky, 1989). Initially, vegetative growth of the colony begins with a germinated macroconidial spore or hypha, which gives rise to primary hyphae that spread radially along the surface of the substrate. At the tip, hyphal growth occurs by the addition of vesicles that contain cell wall precursors and metabolites (Collinge and Trinci, 1974). Lateral branches arise from subapical positions on hyphae. The colony switches to the production of

asexual spores when triggered by such environmental cues as desiccation and starvation. New branches emerge which grow upwards, perpendicular to the substrate. Initially, these aerial hyphae grow at their tips by apical extension like their predecessor hyphae. After several hours, elongation ceases and further growth of some of them occurs by repeated apical budding to produce conidiophore structures. Each bud is separated by a subtle, minor constriction. Development of the daughter bud begins by localized growth at the cell surface of the parent cell, the bud is initiated, and then grows to a pre-determined size. Cessation of growth is followed by nuclear migration into the new proconidium bud. These buds resemble beads on a string and collectively form a proconidial chain (Turian and Bianchi, 1972). During the minor constriction stage, growth can interchange between apical budding and hyphal elongation. However, a distinct developmental stage occurs at the transition between minor constriction chain formation and the major constriction stage. Once the commitment has been made to produce major constriction chains, new growth proceeds solely by apical budding. As well, a surge in the expression of conidiation (*con*) genes marks the transition into this new stage (Sachs and Yanofsky, 1991). After the nucleus has migrated into the new bud, thick double crosswalls are laid at the interconidial junctions. The gap between the septal doublet is gradually modified to form a fragile, connective thread between the macroconidia allowing individual spores to be easily detached and dispersed by air currents. The

entire developmental process from induction of conidiation to macroconidial separation can be completed in less than 24 hours.

Sexual reproduction requires two strains of different mating types, designated *A* and *a*. Fertilization occurs when the nuclei from each strain are united in the perithecium, a mating-structure consisting of differentiated, coiled hyphae, to form a dikaryon (see review by Springer, 1993). The nuclei undergo cycles of division and meiosis until the final product is created; numerous asci each containing 8 binucleate haploid ascospores. The ascospores are then ejected from the perithecium to complete the cycle.

Mutagenesis – a tool for discovering gene function

Historically, an important approach to the study of gene function has involved the use of randomly induced mutations. A gene, which is altered by a mutational event, is recognized by a change in an inherited characteristic that can be measured qualitatively or quantitatively. The first source of mutants for scientific study originated from nature where mutant organisms arose from mutations caused by spontaneous or transposition events. However, characterization of many genes is difficult because the frequency of spontaneous mutation is low and many naturally occurring aberrant strains are not able to survive in nature due to a slow growth rate or auxotrophic requirements. Therefore it is not likely that a collection of these mutants would represent the many genes in the genome. Generation and maintenance of

Figure 1: Life cycle of *Neurospora crassa*. *Neurospora* has both an asexual (a-e) and a sexual (f-k) stage of development. Germination of a spore (a) produces primary hyphae that branch and grow by apical extension (b). The hyphal network forms a mycelium (c). Conidiation, induced by stress conditions, is characterized by the production of chains of conidia on aerial hyphae (d). Free spores are easily separated from the chain (e) and are capable of colonizing new substrate. In the sexual stage, protoperithecia are produced (f). Nuclei from a strain of the opposite mating type are led into the protoperithecium through a specialized hypha (trichogyne). A three-celled crozier is produced (g) after a combination of nuclear division and septum formation. Within the dikaryon compartment, the two nuclei fuse (h). Two meiotic divisions and a single mitotic division of this diploid nucleus produce eight haploid nuclei that are linearly arranged within an ascus (i). From the beaked perithecium (j), ascospores from each ascus are shot and then await germination.

mutant strains is more feasible in a laboratory environment since the rate and method of mutation can be controlled and selection can be optimized.

Experimentally, mutant strains can be created by exposing the model organism to mutagenic agents such as UV light, X-rays or chemicals including nitrous acid, diethyl sulfate and methylcholanthrene. However, this approach has limitations: mutations are usually elicited at random locations in the genome, there may be numerous occurrences in the same genome, and the site of alteration may not be easily located.

Classical morphological mutants of *Neurospora crassa*

The altered phenotype of some morphological mutants, especially strains exhibiting highly restricted colonial growth, has been speculated to be the result of a defect in a single gene that ultimately affects cell wall composition. In the past, it was hypothesized that colonial growth was the result of changes in the relative levels of some of the structural components that comprised the cell wall (Mahadevan and Tatum, 1965). The colonial mutant, *col-1*, was found to contain 0.1x less glucose and as much as 2.5x more glucosamine per unit weight of cell wall compared to wild-type but total polysaccharide amounts did not vary (de Terra and Tatum, 1963). The importance of peptides in hyphal wall structure led Wrathall and Tatum (1974) to discover reduced amounts of peptides in the hyphal walls of all 23 morphological mutants they examined. Some of the strains investigated included colonial strains *balloon*, *col-8* and *col-*

11, semi-colonial and spreading colonial strains and miscellaneous strains such as *ropy-4* and *doily*.

Since it was shown that morphological mutants were truly distinctive in the cellular amounts of many key structural components, these observations then had to be explained. Decreased peptide content of hyphal walls might cause structural fragility resulting in an increased frequency of branching and slower rate of elongation at the hyphal tip (de Terra and Tatum, 1961). An alternative hypothesis instead focused on the limited quantity of another cell wall component that would, in turn, reduce the capacity of peptide binding to the cell wall. Support for this latter hypothesis emerged from analysis of mutants such as *balloon*, which was shown to have reduced activity of the glucose 6-phosphate dehydrogenase enzyme (Scott and Tatum, 1970). The consequent reduction in NADPH levels is thought to result in an alteration in the levels of various fatty acids in the cell. This could then disturb the composition of the cell membrane, which in turn could affect the carbohydrate portion of the adjacent cell wall. It is possible that changes in hyphal wall carbohydrates will support fewer sites for peptide binding (Wrathall and Tatum, 1974).

As an attempt to understand the circumstances that bring about colonial morphology, many researchers have attempted to induce an aberrant morphology by supplementing the growth medium with chemical agents (see review by Mishra, 1977). The best known agent is L-sorbose, which has been shown to cause colonial growth and altered cell wall composition in wild-type

strains (de Terra and Tatum, 1963). As a consequence of sorbose uptake, branching frequency was increased and their cell walls were found to contain more than double the amount of glucosamine and nearly half the amount of glucose of strains grown on normal media. Examination of four truly colonial strains revealed a similar change in the ratios of glucosamine and glucose even in the absence of sorbose supplementation. The sorbose-induced morphological changes in wild-type were later found to correlate with inhibition of the enzyme activity of polysaccharide synthetase (Mishra and Tatum, 1972), which is required for synthesis of cell wall polymers.

Another enzyme essential for cell wall biosynthesis, carbohydrate synthetase, is regulated by cAMP. Mishra (1976) observed that supplementation of the growth medium with excess cAMP elicited colonial morphology in wild-type strains.

In a different, but relevant, type of experiment, Reissig and Glasgow (1971) isolated mucopolysaccharide (MP) from the growth medium of cultures of the conditional colonial mutant, *cot*, when grown at the restrictive temperature (37°C). When MP was exogenously supplied in the medium of *cot* strains at the unrestricted temperature (25°C), the colony displayed a phenotype quite similar to *cot* grown at 37°C: colonial, restricted growth, agglutination of its cells, and increased vacuolization. Supplementation of the wild-type strain with MP showed comparable results. The slow-growth phase of *cot* (at 37°C) was associated with MP production whereas under standard growth conditions,

endogenously produced MP was detectable in the medium of wild-type strains in stationary phase cultures, well after the culture had established an extensive, hyphal network. The major component of MP was found to be galactosamine having many unacetylated amino groups and was believed to occur in the cell wall fraction of *Neurospora* (Mahadevan and Tatum, 1965). The acetylating agent, polyphosphate, was shown to bind and inhibit some of the effects of MP resulting in growth of *col*. In light of these observations, Reissig and Glasgow (1971) have suggested a role for MP in growth and morphogenesis of *Neurospora* with possible involvement in the cell membrane.

With so many agents capable of inducing colonial growth by targeting different biological components of *Neurospora* morphogenesis, it becomes increasingly obvious that morphology is the result of many pleiotropic processes ranging from biochemical pathways to structural and regulatory components of cell wall biosynthesis. This realization brings into focus the difficulty of assigning a morphological phenotype to a defect in a single gene.

Of all the colonial mutants that have been studied, only a few have been attributed to a defect in a known gene. The colonial mutants, *col-2* and *balloon*, have defects in the genes encoding subunits for the glucose-6-phosphate dehydrogenase (G6PD) enzyme resulting in increased thermolability and altered kinetic properties (Scott and Tatum, 1970). Both *col-3* and *col-10* have changes in the kinetic parameters and heat stability of 6-phosphogluconate dehydrogenase (6PGD) resulting from a mutation in that gene (Scott and

Abramsky, 1973). These four mutants are defective at two consecutive stages of the same pentose phosphate pathway, which is primarily required for generating reducing power (NADPH) for subsequent biosynthetic reactions.

Similarly, the *col-1* mutant also exhibits colonial morphology (at the restrictive temperature of 32°C or above) due to a defect in a single gene. However, the mutation likely affects the regulation of many other genes involved in the signal transduction pathway since the *col-1* gene encodes a catalytic subunit of cAMP-dependent protein kinase A (PKA) (Yarden *et al.*, 1992). A regulatory subunit of PKA is encoded by the *mcb* gene. When the *mcb* gene is knocked-out, the resulting mutant lacks polarized growth at the restrictive temperature (Bruno *et al.*, 1996). The remainder of the colonial mutants can be described as having striking anomalies in growth rate (*col-17*), ascospores (*col-6*, *col-12*, and *le-1*), or conidiation (*col-1*, *col-4*, *col-5*, *col-8*, *col-13*, *col-15*, *balloon* and *le-1*).

Not only are many colonial mutants defective in conidiation, a number of other, non-colonial morphological mutants share similar defects. The best described conidiation mutants may lack conidia (aconidiates: *acon-2* and *-3*), have aberrant conidiation structures (granular - *gran*, tangerine - *tng*, fluffy - *fl* and fluffyoid - *fld*) or the macroconidia are unable to fully separate from the chain (conidiation separation defective - *csd-1* and *-2*). Both *acon-2* and *fld* may be defective at or before the start of the minor constriction stage, *acon-3* and *fl* are blocked at the minor constriction stage and do not produce macroconidia

while *gran* produces numerous, unusually small buds and *tng* produces unusually large buds that are unable to cease growth (see reviews by Springer and Yanofsky, 1989 and Springer, 1993).

Likewise, mutants have also been identified in sexual development (see summary by Russo and Pandit, 1992). Defects have been found in all stages including development of perithecia, asci, ascospore morphology (i.e. *col-2*, *col-10*, *peak*) and ascospore germination (i.e. *le-1*, *le-2*). It is interesting to note that many of the mutants exhibiting defects in the sexual cycle are also affected in other facets of growth or development. For instance, the strains listed above have also been identified as morphological mutants. In contrast, there are fewer mutants in which the developmental defect only affects one morphogenic step.

Since so many morphological mutants share certain characteristic abnormalities in morphology, it is apparent that mutations in single genes may affect many aspects of development. Unfortunately, this presents a great challenge in characterization of a new mutant especially if the gene is not known.

Insertional mutagenesis

With the recent advances in molecular biology and automated sequencing, the DNA sequences of many genes are quickly being uncovered while their functions are initially unknown. To gain insight into the gene's function, one approach involves altering the activity of a known gene, either by

replacement or disruption, using tagged homologous DNA. This procedure has the disadvantage that the gene disruption may not necessarily result in a noticeable phenotype and a function may still be difficult to assign. As well, only approximately 10% of integration events result in homologous insertion of linear transforming DNA in *N. crassa*, whereas multiple, ectopic (heterologous) integrations occur more frequently (Paietta and Marzluf, 1985).

Hence, an efficient alternative is to disrupt genes randomly using tagged heterologous DNA and then to screen the transformants for mutant phenotypes. Once the tag has been located, the disrupted gene is identified and a function may be allocated based on the phenotype.

The natural mutagenizing ability of transposons make them an excellent candidate for use in insertional mutagenesis. These short, mobile DNA sequences are capable of inserting into the genome without requiring the host's recombination system. The expression of a gene is modified by the presence of the transposon and the transposon also "tags" this modification. A limitation of transposon usage is that the gene is usually knocked-out completely and conditional mutants are rare, which poses a problem when essential genes are involved.

Much of the cloning strategy of some higher eukaryotes such as *Drosophila melanogaster*, *Zea mays* and *Arabidopsis thaliana* involves insertional mutagenesis using transposable elements. Some of the best characterized transposon systems were discovered in maize by McClintock in

the late 1940's. Transposon tagging can be credited for identification of many maize genes concerned with regulatory function, pigmentation, and chloroplast development (see review by Gierl and Saedler, 1992).

In some experimental organisms, including *A. thaliana*, endogenous transposons have not been characterized well enough to use as mutagenic tags. To solve this problem, systems for gene tagging with transposons originating from other organisms (heterologous system) have been developed. One of the most successful strategies has involved using the Activator (Ac) and Dissociation (Ds) transposable elements from maize. Modified maize transposable elements have successfully allowed identification of functions for numerous genes in *A. thaliana* (Bancroft *et al.*, 1993 and Long *et al.*, 1993), *Petunia* (Chuck *et al.*, 1993), and rice (Izawa *et al.*, 1997) to name a few.

Even fungi have not been overlooked as transposon targets: a large scale identification of *Saccharomyces cerevisiae* genes involved in cell surface biosynthesis and architecture were identified using non-targeted transposon tagging (Luisser *et al.*, 1997).

Endogenous transposons have been recently identified in *N. crassa*. Tad, a nuclear retrotransposon, has only been found in wild isolates from the Ivory Coast and can not be used for insertional mutagenesis due to its instability (Kinsey and Helber, 1989 and Kinsey, 1990). The much smaller transposon, Guest, was recently found by Yeadon and Catcheside (1995) but needs more characterization before it can be considered as a tool for mutagenesis. Use of

heterologous transposons may be possible in *N. crassa* if a suitable transposon tagging system could be developed. One such future possibility is a system currently being devised using Restless, an active Ac-like transposon from the filamentous fungus *Tolypocladium inflatum* (Kempken and Kuck, 1996). For the time being, this idea is speculative since its success depends both on the development of an efficient system in *T. inflatum* and its potential activity in the new host, *N. crassa*.

Although a transposon tagging system for *Neurospora* is not feasible at this time, some fungal mutants have been generated by insertional mutagenesis using selectable marker constructs. The first reported case of this in *N. crassa* was the accidental creation of a colonial mutant by Kinsey and Rambosek (1984) when DNA from a Lambda clone containing the *am* gene integrated at a random, non-homologous site in the genome. More recently, a *preg* mutant was recovered after the disruption of this gene by ectopic integration of plasmid vector DNA (Kang and Metzenberg, 1993).

Similar results have been reported for other fungi as well. *Aspergillus nidulans* mutants produced by heterologous insertion of transforming DNA have been created by Durrens *et al.* (1986) and the *uapA* and *wA* genes have been cloned by Diallinas and Scazzocchio (1989) and Tilburn *et al.* (1990) respectively. Insertional mutagenesis of the dimorphic basidiomycete, *Ustilago maydis*, led to the isolation of the *myp1* gene that is believed to be responsible for the pathogenic mycelial phenotype (Giasson and Kronstad, 1995).

The ideal transformation vector couples a way of selecting positive transformants with DNA that can be easily recovered. Selection of transformants can be simplified using dominant selectable markers: antibiotic resistance genes such as benomyl (Orbach *et al.*, 1986) or hygromycin (Staben *et al.*, 1989), expression of visual indicators such as blue/white screening using the β -galactosidase assay or conversion of a phenotype from auxotrophy to prototrophy (or vice versa).

The basis of this procedure requires the ectopic integration of characterized exogenous DNA into a gene resulting in insertional inactivation. Since the identity of the transforming DNA is known, it then serves as a marker to pinpoint the location of the disrupted gene. Once the tag is isolated, the flanking DNA can be sequenced and characterized.

This tool has particular utility for generating morphological mutants since new mutants are visually scored, especially when a selectable marker is incorporated into the tag. An additional benefit is that the effects of a disrupted gene are already apparent prior to molecular characterization. One disadvantage is that auxotrophic and lethal mutations may avoid detection.

N. crassa is an organism that encompasses a wealth of uncharacterized morphological and biochemical processes. Complex processes such as maintenance of the cell shape, cell wall, conidiation and their roles in *Neurospora*'s biochemical network can be elucidated with the help of a tool such as insertional mutagenesis.

A new mutant of *N. crassa* is created

With the prospect of creating and studying new *N. crassa* mutants, Vierula and Sallmen (unpublished) utilized the insertional mutagenesis strategy. Wild-type *N. crassa* spheroplasts were transformed with the hygromycin resistance construct from the PCSN43 plasmid vector presented in Figure 2. One of the mutants that was produced from this ectopic integration exhibited slow, highly restricted mycelial growth (Figure 3). Single colonies form rough-edged circular mounds (up to 8 mm in diameter) marked with a glistening, irregular surface. The orange-brown colour of the mutant differed from the pink-orange colour of wild-type. Given these phenotypic characteristics, the new mutant was named *sbr* (small, brown) pending identification of the disrupted gene.

Sbr is best described as a colonial, morphological mutant. Perkins *et al.* (1982) describe a colonial mutant as having radial, restricted mycelial growth (typically only a few millimetres in diameter) with a high incidence of hyphal branching and which is self-limiting on agar medium. To date there are at least 80 documented colonial mutant strains that vary in pigmentation, conidiation, texture, density, and fertility. Some of these strains have been characterized and are available from the Fungal Genetics Stock Center (FGSC) (Table 1).

Figure 2: Map of plasmid PCSN43: insertional mutagenesis tool and tag. This plasmid was used as an insertional mutagenesis tag to transform wild-type *N. crassa* strain 988. To create PCSN43 for use as a transforming vector in *N. crassa*, Staben et al (1989) used a modified version of plasmid pDH25 that was created by Cullen et al (1987). Staben ligated the 2.4 kb *Sa*I fragment of pDH25 (thick line) to the *Sa*I site of pBSSK+ (Stratagene). This *Sa*I fragment encodes the bacterial hygromycin B phosphotransferase gene (*hph*) (hatched arrow) under the control of promoter and terminator elements of the *Aspergillus nidulans trpC* gene (thick black regions) and is capable of conferring hygromycin resistance to the host when expressed. Some restriction sites are indicated by approximate location. Enzymes in bold cut at unique sites. [map adapted from Staben et al (1989)] All plasmid maps in the thesis were drawn with the aid of DNAMAN™ software ©1994-1997.

Figure 3: Colony morphology of 48-hour *Neurospora crassa* cultures of (a) wild-type and (b) *sbr* strains. Petri dishes containing Vogel's Minimal agar media were spot inoculated with conidial spores and hyphal fragments and were incubated at room temperature.

Table 1: Colonial mutants of *N. crassa*: description of phenotype and possible genotype. The list represents a sampling of some of the isolated colonial mutants that are available through the Fungal Genetics Stock Center (FGSC). The name of each mutant corresponds to the affected locus and is accompanied by a description of its phenotype, the mutagen responsible and a possible function of the gene affected. Information was summarized from Perkins et al (1982) and the Catalogue of Strains supplement to Fungal Genetics Newsletter no. 43 (1996). Abbreviations: *col* = *colonial*, MC = methylcholanthrene, na = not available, G6PD = Glucose-6-phosphate dehydrogenase, 6PD = 6-Phosphogluconate dehydrogenase, BP = beta-propiolactone

<i>col-1</i>	colonial, no macroconidia	MC	na
<i>col-2</i>	colonial	MC	altered structure of NADP-specific G6PD, accumulation of G6P, reduced NADPH level, linkage group VII
<i>col-3</i>	colonial	na	altered 6PD, reduced NADPH level, linkage group VII
<i>col-4</i> , (many alleles)	spreading colonial morphology with dense conidial balls high in slant	UV	reduced amount of cell wall peptides
<i>col-5</i> (same as <i>col-1</i> ?)	dense, non-conidiating, poorly pigmented, slow spreading reverts readily	UV	na
<i>col-6</i>	colonial, slow ascospore germination	X-rays	na
<i>col-8</i>	colonial, fluffs of hyphae at top of slant	UV	reduced amount of cell wall peptides
<i>col-12</i>	colonial, slow growth from ascospores	UV	na
<i>col-13</i>	usually non-conidiating but may form puffs of aerial hyphae	UV	na
<i>col-15</i>	resembles <i>col-13</i>	spontaneous	na
<i>col-16</i>	colonial	UV	na
<i>col-17</i>	very slow growing colonies	UV	na
<i>bal</i> (balloon)	colonial, conidia rarely produced at the top of the slant	spontaneous	defective in G6PD, linkage group II
<i>le-1</i> (lethal-ascospore)	ascospores appear normal but do not germinate, highly restricted colonial growth, lacks conidia and colour deepens with age	BP	linkage group IV

Objectives

Ectopic integration of the *hph* construct into an essential gene has manifested striking morphological abnormalities in *sbr*. However, what is the primary defect in *sbr*? Does the affected gene encode a structural protein, an enzyme of some biochemical pathway or a regulator of gene expression? Clues provided by its morphology, development and DNA sequence supply the answers to these questions. The greatest challenge is to interpret these answers and to integrate them into the final picture of the gene.

The aim of this project is to further characterize and understand *sbr*'s colonial morphology at the microscopic, reproductive and molecular levels.

Both fluorescence microscopy and Scanning Electron Microscopy (SEM) were employed to characterize the sub-cellular and cellular morphology of *sbr* compared to wild-type. DNA, chitin and tubulin distribution were examined to determine whether the disrupted gene has any phenotypic consequences on nuclei number and/or distribution, the size or shape of septa-determined cellular compartments and microtubule organization respectively. Once the gene is identified, these phenotypic clues may aid in assigning a gene function.

In order to discover other possible consequences of the mutated gene, the fertility of *sbr* was tested. Often, stages of the reproductive cycle of *N. crassa* are secondarily affected by genetic mutations. To test this, mating experiments involving *sbr* were performed to see whether it is sexually compatible and capable of producing viable ascospores.

The final step involved identification of the mutated gene by molecular characterization. This entailed the construction of a genomic library representing all of the genes of *sbr* in a vector that would make rescue of the tagged gene simple. Once retrieved, the DNA was subcloned to narrow down the size of the gene and then sequenced. A search in the genetic database for DNA or protein sequences that share significant homology to *sbr* gene was performed in hopes of gaining insight into its functional role.

II. MATERIALS AND METHODS

Culturing of Experimental Organisms

Neurospora crassa

The *sbr* mutant was originally created in 1992 by Sallmen and Vierula (unpublished) using insertional mutagenesis with plasmid vector DNA. The transformation vector, PCSN43 (Staben *et al.*, 1989) is described in Figure 2 of the Introduction. All other strains of *N. crassa* will be referred to by their Fungal Genetics Stock Center (FGSC) number (where applicable). The strains used in these experiments are summarized in Table 2. The summary includes information on genotype and mating type.

For growth of *N. crassa* on solid medium, hyphal fragments or spores were aseptically transferred to 16 x 150 mm glass culture tubes containing 5 ml of Vogel's Minimal Medium (VMM) [per litre: 15 ml 50x Vogel's stock medium (per litre: 127 g $\text{Na}_3\text{citrate}\cdot 2\text{H}_2\text{O}$, 250 g KH_2PO_4 , 100 g NH_4NO_3 , 10 g $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$, 5 g $\text{CaCl}_2\cdot 2\text{H}_2\text{O}$ predissolved in 25 ml dH_2O added dropwise), 1 ml biotin stock (5 mg biotin in 100 ml of 50% v/v ethanol), 1 ml trace elements (per litre: 50 g citric acid $\cdot 1\text{H}_2\text{O}$, 50 g $\text{ZnSO}_4\cdot 7\text{H}_2\text{O}$, 10 g $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2\cdot 6\text{H}_2\text{O}$, 2.5 g $\text{CuSO}_4\cdot 5\text{H}_2\text{O}$, 0.5 g $\text{MnSO}_4\cdot 1\text{H}_2\text{O}$, 0.5 g H_3BO_3 , 0.5 g $\text{Na}_2\text{MoO}_4\cdot 2\text{H}_2\text{O}$ dissolved in dH_2O), 15 g sucrose and 15 g agar] solidified on a slant. Tubes were incubated at room temperature (RT, 20-25°C) to promote growth.

In order to grow cultures in liquid medium, hyphal fragments recovered from solid media were resuspended in dH₂O (~ 5 ml) and inoculated into 50 ml of VMM liquid media. Cultures were incubated at RT with shaking at 200 rpm for 24 hours to 10 days.

***Escherichia coli* bacteria strains**

Strains used are summarized in Table 3.

Bacteria from either a glycerol stock stored at -70°C or from a colony on an LB plate were streaked across the surface of a 90 mm LB plate, containing 1.5% agar in 20 - 25 ml LB media (per litre: 10 g NaCl, 10 g bactotryptone, 5 g yeast extract, pH 7.5), in order to isolate single colonies. The plate was incubated at 37°C overnight in an inverted position.

To select for colonies with ampicillin resistance, the bacteria were streaked onto LBA plates (LB media supplemented with ampicillin to a final concentration of 100 ug/ml with 1.5% agar).

To grow bacteria in broth, a single colony isolated from a freshly prepared overnight plate culture was inoculated into 2-5 ml LB broth that may have been supplemented with antibiotics when selection of antibiotic resistant bacteria was desired. Cultures were grown overnight (approximately 16 hours) at 37°C with shaking at 250 rpm.

Table 2: *Neurospora crassa* strains used. Strains with a Fungal Genetics Stock Center (FGSC) number were originally obtained from the FGSC. Strain designations were taken from the Catalogue of Strains supplement to The Fungal Genetics Newsletter no. 43 (1996). na = not applicable

Table 3: Strains of *E. coli* used in experimentation. Both the genotype and any known antibiotic resistance genes are also indicated.

★ Sambrook *et al.* (1989)

◆ Stratagene catalog (1995)

★ Elledge *et al.* (1991)

wild-type	987	A	74-OR23-1A
wild-type	988	a	74-OR8-1a
<i>sbr</i>	na	a	na

DH5 α	na	<i>supE44</i> Δ <i>lacU169</i> (Φ 80 <i>lacZ</i> Δ M15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i> *
XL1-Blue (Stratagene)	Tetracycline	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44</i> <i>relA1 lac</i> [F' <i>proAB lac</i> ^r Z Δ M15 Tn10 (Tet ^r)] ♦
XL1-Blue MRA (Stratagene)	na	Δ (<i>mcrA</i>)183 Δ (<i>mcrCB-hsdSMR-mrr</i>)173 <i>endA1 supE44 thi-1 gyrA96 relA1 lac</i> ♦
XL1-Blue MRA (P2) (Stratagene)	na	XL1-Blue MRA (P2 lysogen) ♦
BB4	Tetracycline	<i>e14</i> ⁺ (<i>McrA</i> ⁺) <i>hsdR514 supE44 supF58</i> <i>lacY1 galk2 galT22 metB1 trpR55</i> Δ (<i>argF-lac</i>)U169 [F' <i>lac</i> ^r Z Δ M15 <i>proAB</i> Tn10 (Tet ^r)] ♦
BNN132	Kanamycin	<i>endA1 gyr96 hsdR17 supE44 relA1</i> Δ (<i>lac-proAB</i>) [F' <i>traD36 proAB</i> ⁺ <i>lac</i> ^r Z Δ M15] λ KC(kan-cre) ★

Mating Compatibility of *sbr*

Matings between three strains of *N. crassa* were attempted using procedures adapted from Pandit and Russo (1993). Wild-type strains 987 and 988 (mating types A and a resp.) were crossed with each other and *sbr* (mating-type a) on synthetic crossing media (SC media per litre: 1 g KNO₃, 0.72 g K₂HPO₄, 0.48 g KH₂PO₄, 0.48 g MgSO₄•7H₂O, 0.1 g CaCl₂, 0.1 g NaCl, 100 ul d-Biotin and 100 ul trace elements in dH₂O) with 1.5% agar. 20 ml of media were added to petri plates and allowed to solidify. Once hardened, 1 cm x 2 cm squares of 3MM filter paper were set on the surface to act as the sole carbon source.

N. crassa cultures, at least 7 days old, were vortexed with 5 ml dH₂O. The conidial suspension was collected with a Pasteur pipette and added dropwise (1-2 drops) onto the filter paper squares in the mating plates. For each mating experiment, an aliquot of each of the two strains was added to opposite sides of the plate. In the centre of the plate, aliquots of both strains were mixed. Three crosses were performed: 987 x 988, 987 x *sbr*, 988 x *sbr*. The plates were incubated at RT to promote growth. Mating structures were viewed with a Nikon binocular dissecting microscope.

Ejected ascospores were collected by inverting the mating plate over a second plate containing 25 ml of TE medium (100 mM Tris-Cl; pH 8, 2 mM EDTA, 3% (w/v) agar). Each of the 8 spores from a single ascus were transferred individually to their own culture tube containing 2 ml of VMM solid

spores were heat-activated in a 65°C waterbath for 60 minutes. Ascospores were allowed to germinate at RT.

Microscopic Characterization of *N. crassa*

Nuclei and septa staining

N. crassa liquid cultures of *sbr* and 987 were grown in 50 ml VMM media at RT for 16 hours. Cells were harvested and fixed in a solution of 3.7% formaldehyde in PBS (per litre: 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄ and 0.25 g KH₂PO₄ in dH₂O, pH 7.4) with 0.2% (v/v) Triton X-100 at RT for 45 minutes. Distilled water was used to wash the cells before they were stained with 10 ug/ml Calcofluor (Sigma), 800 ng/ml DAPI in dH₂O. After a 5 minute incubation at RT, the cells were rinsed with dH₂O.

A 10 ul sample was dispensed onto a pretreated coverslip and allowed to dry. Coverslips were pretreated by etching in 3 M HCl for 10 minutes. They were then rinsed five times in dH₂O, rinsed once in acetone and then coated with a thin film (about 10 ul per coverslip) of poly-L-lysine (1 mg/ml). Coverslips with dried cell samples were mounted in Vectashield (Vector) using 20 ul per coverslip. Edges of the coverslip were sealed to the glass slide with clear nail polish. Protocols were taken from Harris *et al.* (1994).

Samples were viewed within 48 hours of preparation under the Zeiss Photomicroscope III Fluorescence microscope equipped with a mercury burner that delivered UV light at 340 nm.

Immunofluorescent labelling of microtubules

N. crassa cells were processed for indirect immunofluorescence microscopy using the method of Oakley *et al.* (1990).

N. crassa liquid cultures of *sbr* and 987 were grown in 50 ml VMM medium for 16 hours at RT. Cells were harvested and then fixed in a solution of 3% formaldehyde in PEM [100 mM Pipes, 25 mM EGTA, 5 mM MgCl₂, pH adjusted to 6.9 and dimethylsulfoxide (DMSO) added to 5% (v/v)] at RT for 90 minutes. Cells were washed three times for 5 minutes each with 2 ml PEM. They were then resuspended in an equal volume of a mixture of Novozyme 234 (10 mg/ml), 2.5 mM EGTA, egg white (~1 ml) and protease inhibitors (1 ug/ml each of Aprotinin, PMSF, TPCK, Leupeptin, Pepstatin and Benzamidine). After incubation at RT for 90 minutes, cells were washed four times as before.

Cells were extracted in 2 ml 1% Triton X-100 (in PEM) for 20 minutes followed by four washes in PEM. They were then resuspended three times for 5 minutes each in 2 ml fresh sodium borohydride (1 mg/ml). Three washes in PEM were performed before cells were resuspended in 1 ml PEMBAL (PEM with 0.1 M L-lysine, 1% globulin free BSA, 0.1% sodium azide) for 30 minutes.

Microtubules were labelled with the primary antibody (1/150 dilution of anti-tubulin YOL IgG rat monoclonal antibodies) by transferring 50 μ l of cell suspension to a microfuge tube containing 50 μ l PEMBAL with antibody and incubating at RT for 90 minutes.

Cells were rinsed four times for 5 minutes each with 250 μ l PEM. The secondary antibody, CY3 (cyanine)-conjugated donkey anti-rat IgG, was added to a final concentration of 1/150 and incubated at RT for 90 minutes. The labelled cells were rinsed four times with 2 ml PEM. Aliquots of the cell suspension (10 μ l) were added to pretreated coverslips and allowed to dry. Each coverslip was mounted onto a 20 μ l drop of Vectashield on a glass slide and the edges of the coverslip were sealed with clear nail polish.

Samples were viewed within 48 hours of preparation using the Zeiss Photomicroscope III Fluorescence microscope. The light source was provided by a mercury burner and cells were viewed on the rhodamine channel (550 nm).

Scanning Electron Microscopy (SEM)

N. crassa slant cultures of *sbr* and 987 were vortexed with 2 ml sterile dH₂O in order to free hyphae and spores. A few drops of each suspension were added to plates containing VMM solid media overlaid with sterile Spectra/Por (MWCO 12,000-14,000) dialysis membrane. Cultures were allowed to grow at RT for 3 to 7 days.

Small squares ($\sim 0.5 \text{ cm}^2$) of dialysis membrane containing mycelium were cut, transferred to a block and quick-frozen in liquid nitrogen. Samples were coated with pure aluminum and then viewed using the JEOL JSM 6400 Scanning Electron Microscope.

DNA Analysis Methods

Extraction of *N. crassa* genomic DNA

Liquid cultures (50 ml) of wild-type and *sbr* were grown at RT for 2-7 days with shaking at 200 rpm. Mycelia were collected under vacuum by filtering onto 1 MM filter paper lining a Buchner funnel. They were washed with cold dH_2O , dried again by filtration, collected, placed into 15 ml polypropylene tubes, and frozen at -70°C .

Frozen mycelia were ground with a pinch of acid-washed sea sand using a cold mortar and pestle. About 3 ml of extraction buffer (0.2 M Tris; pH 8.5, 0.25 M NaCl, 25 mM EDTA, 0.5% SDS) were added to the ground material. Pronase was added to a final concentration of 0.5 mg/ml and incubated at 37°C for 30 minutes. One volume of phenol:chloroform (1 : 1) was mixed with the solution and centrifuged at 13,000 rpm in the SM-24 rotor of the Sorvall RC-5B fixed-angle centrifuge, at 4°C for 60 minutes. The upper aqueous layer was transferred to a new tube and RNase A was added to a final concentration of 300 ug/ml. After incubation at 37°C for 30 minutes, 1 volume of chloroform :

isoamyl alcohol (25 : 1) was added, mixed, and the solution was spun at 7,500 rpm, at 4°C for 15 minutes. DNA in the upper aqueous phase was precipitated by adding 0.54 volumes of cold isopropanol. The tube was left on ice for 1 hour. DNA was pelleted at 7,500 rpm at 4°C for 10 minutes. The pellet was washed with 70% ethanol and dried before it was resuspended in 100 µl TE (10 mM Tris; pH 8, 1 mM EDTA) and stored at 4°C. (Raeder and Broda, 1985)

DNA concentrations were estimated using the absorbance measured at the 260 nm wavelength generated by the Hewlett Packard 8452A Diode Array Spectrophotometer. DNA retrieved using this extraction method was suitable for digestion with restriction enzymes, Southern hybridization, and cloning.

Extraction of plasmid DNA from *E. coli*

Broth cultures (2 ml) of bacteria containing the desired plasmid were grown overnight. The culture was transferred to a 1.5 ml microfuge tube and centrifuged at 14,000 rpm for 2 minutes. The supernatant was discarded and the cell pellet was resuspended in buffer. One of the following methods was used to extract plasmid DNA from contaminating proteins, bacterial DNA and RNA.

Wizard™ mini-preparations (Promega):

When high DNA purity was desired for projects such as sequencing, this alkaline-lysis method was used. The manufacturers instructions were followed (for details consult that protocol).

PlasmidPURE™ mini-preparations (Sigma):

This kit also used an alkaline lysis method and delivered high DNA purity but tended to have higher yields than Wizard™. The manufacturers instructions were followed.

Rapid plasmid mini-preparations:

When this type of alkaline-lysis preparation was used it delivered good yields but with lower purity. The cell pellet was resuspended in 100 ul TE buffer (pH 8) and remained at RT for 5 minutes. Cells were lysed with 200 ul of 0.2 M NaOH, 1% SDS solution and left on ice for 5 minutes. The lysis solution was neutralized by adding 150 ul of 3 M potassium acetate (pH 4.8), vortexed at maximum speed for 2 seconds and returned to ice for 5 minutes. Bacterial DNA was pelleted by centrifugation at 14,000 rpm at RT for 4 minutes. Supernatant containing plasmid DNA was purified by PCI extraction followed by ethanol precipitation. After 1 hour, the plasmid DNA was pelleted at 14,000 rpm for 4 minutes. The pellet was washed 70% ethanol, dried and then resuspended in 20–50 ul dH₂O or TE. For removal of contaminating RNA, RNase A was added to a final concentration of 10 ug/ml and was incubated at RT for 30 minutes.

Digestion with restriction enzymes

A typical digest with a restriction enzyme involved 1-5 ug of DNA digested with 1 ul of enzyme stock (5-20 U/ul) with 1/10 final volume of 10x enzyme buffer, 1/10 final volume of acetylated bovine serum albumin (aBSA), which was

only required by some enzymes, in a reaction volume of 20 μ l made up with dH_2O . Enzymes were purchased from New England Biolabs (NEB), Gibco/BRL or Boehringer Mannheim (BM) and the appropriate buffering system was used according to the manufacturers guidelines. Reactions were incubated at 37°C for 2 hours or overnight unless otherwise specified. Reactions were stopped by freezing or adding 6x loading buffer I (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 40% (w/v) sucrose in dH_2O) containing 10 mM EDTA.

Agarose gel electrophoresis

Agarose gel electrophoresis was performed essentially as described by Sambrook *et al.* (1989). The gels varied in agarose concentration from 0.4% to 1.2% depending on the size of the fragment of interest. Agarose (Boehringer Mannheim) was dissolved by heating in 0.5x TBE (45 mM Tris-borate, 1 mM EDTA). After the solution had cooled to $< 60^\circ\text{C}$, 0.05% (w/v) ethidium bromide was added prior to casting the gel. The electrophoresis unit was filled with enough 0.5x TBE to just cover the surface of the gel.

DNA samples (0.1 μg -0.5 μg DNA) were mixed with 2 μ l of 6x loading buffer I and dH_2O to a final volume of 12 μ l and the entire volume was loaded into the well of an agarose gel. Commercially-prepared Lambda phage DNA cut with *Hind*III (NEB) was diluted (0.6 μ l of 500 $\mu\text{g}/\text{ml}$ stock in 0.9 μ l, 10x NEB 2 buffer, 7.8 μ l dH_2O and 2.7 μ l 6x loading buffer containing 10 mM EDTA) and loaded into a well to act as a size marker.

Voltage was applied to the electrophoresis unit using the Bio-Rad Model 200/2.0 power supply at approximately 5 V/cm. After the DNA had migrated an appropriate distance, the gel was viewed using 254 nm UV light from a hand-held UV wand or on a transilluminator (Fotodyne Inc.) equipped with a Polaroid camera. Band sizes were estimated with the aid of DNAfrag™ software ©1989 (Schaffer *et al.*, 1981).

DNA recovery from agarose gels

Recovery of DNA from agarose gels was accomplished using the protocol of Boyle and Lew (1995). Agarose gels were viewed using long wave UV light (366 nm) from a hand-held UV wand. The desired band was excised with a razor blade. The weight of the excised gel slice was determined after transfer to a pre-weighed microfuge tube. The slice was dissolved in 2.5-3 volumes of 6 M NaI at 50°C. Silica matrix suspension (5 ul) was added, mixed by tapping and left on ice for 5 minutes. The matrix, with bound DNA, was pelleted by centrifugation at 14,000 rpm for 5 seconds in the Eppendorf centrifuge 5415 C. The pellet was resuspended in 400 ul of cold, wash solution (50 mM NaCl, 10 mM Tris-Cl, 2.5 mM EDTA, 50% (v/v) ethanol), repelleted by centrifugation, and the supernatant was discarded. This was repeated twice before the DNA pellet was resuspended in 10 ul sterile dH₂O. The DNA was released from the silica matrix by incubation at 50°C for 5 minutes. The silica beads were pelleted when centrifuged for 30 seconds and the DNA in the supernatant was transferred to a new tube for storage at 4°C.

Construction of *sbr* genomic DNA library

A *sbr* genomic DNA library was created by ligating *sbr* genomic DNA fragments to the λ FixII replacement vector (Stratagene). The genomic DNA insert was partially digested with the 4-bp cutter restriction enzyme, *Sau*3A1, in order to produce large, clonable-sized fragments that represented the genome in as few clones as possible. The *Sau*3A1 ends were partially filled-in with the first two nucleotides leaving 5'-GA-3' overhangs.

The λ FixII vector was commercially prepared by digestion with *Xho*I and was partially filled-in with the first two nucleotides of the *Xho*I site leaving 3'-CT-5' overhangs that were compatible with the insert. The vector was able to accommodate inserts from 9-23 kb, which caused it to become red⁻ gam⁻, and was not capable of growth on the P2 lysogenic strain of *E. coli*, XL1-Blue MRA (P2).

Procedures used to construct the library were adapted from protocols accompanying the λ FixII vector kit (Stratagene) unless otherwise specified.

Each partial digestion reaction consisted of approximately 8.5 ug of *sbr* genomic DNA, 1x *Sau*3A1 buffer, 0.1 ug/ul aBSA, 5 ul of a 1200x dilution of *Sau*3A1 enzyme (total of 0.02 U/reaction) in a total reaction volume of 50 ul made up with dH₂O. Four identical reactions were prepared and incubated at 37°C for 1.5 hours. The enzyme was heat inactivated at 65°C for 25 minutes. This procedure was adapted from the Promega Protocols and Applications Guide (1991).

Size selection of DNA using sucrose gradients

Non-linear sucrose gradients were prepared using an apparatus adapted from Dreyer *et al.* (1991). Sterile solutions of 10% and 40% sucrose (w/v) were made in sucrose buffer (10 mM Tris-Cl, 10 mM NaCl, 1 mM EDTA, pH 8).

A 20 ml syringe barrel with a small, 1 cm long magnetic stir bar was positioned above a stir plate using a retort stand. A 30 cm long piece of plastic tubing was attached to the end of the barrel and served as a link between the gradient-making syringe and the 13.5 ml polyallomer centrifuge tube. At the end of the tubing, a silanized glass capillary tube (1.5-1.8 x 100 mm) was inserted and reached to the bottom of the centrifuge tube. A clamp on the tubing was used to adjust the flow rate of the sucrose solution.

The 10% sucrose solution (6.5 ml) was added to the bottom of the syringe before the plunger (with a 1 mm² rectangular notch cut out) was inserted down to meet the first solution. The flow of the gradient was started and 6.5 ml of 40% sucrose solution was added into the chamber above the plunger. A gradient from 10% to 40% (top to bottom) was gradually formed in the polyallomer centrifuge tube.

The centrifuge tube was topped up with 200 μ l (~30 μ g) of the *sbr* partially-digested genomic DNA solution. The sucrose gradient tube was loaded into a Beckman swinging-bucket SW40 rotor and spun in a Beckman Model L5-50 Ultracentrifuge at 22,000 rpm at 20°C for 22 hours.

After centrifugation, the gradient was collected from the bottom of the polyallomer tube (which contained the largest fragments) to the top in 400-500 ul fractions.

DNA in each fraction was precipitated by adding approximately 400 ul TE (pH 8), 80 ul of 3M sodium acetate (pH 5.2), 0.5 ul of yeast tRNA (10 ug/ul) and 600 ul of isopropanol. The mix was left on ice for a minimum of 3 hours. The fractions were centrifuged at 14,000 rpm for 10 minutes. Pellets were rinsed with 70% ethanol, dried and resuspended in 30 ul TE.

The concentration of DNA was too low in each individual fraction so fractions 1-9 and 10-18 were pooled, precipitated and resuspended in a volume of 50 ul TE. After visualization of DNA on a 0.4% agarose gel, the pooled sample containing fractions 1-9 was prepared for ligation to the λ FixII vector.

The pooled sample (approximately 15 ug DNA) was added to 20 ul of 10x Klenow buffer, 3.5 ul each of 10 mM dATP and dGTP, 7 ul Klenow enzyme (NEB, 2 U/ul) and dH₂O to a total volume of 200 ul. The reaction was incubated at 37°C for 30 minutes in order to partially fill-in the ends of the insert. PCI extraction was performed followed by ethanol precipitation. The pellet was resuspended in 30 ul dH₂O with an approximate DNA concentration of 0.5 ug/ul.

About 1.5 ul of the partially filled-in *sbr* insert (~1 ug) was added to 0.5 ul (0.5 ug) λ FixII vector (Stratagene), 0.5 ul of 10x ligase buffer (500 mM Tris-Cl; pH 7.5, 70 mM MgCl₂, 10 mM DTT), 1.5 ul dH₂O, and 1.0 ul T4 DNA ligase (5

U/ul) to make a ligation reaction of 5 ul. The reaction was incubated overnight at 12°C to promote ligation of the insert and vector DNA.

In order to package the ligated plasmid into λ heads, the Gigapack III Gold Packaging Extract (Stratagene) was used and the manufacturers procedure was followed. To the immediately thawed packaging extract, 4 ul of ligation mix (<1.5 ug DNA) was added and mixed with a pipette tip. The reaction was incubated at RT for 99 minutes. A total of 500 ul SM buffer (per litre: 5.8 g NaCl, 2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 50 ml of 1 M Tris-Cl; pH 7.5, 5 ml of 2% (w/v) gelatin and dH_2O) and 20 ul chloroform were added and mixed. The tube was centrifuged briefly to sediment debris prior to transferring supernatant to a new tube for storage at 4°C.

Preparation of host bacteria for phage infection

A single colony from a freshly prepared plate of the XL1-Blue MRA (P2) strain of *E. coli* was inoculated into 3 ml LB broth supplemented with 10 mM MgSO_4 and 0.2% (w/v) maltose. Cells were grown at 37°C overnight without exceeding an OD_{600} of 1. The cells were pelleted at 2,000 rpm in an SS-34 rotor at 4°C for 10 minutes. Cells were resuspended in 1.5 ml of 10 mM MgSO_4 after discarding the supernatant. The 10 mM MgSO_4 was used to dilute the bacteria to an OD_{600} of 0.5. Cells were stored at 4°C for a maximum of 48 hours.

Infection of bacteria with recombinant phage

The packaged DNA was diluted (i.e. 10^0 to 10^{-6}) in 100 μ l aliquots of SM buffer. To infect the host bacteria, 200 μ l of host cells XL1-Blue MRA (P2) at OD₆₀₀ of 0.5 were mixed with one of the above dilutions per reaction. Each of the reactions (including a negative control) was incubated at 37°C for 20 minutes with gentle shaking. Each sample was added to 4 ml of 48°C LB top agar (0.7%), mixed, poured onto a prewarmed 90 mm LB plate and incubated overnight at 37°C.

Amplification of *sbr* genomic DNA library

XL1-Blue MRA (P2) cells were prepared as before and were diluted to OD₆₀₀ of 0.5 in 10 mM MgSO₄. The cells (600 μ l) were infected by adding 25 μ l (2,000 pfu/ μ l) of undiluted packaged insert and incubated at 37°C for 20 minutes with gentle shaking. The infection reaction was added to 6.5 ml of top LB agarose (0.7%) at 48°C, mixed, layered onto a 150 mm LB plate, and incubated at 37°C for 8 hours.

To recover the phage library, the plates were flooded with 9 ml of SM buffer and were rocked gently at 4°C for 24 hours. The bacteriophage suspension was harvested from the plate using a sterile Pasteur pipette and pooled into a 15 ml polypropylene tube. The plate was rinsed with an additional 2 ml of SM buffer and then was pooled into the same tube. Chloroform was added to a final concentration of 5% and the cell debris was concentrated by

centrifugation at 2,000 rpm at 4°C for 10 minutes. The supernatant containing the bacteriophage was recovered and DMSO was added to 7% (v/v). The suspension was aliquotted into 1.5 ml microfuge tubes and stored at -70°C.

Plaque-lift

Unamplified bacteriophage from the *sbr* genomic DNA library (50,000 pfu) were allowed to infect 600 ul of freshly prepared XL1-Blue MRA (P2) cells at 37°C for 20 minutes. The suspension was added to 6.5 ml top LB agar (0.7%) at 48°C and was poured onto a 150 mm LB plate and incubated at 37°C for 6.5 hours.

After incubation, the plate was chilled at 4°C to harden the top agar. DNA within bacteriophage plaques were transferred to Hybond-N nylon membrane (Amersham) by setting the membrane onto the surface of the plate for 60 seconds while marking the orientation of the membrane relative to the plate. DNA from the same plate was transferred a second time to a "sister" membrane for 2 minutes.

After transfer, the membranes were denatured for 5 minutes in 1 M NaCl, 0.4 M NaOH, neutralized for 5 minutes in 1 M Tris-Cl; pH 7.4-7.5, 1.5 M NaCl and were washed for 30 seconds in 2x SSC (20x SSC: 175.3 g NaCl, 88.2 g sodium citrate and dH₂O to 1 L, pH 7) at RT. The membranes were allowed to dry on paper towel for 30 minutes prior to oven drying at 80°C for 1.5 hours.

Membranes were then used in hybridization experiments and the plaque-plate was stored at 4°C.

Southern transfer

The protocol for a Southern transfer was adapted from Sambrook *et al.* (1989). After agarose gel electrophoresis of the DNA samples, the gel was soaked in 1 M NaCl, 0.4 M NaOH at RT for 45 minutes with gentle rocking to denature and separate DNA into single strands. This was followed by neutralization in 1 M Tris-Cl; pH 7.4, 1.5 M NaCl for another 45 minutes.

A support for the transfer was constructed by placing a 1 cm high Plexiglas block wrapped in 3MM filter paper into a 3 L glass baking dish. Enough 20x SSC was added to the dish to just reach the top of the Plexiglas support. The gel was arranged with wells facing down onto the support and a piece of nylon membrane (Amersham), slightly larger than the gel, was set on top. Two pieces of 3MM filter paper, the same size as the gel, were stacked on top of the membrane followed by about a 10-15 cm high stack of paper towels that were slightly smaller than the gel. The capillary transfer of the 20x SSC buffer through the gel and up into the paper towels was allowed to proceed for a minimum of 14 hours.

After transfer, the membrane was briefly soaked in 6x SSC to remove residual agarose and then was dried in an 80°C oven for 80 minutes. The transfer efficiency was checked using UV light before storage at RT.

Radiolabelling a DNA probe

In order to label a probe that is required for hybridization experiments, a Ready to go-DNA labelling bead (-dCTP) kit (Pharmacia) was used and the manufacturers instructions were followed.

Linear DNA (25-50 ng) in less than 45 μ l TE (pH 8) or dH₂O was denatured by heating at 95-100°C for 5 minutes and quick-chilled immediately in an ice bath for 5 minutes. The DNA was added to the Reaction mix bead before the [α -³²P] dCTP (2-5 μ l) was added. The final volume was brought up to 50 μ l with dH₂O. The contents were mixed gently and then incubated at 37°C for 30 minutes.

The unincorporated nucleotides were removed using spun column chromatography (Sambrook *et al.*, 1989). The bottom of a 1 ml plastic syringe was plugged with glass wool. The column was filled with Sephadex G-50 beads equilibrated in 1x STE (100 mM NaCl, 20 mM Tris-Cl; pH 7.5, 10 mM EDTA). The gel matrix was packed down to a volume of 0.9 ml by repeated spins at 3,000 rpm in a swinging-bucket centrifuge. STE (50 μ l) was added to the radiolabelling reaction and the entire solution was loaded onto the top of the spun column. The column was centrifuged at 3,000 rpm for 4 minutes forcing the radiolabelled probe to be eluted from the column into a 1.5 ml tube. The probe was stored at -20°C until it was required.

Hybridization using radiolabelled DNA probes

The nylon membrane with transferred DNA was prehybridized by a brief dip in 0.1% (w/v) gelatin solution in dH₂O. After the membrane was dry, it was inserted into a glass screw-cap hybridization tube. Hybridization solution (6x SSC, 5x Denhardts, 0.5% (w/v) SDS) was added at 0.05 ml per cm² of membrane. The tube was loaded into the rotating Hybridization Incubator Model 310 (Robbins Scientific) and incubated at 65-68°C for 1 hour.

The DNA probe (500,000 cpm/ml hybridization solution) was combined with salmon sperm DNA (20 ug/ml hybridization solution) and was denatured at 95-100°C for 5 minutes. The mixture was quick-chilled and then added to the hybridization tube. Hybridization was performed at 65-68°C for a minimum of 12 hours.

Stringency washes were performed at the conclusion of the incubation. The hybridization solution was discarded, 50 ml of 2x SSC were added to the hybridization tube and incubated at 68°C for 15 minutes. The procedure was repeated using 2x SSC, 0.1% SDS for 60 minutes followed by a wash of 50 ml of 0.1x SSC for 15 minutes.

The membrane was allowed to air dry slightly prior to exposure to Kodak autoradiographic film. The membrane and film were stored at -20°C in a light-tight cassette until the desired signal was reached.

Developing autoradiographic film

After sufficient exposure of the film to a radiolabelled membrane, the film was developed for 1-5 minutes in GBX developer and replenisher (Kodak). The film was rinsed in water for 1-2 minutes and it was then soaked for 5 minutes in GBX fixer and replenisher (Kodak). The film was rinsed for 5 minutes in water before it was left to dry.

Screening *sbr* genomic DNA library

About 5.0×10^4 clones on a single plaque-lift membrane were screened with a 600 bp ^{32}P -labelled probe. In order to make the probe, plasmid PCSN43 was digested with *Sa*I and *Eco*RI and the 600 bp fragment (corresponding to the *trpC* promoter and part of the hygromycin gene) was recovered from an agarose gel, labelled and hybridized to the membrane.

Once a putative clone was identified, it was cored from the agar plate and resuspended in 1 ml of SM buffer containing 2 drops of chloroform. Three subsequent rounds of screening were required in order to isolate a pure population of phage that showed homology to the PCSN43 probe. This clone was named $\lambda\text{sbr-4TC2}$ and was stored at 4°C.

The Plate Lysate method (Sambrook *et al.*, 1989) was used to extract the DNA from the λ bacteriophage clone that was isolated from the final screening of the *sbr* genomic DNA library.

50 μ l of the phage clone suspension, λ sbr-4TC2, were used to infect 100 μ l of XL1-Blue MRA (P2) cells (OD_{600} of 0.5) at 37°C for 20 minutes. The mixture was added to 4 ml top LB agarose (0.7%) and poured onto a 90 mm LB plate containing 1.5% agarose. After incubating the plate overnight at 37°C, 3 ml of λ Diluent (10 mM Tris-Cl; pH 7.5, 10 mM $MgSO_4$) were added to the plate surface. The plate was incubated at RT for 1.5 hours with gentle rocking. The λ Diluent was harvested and transferred to two 15 ml polypropylene tubes and spun at 5,700 rpm in an SS-34 rotor at 4°C in order to pellet bacterial cell debris. The supernatant was transferred to a microfuge tube and 1 μ l of RNase A (1 mg/ml) and 1 μ l of DNase I (5,000 U/ml) were added before incubation at 37°C for 30 minutes. About 600 μ l of a solution containing 20% PEG 8000 and 2 M NaCl in λ Diluent buffer were added and the mixture was incubated in ice water for 60 minutes. The mixture was centrifuged at 12,000 rpm at 4°C for 10 minutes. The resulting bacteriophage pellet was resuspended in 500 μ l TE (pH 8) by vortexing. Any debris incapable of resuspension was discarded. The mixture was incubated at 68°C for 6 minutes after the addition of 5 μ l of 10% (w/v) SDS. 10 μ l of 5 M NaCl were added and the bacteriophage DNA was purified by PCI extraction. Isopropanol (500 μ l) was used to precipitate the DNA at -70°C for 30 minutes. Centrifugation at 14,000 rpm for 15 minutes recovered the DNA and 70% ethanol was used to wash the pellet. The λ bacteriophage clone DNA was finally resuspended in 25 μ l TE (pH 8) and stored at 4°C.

Transformation of *E. coli*

Protocols from Sambrook *et al.* (1989) were followed. In order to make cells competent for transformation, a single colony of *E. coli* DH5 α from a fresh plate culture was inoculated into 2 ml of LBA broth and grown overnight at 37°C with shaking. The culture (1 ml) was added to 100 ml of LB and grown at 37°C until an OD₆₀₀ of 0.5 was reached.

Cells were transferred into two cold, 50 ml polypropylene tubes and incubated on ice for 10 minutes. The cells were pelleted at 4,000 rpm in an SS-34 rotor at 4°C for 10 minutes. The supernatant was discarded and 2 ml of cold 0.1 M CaCl₂ were used to resuspend cells. DMSO and glycerol were added to final concentrations of 3% and 15% respectively. Competent cells were dispensed in 200 μ l aliquots into 2 ml screw-cap tubes and stored at -70°C.

Insert and vector DNA were linearized with the appropriate restriction enzymes and then purified by either PCI or silica matrix extraction. Ligation was performed by mixing about 150 ng of insert DNA, 50 ng of vector DNA, 1/10 final volume of 10x T4 DNA ligase buffer (NEB), 1 μ l of T4 DNA ligase (5 U/ μ l) and dH₂O up to 20 μ l. The ligation reaction was incubated at 12°C for at least 20 hours.

Approximately 50 ng of transforming DNA (in no more than 10 μ l) were added to a 200 μ l aliquot of competent cells. The suspension was mixed and held on ice for 30 minutes. The transformation reaction was incubated at 37°C for 45 minutes after the addition of 800 μ l LB broth. Up to 200 μ l of the reaction

were spread onto LBA plates and incubated overnight at 37°C. The viable colonies were screened for bacteria harboring recombinant plasmids using blue-white screening. Bacteria were streaked onto 90 mm LB plates containing the appropriate antibiotic (if required), 10 ul of 0.1 M IPTG and 50 ul of 2% X-gal. Plates were incubated overnight at 37°C. Colonies carrying putative recombinant plasmids appeared white while colonies with non-recombinant plasmids were blue due to a colorimetric reaction between IPTG and a functional β -galactosidase gene product.

Screening λ BARGEM7 *N. crassa* genomic DNA library

The λ BARGEM7 *N. crassa* genomic library (Pall and Brunelli, 1994) was obtained from the FGSC and screened for the wild-type copy of the disrupted *sbr* gene. The library contained inserts with average sizes of 5-6 kb that were cloned into the *EcoRI* site of the fungal transformation vector pBARGEM7-2 (Pall and Brunelli, 1993) that had λ arms attached at the *lox-NotI-lox* site. The vector had many useful features including: *bar* and *amp^r* genes as selectable markers, *Cre/lox* mediated recombination (automatic excision), a polylinker containing unique restriction sites and the *lacZ α* sequence from pGEM7Zf(+) for enabling blue/white screening.

200 ul of freshly prepared *E. coli* BB4 cells diluted to an OD₆₀₀ of 0.5 in 10 mM MgSO₄ were mixed with 1 ul of a dilution from the λ BARGEM7 wild-type genomic DNA library (10⁻³ to 10⁻⁶ dilutions in SM buffer). Cells and phage were

incubated at 37°C for 20 minutes and then added to 4 ml of LB top agar. The mixture was poured onto a LB plate and incubated overnight at 37°C.

Plaque-lifts were performed on plates having between 30 and 300 pfu. Membranes were incubated with a 600 bp ³²P-labelled probe from plasmid KSSBR0 that was cut with *Ava*I (containing sequence believed to originate from *sbr*) at 65°C for at least 12 hours for hybridization. For simplicity, this radiolabelled probe will be referred to as Ava600 throughout the remainder of the thesis.

Membranes were washed and exposed to autoradiographic film for 16-48 hours before developing. Positive hybridizing plaques were cored from the original agar plate and stored at 4°C in 1 ml of SM buffer containing 2 drops of chloroform.

Two subsequent screenings were necessary to isolate of pure sample of a phage clone that showed homology to the KSSBR0 probe.

Excision of plasmids from λBARGEM7 cloning vector

The automatic excision feature dictated by Cre/*lox* mediated recombination allowed easy recovery of the recombinant DNA. When *E. coli* strain BNN132 carrying the *cre* gene was infected with the recombinant phage, the plasmid was separated from the λ vector. The plasmid vector carrying the genomic insert was selected by blue/white screening of *E. coli* colonies. The details of this procedure are found below.

200 μ l of BNN132 *E. coli* cells at OD₆₀₀ of 0.5 were infected with 1 μ l of a dilution (10^0 to 10^{-2}) of the phage clone isolated from the λ BarGem7 library ($\sim 2 \times 10^5$ pfu/ μ l) at 30°C for 30 minutes. LB broth (2 ml) was added to each tube and they were incubated at 30°C for 1 hour with shaking. Aliquots of 100 μ l and 50 μ l from each tube were spread onto LBA plates and were incubated at 37°C overnight. Selected viable colonies were inoculated into 2 ml of LBA broth and grown overnight in preparation for plasmid DNA extraction.

Screening nitrate induced *N. crassa* cDNA library

A nitrate-induced cDNA library constructed by R. H. Garrett was made from poly(A)⁺ RNA isolated from mycelia grown on VMM supplemented with 20 mM glutamine. The culture was induced for nitrate assimilation by transfer to a medium containing 20 mM nitrate as the sole nitrogen source. cDNA was ligated to *EcoR*I adapters and cloned into λ ZAPII cloning vectors.

Approximately 1.5×10^5 clones from the cDNA library (previously immobilized on nylon membranes) were screened using the Ava600 probe.

Three subsequent rounds of screening were performed to isolate a pure population of a single clone using XL1-Blue MRA cells as the host.

Excision of phagemids from λ ZAPII cloning vector (cDNA clone)

The λ ZAPII phage clone isolated from the third screening of the cDNA library was named cNSBR and was stored in SM buffer at 4°C. In order to excise the pBluescript phagemid from the λ clone, 200 μ l of XL1-Blue cells at

OD₆₀₀ of 1, 200 ul of λ ZAPII phage clone, cNSBR ($\sim 1 \times 10^6$ pfu), and 20 ul of R408 helper phage (1×10^{11} pfu/ml) were added to a 15 ml polypropylene tube and incubated at 37°C for 15 minutes. LB broth (5 ml) was added and the mixture was incubated at 37°C for 4 hours with shaking. Bacteria and parent λ phage were then inactivated by heating the mixture at 70°C for 20 minutes. The tube was centrifuged at 3,000 rpm in a SM-24 rotor for 5 minutes. Supernatant containing the phagemid packaged in the f1 phage particles and the f1 helper phage was transferred to a new tube and stored at 4°C.

Release of the phagemid was achieved when 200 ul of XL1-Blue cells at OD₆₀₀ of 1 and 20 ul of a 100x dilution of the phagemid stock were incubated at 37°C for 15 minutes. Samples (100 ul) were removed and plated on 90 mm LBA plates. Plates were incubated at 37°C overnight to allow growth of ampicillin resistant colonies. The protocols by Short *et al.* (1988 and 1992) were followed.

Resistant colonies were picked and grown in LBA broth in order to perform plasmid mini-preps.

Generation of cNSBR deletion clones using Exonuclease III

New, smaller clones originating from cNSBR were created by Exonuclease III digestion of DNA that progressed from a 3' recessed end in the MCS (generated by a restriction enzyme) into the cDNA insert using protocols from Sambrook *et al.* (1989). Overlapping clones produced in this manner

allowed easier sequencing of the entire insert using a minimal number of new primers.

DNA from plasmid clone cNSBR (5-10 ug) was digested with *Sst*I followed by digestion with *Xba*I producing a 3' overhang and a 3' recessed end respectively. Digested DNA was purified by PCI extraction and then 1/10 volume of 3M sodium acetate (ph 5.2) was added. The DNA was precipitated in 2 volumes of absolute ethanol, washed in 70% ethanol and resuspended in a volume of 20 ul of dH₂O. The DNA solution was mixed with 6 ul of 10x Exonuclease III buffer (0.66 M Tris-Cl; pH 8, 66 mM MgCl₂) and dH₂O to a total volume of 60 ul. This Exo-DNA mix was incubated at 37°C for 5 minutes and then a 7.5 ul aliquot was removed and added to a microfuge tube containing 25 ul of S1 mix. The S1 mix was prepared to a final volume of 200 ul and contained 27 ul of 10x S1 buffer [Gibco/BRL: 300 mM sodium acetate; pH 4.6, 10 mM zinc acetate, 50% (v/v) glycerol], 20 ul of 3M NaCl, 152 ul dH₂O and 60 U of nuclease S1 (Gibco/BRL).

After the first aliquot was removed from the Exo-DNA mix, 130 U of Exonuclease III enzyme (Gibco/BRL) was added and the incubation was continued at 37°C. At 45 second intervals, a 7.5 ul aliquot was transferred to a new tube containing 25 ul of the S1 mix and placed on ice until a total of 8 aliquots had been removed. All 8 tubes containing the Exo-DNA/ S1 mix were incubated at 30°C for 30 minutes. The S1 enzyme was inactivated by the

addition of 3 ul of S1 stop mix (0.3 M Tris, 50 mM EDTA; pH 8) followed by incubation at 70°C for 10 minutes.

The sizes of the Exonuclease products were checked on an agarose gel and samples that contained similar sized products were pooled, ethanol precipitated and resuspended in a smaller volume of 10 ul dH₂O. In order to clean up the DNA ends of the products, 1 ul of Klenow mix [10 mM Tris-Cl; pH 7.6, 200 mM MgCl₂, 3 U Klenow enzyme (BM)] was added and the solution was incubated for 5 minutes at 37°C. The tubes were incubated at RT for 20 minutes after the addition of 1 ul of 0.5 mM dnTP. 10 ul of Ligation mix [8ul of 10x Ligation Buffer (NEB: 500 mM Tris-Cl, 100 mM MgCl₂, 100 mM DTT, 10 mM ATP, 500 ug/ml BSA, pH 7.8), 32 ul dH₂O, 1 ul Ligase enzyme (NEB)] was added and the reaction was left overnight at RT. *E. coli* DH5α competent cells were transformed with 5ul of each ligation product.

Sequencing

Sequencing reactions

Sequencing reactions were performed using either the Fidelity™ DNA sequencing system (Appligene) or the T7 Sequenase v2.0 sequencing kit (Amersham). The manufacturers instructions were followed. Both methods utilized the Sanger di-deoxy chain termination strategy. All enzymes and buffers were supplied with the kits. Different templates and primers (Table 4) were used but the approximate concentrations remained the same for each reaction; 1-5 ug DNA

template and 1-15 pmols primer. Plasmid DNA was prepared for sequencing using the Wizard™ mini-prep DNA purification system or the PlasmidPURE™ miniprep kit (Sigma). Completed reactions were stored at -20°C until required.

Just prior to loading the reactions onto the sequencing gel, they were heated at 75°C for 5 minutes.

Assembly and running of sequencing gel

One long (41.9 cm x 33.5 cm) and one short (39.5 cm x 33.5 cm) glass plate were cleaned with 0.1% SDS and 70% EtOH, dried, silanized with dichlorodimethyl silane, and then assembled on top of one another while separated by mylar spacers (Life Technologies): two side spacers (0.35 mm) and a bottom spacer (0.4 mm). Clamps were arranged along the sides and bottom to make a tight seal between plate and spacer.

For a 6% glycerol-tolerant denaturing polyacrylamide gel, 15 ml of 40% (v/v) Acrylamide/Bisacrylamide (19:1), 48 g urea, 10 ml of 10x glycerol tolerant gel buffer (per litre: 108g Tris, 36 g Taurine, 2.1 g Na₂EDTA •2H₂O and dH₂O up to 1 litre) and dH₂O up to 100 ml were mixed together and degassed for 15 minutes. Just prior to pouring the gel, 600 ul of freshly prepared 10% APS and 25 ul TEMED were added.

The solution was injected between the plates using a small squirt bottle. A sharktooth comb (62 well, 0.35 mm) was inserted at the top of the gel with teeth facing up. Clamps were placed along plate over the comb and then the gel was allowed to polymerize in a horizontal position for a minimum of 1 hour.

After the gel had set, clamps, comb and bottom spacer were removed to allow the outside of the plates and spaces left by combs to be cleaned of excess and unpolymerized acrylamide. The plates were assembled vertically in the BRL Model S2 sequencing apparatus and 500 ml of 1x glycerol tolerant buffer was poured into each of the upper and lower reservoirs. The space between the plates was flushed out with buffer and the comb was reinserted teeth down until contact with the gel was made.

The pre-run consisted of running the gel 30 minutes at 60 W constant power, 1500-1900 v and 30-45 mA controlled by the Model 3000Xi Programmable Power Supply (BioRad). Pre-heated samples were loaded (5 ul) into the flushed-out wells and the power was returned to the previous setting for 2-5 hours.

The gel was fixed (10% methanol, 10% acetic acid) for 20 minutes after removing side spacers, comb and one of the plates. The gel was removed from the remaining plate by adhering a sheet of 3 MM filter paper to the surface and then pulling away from the plate. The gel / filter paper were covered with plastic wrap and dried in a BioRad Model 583 Vacuum gel dryer at 80°C for 1 hour. The gel was exposed to Kodak XAR 5 film and stored in a film cassette for at least 60 hours prior to developing.

Table 4: DNA primers required for sequencing genomic and cDNA. The name, number of bases, sequence and priming location of each are summarized. All primers were custom made by Gibco/BRL or ★ Carleton University using automated synthesizers.

pcsbr-01	14	ACCGTGAAAAC TGC	<i>sbr</i> DNA of KSSBR0
ptrp273	20	GCCTGGACGACTAAACCAA	<i>trpC</i> promoter of <i>A. nidulans</i> in <i>hph</i> construct
reverse★	19	GGAAACAGCTATGACCATG	Bluescript MCS
sbrL-02	20	AGCAATGTGAGCGAACAGAG	<i>sbr</i> DNA of KSSBR0 and cNSBR
sbrL-02RC	20	CTCTGTTCGCTCACATTGCT	reverse complement of sbrL-02 primer
sbrL-03	16	TTGATGAGGATGGGCC	cNSBR clone
sbrL-04	18	GGCAGAGGCATCTTGGTC	<i>sbr</i> DNA of KSSBR0 and cNSBR
T3	18	ATTAACCCTCACTAAAGG	Bluescript MCS
T7	17	TAATACGACTCACTATA	Bluescript MCS
trpC★	20	GAGGAAGGGCGAACTTAAGA	<i>trpC</i> promoter of <i>A. nidulans</i> in <i>hph</i> construct

RNA Analysis Methods

Northern hybridization

A pre-made Northern blot with RNA originating from wild-type *N. crassa* mycelia cultures (stored at RT since February 1995) was prehybridized at 42°C for 2 hours in 0.1 ml of hybridization solution [5x SSPE (20x: 175.3 g NaCl, 27.6 g NaH₂PO₄•H₂O, 7.4 g EDTA and up to 1 litre with dH₂O, pH 7.4), 50% (v/v) formamide, 2x Denhardt's, 0.5% SDS, 10% dextran sulfate] per cm² of membrane.

The membrane was then hybridized with the Ava600 probe. The probe (500,000 cpm per ml of hybridization solution) was mixed with 20 ug/ml salmon sperm DNA, denatured at 95-100°C for 5 minutes and then quick-chilled on ice before adding to the hybridization solution. Hybridization was carried out at 42°C for 18 hours.

The membrane was washed in 50 ml of 5x SSPE at 42°C for 20 minutes and then repeated. A wash in 50 ml of 1x SSPE, 0.1% SDS at 42°C for 90 minutes was followed by a wash in 200 ml of 0.1x SSPE, 0.1% SDS at RT for 30 minutes. The membrane was allowed to dry briefly before it was exposed to Kodak Biomax MS autoradiographic film with a Biomax MS intensifying screen. The film was exposed at -20°C for 6 days before it was developed.

Protein Analysis Methods

Extraction of protein from *N. crassa* cultures

N. crassa hyphae, from at least a 7 day old slant culture, were resuspended in dH₂O, inoculated into 50 ml VMM liquid media and grown at RT for 24 hours with shaking at 200 rpm. The hyphae were aseptically transferred to a Buchner funnel lined with Whatman filter paper and collected by suction filtration. The hyphae were reinoculated into 100 ml fresh VMM liquid media and grown for another 24 hours under the same conditions. Hyphae were harvested again and frozen at -70°C in a 15 ml polypropylene tube.

Approximately 50 mg of frozen mycelia were ground in a microfuge tube (containing a pinch of acid-washed sea sand) using a plastic pestle while on ice. TE (200 ul, pH 8) was added and the tube was incubated on ice for 1 hour. The mixture was centrifuged at 7,000 rpm at 4°C in an eppendorf Centrifuge 5415 C for 5 minutes and then 14,000 rpm for 15 minutes. The proteinaceous supernatant was collected and the protein concentration was determined.

Approximately 25 ug of protein was mixed with an equal volume of 2x SDS loading buffer and boiled at 95-100°C for 5 minutes just prior to loading into a well of a polyacrylamide gel.

SDS-Polyacrylamide Gel Electrophoresis (PAGE)

Two 0.75 mm thick polyacrylamide gels were made using the Mini-PROTEAN^R II Dual Slab Cell (BioRad). The instructions accompanying the apparatus for preparation of the 10% resolving gel and 4% stacking gel were followed. A comb (10 wells, 0.75 mm thick) was used to make the wells. A mid-range protein molecular marker (Promega) was loaded (1.5 ug in 2x SDS) into a well of each gel to act as a molecular weight standard. The gel was run at 180 v for 30-50 minutes powered by the Bio-Rad Model 200/2.0 power supply.

Protein gels were stained in Coomassie (0.1% Coomassie Brilliant Blue R-250 in fixative (10% acetic acid, 40% methanol)). Background staining was removed with destain (10% acetic acid, 40% methanol). Gels were dried at 80°C for 2 hours in a Model 583 Vacuum Gel Dryer (BioRad) while sandwiched between 2 sheets of cellophane that were equilibrated in destain with 3% (v/v) glycerol.

Western transfer

The gel and nitrocellulose membrane (Protran) were equilibrated in transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol) for 30 minutes.

Using the Transblot^R SD Semi-Dry Electrophoretic Transfer Cell (BioRad), 3 pieces of 3 MM filter paper (the same size as the gel) saturated in transfer buffer were stacked onto the anode. The membrane (slightly larger

than the gel) was layered next followed by the gel. An additional 3 pieces of filter paper were stacked on top. Bubbles were removed at each layer. The cathode was placed on top and transfer was performed at 10 v for 40 minutes.

Transfer efficiency of proteins onto nitrocellulose membranes was checked by staining the membrane for 1 minute in 0.2% Ponceau S in 0.3% (w/v) trichloroacetic acid (TCA). Excess stain was removed with a dH₂O wash. Molecular weight marker bands were traced onto the membrane with a pencil. Storage of the membrane was at 4°C in plastic wrap.

Western immunoblotting

The nitrocellulose blot was blocked in 20 ml of 1x TBS (per litre: 8 g NaCl, 0.2 g KCl, 3 g Tris (pH adjusted to 7.4) and dH₂O to 1 L), with 5% (w/v) skim milk powder for 60 minutes with shaking at RT. Two 5 minute washes were performed in 1x TBS, 0.05% (v/v) Tween-20. A 60 minute incubation with 1:10,000 dilution of rabbit A cell wall serum (primary antibody) in 1x TBS, 2.5% skim milk was followed by 3, 5 minute washes. The membrane was incubated with 1:5,000 dilution of 1 mg/ml anti-rabbit/HRP (secondary antibody) in 1x TBS, 2.5% skim milk for 1 hour. Excess antibody was removed by washing 4 times for 5 minutes with 1x TBS, 0.05% Tween-20.

The BM Chemiluminescence Blotting System was used for detection of membrane-bound proteins labelled by antibodies. Both the kits reagents and instructions were used. Substrate solution A and Starting solution (100:1) were

added (0.5 ml) to the blot that was sandwiched in plastic wrap.

Autoradiographic film was exposed to the membrane for 10 seconds to 1 minute and developed immediately.

III. RESULTS

A. Characterization of the *sbr* phenotype

***Sbr* has a colonial morphology on solid and liquid media**

Compared to most other filamentous fungi, *Neurospora* has a very rapid growth rate. Its hyphae are capable of colonizing new substrate at an apical growth rate of 5 mm/hr under ideal conditions (Ryan *et al.*, 1943).

Following germination, wild-type vegetative hyphae extend radially from the site of inoculation along and within the solid minimal medium (Figure 3a). Within a few hours, these rapidly growing primary hyphae branch at acute angles and the secondary hyphae soon branch in turn. The combination of tip growth and repeated branch formation establishes a mycelial front consisting of many growing hyphal tips (Murray and Srb, 1962). The formation of asexual spores (conidia), from this vegetative feeding mycelium, is usually triggered by dessication or carbon starvation and is characterized by extensive growth of numerous aerial hyphae upwards and away from the nutritive medium. The tips of the aerial hyphae develop a fluffy, orange appearance after a couple of days due to the development of macroconidial chains and clusters that contain carotenoid pigments. With age, these conidiophores become powdery and dry and the macroconidial spores detach and are disseminated by air currents to allow colonization of new substrate.

Upon transfer to solid minimal medium, fragments of the *sbr* mycelium (usually containing more than a single cell) initially grow slowly and radially as thick hyphae that branch at most subapical cell compartments (not shown). Within 12 hours, these club-shaped cells begin to form spherical buds. The budding growth (both aerially and laterally) increases the density of the colony starting at the colony edge and progressing towards the centre while gradually increasing the height of the colony. This restricted growth eventually produces a single, small, circular colony that reaches a maximum diameter of 2-8 mm and height of 1-2 mm (Figure 3b). At this stage, the colony has an off-white translucent colour and its mottled, bumpy- textured surface glistens.

As early as two days after inoculation, the colony has a yeast-like, mound morphology, which is likely to be the result of vertical budding growth that appears most extensive at the colony center and tapers towards the colony periphery. Upon dessication, clumps and clusters of budded hyphae can be observed protruding from the surface of the dense colony. These clusters may consist of aberrant macroconidia exhibiting a defect in conidial separation as seen in the conidial separation mutants, *csp-1* and *csp-2* (Perkins *et al.*, 1982). This development is usually accompanied by disintegration of the colony center, which likely signifies the cessation of the colony's growth. After the first week, the colour progressively deepens from pale yellow to orangish-brown and the surface sheen is reduced as the colony becomes drier. Although, the *sbr* mutant does not produce normal macroconidia, it can be propagated by

breaking off fragments of an existing colony and transferring them to new substrate.

After 2-3 days growth with shaking in liquid media at RT, the wild-type strain forms a single, thick, off-white coloured, mycelial mat. Unlike wild-type, *sbr* hyphae do not appear to fuse to form a single mycelium structure but instead exist as numerous individual colonies, which resemble imperfect spherical pellets that are less than 1 mm in diameter. In addition, the progressive change in pigmentation remains consistent with its appearance on solid medium.

Although *sbr* is a colonial mutant, its phenotype and development are distinct from most other colonial strains.

Chitin deposition and cell morphology in the mutant strain

Staining of nuclei and chitin in wild-type and *sbr* (Figure 4) revealed more differences between the two strains. Wild-type hyphae were thin and tubular with an average diameter of 4 μm . In contrast, *sbr* hyphae were swollen and spherically-shaped exhibiting an average diameter of 20 μm , which is nearly 5 times wider than wild-type hyphae. Calcofluor staining revealed that the chitin distribution was limited to septa in wild-type. However, both walls and septa were stained with equal intensity in *sbr*. A single septum marked the boundary between adjacent cells.

Cell compartments in both strains were de-limited by these septa. Although *sbr* hyphae were wider, its cell compartments were shorter with the

Figure 4: *N. crassa* hyphae visualized by fluorescence microscopy. **(a-b)** wild-type and **(c-d)** *sbr* hyphae were grown in liquid media for 16 hours, fixed and then stained with DAPI (4',6-diamidino-2-phenylindole) and calcofluor to localize DNA in nuclei (n) and cell wall septa (s) respectively. The length of the bar represents 25 μ m.

distance between septa ranging from 5 to 10 μm . In comparison, wild-type compartments were 30 to 50 μm in length.

Nuclei number and distribution were consistent between the two strains. A cell compartment of either strain was occupied by as many as 10 evenly spaced nuclei.

***Sbr* microtubule distribution was irregular**

The arrangement of microtubules in both wild-type and *sbr* tends to correspond to the overall shape of the cell (Figure 5). Microtubules of wild-type were parallel to the direction of hyphal growth and extended from the tip of the hyphal filament along its entire length. In *sbr*, there were numerous randomly arranged, short microtubules that often seemed to criss-cross each cell with no discernible pattern. The majority of microtubules were concentrated in the central area of the cells with fewer bordering the cell perimeter. This arrangement is very different from that of *S. cerevisiae* where microtubules are attached at one end to the spindle pole body in the nuclear membrane and extend from the cell center towards the pole into the budded daughter cell (Barnes *et al.*, 1990). The arrangement of microtubules in *sbr* is similar to that in the microcycle blastoconidiation (*mcb*) growth polarity mutant of *N. crassa* (Bruno *et al.*, 1996).

Figure 5: Arrangement of microtubules (t) in 16-hour *N. crassa* hyphae of (a) wild-type and (b) *sbr*. Fixed cells were incubated with YOL IgG rat monoclonal antibodies (primary) that were specific for tubulin. The secondary antibody was CY3 (cyanine)-conjugated donkey anti-rat IgG. Labelled cells were viewed using fluorescence microscopy. The length of the bar represents 10 μ m.

Unusual budded-hyphae morphology of *sbr*

The most intricate details of *sbr* morphology are revealed by Scanning Electron Microscopy (SEM). Unlike the thin tubular hyphae of wild-type with diameters of 4 μm , *sbr* colonies consist of fat, rounded hyphae with diameters ranging from 20 to 80 μm (Figure 6). From these tuberous projections arise smaller yeast-like buds that are reminiscent of *S. cerevisiae*.

Another obvious distinction occurs in the conidiation structures of *sbr*. Wild-type cultures displayed in Figure 6b exhibit both developing conidiophore structures and macroconidia (2-3 μm diameter) typical of conidiating hyphae. *Sbr* appears to lack conidiophore chains in these young cultures although major constrictions at interconidial junctions are apparent in Figure 6d. Numerous buds are observed protruding from a single larger mother cell (Figure 6c).

***Sbr* is capable of mating**

Three mating experiments were conducted in order to determine whether the mating compatibility of *sbr* was influenced by the gene disruption. Crosses were made with wild-type strains (987 x 988) and *sbr* with each of the wild-type strains (987 x *sbr* and 988 x *sbr*). Only the cross between 988 and *sbr* involved strains of identical mating type.

Black perithecia indicative of mating were observed 7-8 days after the strains were plated in the 987 x 988 and 987 x *sbr* crosses. About 14-15 days after plating, ascospores were shot from beaked perithecia in the 987 x *sbr*

cross (1-2 days later than in 987 x 988). Ascospores were black in colour and appeared normal in morphology. There was no evidence of any mating structures as a result of the 988 x *sbr* cross.

Ascospores were collected from the two successful crosses and germination was attempted. After 24 hours, new hyphae were observed arising from ascospores originating from both crosses. These hyphae formed spreading colonies exactly like wild-type in morphology. No evidence of *sbr* colonies were recovered from any of the germinating ascospores. The experiment was repeated using only the 987 x *sbr* mating and this time all 8 spores arising from a single ascus were collected. Each spore was isolated into its own culture tube and heat-shocked to induce germination.

Two full sets (and one partial set) of ascospores were analyzed and the results are summarized in Table 5. Three types of results were seen: germination producing wild-type hyphae, germination producing *sbr*-like growth, and no germination. Germination attempts with one of the spore sets resulted in 50% of the spores giving rise to hyphae that were wild-type in morphology and a single spore arresting in *sbr*-like growth. The other two spore sets did not give rise to *sbr* colonies and had poorer overall germination.

Figure 6: Scanning Electron Microscopy of *N. crassa* hyphae from 3 day cultures of **(a-b)** wild-type and **(c-d)** *sbr*. A developing wild-type conidiophore (c) and a macroconidium (m) are labelled in frame (b). Samples were quick frozen in liquid nitrogen and coated with pure aluminum as preparation for microscopy. The length of the bar represents 10 μ m.

Table 5: Germination of ascospores from the mating of wild-type (987) x *sbr*.

Each group represents ascospores from a single ascus. The spores were scored for germination ability seven days after heat-shock induction. At this time, ascospores were assessed based on the appearance and development of hyphae. Spores produced wild-type growth (wt), *sbr*-type growth or no signs of germination.

		sb	none	
1	4	1	3	8
2	2	0	6	8
3	1	0	5	6

B. Isolation of the *sbr* gene using tools of molecular biology

Confirmation of tag integration

From phenotypic observations alone one could conclude that the insertional tag has successfully integrated. However, the phenotype cannot distinguish between single and multiple insertions. In order to confirm the number of integrated copies, *sbr* genomic DNA was digested with an assortment of restriction enzymes and the resulting digestion products were probed with the 2.4 kb *SacI* fragment of PCSN43 containing the *hph* construct (Figure 7).

The enzymes *SacI*, *EcoRV*, *XhoI* and *XbaI* were known to lack restriction sites within the 2.4 kb *hph* construct but they could cut at sites flanking it in the genome. Sites for these restriction enzymes also exist in the MCS of PCSN43 bordering the construct. Digestion with each of these enzymes alone resulted in single bands suggesting that there was only one copy of the tag in the genome. Additional evidence for integration was easily corroborated by examining the *PstI/BamHI* digest since the sizes of some of the products could be predicted from the map of the plasmid tag (refer to Figure 2). The 700 bp and 500 bp bands from this digest likely correspond to portions of *TtrpC* and *hph* respectively. As well, the 700 bp band was also seen in the *BamHI* single digestion as expected.

There is reason to suspect that the MCS downstream of *hph* (and possibly pBR322) is no longer associated with the tag. Double digestions involving *Pst*I and each of the enzymes (except *Bam*HI) produced 2 bands that were all larger than 2.5 kb. However, one of the expected products of each double digestion between *Pst*I and *Sac*I, *Eco*RV or *Xba*I was a 1.7 kb band corresponding to the fragment defined by the *Pst*I site in *hph* to the restriction site in the downstream MCS. As well, digestion using *Bam*HI alone should have yielded a third band (500 bp) corresponding to the pBR322 fragment.

The clone, λ sbr-4TC2, is isolated from the *sbr* genomic library

After it was confirmed that only a single copy of the insertional tag had become incorporated into the genome of *sbr*, the recovery of the tag and flanking genomic DNA was attempted. This was accomplished by screening the λ sbr genomic library using the tag as the probe.

In the primary screening, 7 putative clones were identified (Figure 8a) but only one clone was chosen for further study. Four rounds of screening were necessary to isolate a pure population of the clone that had homology to the insertional tag. This clone was named λ sbr-4TC2. Digestion of its DNA with *Not*I resulted in 5 bands. There were two *Not*I sites encoded in the Lambda arms flanking the *sbr* insert and therefore the arms represented the largest bands: 20 and 9 kb. The remaining three bands had sizes of approximately 6.7, 4.8 and 2.4 kb suggesting that the insert was about 14 kb.

Figure 7: Digestion of *sbr* genomic DNA with various restriction enzymes and corresponding Southern blot. The digested DNA was electrophoresed on an agarose gel **(a)** and the Southern blot of the gel was probed with the PCSN43 tag **(b)**. Lanes #1- λ *Hind*III molecular weight marker in kb, 2-*Pst*I, 3-*Xba*I, 4-*Xba*I and *Pst*I, 5-*EcoRV*, 6-*EcoRV* and *Pst*I, 7-*Sac*I, 8-*Sac*I and *Pst*I, 9-*Xho*I, 10-*Xho*I and *Pst*I, 11-*Bam*HI, 12-*Bam*HI and *Pst*I, 13-*Pst*I.

a

b

Figure 8: Positive hybridizing clones from plaque lifts during primary screening of *N. crassa* DNA libraries. **(a)** λ sbr genomic library probed with 600 bp *SalI/EcoRI* PCSN43 tag, **(b)** λ BARGEM-7 wild-type genomic library probed with 600 bp *AvaI* fragment from KSSBR0 (Ava600) and **(c)** nitrogen-induced cDNA library probed with same probe as in b. Arrows indicate plaques selected for further rounds of screening.

The insert was too large to subclone into a plasmid vector directly so the insert was digested with different restriction enzymes in combination with *NotI* (Figure 9a). A Southern transfer of the digestion products was hybridized with the same probe used to screen the library (Figure 9b). The Southern was analyzed to determine which DNA fragment was the best size and had the most convenient ends for ligation into the Bluescript plasmid cloning vector (Stratagene). The 3.2 kb *NotI/HindIII* band was chosen since it was large enough to accommodate both the tag and *sbr* genomic DNA but was also small enough to sequence easily.

This Southern also provided additional confirmation that λ sbr-4TC2 carried the tag. The *BamHI* digestion revealed a 700 bp band that was the same size as the one seen in the Southern of *sbr* genomic DNA in Figure 7. As well, both the 10.5 kb *SacI* and 7.4 kb *XbaI* bands were present in both Southern.

Using this information, the *NotI/HindIII* band was isolated, ligated into the *NotI/HindIII* sites of plasmid pBSKS(+) and transformed into an *E. coli* host. Plasmid DNA from putative clones was analyzed by digestion with *NotI* and *HindIII* (Figure 10). All of the clones selected contained a 3.2 kb insert. This new clone was named KSSBR0.

Figure 9: Agarose gel and corresponding Southern blot of Lambda clone (λ sbr-4TC2) DNA after digestion with restriction enzymes **(a)** and hybridization with PCSN43 tag probe **(b)**. Lanes #1 and 7- λ *Hind*III MW standard in kb, 2-*Bam*HI, 3-*Not*I, 4-*Bam*HI and *Not*I, 5-*Sac*I, 6-*Sac*I and *Not*I, 8-*Xba*I, 9-*Xba*I and *Not*I, 10-*Hind*III, 11-*Hind*III and *Not*I. The smallest bands in lanes 2 and 4 of (b) were manually enhanced for better visibility.

Figure 10: Identification of positive recombinant clones resulting from ligation of 2.9 kb pBSKS+ cloning vector and 3.2 kb *NotI*/*HindIII* digested λ sbr-4TC2 DNA. Lanes #1-6 represent plasmid clones cut with *NotI* and *HindIII*, 7-pBSKS+ vector digested with *NotI* and *HindIII*, 8- λ *HindIII* MW standard in kb, 9-undigested plasmid clone DNA, 10-undigested pBSKS+ vector DNA. The recombinant clone was later named KSSBR0.

1 2 3 4 5 6 7 8 9 10

The KSSBR0 clone harbors *sbr* genomic DNA flanking the tag

A plasmid map of KSSBR0 (Figure 11) was generated by combining information discovered through restriction enzyme digestions and DNA sequencing. The clone is divided into three main fragments: *sbr* genomic DNA (1-715), the insertional tag (715-3200) and the pBluescript cloning vector (3200-6200).

The *sbr* genomic DNA sequence is presented in Figure 12 and has a 57% GC composition over its 715 bases. Some sequence features are outlined in Table 6, which were determined with the aid of DNAMAN™ Sequence Analysis Software ©1994-1997. The sequence encodes unique restriction sites for *Aval*, *BalI*, *BglI*, *BspHI*, *HaeII*, *SphI*, and *StuI* and two sites for each of *NaeI*, *NcoI*, and *PvuII*. However, only the *Aval* and *PvuII* sites were tested by restriction enzyme digestion. There are also a significant number of direct repeats from 8 to 18 bases in length and a hairpin loop that forms an 8 bp stem and 17 bp loop. Translation of all reading frames by DNAMAN™ determined that only the +2 frame lacks termination sequences while all other reading frames have a minimum of 2 stop codons. Further analysis of the putative amino acid sequence and protein structure was derived from the cDNA sequence. *Neurospora* consensus sequences (Bruchez *et al.*, 1993ab) for 5' / 3' regulatory sequences and intron splice sites do not appear to be encoded in this portion of the sequence.

Figure 11: Map of 6.2 kb plasmid clone, KSSBR0. The plasmid is composed of the 3.2 kb *NotI/HindIII* fragment from λ sbr-4TC2 cloned into the pBSKS+ (Bluescript) vector. The insert is composed of a 715 bp fragment of *sbr* genomic DNA (solid grey) and a portion of the PCNS43 insertional mutagenesis tag (hatched). The tag encompasses a portion of the pBSSK vector, *trpC* promoter (not indicated) and the hygromycin resistance gene (white arrow). Some restriction sites have been indicated by approximate location.

Figure 12: Sequence of cloned *sbr* genomic DNA (KSSBR0) flanking the insertional tag. The sequence encompasses 715 bases and is presented from the genomic DNA region adjacent to the PCSN43 insertional tag towards the *Hind*III site in the KSSBR0 multiple cloning site (bases 715 to 1 on the map of KSSBR0). Some features are indicated on the sequence including an 18 base direct repeat (double underline) and a hairpin loop (dashed underline with arrows). The *Ava*I restriction site is indicated and an arrow represents the side of the site that was used to generate the Ava600 probe that was used to screen the other libraries.

1 GCCGGATGTT GACGCCGAGA TGGAGGACCA AGATGCCTCT GCCGCTGCGA

51 ACTCGGGACT CGGCGGCCAT GTTGACCTTA GTGGTGCCGG CATGCTAGGT
Ava600 probe→

101 AACCCAGGTG CCCCTGGTGG TGTGCCTCAA CCCTCTCCCC AGGACCACTC

151 TCAACATCAA CAACACCAGC ACCAGCAGCA GCAACATCAA CACCATCAGC

201 AACAAACAGCA GCAGCAGCAT CAACAGCAAC AGCAGCATCA ACAGCAACAG
* * *

251 CAGCAGGGCC GATATGCTTC AGGCCTTGGA CAGCCCCAGG CCCATCCTCA

301 TCAACATCAG CACCAACCCC CTGTTGCCCC CATGGGCCAG CCCCAGCAGA

351 TGCAGCCGTC CCCTCATCAG CACCCAATGG ACCCTCAGCT ATCCAAGAGC

401 ACGGAGGATA TGGCTCATGA ATCCGGTTAT GGCCAGCTAA ACGTCGAGAG

451 TGCTCTTGCC AAGAGATTGG CTCGTGAGCC GGGTCATCGT CTTGCCCAGC

501 AGAGGCGCCC TGAGCAAGTG CTGAACTTGG CACGTAGAAG CAATGTTGAA
→

551 GCTCTGTTTCG CTCACATTGC TGGTGAGCCG GCTCGCGTCC CTTGTAAGAA
←

601 TTGCCACAAG GGCCACGGTC CATGGACCAG CTGTATTGTG GTGGACGGCC

651 AGATGTGCGG CAGCTGTGCC AATTGCTGGT TTAATGCCAG TGGTGCCAGA

701 TGCAGTTTTTC ACGGT

Table 6: Features of the *sbr* genomic DNA sequence of KSSBR0. Locations and sequence of restriction enzyme sites (that cut at a maximum of 2 sites), direct and a hairpin loop are indicated. For the latter two features, the location represents the first base involved in each incidence of the feature. DNAMAN™ was used to analyze the sequence.

Restriction sites:

<i>Ava</i> I	52	C/TCGGG
<i>Ba</i> II	432	TGG/CCA
<i>Bg</i> II	609	GCCACAA/GGGC
<i>Bsp</i> HI	415	T/CATGA
<i>Ha</i> ell	508	GGCGC/C
<i>Sph</i> I	94	GCATG/C
<i>Stu</i> I	273	AGG/CCT
<i>Nae</i> I	88 and 579	GCC/GGC
<i>Nco</i> I	330 and 620	C/CATGG
<i>Pvu</i> II	630 and 663	CAG/CTG

Direct repeats:

18 bases	213 and 231	AGCAGCATCAACAGCAAC
12 bases	203 and 245	CAACAGCAGCAG

Hairpin	539	AGCAATGTtgaagctctgttcgctc ACATTGCT
---------	-----	---------------------------------------

The PCSN43 tag portion of the KSSBR0 insert consists of 0.9 kb of cloning vector pBSSK(+) and 1.6 kb of the *hph* construct, which includes the *trpC* promoter, entire *hph* gene, *trpC* terminator and possibly some of the pBR322 plasmid. Regions of both the *trpC* promoter and pBSSK were sequenced and found to agree with the published sequences of Mullaney *et al.* (1985) and the Genbank sequence (accession number X52325) respectively.

In addition to its contribution to sequence data, the *sbr* region of KSSBR0 was essential for screening the λ BARGEM-7 genomic library, cDNA library and Northern blot of wild-type RNA. This was permitted by the discovery of the unique *Ava*I restriction site, which allowed the isolation of a 600 bp probe of genomic DNA (which will be referred to as *Ava*600).

A clone is isolated from the λ BARGEM-7 genomic library

Two clones were identified in the primary screening of the library (Figure 8b) using the *Ava*600 probe. Only one of these clones was involved in additional rounds of screening: clone pBG3b1. Restriction enzyme analysis with single digestions of *Bam*HI, *Kpn*I and *Xho*I revealed 2 sites for each enzyme with one of each pair encoded in the vector MCS. Double digestions involving *Bam*HI and *Kpn*I with *Xho*I allowed construction of the plasmid map in Figure 13. The genomic insert, cloned into the *Eco*RI site of the 4.9 kb pBARGEM-7 vector (Pall and Brunelli, 1993), was determined to be about 7 kb. A southern blot of these digestions was probed with *Ava*600 (not shown). Unfortunately, the

Figure 13: Map of the plasmid clone (pBG3b1) that was isolated from the λ BARGEM-7 wild-type genomic library. It was recovered after screening the library with the Ava600 probe. The entire plasmid is about 11.9 kb and harbors a 7 kb genomic DNA insert cloned into the *EcoRI* site of the pBARGEM 7-2 cloning vector (Pall and Brunelli, 1993). Restriction enzyme digestion of the clone predicts the restriction sites indicated (approximate). Each of the three enzymes are encoded once in the MCS of the vector and once in the insert. Southern analysis did not reveal the relative location of the probe in the insert.

location of the probe sequence could not be confirmed. In retrospect, the hybridization signals from the secondary and tertiary screenings of the library were weaker than those observed during the screening of the cDNA library (which was performed after the λ BARGEM-7 screening). As a result, we are uncertain whether this clone encodes the same genomic DNA corresponding to the portion in the KSSBR0 clone or whether it only shares a small region of similarity. Therefore, more experimentation will be required to properly characterize pBG3b1.

C. Characterization and sequence of the cDNA clone

The cNSBR clone encodes *sbr* cDNA

A single clone was isolated from the nitrate-induced cDNA library and was named cNSBR. Initial characterization by restriction enzyme digestion revealed that the clone harbored a 1.9 kb insert in the 2.9 kb pBluescript cloning vector (Stratagene). The plasmid map is featured in Figure 14. Given the large size of the insert, it was necessary to subclone it into smaller sized clones. The first strategy involved clone shrinking using restriction sites that were encoded in both the vector's MCS and the internal sequence of the insert. However, there were no sites that fit these criteria. The second approach entailed the construction of exonuclease III deletion clones. This involved digestion of cNSBR with *Sst*I (producing a 3' overhang) followed by *Xba*I (producing a 3'

recessed end that was vulnerable to exonuclease III digestion). DNA digestion by exonuclease III proceeded in the 3' to 5' direction from the *Xba*I site into the insert to produce new overlapping clones of successively smaller sizes.

The sequence of the cDNA insert featured in Figure 15 was determined by the sequencing strategy summarized in Figure 16, which involved combining sequence information from the exonuclease III deletion clones and the original cNSBR clone using some of the primers presented in Table 4. The entire insert is 1881 bases and has a GC composition of 53%. At the T3 priming end of the insert, a string of 8 adenines [poly(A) tail] define the end of the insert and dually confirm the identity of the insert as cDNA. At the 5' end of the cDNA, a translation start site (ATG) is expected to lead the protein coding sequence, however, the size of the insert does not coincide with the predicted size of the mRNA (discussed later in results) suggesting that the 5' end of the cDNA may be truncated hence the 5' translation consensus sequences may not be encoded in this clone. The sequence of the *sbr* genomic DNA from KSSBR0 was nearly identical to the corresponding region of cNSBR. The genomic DNA differed by only a couple of bases at the sequences corresponding to the tag and MCS junctions.

The amino acid sequence of all reading frames was determined using DNAMAN™. All reading frames, except +2, were found to encode numerous (>15) stop codons that extended throughout the length of the sequence. Reading frame +2 encodes its first stop codon (TAG) at position 1298 followed

by 5 more in the last 580 bases of the sequence. This strongly suggests that +2 is the correct open reading frame (ORF). The maximum ORF spans base 2 to 1297 and encompasses 432 amino acids (not including the stop codon). The GC composition of the potential ORF is significantly different from that of the termination region after the stop codon. The coding region is 57% GC, whereas the 3' noncoding region is only 45% GC. Edelman and Staben (1994) found a similar GC distribution of 58.6% (coding) and 49.1% (noncoding) in the *N. crassa* genes they analyzed. Other features of both the DNA and amino acid sequence are presented in Table 7.

At the 3' end, many *Neurospora* genes have been found to encode the 3' polyadenylation consensus sequence, AATAA, located 10 to 30 bases upstream from the poly(A) tail (Bruchez *et al.*, 1993b) but this sequence does not appear to be conserved in this gene although its 3' region is highly AT rich and many instances of multiple adenines and multiple thymines occur. The closest match to the polyadenylation signal is AAGAAAA, which is located 572 bases upstream from the poly(A) tail and only 2 bases after the TAG stop codon. Although this same sequence was found to be the polyadenylation signal in *cys-3* (Bruchez *et al.*, 1993b), its relative location to the stop codon and poly(A) tail appears to differ greatly from other *N. crassa* genes.

Figure 14: Map of plasmid clone (cNSBR) harboring the cDNA insert. cNSBR was recovered after successful screening of the nitrate-induced cDNA library with the Ava600 probe. The 1.9 kb insert is situated in the middle of the vector's MCS. The 5' most region of the cDNA is located adjacent to the T7 priming site and the 3' termination region borders the T3 priming site. Within the coding sequence (black arrow), some restriction sites are indicated (determined by DNAMAN™). To simplify sequencing, exonuclease III deletion clones were produced from the 3' end by digestion of the plasmid with *Sst*I and *Xba*I (bold) followed by exonuclease III treatment.

Figure 15: Nucleotide and deduced amino acid sequence of *sbr* from the cDNA clone. The entire cDNA was sequenced and found to be 1881 bases. The sequence presented here originates from the T7 priming site and proceeds to the T3 priming site (refer to Figure 14). The predicted coding sequence (+2 reading frame) encompasses 432 amino acids (bases 2-1297) and terminates at a TAG stop codon. Noncoding sequences are indicated in lowercase letters. The site of disruption by the insertional mutagenesis tag in the *sbr* genomic DNA is indicated by the arrow (↑). At the 3' terminus, a poly(A) tail of 8 consecutive adenines (wavy lines) is apparent. Other features of interest include a poly-alanine stretch (single underline), glutamine-rich region (double underline) with a polyglutamine stretch (bold), helix-turn-helix motif (thick underline) and cysteine-rich domain (highlighted).

1 GCATCAACAGGAGCAACATCAGGAGCCTGATATTGACCAGGATTC
1 H Q Q E Q H Q E P D I D Q D S
46 CCTTAAACCAGACCCGTCGCTACGACGACCCTACGGCCAGGAT
16 L K P D P S A Y D D P T A R M
91 GGGCACTGAAGAGTATGCGGCCGCAGCTGCTGCTGCCGTCATGGA
31 G T E E Y A A A A A A A V M E
136 GACGGGTCTTGGTCAACCCGGCTCCCTGAATGATGGCACTGGCGA
46 T G L G Q P G S L N D G T G E
181 GCCGGATGTTGACGCCGAGATGGAGGACCAAGATGCCTCTGCCGC
61 ↑ P D V D A E M E D Q D A S A A
226 TGCGAACTCGGGACTCGGCGGCCATGTTGACCTTAGTGGTGCCGG
76 A N S G L G G H V D L S G A G
271 CATGCTAGGTAACCCAGGTGCCCCTGGTGGTGTGCCTCAACCCCTC
91 M L G N P G A P G G V P Q P S
316 TCCCCAGGACCACTCTCAACATCAACAACACCAGCACCAGCAGCA
106 P Q D H S Q H Q Q H Q H Q Q Q
361 GCAACATCAACACCATCAGCAACAACAGCAGCAGCAGCATCAACA
121 Q H Q H H Q Q Q Q Q Q Q H Q Q
406 GCAACAGCAGCATCAACAGCAACAGCAGCAGGGCCGATATGCTTC
136 Q Q Q H Q Q Q Q Q Q Q G R Y A S
451 AGGCCTTGGACAGCCCCAGGCCCATCCTCATCAACATCAGCACCA
151 G L G Q P Q A H P H Q H Q H Q
496 ACCCCCTGTTGCCCCCATGGGCCAGCCCCAGCAGATGCAGCCGTC
166 P P V A P M G Q P Q Q M Q P S
541 CCCTCATCAGCACCCAATGGACCCTCAGCTATCCAAGAGCACGGA
181 P H Q H P M D P Q L S K S T E
586 GGATATGGCTCATGAATCCGGTTATGGCCAGCTAAACGTCGAGAG
196 D M A H E S G Y G Q L N V E S
631 TGCTCTTGCCAAGAGATTGGCTCGTGAGCCGGGTCATCGTCTTGC
211 A L A K R L A R E P G H R L A
676 CCAGCAGAGGCGCCCTGAGCAAGTGCTGAACTTGGCACGTAGAAG
226 Q Q R R P E Q V L N L A R R S

721 CAATGTTGAAGCTCTGTTTCGCTCACATTGCTGGTGAGCCGGCTCG
 241 N V E A L F A H I A G E P A R

 766 CGTCCCTTGTAAGAATTGCCACAAGGGCCACGGTCCATGGACCAG
 256 V P C K N C H K G H G P W T S

 811 CTGTATTGTGGTGGACGGCCAGATGTGCGGCAGCTGTGCCAATTG
 271 C I V V D G Q M C G S C A N C

 856 CTGGTTTAATGCCAGTGGTGCCAGATGCAGTTTTTCACGAAACGCG
 286 W F N A S G A R C S F H E T R

 901 CAACCCCCAAAATTCTCAACAGCAGCAGCACAAATGCGGCGATCCT
 301 N P Q N S Q Q Q Q H N A A I L

 946 GCCGAACGGGGCGAACGGTGAATTAATATTTTCAGCTGACCCCAA
 316 P N G A N G G I N I S A D P N

 991 TGCCTTTAGGTTTGGCACTCCTCACCCGCTTGGTACTCATGCTGC
 331 A F R F G T P H P L G T H A A

 1036 CCTTGCTGCTCCTGCTCCCGTGAGTGCTATCGCTCCAGCAGCTCC
 346 L A A P A P V S A I A P A A P

 1081 CTCGATGAGCAACCCAATGCTTCAGCACATGTTGAATCGGGCCCT
 361 S M S N P M L Q H M L N R A L

 1126 GTCCGAGGTACGGAGCGCTGACAAGGCTACCCGGCAACTGATTCT
 376 S E V R S A D K A T R Q L I L

 1171 CATGGAATGCGCTGCCAAAAAACTGGCGTTGAAGATGGTCGATTA
 391 M E C A A K K L A L K M V D Y

 1216 TGAGGAGCTTATTAGCTCCCAGGAGCAGTCAGGGAACGCCGGAGG
 406 E E L I S S Q E Q S G N A G G

 1261 ACCTGGCCAGCAGGGCTTGGGTGAAGATGCGGGTGCATAGgaaga
 421 P G Q Q G L G E D A G A **stop**

 1306 aaacgctcctgatttacaaatggagaacttctcactcgttccaga

 1351 cttagaggacttttatgggcatacatatggcccgtcggcttgcatc

 1396 cctgatcgtgcggcttctttttcangacgcaaaagtnttcccgtcc

 1441 tgcaactngaccatcaatncaggaccagcatgggtcatgactgt

1486 ccgcacaaccacgcagttgcgtggatatactggtgctacccgatt
1531 cgcatgtgaaggatcatcaccccatatttaacctttcgacatat
1576 cacagcagaaatttgattcctttcgaaggatcaagggcatgaag
1621 agccttatccacatagaaccactaccaacaccttttcgtttcttc
1666 gtttgggggcttcctagagaatttatcattaggctgattggatgg
1711 aatgggggaaaagacaaatcttagccgggacagatactttgatat
1756 tttttcttcagactcgtatgtcgatagtggcacggatgcaaattct
1801 accagcgatacccaagtcccgccccaaaccctcctcttcaaggca
1846 ggaagcaatttgacaatgcctccagcctaaaaaaaa

Figure 16: Sequencing strategy used to determine the sequence of the *sbr* cDNA clone. The cDNA sequence is represented as the thin line between base 1 and 1881 with its coding sequence indicated (cross-hatched arrow). Black arrows represent either sequencing data obtained from exonuclease III deletion clones (cNSBR.x) or primers (Table 4). Most of the sequencing proceeded in the 3' to 5' direction with only two of the runs reading the complementary sequence. Much of the sequence of the coding region was determined by manual sequencing using the Sanger dideoxy-mediated chain-termination method (see Sambrook *et al.*, 1989 for summary) from overlapping runs.

sbr cDNA clone

Table 7: Features of the cDNA sequence of *sbr*. DNA and protein sequences of interest are identified with their location (by either base or amino acid (aa) number). The CONSENSUS column examines sequences from other genes that share some similarity to the *sbr* feature. Subscripts indicate the number of times that a particular amino acid consecutively occurs and "X" represents any amino acid other than cysteine (C). ZnF = Zinc Finger

poly(A) tail	1874-1881	AAAAAAAAA	
stop codon	1298	TAG	TAG occurs in 11% of <i>N. crassa</i> cases (Bruchez et al, 1993a)
poly-alanine stretch	aa 36-42	AAAAAAAA	
glutamine-rich	aa 103-189	46% glutamine over 87 aa	
poly-glutamine (poly-histidine) stretch	aa 111-145	QHQQHQHQ ₄ HQH HQ ₇ HQ ₅ HQ ₆	WC1 (Ballario et al, 1996) NIT4 (Yuan et al, 1991) DAL81 (Bricmont et al, 1991)
cysteine-rich	aa 258-294	CX ₂ CX ₉ CX ₇ CX ₂ CX ₂ CX ₈ C	WC1 - class IV ZnF CX ₂ C ₁₈ CX ₂ C (Ballario et al, 1996) NIT4 – class III ZnF (Zn(II) ₂ Cys ₆) CX ₂ CX ₆ CX ₆ CX ₂ CX ₇ C (Yuan et al, 1991) DAL81 – class III ZnF (Zn(II) ₂ Cys ₆) CX ₂ CX ₆ CX ₈ CX ₂ CX ₆ C (Bricmont et al, 1991)
helix-turn-helix	aa 194-211	TEDMAHESGYGQL NVESA	DAL81 (Bricmont et al, 1991 – contains more references)

Features of the deduced amino acid sequence

The amino acid sequence (incomplete) has 4 areas of interest: a poly-alanine stretch of 7 residues (amino acids 36-42), a glutamine-rich region (aa 103-189) which is 46% glutamine over 87 amino acids, a cysteine-rich region (aa 258-294) arranged $C^1-X_2-C^2-X_9-C^3-X_7-C^4-X_2-C^5-X_2-C^6-X_8-C^7$ and a helix-turn-helix DNA binding motif (aa 194-211).

The secondary structure of the putative SBR protein, determined using a combination of prediction methods, is suggested in Figure 17. Much of the sequence predicts long intervals of alternating coiled and α -helical regions occasionally interrupted by small stretches (3-5 amino acids) of β -sheet structure. Helical secondary structure is predicted for both the poly-alanine and glutamine-rich regions. The cysteine-rich domain has a small β -sheet region encompassing about 6 amino acids at the third cysteine residue (C^3) and the pairs of cysteines ($C^1-X_2-C^2$ and $C^4-X_2-C^5$) surrounding it are predicted to participate in a bend or turn. The helix-turn-helix motif (previously determined by amino acid sequence homology to regulatory proteins identified in Bricmont *et al.*, 1991) coincides to the predicted secondary structure: two α -helical regions surround a turn at the conserved glycine residue. Another structure of interest is a long helical stretch (aa 365-408) involving 44 amino acids near the carboxyl terminal.

The cDNA encodes a novel gene

DNA and amino acid sequence comparisons revealed no overall homologies with other known sequences from the Genbank, *S. cerevisiae* and *Neurospora* databases using the BLAST search algorithm (Altschul *et al.*, 1997). However, searches for protein homology domains revealed that the glutamine-rich, cysteine-rich, and helix-turn-helix regions were common to other proteins (Figure 18).

The glutamine-rich region is 46% glutamine over 87 amino acids and primarily features a poly-glutamine (poly-histidine) stretch of 35 amino acids that is periodically interrupted by histidine residues. This stretch is similar (but not identical) to glutamine-rich regions from other *N. crassa* proteins such as WC1 (Ballario *et al.*, 1996) and NIT4 (Yuan *et al.*, 1991) and the *S. cerevisiae* protein, DAL81 (Bricmont *et al.*, 1991). In all these regulatory proteins, the glutamine-rich domains are suspected to be involved in transcription activation.

Coincidentally, the WC1, NIT4 and DAL81 proteins encode putative zinc-fingers due to their cysteine-rich residues. Although the putative SBR protein also displays a single, cysteine-rich region, its sequence does not agree with any known zinc-finger domains (Todd and Andrianopoulos, 1997; Ballario *et al.*, 1996), the RING-finger motif (Saurin *et al.*, 1996) or the LIM domain (Sanchez-Garcia and Rabbitts, 1994) which all exemplify highly conserved cysteine-rich motifs that are capable of binding zinc ions.

Figure 17: Secondary structure prediction of SBR protein (incomplete) encoded by the cNSBR clone. The analysis was performed using Network Protein Sequence Analysis at IBCP, France (http://www.ibcp.fr/serv_pred.html) and includes predictions from different methods: Gibrat (Gibrat *et al.*, 1987), Levin Homologue method (Levin *et al.*, 1986), Double Prediction Method (Déleage and Roux, 1987), and Self Optimized Prediction Methods from Alignment (Geourjon and Déleage, 1995). The last prediction method (consensus) averages the results of the previous four methods. SBR protein domains in bold are discussed in the text. Abbreviations: C=coil, ■=α-helix, E=extended β-sheet, S/T=bend/turn

Figure 18: Homology between the predicted SBR protein and regulatory proteins containing conserved domains of **(a)** glutamine-rich residues, **(b)** cysteine-rich residues and **(c)** the helix-turn-helix motif. Highlighted residues indicate conserved amino acids. See the text for references.

WC1	QHQMHHQHQQQQQQQQQQQQQQQQQQQQQQQQQQQHQHQQQQ
NIT4	QQQQQRQQQQQQQQQQQQQQQQQQQQQQQQQQ
DAL81	QQQQHQQQQQQQQQQQQQQQQQQ
SBR	QHQQHQHQQQQQHQHQQQQQQQHQQQQQHQQQQQQ

WC1	CANCHTRNTP EWRRGPSGNRDL CNSC
NIT4	CIACRRRKS KCDGALPSCAACASVYGTEC
DAL81	CNQCRLKKTKCNTFP CLECETSR TKC
SBR	CKNCHKGHGPWTSCIVVDGQMC GSCANCWFNASGARC

λ REP	Q	E	S	V	A	D	K	M	G	M	G	Q	S	G	V	G	A	L
λ CRO	Q	T	K	T	A	K	D	L	G	V	T	Q	S	A	I	N	K	A
CAP	R	Q	E	I	G	Q	I	V	G	C	S	R	E	T	V	G	R	I
434R	Q	A	E	L	A	Q	K	V	G	T	T	Q	Q	S	I	E	Q	L
434CRO	Q	T	E	L	A	T	K	A	G	V	K	Q	Q	S	I	Q	L	I
P22R	Q	A	A	L	G	K	M	V	G	V	S	N	V	A	I	S	Q	T
MAT α 2	K	E	E	V	A	K	K	C	G	I	T	P	L	Q	V	R	V	T
DAL81	D	K	T	C	A	L	N	E	G	R	Q	S	H	L	I	L	G	R
SBR	T	E	D	M	A	H	E	S	G	Y	G	Q	L	N	V	E	S	A

D. Analysis of RNA and proteins

***sbr* gene shows homology to mRNA transcript**

A Northern blot of wild-type RNA (Figure 19) including both whole and poly(A) enriched RNA was probed with Ava600 to determine: 1) whether the *sbr* gene was expressed 2) it's level of expression and 3) the size of its mRNA transcript. Although there was some non-specific hybridization to the abundant rRNA, the probe also hybridized as two bands to the poly(A) enriched RNA. Northern hybridizations to other blots (not shown) revealed a faint banding pattern that was the same as the one in Figure 19 suggesting that the transcript may be expressed at low levels. The predicted size of the hybridizing mRNA could be as small as 1.7 kb but is likely closer to 2.9 kb since this larger band on the blot had stronger hybridization than the 1.7 kb signal. This finding also suggests that we should expect *sbr* cDNA of the same size, however, sequence analysis determined that the cDNA is 1.9 kb. Therefore, it is likely that cNSBR is truncated at the 5' end and is probably missing both the 5' regulatory sequences and the amino terminal of the putative SBR polypeptide.

Figure 19: Northern blot of wild-type RNA probed with Ava600 (*sbr* genomic DNA). **(a)** Gel electrophoresis and **(b)** Northern hybridization of wild-type whole RNA (lane 1) and poly(A) enriched RNA (lane 2). Bands of ribosomal RNA (rRNA) act as an approximate molecular weight standard.

Proteins of *sbr* and wild-type are indistinguishable

Total cell protein extracts of *sbr* and wild-type were analyzed by SDS-PAGE to visualize proteins that spanned 25 to 98 kDa. There were no significant differences between the protein compositions of the two strains (not shown). The only anomaly was that *sbr* exhibited a 37 kDa protein band that stained less intensely than wild-type. This inconsistency may be attributed to a difference in the amount of protein loaded or possibly due to degradation.

Western immunoblotting with cell wall antisera led to a similar general result. There were no distinguishable differences in antibody binding to proteins of *sbr* and wild-type (not shown). In both strains, numerous cellular proteins showed affinity to the antiserum against cell wall proteins.

IV. DISCUSSION

Insights into the colonial morphology of *sbr*

The *sbr* mutant was generated by insertional mutagenesis using an *hph* gene construct. It has a highly restricted, colonial morphology under all growth conditions tested. Superficially, *sbr* resembles a large class of morphological mutants all classified as colonials (Garnjobst and Tatum, 1967). They share the main attributes of restricted, radial growth and increased hyphal branching but may vary in texture, conidiation, pigment, density, and fertility.

All of the colonials included in Table 1 and others such as *doily* and *peak* exhibit this restricted colonial growth. The texture of these colonies may be smooth (*balloon*), bumpy (*cot-2*) or even fuzzy (*doily*). The complex arrangement of the hyphae that constitute the overall colonial morphology also varies widely. Microscopic examination of hyphae reveals that *sbr* hyphae are not only 4 to 20 times wider than wild-type, but also its swollen, budded cells greatly differ from the conventional narrow, tubular morphology of wild-type hyphae.

Phenotypically, *sbr* most closely resembles a colonial strain designated as *le-1*, for ascospore lethal (Murray and Srb, 1961). The hyphae of *le-1* appear swollen and highly articulated with bud-like protrusions. With age, the compact aerial mycelium darkens yet remains paler than *sbr*. From the aerial mycelium, a few sparsely arranged filamentous branches (similar in appearance

to wild-type) may protrude, which have not been observed in *sbr*. As well, the *le-1* colonies are nearly twice as large and fragment more easily.

In comparison, the colonial morphology of strains, *col-1* (de Terra and Tatum, 1963), *col-5*, *cot-1*, *peak* and *doily* (Vierula, 1996) are formed by a network of hyphae: each hypha is similar in shape and width to wild-type but differs by an increase in branching frequency. Similarly, *sbr* also exhibits a high degree of branching eventually giving rise to spherical, bud-like cells.

The increased density of the mycelium of colonial mutants appears to be the result of increased branching and budding. An older theory speculated that new branch formation was a result of turgor pressure distending a portion of the cell wall which had weakened structural integrity (de Terra and Tatum, 1961). The theory was modified slightly upon the discovery of localized cell-wall-bound enzymes, which were capable of hydrolyzing cell wall polymers such as proteins and polysaccharides, that could weaken the wall sufficiently for branch initiation (Mahadevan and Mahadkar, 1970). As an example, the spreading colonial mutant, *spco-1*, showed an increase in both the branching frequency and cell wall autolytic activity (Mahadevan and Mahadkar, 1970).

More recently, a model of bud-site selection in *S. cerevisiae* has been elucidated and may reflect a relevant mechanism for localized growth at hyphal tips and branch sites in *N. crassa*. In yeast, both the genotype and nutrient availability determine the budding pattern (Drubin and Nelson, 1996). Once a target site has been chosen, a signalling network comprised of GTP-binding

proteins, kinases, phosphatases, structural proteins and the cell wall biosynthetic machinery localizes to this site and initiates polarized growth (Drubin and Nelson, 1996). Similarly, signal transduction pathways involving Ras-like protein (Kana-uchi *et al.*, 1997), kinase (Yarden *et al.*, 1992; Bruno *et al.*, 1996) and calmodulin-binding protein (Prokisch *et al.*, 1997) have been found to influence cell growth and morphology in *N. crassa*.

One signalling pathway that has received some serious attention has been one that involves calcineurin, a Ca^{2+} /calmodulin-regulated serine/threonine phosphoprotein phosphatase. Inhibition of calcineurin function results in loss of the high Ca^{2+} gradient at the hyphal tip, and consequent changes in hyphal morphology and an increase in branching (Prokisch *et al.*, 1997).

In the case of *sbr*, it is possible that there may be more enzyme activity associated with its cell wall due to an altered proportion of cell wall polymers to enzymes; or its hyphal morphology may be a result of disruption of a signalling pathway causing an altered Ca^{2+} gradient in the hyphal tips. It should be noted however, that the mycelial and cell-wall-associated proteins of *sbr* did not significantly differ from those of wild-type extracts (unpublished data). The extremely compact morphology of *sbr* precluded any measurement of Ca^{2+} gradients.

In addition to the repercussions of branching on growth, a mucopolysaccharide (MP) produced and secreted by *N. crassa* has been

associated with the slow-growth phase of a temperature sensitive *cot* strain (Reissig and Glasgow, 1971). It is suspected that the growth rate and morphology have been effected by the agglutination ability of MP. The aberrant morphology can be partially reversed when polyphosphate binds and acetylates MP. In wild-type strains, both MP and polyphosphate levels are detected in the stationary growth phase, well after the extensive mycelium has been established. It is conceivable that the glistening texture and dense, clumpy appearance of *sbr* is a result of MP production early in the vegetative life cycle. To confirm this, either the growth medium could be analyzed for MP or polyphosphate could be added to induce growth.

Both asexual and sexual spore development is disturbed

The *sbr* mutation impacts upon all stages of the life cycle including vegetative hyphal growth, conidiation and the germination of sexual spores. In asexual spore development, it appears that conidiation still proceeds after desiccation but aerial hyphae do not project from the surface of the dense colony. Instead, development of conidiophores remains tightly associated with the surface. As the colony becomes increasingly dry, clusters of large buds may coat and stipple the exterior of the colony. As observed by SEM, these buds do not resemble the chains of macroconidia from wild-type cultures but instead appear rounder, resembling the cells of budding-yeast before separation. The fact that *sbr* does not produce free conidia suggests that there is a defect in

conidial separation as seen in *csd-1* and *-2*. It is possible that the defect may occur even earlier in the developmental pathway. However, constrictions at bud junctions are evident, which suggests that the major constriction stage of development is not blocked.

The unusually large size and great numbers of buds are reminiscent of the *tng* and *gran* mutants respectively. Both of these mutants are believed to have defects that occur in asexual development, arising either at the stage of growth localization (*gran*) or growth cessation (*tng*) during repeated apical budding. The *gran* strain produces unusually round macroconidia due to aberrant conidiation (Springer and Yanofsky, 1989). A single *gran* proconidium may give rise to numerous (5 to 10) unusually small buds compared to wild-type (1 to 3 buds). Some conidia of *tng* have been found to reach sizes of 100 μ m or more and lack an appreciable cell wall; as a result they are quite fragile (Springer and Yanofsky, 1989). Unlike *tng* conidia, *sbr* buds are quite robust and they stain brightly with calcofluor, which is indicative of a chitinous cell wall. Apparently, the primary defect of *sbr* cannot be attributed to a single structural abnormality in either of the vegetative or conidiation stages.

Other genes involved in conidial development are the conidiation (*con*) genes. Many are highly expressed during conidiation and become transcriptionally active during the transition from minor to major constriction (Sachs and Yanofsky, 1991). Both *con-6* and *-10* transcripts and proteins are not detectable during vegetative mycelial growth but are elevated during

conidiation (Sachs and Yanofsky, 1991). Both genes have an upstream enhancer regulatory sequence that may interact with binding factors that have been found in extracts of wild-type mycelia but not conidia (Springer, 1993). Therefore, it has been suggested that some *con* genes may be repressed during the mycelial stage by *trans*-regulatory proteins. Madi *et al.* (1994) attempted to identify regulatory genes that controlled the development of conidia. To do so, they generated mutants that expressed either of the *con-6* or *-10* genes during vegetative growth. Some of the resulting mutants were blocked in conidiation or conidial separation and had phenotypes that resembled *acon-2* and *-3*, *fl*, *fld*, *csd-1* and *-2*. It was suggested that the altered genes likely encoded proteins that were not normally expressed at high levels (or possible at all) during vegetative growth and therefore may have interfered with normal growth (Madi *et al.*, 1994). They also suggested that *con-6* and *-10* are regulatory genes since the mutations not only altered the expression of genes that are normally highly expressed during conidiation (*con* genes) but also influenced conidial development.

Based upon the phenotype alone, it is possible that the *sbr* gene product also encodes a regulatory protein that functions at a specific developmental stage. During *sbr* development, the colony radius reaches a limited size and then growth by intense budding and branching increases the density and height of the colony. It is possible that this seemingly early "aerial" development may be due to early expression of genes that are normally active in later stages. The

switch to vertical growth does not appear to directly correspond to desiccation or carbon starvation and the actual appearance of conidial clusters does not follow until many days later.

Although the asexual conidia are capable of germinating, the sexual spores (ascospores) are not as viable. Ascospores produced from a mating between wild-type and *sbr* produced perithecia and ascospores that were normal in morphology. Surprisingly, germination frequency of the spores was poor and nearly all successfully germinating spores gave rise to spreading colonies that looked exactly like wild-type. Only a single spore out of 22 produced a compact colony that resembled *sbr* and more than half the spores did not germinate. If the gene were lethal then we would expect 2 pairs out of the 4 pairs of spores (originating from a single ascus) to fail germination. Although the observations are not in agreement, the sample size is too small to make any solid conclusions.

Many other mutants have been found to be defective in ascospore germination including *le-1* (Murray and Srb, 1961), a colonial mutant with similar morphology to *sbr*. Germination of *le-1* spores did not result in any *le-1*-like colonies using routine heat-shock activation. A small number of spores (1.5%) were induced to germinate when washed with a 1:10 dilution of bleach and ground with a mortar (Garnjobst and Tatum, 1967). Since germination of some ascospores can be rescued by mechanically or chemically disrupting the spore

coat, there may either be a defect in the lytic enzymes responsible for wall degradation or, the spore coat is unusually resistant to lysis.

The resemblance between *sbr* and *le-1* in spore germination marks yet another similarity between the two strains, which may infer that they are both suffering from defects in the same gene. The *le-1* strain was isolated after treatment of wild-type cells with a chemical agent (Murray and Srb, 1961). Often chemical mutagens induce random point mutations, which in this case, may have partially disrupted the gene causing the *le-1* phenotype. In contrast, it is probable that the more severe *sbr* phenotype is a result of complete gene inactivation. Several experiments were undertaken to localize *sbr* by RFLP mapping but no suitable polymorphisms were detected in the cloned sequences.

Does the cytoskeleton influence the morphology of *sbr*?

Since both the vegetative and reproductive morphology is altered in *sbr*, it was of interest to examine the structural component of the cell that has been shown to define the cell shape in other organisms: the cytoskeleton. The two main cytoskeletal components of interest are microtubules and microfilaments (actin). In the cells of higher eukaryotes, microtubules have been implicated in cell shape determination, movement of most (if not all) organelles, chromosome movement, and organization of other cytoskeletal components. In yeast, microtubules may determine the polar axis of growth and have a role in mitosis, nuclear migration, nuclear fusion, and organelle transport (Adams and Pringle,

1984 and Barnes *et al.*, 1990). Microtubules of *A. nidulans* are likely involved in the nuclear cell cycle but have not been shown to directly influence hyphal morphogenesis or growth polarity (Harris, 1997). However, inhibition of microtubule spindles prevents septum formation (Harris, 1997).

In *Neurospora*, microtubules and their associated proteins are believed to play a role in nuclear migration (Plamann *et al.*, 1994 and Robb *et al.*, 1995), organelle movement (Steinberg and Schliwa, 1993), and hyphal growth polarity (Seiler *et al.*, 1997). Wild-type microtubules are aligned in the direction of hyphal growth in both subapical hyphal regions and hyphal tips as seen in Figure 5 and Bruno *et al.* (1996). Nuclei migrate along microtubule tracts resulting in an even spacing of nuclei along the subapical hypha. Although microtubules in *sbr* appear to be functional as suggested by the distribution of nuclei, the microtubules are arranged as short, scattered cables whose ends are not aligned towards the cell junctions.

A similar phenotype was observed in the *N. crassa mcb* mutant which has a defect in polarized growth resulting in bulbous cells that grow isotropically until they rupture (Bruno *et al.*, 1996). This isotropic growth is likely due to mis-localization of new cell wall materials delivered by vesicles.

The mode of vesicle transport and growth polarization is still a subject of great debate. Early experiments using anti-tubulin agents suggested that microtubules were not essential for apical growth (That *et al.*, 1988). However, Steinberg and Schliwa (1993) have determined by video microscopy that

microtubules support organelle transport in *Neurospora*. More recently, Seiler *et al.* (1997) used Transmission Electron Microscopy (TEM) to show that polarized growth is dependent on the activity of kinesin (a microtubule motor) to transport vesicles to the growing tip. These mutants were found to lack the apically-associated Spitzenkörper vesicle docking centre and had far fewer vesicles at the tip. In contrast, Bruno *et al.* (1996) insinuated that polarized growth was an actin-dependent process due to the correlation between loss of growth polarity at the hyphal tip and the corresponding arrangement of actin patches in the *mcb* mutant.

Actin organization in yeast is responsible for localized growth of the cell surface and in directing organelle transport from mother cell to bud (Barnes *et al.*, 1990). Filamentous actin of *S. cerevisiae* is organized into cables and patches where cables traverse the yeast cell in accordance to the direction of polarization while patches are concentrated at sites of cell surface growth (Adams and Pringle, 1984). Actin has differential localization during the yeast cell cycle: it is concentrated at the bud site just prior to bud emergence, is then dispersed throughout the new bud during its phase of isotropic growth, and finally becomes relocalized at the septum of the mother-bud junction at separation (Adams and Pringle, 1984).

In contrast, *A. nidulans* shows simultaneous co-localization of actin binding to both the growing hyphal tip and septa (Harris *et al.*, 1994) but is also believed to be the central cytoskeletal component involved in the delivery of

vesicles to areas of active cell wall deposition (Harris, 1997). In *Neurospora*, actin patches are also concentrated at hyphal tips and septa (Bruno *et al.*, 1996). This is not completely true for the *mcb* mutant where actin is not localized to the tip, but rather, is distributed uniformly over the cell cortex and concentrated at mis-localized septa (Bruno *et al.*, 1996).

It has been speculated that vesicle transport to the hyphal tip and septum may depend upon separate secretory pathways involving different Golgi bodies or sorting signals (Bruno *et al.*, 1996; Harris, 1997 and Mondésert *et al.*, 1997). One would expect that as the length of the cell increases, there would be greater demands for an efficient transport system between the site of metabolite synthesis and the site of utilization or incorporation. Hence, it would be reasonable to hypothesize that both microtubules and actin play a joint role in directing vesicles to the site of their incorporation. It is possible that microtubules, which have been found to be associated with Golgi bodies (Fath *et al.*, 1997), transport vesicles over the length of the hyphal cell to its apex and then actin microfilaments deposit them at the membrane/wall surface.

Conceivably, the spherically-shaped cells of *sbr* may be a consequence of the random deposition of vesicles (containing cell wall precursors and metabolites), which are isotropically fed to the surface of the cell membrane/wall by the observed scattered tracks of microtubules. Since the pattern of actin distribution cannot be confirmed at this time, it can not be directly correlated to the *sbr* morphology. However, staining with calcofluor (binds to chitin and other

β -linked polysaccharides) can at least identify areas of the cell that have integrated chitin into its wall. Calcofluor staining of wild-type hyphae is localized to chitinous septa and (to a lesser extent) cell walls. *Sbr* cells displayed strong staining of both septa and walls indicating that septa localization was normal and therefore actin was functional (assuming that actin is required for septa development). The significant incorporation of stain into the walls may indicate an altered composition of the cell wall. As seen by Mahadevan and Tatum (1965), colonial mutant, *col-1*, had more than double the amount of glucosamine incorporated into its wall. Not surprisingly, chitin is a structural polysaccharide of N-acetyl-D-glucosamine residues joined by β 1-4 linkages.

Tag integration is confirmed

The original insertional mutagenesis strategy involved transforming wild-type *N. crassa* with the 2.4 kb *hph* construct that was excised from PCSN43 by *Sa*I digestion. The Southern blot of *sbr* genomic DNA was probed with the tag and confirmed that only a single copy had incorporated. Surprisingly, sequencing of KSSBR0 (carrying cloned *sbr* genomic DNA) revealed that the transforming DNA not only encompassed the *hph* construct but also some of the PCSN43 Bluescript cloning vector that flanked the construct. Such an occurrence was surprising since the cells were transformed with a gel-purified, DNA preparation of the 2.4 kb *Sa*I fragment consisting only of the *hph* construct. Therefore, the actual transforming DNA must have been circular or

incompletely-digested vector DNA, which co-purified with the linear DNA fragment. This was an unexpected result since the frequency of transformation has been found to be reduced when using circular DNA (Paietta and Marzluf, 1985).

Initially, this revelation posed potential problems for subcloning and sequencing since the tag was actually larger than anticipated and encoded an additional T7 priming site and restriction enzyme sites. Fortunately, subclone KSSBR0 (6.2 kb) was found to contain 700 bp of *sbr* genomic DNA flanking the PCSN43 vector, which was subsequently sequenced and used as a probe to screen the cDNA library.

The actual integration of the tag appears to occur in the coding sequence of the gene. It is likely that the gene has been completely inactivated due to the large size of the tag (~3.4 kb) and its location.

Is SBR a regulatory protein?

Despite the fact that *sbr* does not have overall homology to any known gene, a possible function may still be ascertained based on some of the amino acid homology domains that it encodes. The first homology domain corresponds to a helix-turn-helix (hth) DNA binding motif that has been found to occur in numerous regulatory proteins. The motif's sequence has three conserved residues (alanine/glycine, glycine, and valine/isoleucine) that are believed to be important for the stability of the bihelical complex (Brennan and

Matthews, 1989). The middle glycine residue corresponds to a turn in the secondary structure whereas the other two conserved residues are each part of an α -helix that surrounds the turn. Not only does SBR have conservation of these residues but it also has the expected secondary structure. The motif was first proposed for a small number of regulatory proteins expressed by phage and it has since been identified in regulatory proteins of other organisms (see review by Brennan and Matthews, 1989). However, the presence of a motif does not give definitive proof as to its function. For example, the *S. cerevisiae* protein DAL81 also contains this motif but deletion of the sequence did not affect the function of the gene's product (Bricmont *et al.*, 1991).

The poly-glutamine stretch and cysteine-rich residues of SBR are also found in a large number of gene products including other fungal proteins such as WC1, NIT4, and DAL81. WC1 (white collar) is a transcription factor that binds to and affects light regulation of the *albino-3* gene of *N. crassa* (Ballario *et al.*, 1996). The NIT4 protein of the same organism appears to regulate structural genes at the transcriptional level via the nitrogen regulatory circuit. This assertion is based on observations that deletion of the domains results in loss of function, it has sequence homology to *A. nidulans*'s NIRA regulatory protein, and *nit-3* expression requires co-activation by NIT4 and NIT2 (another regulatory protein) (Yuan *et al.*, 1991). In yeast, DAL81 is believed to be a regulator of the allantoin degradation pathway at the transcription level (Bricmont *et al.*, 1989) and also encodes both of these domains. Deletion of one

its polyglutamine stretches results in a 50% loss of activity whereas deletion of zinc finger cysteine residues does not influence function (Bricmont *et al.*, 1991).

The cysteine-rich residues from the above proteins specify putative zinc finger DNA binding proteins that belong to different classes as determined by their amino acid sequence and secondary folding structure. WC1 encodes a single class IV zinc finger protein where a single zinc ion is linked to four cysteines (Ballario *et al.*, 1996). Other class IV members include GATA factors, which are characterized by the occurrence of two zinc finger proteins, which bind a total of two zinc ions (Sánchez-García and Rabbitts, 1994). In contrast, both NIT4 and DAL81 are considered class III zinc fingers, also known as the $\text{Zn(II)}_2\text{Cys}_6$ binuclear cluster DNA binding motif. They are believed to form a cloverleaf structure by arrangement of their six cysteine residues around two zinc ions (Todd and Andrianopoulos, 1997). This binding motif is unique to over 80 fungal proteins, which generally act as transcriptional activators (Todd and Andrianopoulos, 1997).

The cysteine-rich region of SBR does not correspond to either of these classes nor to any other class of zinc finger protein. In addition, its cysteine-rich region is predicted to contain small β -sheet structures and turns (possibly forming anti-parallel sheets) but lacking any α -helical secondary structures. Conversely, many of the known zinc fingers are constructed from both α -helices and β -sheets (Sánchez-García and Rabbitts, 1994). Despite this discrepancy, it is interesting that the cysteine residues in SBR are all clustered together in the

same area of the >430 amino acid protein . Only a single cysteine occurs outside of this region, 100 amino acids downstream of the cysteine-rich region. Since the sequence of this region does not match any known zinc fingers precisely, it may represent a non-functional, evolutionarily conserved motif or it may belong to a new class of zinc finger DNA binding proteins.

In light of the similarities between the SBR protein domains and the domains of the other regulatory proteins, it is reasonable to suspect that SBR also has a regulatory function. Naturally, the best evidence to corroborate this speculation would be experiments such as a DNA binding assays or even deletions of the hth, polyglutamine or cysteine-rich regions.

Whatever the role of *sbr*, Northern hybridization has also confirmed that it is expressed and its transcript is likely around 2.9 kb. A concern with the Northern was the presence of a second fainter band (~1.7 kb) under the main 2.9 kb band. In light of the repetitive nature of the *sbr* DNA sequence, the probe may have non-specifically recognized these repeated sequences or the sequence may encode two promoter sites. Although readily detectable in polyadenylated RNA, hybridization of Northern blots of total RNA showed faint hybridization to the *sbr* Ava600 probe. This indicates that the gene is not highly expressed in mycelial cultures. In order to get a true indication of expression levels, *N. crassa* cultures at different developmental stages (i.e. germinating conidia, mycelial, and conidiating) could be tested. Another useful experiment would be to attempt isolation of *sbr* RNA and to probe its Northern with genes

that are known to be expressed at particular developmental stages (i.e. *con* genes). Such an experiment could determine whether the *sbr* mutation has affected the expression of other genes.

In the cases of the *cot-1* and *mcb* morphological mutants, both encode subunits of cAMP-dependent protein kinase A (PKA). Correspondingly, expression of this regulatory unit has been found necessary for polarized hyphal tip growth (Yarden *et al.*, 1992 and Bruno *et al.*, 1996). As previously discussed, calcineurin is regulated by Ca^{2+} -binding calmodulin and its expression is essential for cell growth and maintenance of the high Ca^{2+} gradient at the hyphal tip (Prokisch *et al.*, 1997). Similarly, the *N. crassa ras2* gene, also involved in a signal transduction pathway, has been found to regulate apical growth of hyphae, cell wall synthesis and conidial formation (Kana-uchi *et al.*, 1997).

There is also evidence for involvement of signal transduction pathways in the development of other fungi. Growth of *A. nidulans* requires expression of both calcineurin (Rasmussen *et al.*, 1994) and the *ras* homolog (Som and Kolaparthi, 1994), whereas *S. cerevisiae* requires Ras-like GTP binding proteins for polarized actin cytoskeleton assembly at the site of bud emergence (Drubin and Nelson, 1996).

A common phenomenon shared by mutants defective in these regulatory pathways is the numerous effects on development and morphogenesis. Such extensive consequences are similarly apparent in *sbr*. Alterations in all stages of development including gross changes in the morphology and disruption of

cytoskeletal arrangement suggest that *sbr* disruption confers many pleiotropic effects. A fitting role for *sbr* could include involvement in a signal transduction pathway that has a regulatory role in morphogenesis.

In this thesis, ectopic integration using heterologous DNA has proved to be an effective tool in generating a new morphological mutant. This *sbr* mutant has a distinctive colonial phenotype, which is linked to defects in hyphal growth and morphology, conidiation, microtubule arrangement and germination of sexual spores. Sequence analysis of the gene suggests that it plays a regulatory role in the cell. The subsequent repercussions of these findings and future work will likely prove beneficial in enhancing our knowledge of the mechanisms of morphogenesis, hyphal growth and development.

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IMAGE EVALUATION TEST TARGET (QA-3)

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