GENETICS AND PHYSIOLOGY OF RESPIRATION IN *ASPERGILLUS NIDULANS*

A THESIS PRESENTED IN PARTIAL FULFILMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR IN PHILOSOPHY
IN
MOLECULAR GENETICS
AT MASSEY UNIVERSITY,
PALMERSTON NORTH, NEW ZEALAND

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2001

ABSTRACT

Obligate aerobes such as *Aspergillus nidulans* primarily use the classical respiratory pathway for ATP production. However, the use of alternative energy-producing processes in *A. nidulans* was first speculated upon when cytochrome *c*-deficient strains were observed to be viable upon fermentable carbon sources. It was therefore postulated that the *cycA*$^-$ strains of *A. nidulans* may use fermentation and an alternative respiratory pathway to compensate for the non-functioning cytochrome *c*-dependent pathway.

Characterisation of the *A. nidulans* cytochrome *c*-deficient strains was carried out. The growth parameters for strain A68 were consistent with other cytochrome *c* mutants; the strain grew more slowly than the corresponding wildtype strain on fermentable carbon sources, and produced higher levels of ethanol. Spectral analysis confirmed the lack of detectable levels of cytochrome *c* in the *cycA*$^-$ strains, and decreased levels of cytochrome *c* oxidase, consistent with the non-functioning cytochrome *c*-dependent respiratory pathway. The presence of a hemoglobin-like molecule in the *cycA*$^-$ and *cycA*$^+$ strains was determined by CO binding assays.

Inhibitor studies determined the presence of an alternative respiratory pathway in *cycA*$^-$ and *cycA*$^+$ strains of *A. nidulans*. An active cytochrome *c*-dependent pathway was found to be present in the *cycA*$^+$ strains, yet absent from the *cycA*$^-$ strains. Results also suggested the presence of a putative third terminal oxidase in the *cycA*$^-$ and *cycA*$^+$ strains. Increased levels of *b*-type heme observed in the redox spectra of the *cycA*$^-$ strains were suggested to be associated with the putative third terminal oxidase.

Therefore, the *cycA*$^-$ strains of *A. nidulans* appear to use fermentation and the alternative respiratory pathway to compensate for the non-functioning cytochrome *c*-dependent pathway. The putative hemoglobin molecule identified in these strains may also function as a terminal oxidase, in addition to the putative third terminal oxidase.

PCR amplification with degenerate primers was carried out to confirm the presence of an *AOX* gene in *cycA*$^+$ and *cycA*$^-$ strains of *A. nidulans*. The product of the *AOX* gene is likely to function as a terminal oxidase in the alternative pathway. A comparison of fungal and plant AOX protein sequences was carried out. A conserved cysteine residue which has

been implicated in dimer formation and pyruvate regulation was found to be absent from the fungal sequences.

A preliminary expression study of the *A. nidulans AOX* gene was carried out by RT-PCR. The putative regulatory elements identified within the *A. nidulans AOX* gene promoter are also located within the promoters of other respiratory-related genes (eg. *A. nidulans cyc*A) and genes involved in reducing the formation of reactive oxygen species (ROS) (eg. *A. nidulans catA-C* and *sod1*). This implies similar mechanisms of regulation, which may be controlled in a coordinated manner.

A functional analysis of the *A. nidulans cyc*A gene promoter was carried out to identify important regulatory elements. Reporter constructs containing *cyc*A-*lacZ* fusion genes were transformed into *A. nidulans*. Although integrated at the *arg*B locus, the constructs had very low levels of *lacZ* expression (with the exception of the positive control). A number of parameters were investigated, and a 'promoter switch' experiment commenced, however the cause of the faulty *cyc*A-*lacZ* expression system was not determined.

Functional expression of the *A. nidulans cyc*A gene promoter in yeast was also attempted, as regulatory mechanisms controlling cytochrome *c* expression are believed to be analogous in *A. nidulans* and *Saccharomyces cerevisiae*. Reporter constructs containing *cyc*A-*lacZ* fusion genes were transformed into *S. cerevisiae*, with resulting low β-galactosidase activity, similar to the results in *A. nidulans*. The wildtype strain of *A. nidulans* from which the *cyc*A gene promoter fragment was amplified was shown by spectral analysis to have low levels of cytochrome *c*, in comparison to other wildtype strains of *A. nidulans*. Therefore, it is possible that the low levels of *lacZ* expression from the *cyc*A-*lacZ* fusion genes may be representative of the level of *cyc*A expression in that strain. However, low levels of *lacZ* expression were also observed for the *S. cerevisiae* positive control, indicating that the expression system was not working properly. Therefore, the expression of the *A. nidulans cyc*A gene promoter in *S. cerevisiae* could not be assessed in this study.

ACKNOWLEDGEMENTS

I would firstly like to thank Dr Rosie Bradshaw, my chief supervisor. Her encouragement and resourcefulness definitely helped during those tough times when nothing seemed to be working! I would also like to thank my co-supervisors, Drs Simon Brown and Kathryn Stowell. Simon, my sincere thanks for all the time and effort you contributed towards my project, I truly appreciated it. I very much enjoyed our little chats (and the coffee)! Your enthusiasum for your work is definitely inspiring. Thanks to Kathryn, too, for your valuable input – even though your expertise was not utilised as much as originally planned. A big thank you must go to Rosie and Kathryn for all the time and effort you contributed towards getting this finally finished!

Thanks to past and present members of the IMBS (MGU), for making Massey an enjoyable place to be, especially Em, Lisa, Beccy, Bec (the honorary RB lab member) and Rich. A mention of thanks must be made here also to Brendon, Branwen, Austen, Sheralee and Jo.

A big thank you to Lisa and Carolyn, for letting me borrow stuff! Your encouragement and useful advice was truly appreciated. Thanks to Beccy, as well, for her well-supplied desk which she insisted I take advantage of. And not forgetting Bec (general dogsbody) for her help over the last couple of months.

Thanks to everyone else who has helped in some way – Fee, for being a good friend; my family, for a welcome distraction from study; Jos & Bry, for the support & cups of tea; and Anne, for your interest and generosity. Lastly (but very importantly!), a huuuge thank you to Marty, for all that you have done to help (and for putting up with me).

ABBREVIATIONS

ANCF	*Aspergillus nidulans* CCAAT binding factor
AOX	alternative oxidase
ATP	adenosine triphosphate
bp	base pair
cAMP	cyclic adenosine monophosphate
CCR	carbon catabolite repression
CCHL	cytochrome *c* heme lyase
CO	carbon monoxide
COX	cytochrome *c* oxidase
CycA	*A. nidulans* gene encoding cytochrome *c*
CYC1	*Saccharomyces cerevisiae* gene encoding iso-1-cytochrome *c*
CYC7	*S. cerevisiae* gene encoding iso-2-cytochrome *c*
DNA	deoxyribonucleic acid
EMP	Embden-Meyerhof-Parnas pathway
ETC	electron transport chain
HAP1	*S. cerevisiae* heme activated protein, mediating oxygen regulation
HAP2/3/4/5	*S. cerevisiae* CCAAT binding factor, mediating carbon regulation
HMC	high molecular weight complex
hr	hour(s)
Kb	kilobase pair(s)
KCN	potassium cyanide
MAI	maximum amount of inhibition
min	minute(s)
NADH	nicotinamide adenine dinucleotide
ORF	open reading frame
PCR	polymerase chain reaction
RPM	repression modules
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcribed PCR
SHAM	salicyl hydroxamic acid
SOD	Superoxide dismutase
TCA	tricarboxylic acid cycle
tsp	transcriptional start site

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CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

Aspergillus is a genus of asexual fungi, some members of which have sexual stages and so are classified among the ascomycetes (eg. *Emericella nidulans*). *Aspergilli*, first discovered by Micheli in 1729, are some of the most abundant micro-organisms found worldwide. The fungi are ubiquitous throughout the environment, and are found in soils, decaying matter, cereals and cereal products (Martinelli & Kinghorn, 1994). The predominant role of fungi as scavengers in the environment has led to an extraordinary metabolic versatility, which has both positive and negative implications for human commerce.

1.1.1 COMMERICAL APPLICATIONS OF *ASPERGILLUS*

Many *Aspergillus* species are used as producers of commerically valuable substances. These products include food products and enzymes, in addition to primary and secondary metabolites such as antibiotics and organic acids. For example, *A. niger* is used for the commerical production of citric acid, an important organic acid used in many industrial and pharmaceutical applications, eg. as an acidulant and preservative in food and beverages. Other industrial enzymes produced by *Aspergillus* species, which are also used as preservatives in the food industry, include glucose oxidase, pectinases and proteases (Park Talaro & Talaro, 1999). *A. oryzae* and *A. soyae* are used in commercial fermentation processes to produce soy sauce and miso (Onions *et al.*, 1981). *A. nidulans* is used extensively for the commerical production of pharmaceuticals (eg. Interferon-α2, Epidermal Growth Factor and Growth Hormone), while *A. niger* is used for the production of Penicillin. Fungal species have been used extensively for these processes, as they synthesise extremely high quantities of product. Another advantage is that several species have a long history of safe use, an important consideration in the manufacture of products which are destined for human consumption (Fowler & Berka, 1991).

1.1.2 ADVERSE EFFECTS OF *ASPERGILLUS*

In some instances, the fungus' metabolic versatility can also result in the production of mycotoxins. *A. parasiticus*, *A. flavus* and *A. nomius* all synthesise aflatoxin, which is responsible for the spoilage of many crop species, including peanuts, corn and cotton-seed (Brown *et al.*, 1999). *A. caelatus* has also recently been reported to produce aflatoxin (Peterson *et al.*, 2000). Aflatoxin has been implicated in causing liver cancer in humans, and produces deleterious effects in cattle when consumed (Brown *et al.*, 1999). *Aspergillus* species have also been implicated in causing allergies (Park Talaro & Talaro, 1999).

Another adverse effect of the fungus' metabolic versatility is the occurrence of mycoses; of the estimated 600 *Aspergilli* species, eight are involved in human disease. Infection by the thermophilic *A. fumigatus* is most common. *Aspergillus* is classified as an opportunistic pathogen, as it mainly infects immuno-compromised hosts such as AIDS, leukemia, and burns patients, and those recovering from organ transplantation (Park Talaro & Talaro, 1999). However, infection also occurs in healthy, heavily exposed individuals. Infection (*Aspergillosis*) occurs through conidial/mycelial exposure, with respiratory and mucocutaneous portal entries. The spores germinate and form 'fungus balls'. Benign, non-invasive infection can occur in the sinuses, ear canals, eyelids and conjunctiva. *Invasive Aspergillosis* primarily occurs in pulmonary regions (Hall & Denning, 1994), resulting in necrotic pneumonia. The fungus often disseminates to the brain, heart, skin and other organs, resulting in a grim prognosis for immuno-compromised patients.

Treatment of non-invasive *Aspergillus* infection involves surgical removal, or local drug therapy with antifungal agents. Amphotericin B and/or Itraconazole are two commonly used antibiotics, which impair components of the fungal cell membrane, thus allowing leakage of intracellular components. The combined therapy of these antifungal agents is presently the only treatment available for *Invasive Aspergillosis*. Although the drugs have only limited success, infection is fatal if left untreated. Experimental antifungal agents also target components of the fungal cell wall, eg. the class of pneumoncandins inhibit the synthesis of 1,3-β-D-glucan (Onishi *et al.*, 2000).

1.1.3 CONTROL OF ENVIRONMENTAL CONDITIONS

Environmental parameters (such as carbon and nitrogen sources, cell growth rate, oxygen availability, temperature and pH) are known to affect the respiratory and metabolic state of the organism (Martinelli & Kinghorn, 1994; Raitt *et al.*, 1994). For some *Aspergilli*, manipulation of these parameters is carried out to increase synthesis of a particular product (Park Talaro & Talaro, 1999). For example, high concentrations of easily metabolised sugars, and a high degree of aeration are critical for increasing citric acid production from *A. niger* (Martinelli & Kinghorn, 1994). Recent papers (Kirimura *et al.*, 1987) have also identified the participation of an alternative respiratory pathway in this process (section 1.4.2).

Regulation of the growth and respiration of the organism would also be highly desirable for controlling the levels of harmful secondary metabolites produced by some of the species. For example, *A. parasiticus* produces the highly toxic secondary metabolite, aflatoxin, from accumulated acetyl CoA. This accumulation occurs when oxidative respiration is restricted, eg. under conditions of high glucose levels and low aeration (Shih & Marth, 1974). Thus the importance of oxygen, in particular, as a ubiquitous regulator of gene expression can be noted. However, the actual mechanism by which this regulation occurs is poorly understood at present (Martinelli & Kinghorn, 1994).

1.1.4 *A. NIDULANS*

The obligate aerobe *A. nidulans* has been used extensively as a model organism. Thus it is well characterised at the molecular and genetic level (Davis *et al.*, 1988). The fungus possesses many advantageous qualities which makes it attractive for such study; firstly, the fungus has a complex life cycle analogous to that of higher eukaryotes, yet cultivation on simple media is possible. *A. nidulans* is also readily analysed by molecular and genetic techniques. One of the most invaluable features of *A. nidulans* is its physiological versatility: it can utilise a vast array of nutrients, and grows over a wide range of environmental conditions (Pontecorvo *et al.*, 1953). Growth of *A. nidulans* at various oxygen concentrations has been shown to have little effect on morphological parameters, such as the degree of branching and the mean length of hyphal segments (Carter & Bull, 1971). However, at low oxygen concentrations, abnormally large, thick-walled mycelia are produced, indicating some physiological stress. Alcohol dehydrogenases have been found

in wildtype strains of *A. nidulans*, and are believed to be important in allowing the organism to survive during periods of anaerobic stress (Kelly *et al.*, 1990; Martinelli & Kinghorn, 1994). The existence of the alcohol fermentation pathway in *A. nidulans* was confirmed recently, when pyruvate decarboxylase activity was discovered (Lockington *et al.*, 1997).

To study respiration, mutants in the cytochrome *c* locus (which encodes a protein that is an integral component of the electron transport chain) have been sought. *Neurospora crassa* mutants have been obtained which produce reduced levels of cytochrome *c* (Bottorff *et al.*, 1994). Petite mutants of the yeast *Kluyveromyces lactis* were isolated which had complete absence of cytochrome *c* transcript, and were only able to grow on fermentable carbon sources (Chen & Clark-Walker, 1993). Cytochrome *c* deficient mutants of *A. nidulans* were isolated at Massey University by the method of direct gene replacement. These mutant strains are the first of a filamentous fungus to be shown to be viable after complete elimination of a functional cytochrome *c* gene. The mutants are slow growing, and capable of growth on glucose and glycerol (Bird, 1996). These results suggest that some of the fungus' energy requirements may be generated by substrate-level phosphorylation.

The presence of an alternative respiratory pathway has also been reported in *A. nidulans* (Turner & Rowlands, 1976). The pathway has been identified in other fungal species, eg. *A. niger* (Kirimura *et al.*, 1987) and *N. crassa* (Bottorff *et al.*, 1994), as well as yeast. The pathway has also been discovered in all plants studied to date, some 800 species (Vanlerberghe & McIntosh, 1997). The activity of the pathway has been observed under conditions in which the cytochrome *c* dependent pathway is incapacitated, although the pathway is constitutive in some organisms. It will be interesting to see if the functioning of this pathway allows *A. nidulans* to survive in the absence of cytochrome *c*.

1.2 ENERGY TRANSDUCTION

1.2.1 BRIEF OVERVIEW

The prevalent source of energy in biological systems is adenosine triphosphate (ATP). ATP is formed when electron carriers (eg. NADH, $FADH_2$) or substrates donate a high energy phosphate bond (P_i) to ADP (adenosine diphosphate).

The primary catabolism of 'fuels' (protein, carbohydrates and lipids) necessary for ATP

production proceeds through a series of three interconnected pathways in many organisms: glycolysis; the tricarboxylic acid cycle (TCA/Krebs cycle); and the respiratory pathway (Figure 1.1) (Park Talaro & Talaro, 1999).

Glycolysis (otherwise known as the Embden-Meyerhof-Parnas (EMP) pathway) involves the catabolism of glucose to form pyruvic acid (pyruvate). A small amount of ATP is formed by the energy released from the substrate going directly to ADP (substrate level phosphorylation). Pyruvate molecules enter the TCA cycle for further processing and energy release (aerobic organisms), or become involved in fermentative metabolism (anaerobes). Electron carriers generated by glycolysis and the TCA cycle (NADH and $FADH_2$) are utilised in the respiratory pathway.

The respiratory pathway consists of a series of redox carriers which receive electrons from electron carriers, and translocate them in an orderly and sequential manner (Figure 1.2). The transfer of electrons causes the expulsion of H^+ ions (protons) into the mitochondrial intermembrane space, thus generating a proton motive force. The free energy stored in the pH gradient drives the synthesis of ATP. The coupling of electron transport with ATP synthesis is known as oxidative phosphorylation.

It is important to remember that cells maintain a constant state of equilibrium. Thus catabolic and anabolic pathways occur simultaneously, with many of the compounds produced in either type of pathway being recycled for use in the other.

1.2.2 AEROBIC RESPIRATION

Organisms which are aerobic heterotrophs (ie require oxygen) use aerobic respiration to meet their energy requirements. Aerobic respiration involves all three pathways (Figure 1.1), with oxygen as the terminal electron acceptor. ATP production is high (36 ATP molecules per glucose molecule), with CO_2 and H_2O also synthesised.

i.e. $C_6H_{12}O_6 + 6O_2 + 36ADP + 36P_i = 6CO_2 + 6H_2O + 36ATP$

Bacteria have a respiratory pathway similar to eukaryotes, although one or more of the redox steps may be omitted, and alternative electron transport schemes may be present (for a review, see Park Talaro & Talaro, 1999). Alternative pathways for the catabolism of

Figure 1.1 **Simplistic Model Showing The Interconnection Of The Three Pathways Involved In Energy Transduction.**

The three coupled pathways are shown: Glycolysis, the Tricarboxylic acid cycle, and the respiratory pathway. The products generated (from 1 glucose molecule) by each of these pathways are indicated in the output summary.

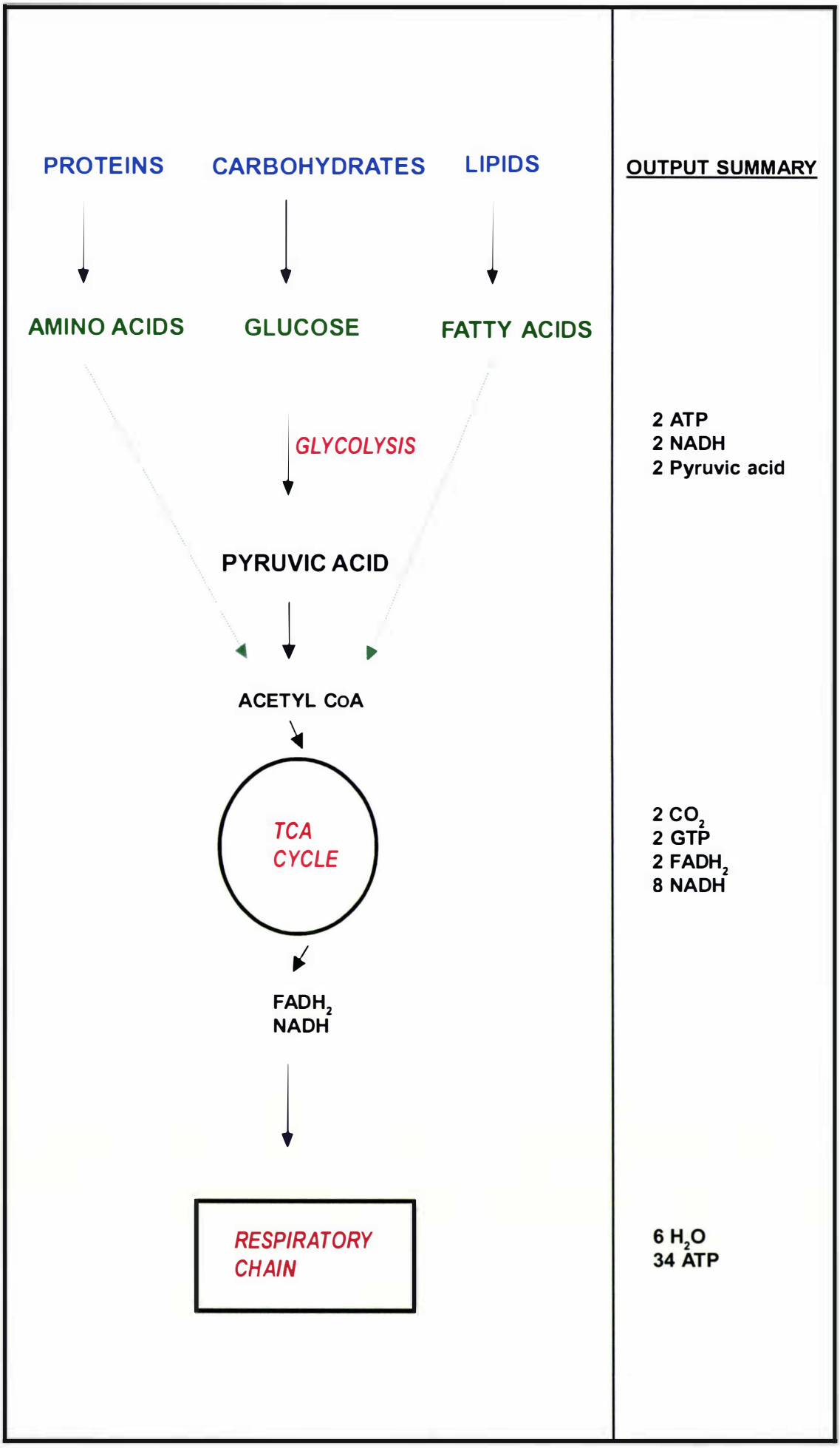
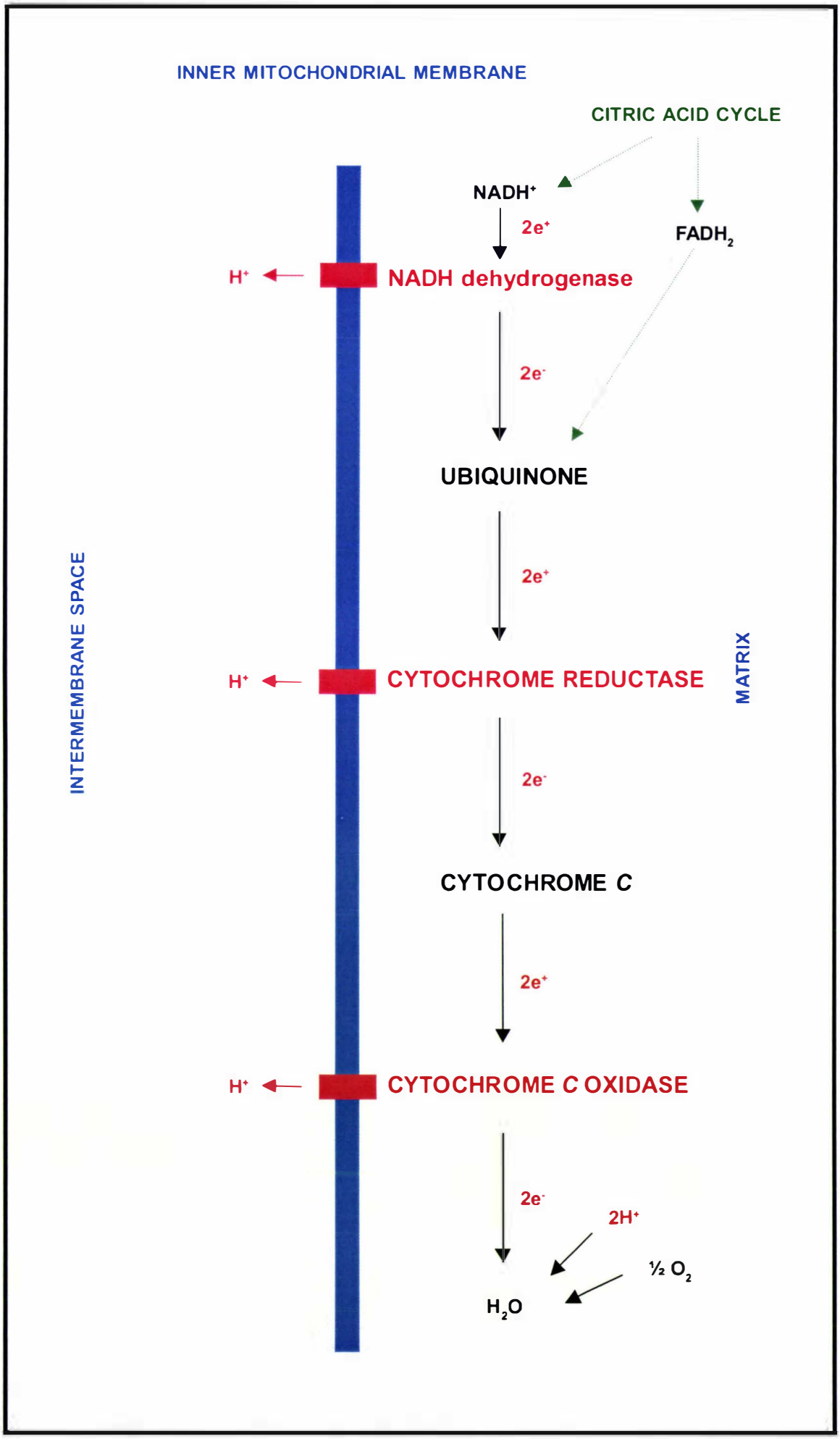

PROTEINS
CARBOHYDRATES
LIPIDS
OUTPUT SUMMARY
AMINO ACIDS
GLUCOSE
FATTY ACIDS
GLYCOLYSIS
2 ATP
2 NADH
2 Pyruvic acid
PYRUVIC ACID
ACETYL CoA
TCA CYCLE
2 CO2
2 GTP
2 FADH2
8 NADH
FADH2
NADH
RESPIRATORY CHAIN
6 H2O
34 ATP

Figure 1.2 The Classical Respiratory Pathway

The entry point of the electron carriers NADH and $FADH_2$ from the TCA cycle, and the transfer of electrons between the four protein complexes is depicted. The three phosphorylation sites, at which proton (H^+) transfer and ATP production occur, are indicated in red.


INNER MITOCHONDRIAL MEMBRANE
CITRIC ACID CYCLE
NADH+
2e+
FADH2
H+
NADH dehydrogenase
2e-
UBIQUINONE
2e+
INTERMEMBRANE SPACE
MATRIX
H+
CYTOCHROME REDUCTASE
2e-
CYTOCHROME C
2e+
H+
CYTOCHROME C OXIDASE
2e-
2H+
½ O2
H2O

carbohydrates are also present in bacteria, including the phosphogluconate pathway (hexose monophosphate shunt). This pathway allows for the oxidation of glucose and other hexoses, in anaerobic conditions. ATP, NAPDH and pentose molecules are generated (the latter being important for nucleic acid synthesis).

1.2.3 ANAEROBIC RESPIRATION

Anaerobic organisms (and some facultative anaerobes) can also use anaerobic respiration to gain ATP. All three pathways (Figure 1.1) are used, however oxygen-containing inorganic salts are used as the final electron acceptors, such as nitrate (NO_3) and nitrite (NO_2).

ie $NO_3^- + H_2 \rightarrow NO_2^- + H_2O$

The end product (eg. NO_2^-) can be further reduced by the process of denitrification, which is carried out by nitrate reductases or hemoglobin (section 3.6.2).

Anaerobic/fermentative metabolism predominantly occurs in facultative anaerobes. In conditions devoid of oxygen, the incomplete oxidation of glucose and other carbohydrates utilises organic molecules as terminal electron acceptors (Figure 1.3).

Ethyl alcohol and lactic acid are produced by yeast and homolactic bacteria, respectively. As only the first pathway (glycolysis) is utilised, only a small amount of ATP is produced (2 ATP molecules per glucose molecule) compared to aerobic respiration (Figure 1.1). Some organisms (eg. *S. cerevisiae*) exhibit the Pasteur effect, in which the rate of glycolysis is increased under anaerobic conditions, to meet energy requirements (Storey, 1985).

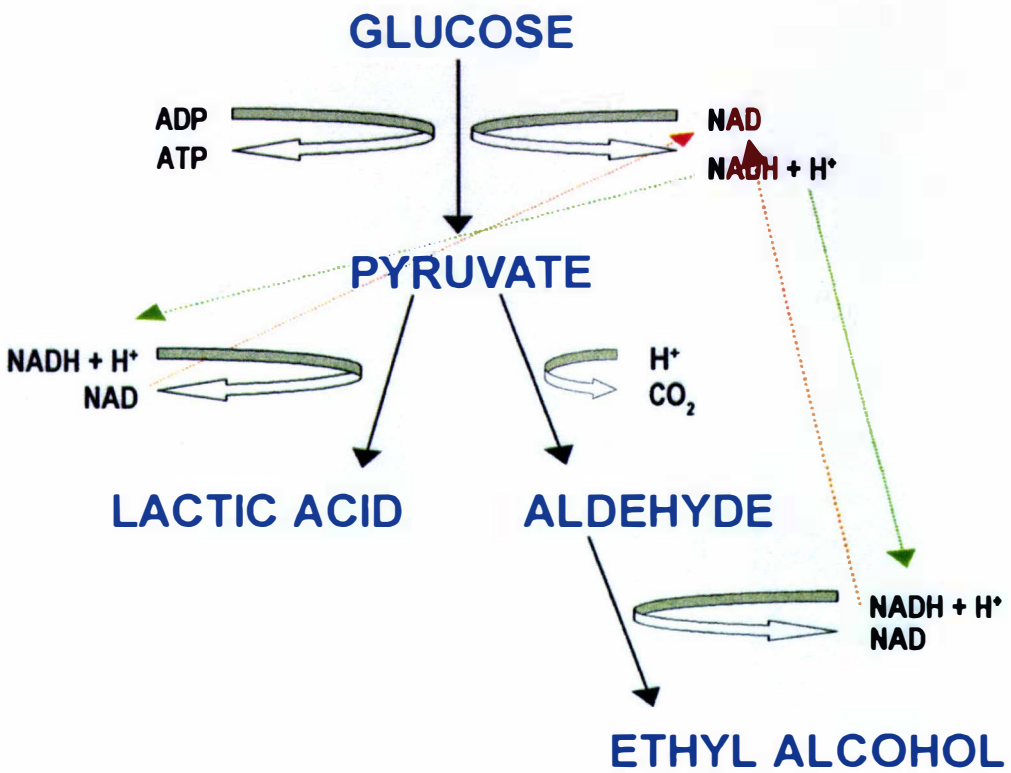


Figure 1.3 Fermentative Metabolism. The conversion of pyruvic acid (formed by glycolysis) to lactic acid and ethyl alcohol is depicted. Recycling of NADH and NAD^+ is carried out.

1.3 THE CLASSICAL RESPIRATORY PATHWAY

Mitochondria are organelles essential for respiration in most cells, and the classical respiratory pathway which drives energy production is located in the inner mitochondrial membrane (Voet & Voet, 1990). There are four protein complexes in the classical respiratory pathway, through which electrons are transported in a sequential manner. Three of these complexes are 'phosphorylation sites', at which ATP synthesis occurs: Complex I (NADH dehydrogenase), Complex II (cytochrome bc_1 complex) and Complex III (cytochrome *c* oxidase; COX).

1.3.1 THE CYTOCHROME *C* PROTEIN

The cytochrome *c* protein is an integral component of the electron transport chain (ETC), and its primary function is to shuttle electrons between cytochrome *c*1 (Complex II) and COX (Complex III), by alternately binding to the two complexes (Figure 1.2). Cytochrome *c* is a small, globular protein of approximately 12.3 kDa, and contains a covalently-bound heme molecule. The protein is highly conserved in over one hundred species from humans to bacteria (Voet & Voet, 1990). In eukaryotes, the protein is generally 103-112 amino acids in length, and approximately 38 of these residues are invariant. These highly conserved residues possess important structural and/or functional roles - eg. both His80 and Met18 covalently bind the redox-active iron atom within the heme group, while nine highly conserved lysine residues surround the heme. The positive charges on the lysine residues are believed to interact with negative charges on cytochrome *c*1 and COX, thus aiding in the electron transfer process (Voet & Voet, 1990). Other residues, while not invariant, are conservatively substituted and generally reside within internal regions, such as the heme-binding pocket.

TRANSLOCATION OF THE CYTOCHROME *C* PROTEIN

The apo-cytochrome *c* protein is encoded by a nuclear gene, synthesised on cytoplasmic ribosomes and subsequently translocated to the mitochondria. The mature cytochrome *c* protein contains a covalently bound heme group, which is attached to the apo-cytochrome *c* protein during or after import into the mitochondrial intermembrane space. The reaction is catalysed by cytochrome *c* heme lyase (CCHL), which is located in the inner mitochondrial membrane (Wang *et al.*, 1996).

The translocation of apo-cytochrome *c* is not entirely understood at present, but it is known to be unique in comparison to other nuclear-encoded proteins which are transported to the same mitochondrial compartment, as the protein does not contain a cleavable targetting sequence. Studies have suggested that a minimal length of polypeptide chain at the N-terminus is important for import, rather than any specific sequence (Wang *et al.*, 1996).

THE ROLE OF THE CYTOCHROME *C* PROTEIN IN APOPTOSIS

Recent studies have discovered a second function for cytochrome *c*, separate to its involvement in oxidative phosphorylation (section 1.3.1) and independent of its redox status; that of an essential cofactor for apoptosis (for reviews, see Reed, 1997; Cai *et al.*, 1998; Gottlieb, 2000). Two pathways have been characterised for apoptosis (programmed cell death): the cytochrome *c* mediated process; and the Fas-mediated process. Both pathways involve cytochrome *c*, and induce the activation cascade of caspases (proteases), which cleave a variety of cellular substrates, ultimately leading to DNA fragmentation and cell death (Cai *et al.*, 1998). In both processes, the non-functioning ETC (due to the loss of cytochrome *c*) leads to the dissipation of the proton motive force, causing the inner mitochondrial membrane to become permeable to small molecules, and ultimately leading to membrane rupturing. Resulting ATP deprivation and the generation of reactive oxygen species (ROS) (section 1.4.2) also leads to cellular damage and death.

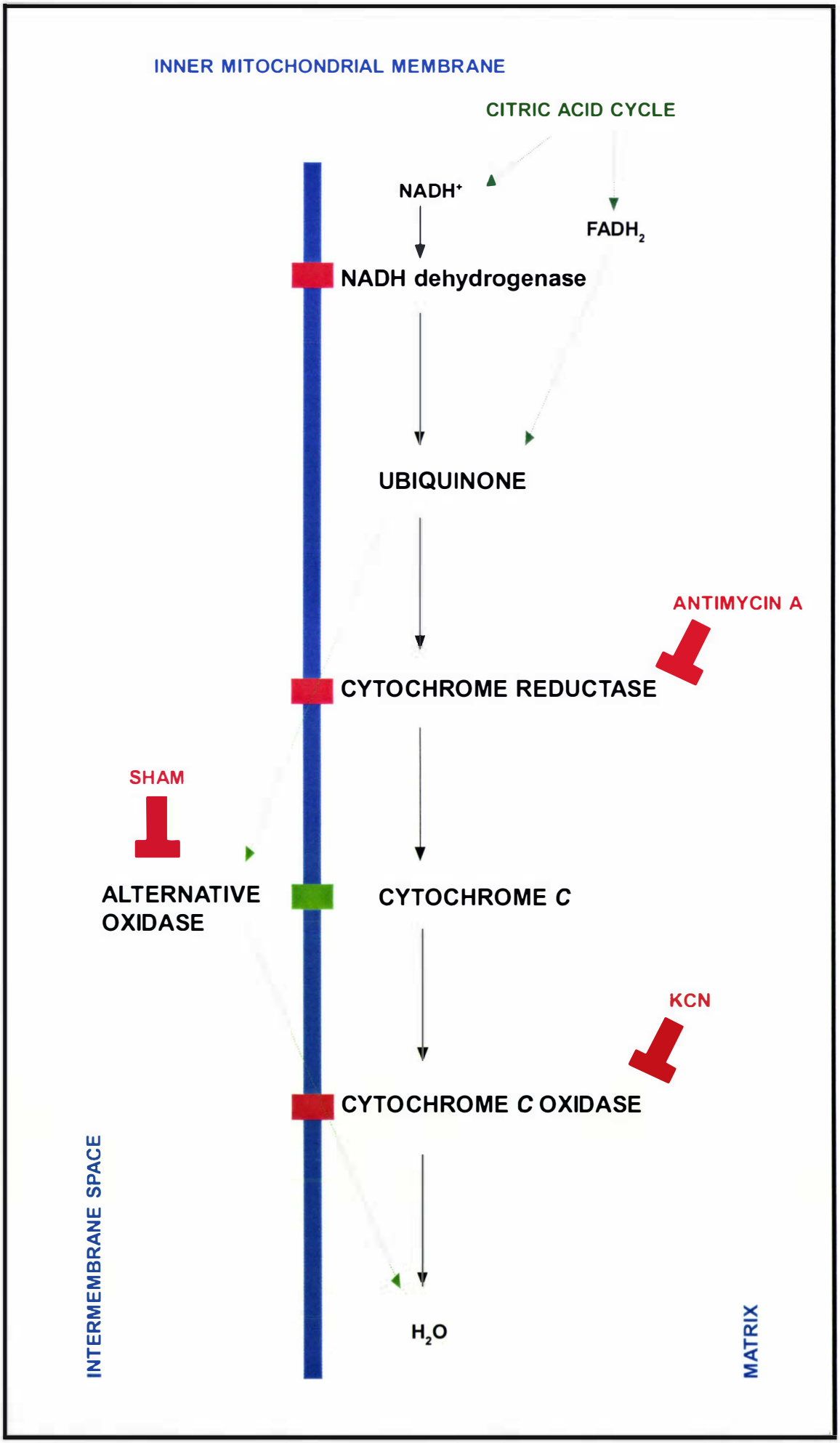
1.4 THE ALTERNATIVE RESPIRATORY PATHWAY

The alternative respiratory pathway has been well characterised in many plants, and a growing number of fungal and yeast species (McIntosh, 1994). The pathway is also present in some algae, as well as in the bloodstream form of the parasitic protozoan, *Trypanosome* (Vanlerberghe & McIntosh, 1997).

The alternative respiratory pathway has generally been found to branch from the classical respiratory pathway at the ubiquinine pool (Figure 1.4), and to consist of a single, novel ubiquinol:oxygen oxidoreductase (Vanlerberghe & McIntosh, 1997). The alternative oxidase (AOX) accepts electrons from ubiquinone and reduces oxygen to water, as does COX, the terminal oxidase of the classical respiratory pathway. However, in most organisms the utilisation of the alternative respiratory pathway does not generally contribute to ATP production, as proton translocation only occurs at Complex I (NADH dehydrogenase) (Figure 1.2). Contrary to these findings, a recent study (Joseph-Horne *et al.*, 1998) reported that oxygen consumption and ATP production were both found to be affected adversely by the addition of salicyl hydroxamic acid (SHAM), a specific inhibitor of the AOX, in the pathogenic fungus *Gaeumannomyces graminis* var. *tritici*. All of these observations have generated much speculation as to the possible role of the alternative respiratory pathway.

Figure 1.4 **Entry point of the Alternative Respiratory Pathway into the Classical Respiratory Pathway**

The entry point of the alternative respiratory pathway into the classical respiratory pathway is generally at the ubiquinone pool. The alternative pathway consists of a single enzyme, the alternative oxidase (AOX). Specific inhibitors to the oxidases in either pathway are indicated; salicyl hydroxamic acid (SHAM) and potassium cyanide (KCN) to the AOX and cytochrome *c* oxidase (COX), respectively. Antimycin A, a potent inhibitor of cytochrome reductase (the bc_1 complex), is also indicated.


INNER MITOCHONDRIAL MEMBRANE
CITRIC ACID CYCLE
NADH+
FADH2
NADH dehydrogenase
UBIQUINONE
ANTIMYCIN A
CYTOCHROME REDUCTASE
SHAM
ALTERNATIVE OXIDASE
CYTOCHROME C
KCN
CYTOCHROME C OXIDASE
H2O
INTERMEMBRANE SPACE
MATRIX

1.4.1 ACTIVITY OF THE PATHWAY

Although both the classical and alternative respiratory pathways accept electrons from ubiquinone and reduce oxygen to form water, early studies (Baher & Bonner, 1973) indicated that activity of the former was kinetically favoured due to the non-phosphorylating nature of the latter pathway. It was therefore suggested that the alternative pathway was used only when electron flow through the cytochrome *c*-dependent pathway was inhibited or saturated; ie. as an 'overflow mechanism', thus alleviating potential repression of the TCA cycle mechanism. However, recent studies have ascertained the ability of the alternative respiratory pathway to compete with the cytochrome *c*-dependent pathway for electrons (Day *et al.*, 1996). Also, electron flow can be re-routed to the alternative pathway (from the cytochrome *c*-dependent pathway) under certain 'stress' conditions (section 1.4.1). Therefore, the primary function of the alternative respiratory pathway is not that of an 'overflow' mechanism, although the possibility of this role cannot be discounted.

Traditionally, the activity of the alternative respiratory pathway has been determined by the use of specific inhibitors to the oxidases in each respiratory pathway (Baher & Bonner, 1973). These inhibitors include potassium cyanide, which specifically targets COX, thus the classical respiratory pathway is said to be 'cyanide-sensitive'. The activity of the AOX is specifically inhibited by various chemicals, including hydroxyamic acids, n-propyl-gallate and disulfiram (Sluse & Jarmuszkiewicz, 1998). The AOX is unaffected by cyanide, thus the activity of the alternative respiratory pathway is denoted 'cyanide-insensitive'.

INDUCING CONDITIONS

Induction of the alternative pathway's activity has been observed to occur under 'stress' conditions in plants, which include wounding, chilling, drought, salt stress, and factors which impair or inhibit the cytochrome *c*-dependent pathway (Vanlerberghe & McIntosh, 1997). For example, induction of the alternative respiratory pathway's activity has been reported during copper-limited growth of *Candida utilis* (Downie & Garland, 1973) and *Podospora anserina* (Borghouts *et al.*, 1997). The induction of this pathway is due to the non-functioning cytochrome *c*-dependent pathway, as copper is a necessary co-factor for COX.

Activity of the pathway has also been reported to occur by altering the carbon source which the organism is utilising, eg. induction of the pathway's activity was observed in *N. crassa* upon culturing the organism on media containing ethanol (Li *et al.*, 1996). Despite this, the activity of the cytochrome-dependent pathway was reported to be predominant in this fungus at all measured growth phases. Other studies have reported carbon source regulation of the alternative respiratory pathway's activity in *Hansenula anomala* and *Moniliella tomentosa* (Li *et al.*, 1996).

1.4.2 FUNCTIONS OF THE PATHWAY

Only one definite function of the alternative respiratory pathway has been ascertained as yet; that of heat volatization by *Sauromatum guttarum* (the voodoo lily), to attract pollinating insects (McIntosh, 1994). However, a number of recent studies have brought to attention the involvement of the alternative respiratory pathway in a variety of cellular processes, such as citric acid fermentation in *A. niger* (Kirimura *et al.*, 1987) and cephalosporin C formation by soybean oil in *Acremonium chrysogenum* (Karaffa *et al.*, 1999).

Activity of the pathway has also been recently implicated in thermoregulation in plants, to aid survival in cold conditions (Breidenbach *et al.*, 1997). Supporting this suggestion is the discovery that tropical plant species, such as *Episces cupreata* Hook, have very little AOX activity. This is in direct contrast to species such as wheat, which have adapted to a wider range of environmental conditions. A recent study by Gonzalez-Meler *et al* (1999) also discovered an increase in mungbean AOX protein levels, with growth at low temperatures.

Recent studies (Wagner, 1995; Popov *et al.*, 1997; Maxwell *et al.*, 1999) suggest the involvement of the AOX in processes which reduce the formation of mitochondrial reactive oxygen species in plant cells, thus suggesting a more general function of the pathway in these organisms. This is postulated to be the only other function of AOX which is supported by experimental evidence, in addition to its role in *S. guttarum.*

A variety of reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide ($\cdot O_2^-$) and the hydroxyl radical ($\cdot OH$), are produced by all organisms as a consequence of normal metabolic processes (Maxwell *et al.*, 1999). These compounds are also formed under conditions in which the classical respiratory pathway is impaired. If chemical

conversion of these compounds does not occur, peroxidation of the lipid membranes can result, ultimately leading to cell death.

Methods evolved to protect the cells against such damage include antioxidants (eg. ascorbate and glutathione) and ROS-scavenging enzymes, such as catalase, superoxide dismutase (SOD) and peroxidase. The recent studies cited above suggest that a third mechanism has evolved to guard against cellular damage by ROS; the use of the alternative respiratory pathway. As one of the main sources of superoxide production is the ubiquinone pool, a defective cytochrome pathway would lead to the accumulation of ROS. However, the presence of a second quinol oxidase would help to oxidise upstream electron-transport components, thus resulting in lowered ROS production. Supporting this hypothesis is the observation that 'stress' conditions result in increased levels of ROS (Maxwell *et al.*, 1999), while independent studies have reported an increased activity of the alternative pathway under such conditions (section 1.4.1). Interestingly, activity of the alternative pathway is induced by ROS (Wagner, 1995). It has therefore been proposed that ROS (eg. H_2O_2) acts as a secondary messenger, for signalling between the nucleus and the mitochondria (Wagner, 1995; Maxwell *et al.*, 1999) (section 1.4.4).

The alternative respiratory pathway has also been suggested to play a role in pathogen defense, as the oxidative burst of H_2O_2 generated at the host cell wall by the invading pathogen would induce the pathway's activity (Maxwell *et al.*, 1999). Interestingly, salicylic acid (which is required for the induction of disease resistance in plants) has been also reported to activate AOX synthesis. Although the mechanism by which this stimulation occurs has not yet been elucidated, it has been postulated that salicylic acid binds catalase enzymes, thus inhibiting their function and facilitating the induction of the alternative oxidase pathway by reactive oxygen species (Wagner, 1995).

However, the alternative respiratory pathway has also been suggested to enable pathogenic fungi surive the defence mechanisms mounted by the host. Nitric oxide (NO) generated by the plant's defenses upon fungal invasion inhibits the COX in the fungal cytochrome pathway. Thus, the alternative respiratory pathway could facilitate the survival of a pathogenic fungus by keeping the ubiquinone pool sufficiently oxidized to prevent the autoxidation of reduced ubiquinone and the subsequent formation of ROS, when electron flow through the cytochrome pathway becomes limited. A similar mechanism could also be employed by mammalian pathogenic fungi, as the presence of an alternative respiratory

pathway has been found in a number of these (e.g. *C. albicans*, *Histoplasma capsulatum*), with increased expression of the alternative oxidase observed upon incubation with ROS (Huh & Kang, 2001; McEwan *et al.*, 2001).

Recent studies have also suggested that the alternative respiratory pathway may provide a mechanism for resistance against fungicides aimed at mitochondrial respiration (Affourtit *et al.*, 2001; Joseph-Horne *et al.*, 2001). For example, the rice blast pathogen *Magnaporthe grisea* is known to acquire resistance against the fungicide SSF126 by inducing the expression of an alternative oxidase. SSF126-induced respiration of *Botrytis cinerea* has also been attributed to the presence of an alternative oxidase (Affourtit *et al.*, 2000).

1.4.3 THE ALTERNATIVE OXIDASE PROTEIN

The AOX protein was first purified from *S. guttatum*, and the monoclonal antibody (AOA) raised against the 32.2 kDa *S. guttatum* polypeptide has since been used extensively to isolate homologous proteins from other organisms, which range from approximately 32 – 40 kDa in size (Vanlerberghe & McIntosh, 1997). The AOX protein is often encoded by a single nuclear gene (*Aox1*), such as that first cloned from *S. guttatum* (Vanlerberghe & McIntosh, 1997). In many studies, PCR amplification with degenerate primers has been carried out to obtain a cDNA fragment which is then used to screen a genomic library. Organisms for which this has been carried out include: *A. niger* (Kirimura *et al.*, 1999); *Candida albicans* (Huh & Kang, 1999); *Pichia stipitis* (Shi *et al.*, 1999) and *H. anomala* (Sakajo *et al.*, 1999).

Some organisms have been found to contain multiple *AOX* genes; eg. *N. crassa* contains two *AOX* genes, *aod-1* and *aod-2* (Li *et al.*, 1996) (section 1.4.4). The *aod-1* gene encodes the functional AOX protein, whilst the *aod-2* gene product regulates *aod-1* gene expression (Li *et al.*, 1996). *C. albicans* also contains two *AOX* genes, although the functions of these have not been ascertained as yet (Huh & Kang, 1999). For many higher plants, multiple protein bands are observed when western blotting is carried out with the *S. guttatum* AOA antibody. Multiple AOX genes have been identified in soybean, tomato, rice, tobacco and mango (McCabe *et al.*, 1998). However, the molecular basis for this observation has only been determined for soybean, in which the three *AOX* genes are developmentally regulated in a tissue-specific manner (Finnegan *et al.*, 1997; McCabe *et al.*, 1998). Thus it has been suggested that multiple *AOX* genes in plants (and some fungi) may be the rule

rather than the exception.

STRUCTURE OF THE AOX PROTEIN

The amino acid sequence of the AOX protein is highly conserved among different species, especially among plants (McCabe *et al.*, 1998). Structural analysis of the AOX from many different organisms reported the enzyme to consist of a membrane bound protein homodimer (Sluse & Jarmuszkiewicz, 1998). However, recent reports suggest that fungal AOX's differ from those of plants, as the former function as monomers (Umbach & Siedow, 2000; Joseph-Horne *et al.*, 2000).

Hydropathy analysis of the protein sequences from six plants and a protozoan revealed that each monomer of the AOX is composed of two membrane-spanning α helices connected by a helical region (Sluse & Jarmuszkiewicz, 1998). The monomers are separated by a sulfhydryl-disulphide system, which has been found to play an important role in the regulation of the dimeric protein's activity in plants (section 1.4.4). The cysteine residues involved in the sulfhydryl-disulphide system are highly conserved amongst plants, yet absent from fungal AOX sequences (Umbach & Siedow, 2000).

While spectroscopic studies of the protein have not revealed a heme or iron centre, the activity of the enzyme would suggest a requirement for iron (Albury *et al.*, 1996). Also, the analysis of cDNA sequences from a variety of organisms have detected the presence of three conserved iron-binding sequence motifs (Glu-X-X-His) which are known to be present in other well-documented bi-nuclear iron-containing enzymes such as the ribonucleotide reductase R2 family (Albury *et al.*, 1996). Two of these motifs are located within the carboxyl-terminal domain of the AOX protein, at the base of the second membrane-spanning α helix. Thus a hypothetical catalytic site located in this region was suggested, given the presence of these motifs and other highly conserved amino acid residues. A recent study by Albury *et al* (1998) involved the mutagenesis of a highly conserved glutamate (Glu-270) in plant AOX, which was postulated to act as a bridging ligand in the putative active site. This suggestion is supported by the resulting inactivity of the mutant protein. The features of the AOX active site are conserved in all organisms investigated to date, including fungi (Siedow & Umbach, 2000).

1.4.4 REGULATION OF THE ALTERNATIVE RESPIRATORY PATHWAY

The mechanisms by which the activity of the AOX protein, and the alternative respiratory pathway, are regulated have been extensively studied recently and a number of contributing factors have been determined. The three different levels of regulation are gene expression, post-translational modifications of the protein, and the action of allosteric effectors (Sluse & Jarmuszkiewicz, 1998).

The level of gene expression, as evidenced by the amount of AOX protein present in the system, is an obvious factor contributing to the activity of the pathway (Ribas-Carbo *et al.*, 1997). As mentioned previously (section 1.4.3), in *N. crassa* the *aod-1* gene product is the functional AOX protein, while *aod-2* encodes a protein which is required for optimal expression of *aod-1*. This regulation appears to be at the level of transcription, as reduced levels of the *aod-1* mRNA transcript are observed in *aod-2* mutants (Li *et al.*, 1996).

Post-translational modifications in plants include regulation of the redox status of the AOX protein's sulfhydryl groups (section 1.4.3). Sulfhydryl-disulphide bonds are formed between the conserved cysteine residues located towards the N-terminus of each protein monomer, thus forming the dimeric AOX protein. Oxidised and reduced forms of dimeric AOX can exist, with the latter being approximately four- to five-fold more active than the oxidised form (Sluse & Jarmuszkiewicz, 1998). The redox status of the protein has also been shown to be regulated in intact mitochondria by the redox state of the NADP(H) pool in the mitochondrial matrix (Wagner, 1995). Fungal AOX's function as a monomer, and lack the conserved cysteine residues involved in the dimerisation process. Therefore fungal AOX proteins appear to be unaffected by this type of regulation.

The presence of pyruvate and some other α-keto acids are also known to affect the activity of plant AOX's. Activation by these compounds involves the formation of a thio-hemiacetal with the sulfhydryl group of a conserved cysteine residue in the AOX monomer. The compounds stimulate the enzyme and alter its kinetics (K_m) so that it can react to lower levels of reduced ubiquinone (Ribas-Carbo *et al.*, 1997). Fungal AOX activity is insensitive to stimulation by pyruvate, yet stimulated by purine nucleotides. Recent studies reported that *C. albicans* and *N. crassa* lack the conserved cysteine residue

involved in the activation response to pyruvate (Joseph-Horne *et al.*, 2000; Siedow & Umbach, 2000).

An interaction between the redox status of the protein and activation of AOX by pyruvate has been reported. The dimeric AOX protein must be reduced for activation by pyruvate to occur, and the reduced form of AOX exhibits low levels of activity without the addition of pyruvate (Sluse & Jarmuszkiewicz, 1998).

The activity of the AOX protein is also affected by substrate availability, which is linked to the concentration and redox status of the quinone pool in the inner mitochondrial membrane, and the oxygen concentration (for a review, see Sluse & Jarmuszkiewicz, 1998). The amino acid residues involved in the AOX binding site for quinone are highly conserved in plants (Sluse & Jarmuszkiewicz, 1998) and in fungi (Joseph-Horne *et al.*, 2000). An interaction between pyruvate activation and ubiquinol concentration has been observed in plants, as stimulation of the AOX by pyruvate leads to a lower concentration of ubiquinol being required.

Therefore, the activity of the AOX is dependent upon the level of gene expression, the redox status of the protein and the presence of allosteric effectors (the last two factors applying to plants only). The activity of the pathway is influenced by the ubiquinone concentration and redox status. Although interactions between some of the regulatory components have been elucidated, the complex mechanism by which all of these factors interact is not yet fully understood. Interestingly, the AOX proteins in plants and fungi appear to be regulated differently, despite structural similarities (Siedow & Umbach, 2000).

Induction of the alternative respiratory pathway often occurs when the cytochrome *c*-dependent pathway is impaired. This implies that the expression of nuclear genes is linked to the activity of the cytochrome pathway, therefore communication between the nucleus and the mitochondria must be taking place. Recent studies have implicated ROS as second messengers in this signal transduction pathway (Wagner, 1995; Popov *et al.*, 1997; Maxwell *et al.*, 1999) (section 1.4.2). Intracellular calcium and cAMP levels have also been suggested to induce activity of the alternative pathway (Tsuji *et al.*, 2000; Medentsev *et al.*, 1999). Some of the transcription factors suggested to regulated *AOX* gene expression (section 4.1.2) have been noted to regulate the expression of other respiratory-related genes, including the cytochrome *c* gene (sections 1.5 & 1.6).

1.5 TRANSCRIPTIONAL REGULATION OF EUKARYOTIC GENE EXPRESSION

Transcriptional regulation of gene expression can only occur when the DNA is accessible to regulatory proteins. Chromatin condensation regulates gene transcription by inhibiting transcription factor access. Chromatin 'priming' (ie unravelling) is regulated by methylation and acetylation. Transcription is generally initiated by the binding of the RNA Polymerase II apparatus to the gene promoter, generating a basal level of expression (Ogbourne & Antalis, 1998). Subsequent activation or repression of gene expression is mediated by the interaction of regulatory factors with specific DNA elements. These elements are generally located within the 5' region of a gene's promoter (ie. upstream of the transcriptional start site), thus they are referred to as UASs (Upstream Activation Sites). Binding of the regulatory factor to the target site facilitates interaction with the transcriptional apparatus for subsequent activation or repression.

Transcription factors which activate gene expression interact with enhancer and *cis*-acting elements (McNabb *et al.*, 1995). The position and orientation of enhancer elements are usually independent of function, while *cis*-acting elements are generally close to the transcriptional start site, in a fixed orientation (Jacobs & Stahl, 1995). Transcriptional activators generally contain two important domains: a DNA-binding domain, and a transcriptional activation domain. The former domain usually contains a DNA-binding motif (eg. helix-turn-helix and zinc finger structures). These motifs specifically recognise and bind sequences within the target DNA. The transcriptional activation domains of regulatory factors are less well characterised, though these regions are generally known to be enriched in acidic amino acids, glutamine or proline (Olesen & Guarente, 1990).

Eukaryotic transcription factors have been shown to possess separate and independent DNA-binding and transcriptional activation domains. In many regulatory proteins, these regions are encoded within a single polypeptide, eg. the yeast zinc finger proteins HAP1 and GAL4 (section 1.7). Such regulatory proteins commonly function as multimers, especially dimers, therefore they also contain dimerisation domains (eg. HAP1, section 1.7) (Davis & Hynes, 1991). An increasing number of regulatory proteins have been characterised which are composed of multiple polypeptides, each encoding a separate function, which specifically interact to form oligomeric complexes. These complexes are capable of recognising and binding to specific UASs (McNabb *et al.*, 1995). This is

exemplified by the *A. nidulans* and yeast heterotrimeric complexes AnCP and HAP2/3/4/5 (respectively) which bind specifically to a CCAAT motif (section 1.8).

Transcriptional repression in eukaryotes is not as well characterised as activation of gene expression, and is less common in filamentous fungi than in other eukaryotes (Davis & Hynes, 1991). Repression is mediated through specific regulatory proteins binding silencer elements and negative regulatory elements (NREs) (Ogbourne & Antalis, 1998). Transcriptional repression can also occur by protein-protein interactions, without DNA binding occuring. The repressor can bind the activator protein, thus inhibiting DNA-binding and/or transcriptional activation by the latter (Struhl, 1995).

1.5.1 CONTROL OF GENE EXPRESSION IN FILAMENTOUS FUNGI

Regulatory elements which are generally located within eukaryotic gene promoters, and transcriptionally activated, include TATA elements and CCAAT boxes. Studies have found filamentous fungi to differ somewhat from *S. cerevisiae* in the organisation of their transcriptional control sequences.

TATA elements (consensus TATAAA) are bound by the TATA-binding protein (TBP), which is a constituent of the RNA Pol II apparatus. For gene promoters lacking TATA elements, it has been suggested that TBP binds in a non-specific manner (Struhl, 1995). Similar to higher eukaryotes, TATA elements are not present in all fungal genes, and when present are only found in single copy, at approximately 60-120 bp from the transcriptional start site (Jacobs & Stahl, 1995). This differs from *S. cerevisiae*, in which the elements are multi-copy and very variable in position (Gurr *et al.*, 1987).

In higher eukaryotes and *S. cerevisiae*, TATA elements determine transcription efficiency, whilst PyAAG/pyrimidine-rich sequences are important in determining transcription initiation sites (Punt & van den Hondel, 1992; Struhl, 1995). In filamentous fungal genes, TATA elements are instrumental in determining transcription efficiency only to a minor extent (Punt & van den Hondel, 1992). Pyrimidine-rich sequences close to the transcriptional start site have not been identified in all genes of filamentous fungi, only those which are strongly-transcribed and constitutive (Jacobs & Stahl, 1995).

CCAAT boxes are known to play an important role in transcription activation in higher eukaryotes. The situation regarding CCAAT motifs in both fungal and yeast genes was previously largely unknown (Gurr *et al.*, 1987), however in both species regulatory CCAAT-binding factors have since been reported (Forsburg & Guarente, 1989; Chen & Clark-Walker, 1993; Bonnefoy *et al.*, 1995) (section 1.8). Activity of the CCAAT-binding factors in fungi has been found to be regulated by carbon source (Forsburg & Guarente, 1989) (section 1.8.2). Bonnefoy *et al* (1995) reported that the CCAAT element present in the *A. nidulans amdS* gene was important in establishing both basal and derepressed levels of expression.

1.6 THE CYTOCHROME *C* GENE (*cycA*) IN *ASPERGILLUS NIDULANS*

The single copy cytochrome *c* gene (*cycA*) in *A. nidulans* was isolated and characterised by Raitt *et al* (1994), and found to contain an ORF (open reading frame) with two conserved introns. However, the published *A. nidulans cycA* nucleotide sequence (encoding 109 amino acid residues) was found to be incorrect - a third, previously undetected intron was discovered, which effectively altered the position of the transcriptional start site and the predicted N-terminal amino acid sequence (McGlynn, 1997). Despite this, the corrected N-terminal region of the *cycA* gene was found to be very similar to the N-terminal region of the *Saccharomyces cerevisiae CYC7* gene, and the position of the additional intron was found to be situated at an identical position to that of an intron in the *N. crassa* cytochrome *c* gene. The corrected *cycA* nucleotide sequence (encoding 112 amino acid residues) displays 72% and 87% similarity to the *S. cerevisiae* iso-1-cytochrome *c* gene (*CYC1*) and the *N. crassa* cytochrome *c* gene, respectively, thus indicating a high level of conservation.

1.6.1 THE PRESENCE OF PUTATIVE REGULATORY ELEMENTS WITHIN THE *cycA* GENE PROMOTER

A number of putative *cis* regulatory elements have been identified within the upstream regulatory sequence of the *cycA* gene (Johnson & Bradshaw, 1998). These include three possible HAP1 binding sites which quite closely fit the "optimal" HAP1 consensus sequence (Figure 1.5). Thus it has been speculated that a HAP1-like protein, homologous to the one known to regulate oxygen-induced transcriptional activation of many yeast respiratory-related genes (section 1.8), will also regulate the *A. nidulans cycA* gene. In

addition, a CCAAT motif detected within the 5' region of the *cycA* gene is considered to be a putative binding site for the *A. nidulans* CCAAT binding factor (AnCF). This heteromeric complex is involved in transcriptionally activating genes involved in respiration, and its activity is induced by carbon source (section 1.9.1). This complex is homologous to the yeast CCAAT-binding factor, the HAP2/3/4/5 complex (sections 1.7.1 & 1.9). Three putative TATA boxes also located in the 5' region of the *cycA* gene were postulated to be involved in transcription initiation.

1.6.2 TRANSCRIPTIONAL REGULATION OF THE *cycA* GENE

Many of the studies on the influence of environmental factors on the expression of the cytochrome *c* gene have been carried out in the baker's yeast, *S. cerevisiae* (section 1.7). This is largely because of the commerical importance of yeast, the relative ease of experimentation, and the organism's facultative anaerobic metabolism. Whilst comparatively less characterisation has been carried out on *A. nidulans*, environmental factors (such as carbon source and oxygen) are known to affect members of the *Aspergillus* genus which are also of commerical importance (Martinelli & Kinghorn, 1994).

Putative HAP1 Binding Site In *A. nidulans cycA*	**Sequence**				
CYCA (-634)	CGGC	**CGG**	GGAGAG	**CGG**	GAAAAGG
CYCA (-669)	TAAT	G**G**C	CGCTAA	G**G**C	ATCAGGC
CYCA (-905)	GATG	**CGG**	GAGGAC	**CGG**	GACAGGA
Yeast HAP1 CONSENSUS		**CGG**	NNNTAN	**CGG**	NNNTA

Figure 1.5 Alignment of the putative HAP1 binding sites located within the *A. nidulans cycA* promoter fragment, with the 'consensus' yeast HAP1 binding site. Sequences containing the conserved CGG triplets and TA nucleotides are indicated by red and blue lettering, respectively.

The regulation of transcription of the *A. nidulans cycA* gene by environmental parameters such as glucose, oxygen and heat-shock conditions has been studied (Raitt *et al.*, 1994).

REGULATION BY OXYGEN

The *cycA* gene was shown to be transcriptionally induced ten-fold upon the introduction of aerobic conditions, while the cytochrome *c* transcript was almost undetectable under anaerobiosis (Raitt *et al.*, 1994). Oxygen regulation in *S. cerevisiae* is mediated through the HAP1 transcription factor (section 1.6.1). Three putative HAP1 sites have been identified within the *cycA* gene promoter (Figure 1.5) (Johnson & Bradshaw, 1998).

Although *A. nidulans* can survive under anaerobic conditions, normal growth does not occur - indicating insufficient ATP production for cell growth, or a requirement for the metabolites synthesised in aerobiosis. It has been postulated that the extent of anaerobic growth observed indicates the fermentative capacity of an organism, in the absence of other limiting factors (Gonzalez-Siso *et al*, 2000). Thus, these observations indicate that *A. nidulans* does not generate energy by means of fermentation, as expected, since the fungus has been classified as an obligate aerobe.

The presence of alcohol dehydrogenases in the fungus is therefore puzzling, as these enzymes are involved in the fermentative metabolic pathway (Figure 1.3). It has been suggested that the enzymes are important in allowing *A. nidulans* to survive during periods of anaerobic stress (Kelly *et al.*, 1990). The presence of pyruvate decarboxylase activity was also recently discovered in *A. nidulans*, thus confirming the existence of the alcoholic fermentation pathway (Lockington *et al.*, 1997). Further study is required before the significance of these findings is fully understood.

Interestingly, a transient induction of the actin transcript was shown in low oxygen concentrations (Raitt *et al.*, 1994), and this was proposed to be associated with alterations in morphology and metabolism of the fungus which occur during periods of anaerobic stress (Carter & Bull, 1971). Alternatively, it was suggested that the induction of the actin transcript could be related to the respiratory adaptations of *A. nidulans* under these conditions.

REGULATION BY CARBON SOURCE

The influence of carbon source on expression of *cycA* has been partly determined: transcription was unaffected by catabolite repression (unlike *S. cerevisiae*; section 1.6.1) (Raitt *et al.*, 1994). This result was not unexpected, given the obligate aerobe nature of the fungus' metabolism.

Carbon catabolite repression (CCR) of other *A. nidulans* genes has been well characterised. CCR, or glucose repression, occurs when the cell preferentially utilises the simple sugar, glucose. Under these conditions, the energy requirements of the cell are supplied by substrate-level phosphorylation (section 1.2.1), and expression of respiratory-related genes are repressed. CCR in *A. nidulans* is mediated through the CREA protein, encoded by the *creA* gene (Davis & Hynes, 1991). Similar to other repressor proteins, the CREA protein contains two zinc fingers (C_2H_2 class), an alanine-rich domain, and a number of S(T)PXX motifs. CREA specifically binds the CREA regulatory element (consensus sequence 5'-SYGGRG-3') located within the target gene promoter, and represses transcription (Dowzer & Kelly, 1991). Putative CREA sites have been identified within the *cycA* gene promoter (McGlynn, 1997).

A putative CCAAT binding site for the AnCF complex, an *A. nidulans* specific transcriptional activator which is induced by carbon source (section 1.9.1), has also been located in the 5' region of the *cycA* gene promoter (Johnson & Bradshaw, 1998). As mentioned above (section 1.6.1), this complex is very similar to the yeast HAP2/3/4/5 protein complex which binds a CCAAT motif found upstream in the *CYC1* gene (section 1.7.1). Transcriptional activation of *CYC1* by HAP2/3/4/5 is observed when cells are shifted from a fermentable carbon source to a non-fermentable one (Forsburg & Guarente, 1989).

HEAT-SHOCK REGULATION

A three- to four-fold induction of *cycA* transcription was shown under heat-shock conditions, very similar to the response observed by the minor isoform (*CYC7*) of the *S. cerevisiae* cytochrome *c* gene in the stationary growth phase (Raitt *et al.*, 1994). Some of the metabolic changes which occur when cells approach stationary phase have been characterised in *S. cerevisiae* (Flattery-O'Brien *et al.*, 1997). Firstly, ethanol derived from

fermentation of glucose in the exponential growth phase is utilised as a carbon source (by means of respiration, thus accounting for the induction of *CYC7* transcription). Secondly, cAMP levels decrease as the supply of nutrients becomes deplenished, and thirdly, the switch to respiration is proposed to lead to an increase in oxidative stress, resulting from greater leakage of reactive oxidative species (ROS) from the electron-transfer chain, especially from the ubiquinone pool.

It is not yet known whether transcription of the *cycA* gene in this instance is specifically induced by heat-shock, or induced by a more general cellular "stress" response. Although no putative binding sites for the heat-shock factor have been identified within the upstream regulatory region of the *cycA* gene, a putative binding site for the stress response element (consensus sequence CCCCT) is present within this sequence (McGlynn, 1997).

1.7 REGULATION OF CYTOCHROME *C* EXPRESSION IN *SACCHAROMYCES CEREVISIAE*

There are two isoforms of the cytochrome *c* gene in *S. cerevisiae*, *CYC1* and *CYC7*, which encode iso-1-cytochrome *c* and iso-2-cytochrome *c*, respectively (Forsburg & Guarente, 1989). While *CYC1* and *CYC7* are 78% identical at the amino acid level (Montgomery *et al.*, 1980), and appear to be isofunctional, the two genes are differentially regulated depending upon physiological conditions, such as carbon source and oxygen. The influence of these environmental parameters is coordinately regulated, and carried out via the specific interaction of regulatory proteins with UAS' located within the upstream regulatory region of each gene. *CYC1* contains two functional UAS sequences, UAS1 and UAS2, while *CYC7* contains only one (Forsburg & Guarente, 1989).

Although transcriptional regulation is the major means of controlling the differential expression of the two isoforms, differential ubiquitin-degradation of the apo-isoforms under certain physiological conditions has also been suggested to play a role (Pearce & Sherman, 1997).

Many duplicated genes (often phenotypically redundant) have been found in *S. cerevisiae*, including the respiratory gene pairs *CYC1/CYC7* and *COX5A/COX5B*, which encode isoforms of Cytochrome *c* and subunits of COX, respectively. These have been suggested in the past to have arisen due to whole-genome duplication/polyploidy. Recent reports

propose a model in which evolutionary divergence of *Saccharomyces* from *Kluyveromyces* occurred some 10^8 years ago, after which *Saccharomyces* became a degenerate tetraploid following a polyploidy event. Many of the gene pairs were subsequently deleted, with only a small fraction being retained, and the gene order was rearranged by many reciprocal translocations between chromosomes. This implies that *Kluyveromyces* homologues of the retained gene pairs (such as *CYC1/CYC7*) should be single-copy, and no contradictory examples to this were found. One of the most important physiological differences between *Saccharomyces* and other yeasts is the former's ability to ferment sugars under anaerobiosis, producing ethanol. It has been proposed that gene duplication was instrumental in the yeast's evolutionary adaptation to anaerobic growth, as several of the gene pairs are differentially regulated under aerobic and anaerobic conditions, and some encode sugar transporters (Wolfe & Shields, 1997).

1.7.1 TRANSCRIPTIONAL REGULATION OF *CYC1* & *CYC7*

Many of the components of the electron transport chain are encoded by nuclear genes, thus efficient expression of these genes requires coordination between mitochondrial conditions and the physiological status of the cell (Forsburg & Guarente, 1989; Poyton, 1999). This signal transduction occurs in two ways; firstly, nuclear-encoded proteins regulate mitochondrial protein levels by controlling the expression of relevant nuclear genes, eg. HAP1 binds to the UAS1 in *CYC1* (section 1.7.1). Secondly, the cytochrome *c* cofactor, heme, activates transcription of these genes, and it mediates this effect via the HAP proteins.

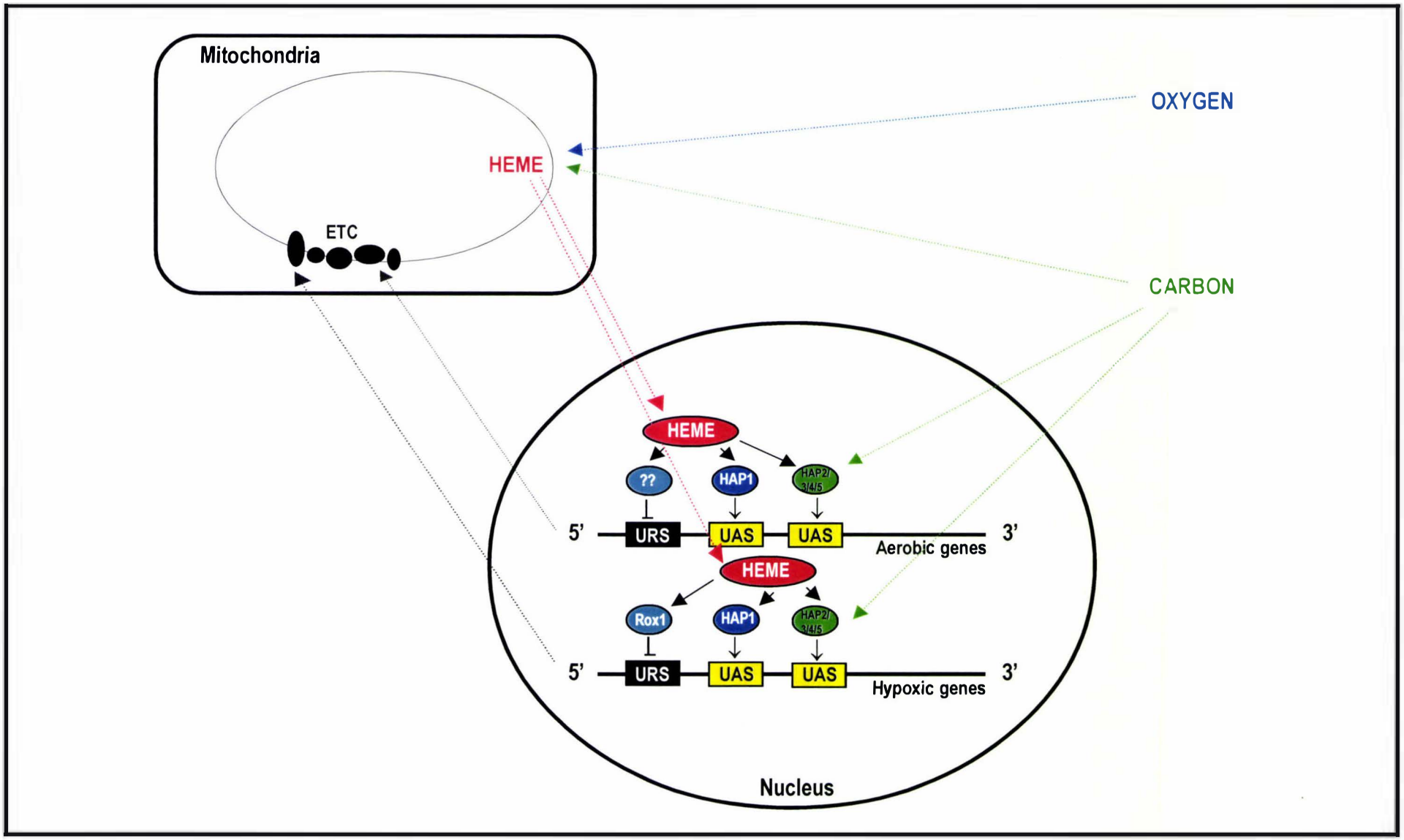
Comprehensive reviews have been published on the transcriptional regulation of *S. cerevisiae* genes by oxygen (Zitomer & Lowry, 1992; Kwast *et al.*, 1999) and carbon source (Gancedo, 1998).

REGULATION BY HEME & OXYGEN

Oxygen availability affects the levels and activities of a large number of yeast proteins, many of which are involved in processes or pathways that use oxygen, eg. the ETC (Poyton & Burke, 1992; Kwast *et al.*, 1999). Heme is believed to play an intermediary role in the oxygen-sensing apparatus of yeast cells, and this is supported by a number of findings. Firstly, heme biosynthesis takes place in the mitochondria (Figure 1.6), and

Figure 1.6 Regulation Of Aerobic And Hypoxic Genes In Yeast

Some of the regulatory processes controlling expression of aerobic and hypoxic genes are indicated. Communication between the nucleus and mitochondria is also shown. Oxygen and carbon are required for heme biosynthesis in the mitochondria; heme then mediates its effect via regulatory factors such as HAP1 and HAP2/3/4/5, which transcriptionally activate nuclear genes. Carbon also exerts its regulation via the HAP2/3/4/5 complex. Repressor proteins (eg. ROX1) are also involved in transcriptional regulation. Activation and repression of nuclear genes by the transcription factors is indicated by arrows and blunt ends, respectively. The proteins encoded by the nuclear genes are transported to the mitochondria (shown by black arrows) where they are generally involved in respiratory processes.


Mitochondria
HEME
ETC
OXYGEN
CARBON
HEME
??
HAP1
HAP2/3/4/5
5'
URS
UAS
UAS
3'
Aerobic genes
HEME
Rox1
HAP1
HAP2/3/4/5
5'
URS
UAS
UAS
3'
Hypoxic genes
Nucleus

requires molecular oxygen, thus the heme concentration in a cell is considered to reflect oxygen availability. Secondly, heme-deficient cells (either wildtype cells grown anaerobically or cells mutant in heme biosynthesis grown without a heme supplement) mimic hypoxia: the 'aerobic' gene *CYC1* is strongly repressed, while 'hypoxic' genes (eg. *CYC7*) are upregulated (Poyton & Burke, 1992). Thirdly, the addition of heme to anaerobic cells reverses the effect of low oxygen tension on aerobic and hypoxic genes. Lastly, the binding of HAP1 to the UAS1 of *CYC1* is stimulated *in vitro* by the addition of heme (Poyton & Burke, 1992), and the gene is subsequently transcriptionally induced 200-fold (Guarente & Mason, 1983).

There are two classes of oxygen regulated genes, for both of which heme plays a role in monitoring oxygen levels. 'aerobic' genes, eg. *CYC1,* are optimally transcribed in the presence of oxygen, while 'hypoxic' genes, eg. *CYC7*, are optimally transcribed in its absence (Kwast *et al.*, 1999) (Figure 1.6). *CYC1* produces the majority (95%) of cytochrome *c* under normal, aerated conditions, while the minor isoform, *CYC7*, makes up the remaining 5%. Under anaerobic conditions, and in stationary phase cultures, *CYC7* is preferentially expressed. The two apo-isocytochrome *c* proteins have differential stabilities, with apo-iso-2-cytochrome *c* found to be stable in the absence of the heme cofactor, while apo-iso-1-cytochrome *c* is undetectable (Guarente & Mason, 1983), due to its rapid degradation. This differential stability has been proposed to poise the cell for the transition from aerobic to anaerobic conditions (Dumont *et al.*, 1990).

Heme works in conjunction with the regulatory protein HAP1 to transcriptionally activate the *CYC1* gene via its UAS1. The *CYC7* gene also positively responds to heme through HAP1 under anaerobic conditions. Transcriptional repression of *CYC1* and *CYC7* is also mediated via heme. Under anaerobic conditions, transcription of *CYC1* is repressed via an unidentified protein. Expression of *CYC7* is repressed via the *ROX1* protein, under aerobic conditions. Although the expression of *ROX1* is heme-dependent, the activity of the protein is not, thus heme *per se* is not directly involved in the repression of *CYC7* (Figure 1.6) (Kwast *et al.*, 1999).

REGULATION BY CARBON SOURCE

Transcription of the cytochrome *c* genes in *S. cerevisiae* is affected by carbon catabolite repression (CCR), unlike *cycA* in *A. nidulans* (section 1.6.2), eg. *CYC1* transcription is

repressed five- to ten-fold in the presence of glucose (Guarente & Mason, 1983). CCR in *S. cerevisiae* is mediated via the HAP2/3/4/5 complex and the MIG1 protein, a homologue of CREA (section 1.6.2). It has not yet been determined whether MIG1 causes transcriptional repression by directly binding to the target site, or by forming a complex with other proteins, as recent studies have reported conflicting findings (Gancedo, 1998).

Transcriptional activation of *CYC1* and *CYC7* by HAP2/3/4/5 is observed when cells are shifted from a fermentable carbon source to a non-fermentable one, as cytochromes are obviously required for respiration under these conditions. For example, the transcriptional induction via the wildtype UAS2 in *CYC1*, upon switching from glucose to lactate, is approximately 50-fold (Forsburg & Guarente, 1989).

S. cerevisiae is classified as a facultative anaerobe, thus it utilises glucose to generate energy both in the presence and absence of oxygen (section 1.2.3). The yeast is said to have a "Crabtree positive" phenotype, as it generates only 10% of its energy requirements by respiration under aerobic conditions (ie. it is limited in its respiratory capacity). The yeast preferentially ferments, whilst repressing respiration in the presence of glucose. *K. lactis* is also a facultative anaerobe, yet it has a "short-term Crabtree negative" phenotype. The main difference between the two yeasts is the extent to which fermentation is carried out under aerobic conditions - *K. lactis* preferentially respires, although fermentation is still carried out at a low rate (Gonzalez-Siso *et al*, 2000). Thus, disruption of the cytochrome *c* gene in *K. lactis* is not lethal, as the yeast can grow fermentatively (Chen & Clark-Walker, 1993). It is suspected that this may also be the case in *A. nidulans*, as replacement of the cytochrome *c* gene is not lethal (Bird, 1996).

1.8 THE YEAST TRANSCRIPTIONAL ACTIVATOR, HAP1

HAP1 is a heme-activated protein encoded by the *HAP1* (*CYP1*) gene, and is known to transcriptionally activate many yeast genes, especially those involved in respiration and electron transfer, eg. cytochrome *c* genes. Although HAP1 belongs to the zinc cluster family of fungal transcriptional regulators, there are a number of unique features that HAP1 possesses which set it apart from the other zinc cluster proteins. Firstly, HAP1 has been shown to carry out its regulatory functions in the absence of heme, which is considered to be its effector. Secondly, HAP1 is unusual as it mediates both positive and negative effects, depending on the target gene and the physiological state of the cell (Figure

1.6) (Defranoux *et al.*, 1994). Thirdly, HAP1 target sequences are degenerate forms of an imperfect direct repeat, unlike the palindromic inverted repeat sequences bound by the majority of other zinc cluster proteins, therefore the protein binds in an asymmetrical conformation (Ha *et al.*, 1996). Despite the difference in target sequence conformation, HAP1 does show a high level of conservation with other zinc cluster proteins (Zhang & Guarente, 1996): HAP1 binds sites containing CGG triplets, and specific amino acids involved in binding are conserved.

1.8.1 STRUCTURE OF THE HAP1 PROTEIN

HAP1 is a protein of 1, 383 amino acid residues (Zitomer & Lowry, 1992), within which are five functional domains (Figure 1.7): an N-terminal C_6 zinc binuclear cluster for DNA-binding, a dimerisation domain, a central heme-binding domain, HRM7 (heme-responsive motif) and a C-terminal acidic region involved in transcriptional activation. Three of these domains are important for heme regulation: the dimerisation domain, a central heme-binding domain and HRM7 (Hach *et al.*, 1999).

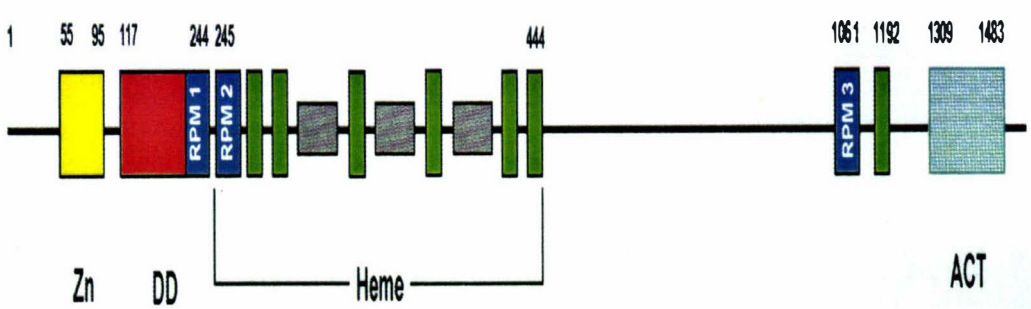


Figure 1.7 Schematic Representation Of The HAP1 Protein. Important domains within the yeast transcriptional activator HAP1 are indicated. Shown are the Zinc cluster (Zn) (residues 55-95), the dimerisation domain (DD) (residues 117-244), the central heme binding domain (residues 245-444), and the transcriptional activation domain (ACT) (residues 1309-1483). Heme-responsive motifs (HRM's) are indicated by a green box; HRM1-6 are located within the central heme binding domain, while HRM7 is located near residue 1192. The three 17-amino acid repeats (light grey boxes) are indicated within the central heme binding domain. The three repression modules (RPM1-3), located within the dimerisation domain, the central heme binding domain, and near HRM7 are also shown.

The binuclear zinc cluster is formed by the cysteine-rich motif (C X_2 C X_6 C X_{5-9} C X_2 C X_{6-8} C) within the protein binding two zinc atoms, thus facilitating the formation of a rigid, compact structure. This type of regulatory protein usually binds as a homodimer, with each zinc cluster interacting with a CGG triplet within the cognate DNA site. The binding specificity of each protein is determined partly by the spacing between the CGG triplets (ie. the linker region and the start of the dimerisation element). For example, the transcriptional activators PUT3 and PPR1 bind CGG triplets with spacings of ten and six bp, respectively (Zhang & Guarente, 1996). The binding specificity of each zinc cluster protein is also determined by the orientation of the CGG triplets within the cognate DNA site.

In the case of HAP1, it has been indicated by DNase I footprinting experiments that the zinc clusters within the protein must be positioned asymmetrically, in a direct repeat orientation, to facilitate proper contact with the cognate DNA site (Figure 1.8 A). The asymmetry in the binding of HAP1 is due to an asymmetric interaction between the zinc cluster in one HAP1 monomer and the residues N-terminal to the zinc cluster in the other HAP1 monomer (Zhang & Guarente, 1996; King *et al.*, 1999).

HAP1 TARGET SEQUENCES

Generally, target sequences for global regulators conform strictly to consensus sequences (Pfeifer *et al.*, 1987). However, the target sequences of HAP1 initially appeared to be dissimilar to other zinc cluster proteins, as they are degenerate forms of an imperfect direct repeat (Figure 1.8 B) (Zhang & Guarente, 1996).

HAP1 binds the *S. cerevisiae CYC1* and *CYC7* genes with equal affinity, yet the protein activates transcription from UAS1 in *CYC1* much more strongly under aerobic conditions (Zitomer & Lowry, 1992). This increased level of activation was proposed to occur due to the UAS1 in *CYC1* bearing a greater similarity to the 'consensus' HAP1 binding site sequence. Importantly, this site contains both CGG triplets: other yeast genes shown in Figure 1.8 B, whose HAP1 target sites lack one/both CGG triplet(s), have been observed to undergo increased transcriptional activation when site-directed mutagenesis was carried out to alter both triplets to CGG (Zhang & Guarente, 1996).

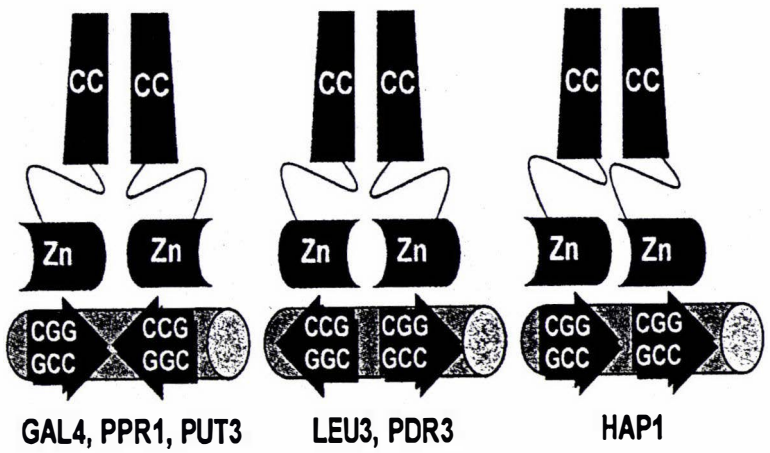
Figure 1.8

A **Binding Of The HAP1 Protein**

A schematic representation of the binding preferences of zinc cluster proteins. GAL4, PPR1 and PUT3 bind to CGG half sites aligned in an inverted orientation, whilst LEU3 and PDR3 prefer sites aligned in an everted orientation. HAP1 is unusual, as it can recognise sites aligned in a direct repeat orientation. The cylinder containing the CGG triplets (of indicated orientation) represents the DNA. CC and Zn (joined by the linker domain) represent the coiled coil dimerisation domain and the zinc cluster, respectively. (Ref: King *et al.*, 1999)

B **Alignment Of Known Yeast HAP1 UAS's With The Consensus HAP1 Target Sequence**

Red and blue lettering indicate nucleotides which match the CGG triplets sequence and the TA repeats (respectively) of the consensus HAP1 target sequence (Ref: Johnson & Bradshaw, 1998).



A

Gene	Sequence of HAP1 site				
CYC1	CGGC	CGG	GGTTTA	CGG	ACGATGA
CYC7	CCCT	CGC	TATTAT	CGC	TATTAGC
CTT1	GGAA	TGG	AGATAA	CGG	AGGTTCT
CYB2	GGCA	CGG	AGATAT	CGG	CAGGCTT
CYT1	CCGC	CGG	AAATAC	CGG	CCGCCCA
KlCYC1	ATTT	CGG	GAACAT	CGG	TCAAGAC
CONSENSUS		CGG	NNNTAN	CGG	NNNTA

Genes with HAP1 binding sites:

CYC1: Iso-1-cytochrome *c* from *S. cerevisiae* (Pfeifer *et al.*, 1987)
CYC7: Iso-2-cytochrome *c* from *S. cerevisiae* (Prezant *et al.*, 1987)
CTT1: Catalase T from *S. cerevisiae* (Winkler *et al.*, 1988)
CYB2: Cytochrome *b*2 from *S. cerevisiae* (Lodi & Guiard, 1991)
CYT1: Cytochrome *c*1 gene from *S. cerevisiae* (Schneider & Guarente, 1991)
KlCYC1: Cytochrome *c* gene from *K. lactis* (Freire-Picos *et al.*, 1995)

B

However, other nucleotides within the HAP1 binding sites are also likely to be important, especially if the rest of the sequence is not optimal. These nucleotides most probably stabilise the interaction between the HAP1 protein and the target DNA sequence. In particular, the importance of the second TA nucleotide pair (Figure 1.8 B) has been shown - an increase in activation was observed for the wildtype *CYC1*, *CTT1* and *CYB2* genes when the second nucleotide pair was altered to TA. Additionally, this nucleotide pair is essential for the activation of the *CYC7* UAS, as any alteration of these nucleotides results in reduced binding and activation of HAP1. In comparison, mutation of the CGG triplets within the *CYC1* UAS1 to CGCs resulted in a 10-fold reduction in binding and activation when compared to wildtype *CYC7*, most probably due to the absence of the second TA pair in the former. Thus, other nucleotides (such as the second TA pair) within a HAP1 target site can compensate for any unfavourable CGG-type triplets present (Zhang & Guarente, 1996).

THE DIMERISATION DOMAIN

The minimal dimerisation domain necessary for dimerisation and DNA binding is between residues 105 – 135 (Hach *et al.*, 2000) (Figure 1.7). Similar to most other zinc cluster proteins, HAP1 contains a coiled coil dimerisation element within its dimerisation domain. Interestingly, this region was recently discovered to be non-essential for DNA binding of HAP1, although it was found to differentially affect transcriptional activation of the *S. cerevisiae* cytochrome *c* genes (Hach *et al.*, 2000; Lukens *et al.*, 2000). It was suggested that the coiled coil dimerisation element may affect transcriptional activation through interactions with putative coactivator(s) or part of the RNA Polymerase II transcriptional apparatus.

The coiled coil dimerisation element also plays an important role in HAP1 homodimer formation, therefore it was surprising that the deletion of this region had no significant effect on DNA binding (Hach *et al.*, 2000). It was postulated that the extreme flexibility of the HAP1 protein allows for functional compensation to occur when certain regions are deleted.

THE HEME-RESPONSIVE MOTIFS

There are seven heme-responsive motifs (HRM) within the HAP1 protein (Hach *et al.*, 1999). The conserved motif [(R/K)CP(I/V)XX] resembles a metal- or heme-binding site, and is also present in CCHL, which catalyses heme attachment to cytochrome *c* (Zhang & Guarente, 1994). Six HRM's (HRM 1-6) are located within the central heme-binding domain, amino acid residues 245-444 (Figure 1.7). HRM7 is located towards the C-terminal end of the protein, commencing at residue 1192 (Figure 1.7).

Results from a recent functional analysis of the HRM's suggest that HRM1-6 plays only an auxillary role in mediating heme regulation, as deletion of this region did not greatly affect activation by heme (Hach *et al.*, 1999). Deletion of the three 17-amino acid repeats contained within the central heme-binding domain (Figure 1.7) did not affect activation or repression by heme, indicating these sequences are probably not involved in heme regulation. As part of this study, the authors determined HRM7 to be essential for heme-dependent activation of the HAP1 protein. Previous studies (Haldi & Guarente, 1995) had implicated this region in mediating heme control of the protein's activity, as mutants were found to be independent of heme when assayed for activation.

THE TRANSCRIPTIONAL ACTIVATION DOMAIN

The DNA-binding domain, dimerisation domain, and heme-responsive motifs have been well characterised (sections 1.8.1), whilst less is known about the transcriptional activation domain (ACT). This domain is located between residues 1309 – 1483 of the HAP1 protein (Figure 1.7). Similar to other zinc cluster proteins, the HAP1 ACT is rich in acidic amino acids (Forsburg & Guarente, 1989).

1.8.2 ACTIVATION & REPRESSION OF THE HAP1 PROTEIN

HAP1 is sequestered in a high molecular weight complex (HMC) in the absence of heme (Fytlovich *et al.*, 1993). Dimeric HAP1 is bound by molecular chaperones, such as Hsp90 and Ydj1, rendering it inactive, with low DNA binding affinity. HAP1 is activated when heme dissociates the molecular chaperones and preferentially binds to HAP1 (Hon *et al.*, 1999).

Three repression modules (RPM1-3), located within the dimerisation domain, the central heme-binding domain, and HRM7 (Figure 1.7), have been recently implicated in the repression of HAP1 in the absence of heme. The modules are thought to function synergistically, as deletion of any one causes the depression of HAP1 in heme-deficient conditions. It has been suggested that the RPMs could repress HAP1 activity by masking the protein's DNA binding and/or activation domains, through intramolecular interactions. Alternatively, the RPMs may be involved in HMC formation. Therefore, a new model for heme regulation of HAP1 has been proposed, which involves two classes of modules involved in mediating heme control: RPMs and HRMs. RPMs are suggested to repress HAP1 activity in the absence of heme, possibly by facilitating HMC formation. HRMs mediate activation of HAP1 by binding heme and thereby causing the dissociation of the HMC (Hach *et al.*, 1999).

1.9 THE YEAST TRANSCRIPTIONAL ACTIVATOR, THE HETEROMERIC HAP2/3/4/5 COMPLEX

The yeast heteromeric HAP2/3/4/5 complex activates transcription through specific interactions with CCAAT 'boxes' found upstream of nuclear genes encoding components of the mitochondrial electron transport chain (Olesen & Guarente, 1990). The yeast CCAAT-binding factor has been shown to be a heteromeric complex, consisting of the heme-activated proteins HAP2, HAP3, HAP4 and HAP5 (Gancedo, 1998).

The consensus sequence for the CCAAT-binding complexes was determined to be (CPuPuCCAATC/GA/GGA/CG), with an absolute requirement for the core CCAAT sequence. The preferred surrounding nucleotides are also given in the consensus sequence (Mantovani, 1998; Brakhage *et al.*, 1999).

1.9.1 HOMOLOGUES OF THE HAP2/3/4/5 COMPLEX

Heteromeric CCAAT-binding complexes appear to have been conserved, in both structure and function, over millions of years of evolution. Homologues of the HAP2/3/4/5 complex's subunits have been found in many other species. For example, the rat CCAAT-binding protein CPF is composed of the CPF-B, CPF-A and CPF-C fractions which are the homologs of the HAP2/3/5 subunits, respectively (Brakhage *et al.*, 1999). Other

CCAAT-binding proteins include: NF-Y, found in humans, mice and *Xenopus*; and AnCF (*Aspergillus nidulans* CCAAT-binding Factor) (Mantovani, 1998; Brakhage *et al.*, 1999).

1.9.2 ACTIVITY OF THE HAP2/3/4/5 COMPLEX

Transcriptional activation by HAP2/3/4/5 is observed when cells are shifted from a fermentable carbon source to a non-fermentable one, ie. in the absence of CCR. The activity of the complex has been shown to be induced by carbon source, as *S. cerevisiae* strains containing mutated *hap2*, *hap3* or *hap4* genes are incapable of growth on non-fermentable carbon sources such as lactate (Forsburg & Guarente, 1989).

The exact mechanism by which the carbon source signal is transduced to the cells is not yet known. The expression of the *hap2* and *hap3* genes is constitutive, while transcription of *hap4* is induced upon a switch to a non-fermentable carbon source (Forsburg & Guarente, 1989). Therefore, the activity of the complex is regulated by HAP4, although this is not always the case. For example, transcription of the *S. cerevisiae ASN1* gene is activated by HAP2/3/5, even in a *hap4* mutant strain (Brakhage *et al.*, 1999). The HAP2/3/5 complex was therefore suggested to activate gene expression by remodelling the chromatin structure, while HAP4 can act as a direct transcriptional activator.

The activity of the complex is also influenced by heme, as is shown by the 10-fold decrease in HAP2/3/4/5 activity at UAS2 in heme-deficient cells, compared to wildtype cells. Thus respiratory genes activated by HAP2/3/4/5 are also considered to be sensitive to the availability of oxygen, as it is required in heme biosynthesis (Figure 1.6) (Forsburg & Guarente, 1989).

1.9.3 THE SUBUNITS OF THE HAP2/3/4/5 COMPLEX

The HAP2/3/5 subunits are necessary for DNA-binding, and accomplish this as a heteromeric complex. All of the subunits therefore contain specific domains for DNA-binding and subunit association. Dimerisation of HAP3 and HAP5 occurs initially, faciliated by the conserved histone motifs contained within the proteins. HAP2 then associates with HAP3/5. The sequential formation of the heteromeric complex occurs without DNA binding, although this event stabilises the protein-protein interactions within the complex (Brakhage *et al.*, 1999). Lastly, the HAP4 protein interacts with the complex.

This protein contains an essential, highly acidic domain at its carboxyl terminus which is functionally homologous to the acidic transcriptional activation domain of the GAL4 protein (Olesen & Guarente, 1990).

1.9.4 THE SUBUNITS OF THE ANCF

Homologues of the HAP2/3/5 subunits have been found in *A. nidulans*; HAPB, HAPC and HAPE, respectively. These subunits have been overexpressed in *E. coli* and found to form a heteromeric complex *in vitro* (Brakhage *et al.*, 1999).

Although an *A. nidulans* homologue to the *S. cerevisiae* HAP4 protein has not been found as yet, analysis of fungal HAP5 homologues (eg. HAPE) have revealed the presence of a domain which is necessary for interaction with the HAP4 protein. Also, electrophoretic mobility shift assays (EMSA's) with *A. nidulans* nuclear extracts and CCAAT-containing probes generate two complexes. One band is analogous to the heteromeric complex formed by HAPB/C/E (approximately 87 kDa), the other is approximately 120 – 130 kDa (eg. HAPB/C/E plus another protein) (Brakhage *et al.*, 1999). Therefore, there is evidence for an *A. nidulans* homologue of HAP4. Interestingly, autoregulation of the ANCF complex was recently reported to occur via its HAPB subunit (Steidl *et al.*, 2001).

1.9.4 ASSEMBLY OF THE HAPB/C/E COMPLEX (ANCF)

A model for the assembly of the HAPB/C/E complex is shown in Figure 1.9. Similar to the HAP2/3/5 complex, HAPC and HAPE interact initially and form a dimer; HAPB then associates. DNA-binding to the CCAAT element by the heteromeric complex is then possible.

Unlike the HAP2/3/5 complex, activity of the AnCF does not appear to be an absolute requirement for transcriptional activation in *A. nidulans*, as strains deficient in *hapb*, *hapc* or *hape* are capable of growth on non-fermentable carbon sources (Brakhage *et al.*, 1999). This result is not surprising, as many of the genes regulated by CCAAT-binding complexes are involved in respiratory processes, and *A. nidulans* is an obligate aerobe, unlike *S. cerevisiae*.

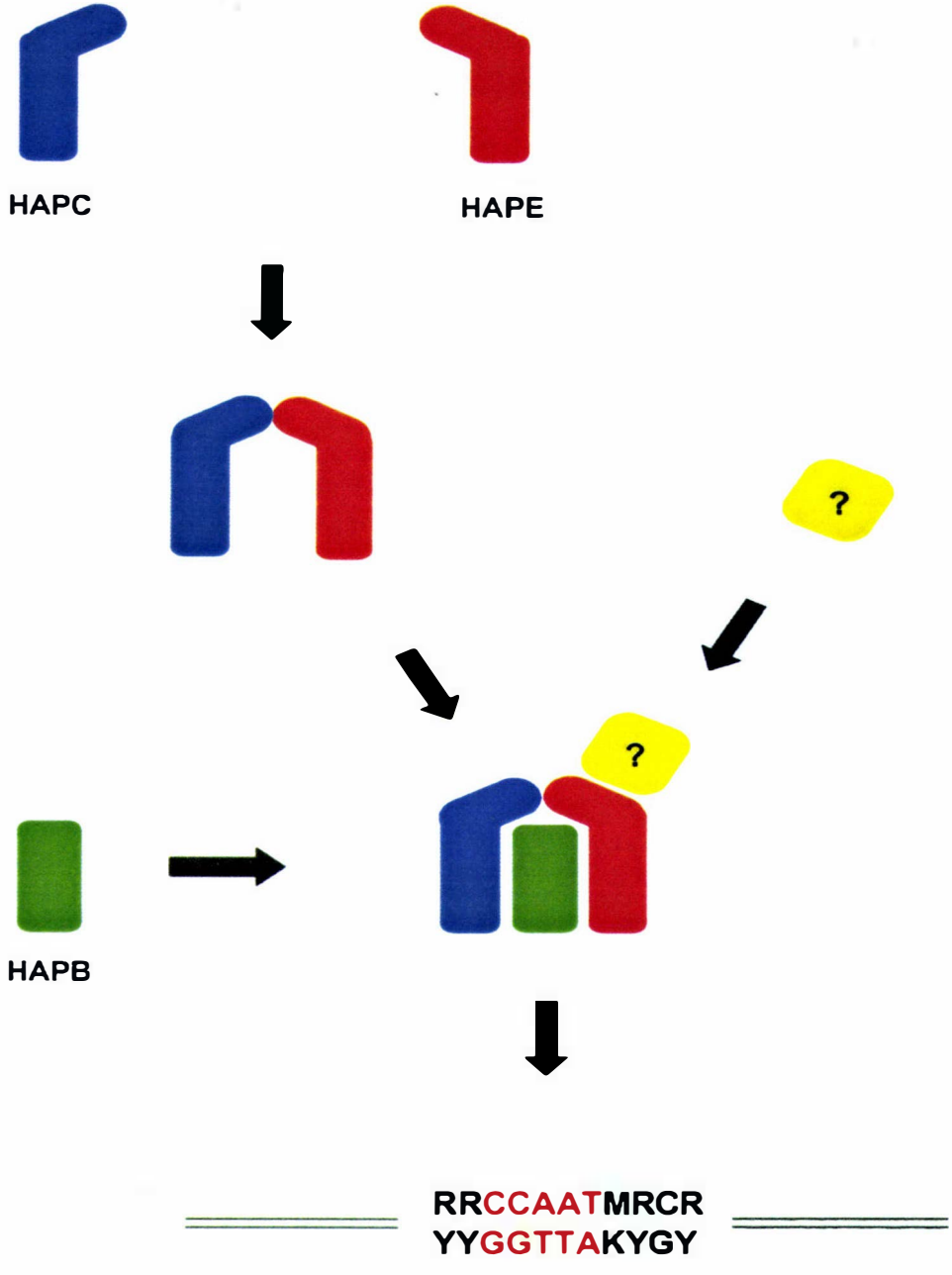


Figure 1.9 Assembly Of The AnCF. Proteins HAPC and HAPE interact via conserved histone motifs to form a dimer. HAPB then associates, and the heteromeric AnCF is formed. A putative fourth subunit, homologous to HAP4, also interacts with the complex. The complex interacts specifically with the CCAAT element within the target gene promoter.

1.10 AIMS OF THE PROJECT

The viability of an *A. nidulans* strain deficient in cytochrome *c* provided the impetus for this project. Other cytochrome *c* deficient mutants had been generated in the past, but none in an obligate aerobe. Therefore, the elucidation of the alternate energy-yielding processes used by this strain was of great interest.

The observation of putative regulatory elements within the *A. nidulans* cytochrome *c* gene (*cycA*) also raised the intriguing question as to why the expression of a seemingly essential gene would be regulated.

Any findings regarding the regulation of respiratory processes in this fungus would have important implications, given the extensive use of filamentous fungi in commerical applications

Determining the activity of an alternative respiratory pathway in *A. nidulans*, and possible mechanisms which may regulate this activity, would also be an important finding of this study. Alternative respiratory pathways have recently been suggested to play an important role in enabling pathogenic fungi to overcome host defence mechanisms upon invasion. Thus, the AOX protein has been suggested to be an attractive target for antifungal therapy. Investigation into the activity of the alternative respiratory pathway in *A. nidulans* would therefore be extremely beneficial, given the predominance of this fungus as an opportunistic pathogen.

Thus the aims of the project were:

1. Characterisation of the cytochrome *c*-deficient mutant of *A. nidulans*, to compare the growth of this strain with wildtype strains of *A. nidulans* and other cytochrome *c* deficient mutants (Chapter 3). Spectral analysis was required to confirm the absence of detectable cytochrome *c* in these strains. The use of the alternative respiratory pathway and fermentative metabolism in the *cycA*$^-$ strains was also investigated (Chapter 3).
2. Characterisation of the alternative oxidase gene of *A. nidulans* (Chapter 4), which may encode the protein involved in the alternative respiratory pathway. Expression of the *A. nidulans AOX* under varying physiological conditions was also investigated (Chapter 4).

3. Functional analysis of the *A. nidulans* cytochrome *c* gene promoter, to determine the significance of putative regulatory elements which were identified previously (Chapter 5). Particular interest was focused on the oxygen regulation of *cycA* gene expression, which may be mediated by a HAP1-like transcription factor.
4. Functional expression of the *A. nidulans* cytochrome *c* gene promoter in *S. cerevisiae* would determine whether yeast proteins are able to regulate *cycA* gene expression (Chapter 6). It had been speculated that this was likely, given the similarities in regulatory mechanisms controlling cytochrome *c* expression in these two organisms.

CHAPTER 2

MATERIALS AND METHODS

2.1 FUNGAL STRAINS, BACTERIAL STRAINS AND PLASMIDS

Fungal strains, bacterial strains and plasmids used in this study are listed in Table 2.1.

2.2 WATER SUPPLY AND STERILISATION

The water (dH_2O) used in the preparation of solutions was purified using a Millipore MilliQ Reagent Water System. All solutions (unless otherwise stated) were sterilised at 121 °C for 25 - 40 minutes, or 110°C for 25 minutes, in commercial autoclaves. This method was also used to sterilise equipment and acid-washed glassware.

2.3 MEDIA

Molten, sterilised solid media were cooled to 55 - 60°C before the addition of supplements and pouring of plates. Bottled media were stored at room temperature, and plates at 4 °C.

Aspergillus nidulans protocols were obtained from Pontecorvo *et al* (1953), and the Fungal Genetics Stock Centre (http://www.fgsc.net/index.html). *Saccharomyces cerevisiae* protocols were obtained from Rose *et al* (1990).

2.3.1 BACTERIAL MEDIA

LIQUID MEDIA

Luria Broth (LB) Medium (g/L): tryptone, 10.0; NaCl, 5.0; yeast extract, 5.0; D-glucose, 10.0. The pH was adjusted to 7.5 prior to autoclaving.

SOLID MEDIA

Luria Agar: Agar (20 g/L) was added to LB media prior to autoclaving.

MEDIA SUPPLEMENTS

When required, ampicillin, isopropylthio-β-D-galactosidase (IPTG) and 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal) in dimethylformamide were added at a final concentration of 100 μg/mL, 30 μg/mL and 60 μg/mL respectively.

2.3.2 FUNGAL MEDIA

LIQUID MEDIA FOR *A. NIDULANS*

Aspergillus complete media (MYG) (g/L): malt extract, 5.0; yeast extract, 2.5; D-glucose, 10.0; and $CuSO_4.5H_2O$, trace.

Aspergillus minimal media (AMM) (g/L): $NaNO_3$, 6.0; $MgSO_4.7H_2O$, 0.52; KCl, 0.52; KH_2PO_4, 1.52; D-glucose, 10.0; $FeSO_4.7H_2O$, trace; and $ZnSO_4.7H_2O$, trace. The pH was adjusted to 6.5 prior to autoclaving.

Media was prepared either as a 1x or 2x concentrated solution, by adjusting the volume of dH_2O added.

LIQUID MEDIA FOR *S. CEREVISIAE*

Yeast Complete Media (YPD) (g/L): bactopeptone, 20.0; yeast extract, 10.0; and D-glucose, 20.0.

Yeast Minimal Media (YMM) (g/L): yeast nitrogen base (without amino acids and ammonium sulphate), 1.7; ammonium sulphate, 5.0; and D-glucose, 20.0.

SOLID MEDIA

For plates: Agar (20 g/L) was added to liquid media prior to autoclaving.

Top Agar: Agar (8 g/L) was added to liquid media prior to autoclaving.

SOLID MEDIA FOR *S. CEREVISIAE*

X-gal indicating Media (/L): 0.45 L of Solution I (100 mL of Phosphate Buffer Stock Solution, 1 mL of 1000x Mineral Stock Solution, 20.0 g of D-glucose) and 0.5 L of Solution II (20 g agar) were autoclaved separately and cooled to 60°C. Solution I was then supplemented as required (section 2.3.2), and 10 mL of 100x Vitamin Stock Solution added. Solutions I and II were mixed together prior to the pouring of plates.

Phosphate Buffer Stock Solution (g/L): KH_2PO_4, 136.1; $(NH_4)_2SO_4$, 19.8; 42.1, KOH. The pH was adjusted to 7.0 prior to autoclaving.
1000x Mineral Stock Solution (g/0.1 L): $FeCl_3$, 3.2 x 10^{-3}; $MgSO_4.7H_2O$, 19.72. The solution was autoclaved prior to use.
100x Vitamin Stock Solution (mg/0.1 L): thiamine, 4.0; biotin, 0.2; pyridoxine, 4.0; inositol, 20.0; pantothenic acid, 4.0. The solution was filter-sterilised through a 0.45 μM filter (Gelman Sciences) prior to use.

MEDIA SUPPLEMENTS FOR *A. NIDULANS*

For strains containing the *pyrG* mutation, uracil (2.2 g/L) was added to the media prior to autoclaving (MU = MYG + uracil). MM was supplemented as required to the following concentrations: arginine, 200 μg/mL; methionine, 1 μg/mL; para-aminobenzoic acid, 1 μg/mL; pyridoxine, 0.05 μg/mL; sodium sulfite, 1 μg/mL.

For regeneration of protoplasts, sucrose (273.8 g/L) was added to solid media. Sucrose was prepared as a concentrated (2x) stock and autoclaved at 110°C for 20 minutes. The sucrose was then added to an equal volume of molten solid media (2x concentration) for osmotically stabilised media. For screening transformants, 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal) in dimethylformamide was added to a final concentration of 60 μg/mL.

Table 2.1 Strains and Plasmids

Strain or Plasmid	Revelant Characteristics	Source or Reference
Fungal Strains		
Aspergillus nidulans		
A18 (R153)	*w*A3, *pyro*A4	Waldron & Roberts (1973)
A57	*cycA*$^{+}$ *pyr*4^{+}	Bird (1996)
A58	*cycA*$^{-}$ *pyr*4^{+}	Bird (1996)
A67	*cycA*$^{+}$ *pyr*4^{+}	Bird (1996)
A68	*cycA*$^{-}$ *pyr*4^{+}	Bird (1996)
A71	*y*A2 *w*A3 *meth*H2 *arg*B2 galA1 *ivo*A1 *s*C12	FGSC, Kansas
1-85	*pyr*G89 *paba*A1 *w*A3 *qut*R16	Bird (1996)
2-124	*arg*B2; *bi*A1	John & Peberdy (1984)
Saccharomyces cerevisiae		
BWG1-7A	*MATa ade1-100 his4-519 leu2-2 leu2-112 ura3-52*	Turcotte & Guarente (1992)
LPY22	*MATa ade1-100 his4-519 leu2-2 leu2-112 ura3-52 Δ HAP1::LEU2 CYC1-ΔUAS2*	Turcotte & Guarente (1992)
Bacterial Strains		
Escherichia coli		
XL-1	*sup*E44 *hsd*R17 *rec*A1 *end*A1 *gyr*A96 *thi*-1 *rel*A1 *lac*F'[*pro*AB+ *lac*I^{q} *lac*ZDM15 *Tn*10(*tet*^{R})]	Bullock *et al* (1987)
Plasmids		
R12	PIC20R	Sambrook *et al* (1989)
R41	2.3 kb *Pst* I-*Hind* III fragment of *A. nidulans cycA* gene in pBS	Raitt (1992)
R108 (pAN923-41B)	Intact *arg*B gene in R117	Van Gorcom *et al* (1986)
R116 (pAN5-d1)	2 kb *Bam* HI fragment of *A. nidulans gpd*A gene promoter in pAN923-42B$_{BglII}$ (R117)	Punt *et al* (1990)
R117 (pAN923-42B$_{BglII}$)	*lac*Z Ap^{r} *trp*C *arg*B-*Bgl*II	Punt *et al* (1990)
R133	2.1 kb *Eco* RI fragment of *A. nidulans cycA* gene in pUC118	McGlynn (1997)
R141 (pLG669Z)	1.1 kb fragment of the *S. cerevisiae* CYC1 gene promoter in Yep24-B	Guarente & Ptashne (1981)
R142 (pLG178)	0.18 kb fragment of the *S. cerevisiae* CYC1 gene promoter in Yep24-B	Guarente & Mason (1991)
R155	1.3 kb *Pst* I-*Bam* HI fragment of *A. nidulans cycA* gene promoter in R12	This study
R159	1.3 kb *Bgl* II-*Bam* HI fragment of *A. nidulans cycA* gene promoter in R117	This study
R160	1 kb *Xho* I fragment of *A. nidulans cycA* gene promoter in R12	This study
R164	0.74 kb *Xho* I-*Bam* HI fragment of *A. nidulans cycA* gene promoter in R12	This study
R165	0.68 kb *Xho* I-*Bam* HI fragment of *A. nidulans cycA* gene promoter in R12	This study
R166	0.65 kb *Xho* I-*Bam* HI fragment of *A. nidulans cycA* gene promoter in R12	This study

Table 2.1. (cont) **Strains and Plasmids**

Strain or Plasmid	Revelant Characteristics	Source or Reference
Plasmids (cont)		
R167	0.56 kb *Xho* I-*Bam* HI fragment of *A. nidulans cycA* gene promoter in R12	This study
R168	0.4 kb *Xho* I-*Bam* HI fragment of *A. nidulans cycA* gene promoter in R12	This study
R169	0.74 kb *Bgl* II-*Bam* HI fragment of *A. nidulans cycA* gene promoter in R117	This study
R170	0.68 kb *Bgl* II-*Bam* HI fragment of *A. nidulans cycA* gene promoter in R117	This study
R171	0.65 kb *Bgl* II-*Bam* HI fragment of *A. nidulans cycA* gene promoter in R117	This study
R172	0.56 kb *Bgl* II-*Bam* HI fragment of *A. nidulans cycA* gene promoter in R117	This study
R173	0.4 kb *Bgl* II-*Bam* HI fragment of *A. nidulans cycA* gene promoter in R117	This study
R182	1 kb *Xho* I fragment of *A. nidulans cycA* gene promoter in R142	This study
R183	1.3 kb *Bgl* II-*Bam* HI fragment of *A. nidulans cycA* gene promoter in R116 (without *A. nidulans gpdA* promoter), correct orientation	This study
R184	1.3 kb *Bgl* II-*Bam* HI fragment of *A. nidulans cycA* gene promoter in R116 (without *A. nidulans gpdA* promoter), reverse orientation	This study
R185	2 kb *Bam* HI fragment of *A. nidulans gpdA* gene promoter in R117 (correct orientation)	This study
R186	2 kb *Bam* HI fragment of *A. nidulans gpdA* gene promoter in R117 (reverse orientation)	This study
R187	0.17 kb fragment of *A. nidulans AOX* gene in pGEM	This study
R188	0.35 kb fragment of *A. nidulans AOX* gene in pGEM	This study
R189	0.5 kb fragment of *A. nidulans AOX* gene in pGEM	This study
puc118	Ap^r *lacZ'*	Messing, J. (1983)

MEDIA SUPPLEMENTS FOR *S. CEREVISIAE*

For strains containing the *ura3* mutation, uracil (2.2 g/L) was added to the media prior to autoclaving. YMM and X-gal indicating Media were supplemented as required to the following concentrations: adenine sulphate, 40 μg/mL; leucine, 40 μg/mL; histidine, 40 μg/mL. For screening transformants, 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-gal) in dimethylformamide was added to a final concentration of 40 μg/mL.

2.4 GROWTH AND MAINTENANCE OF CULTURES

2.4.1 BACTERIAL CULTURES

Pure *Escherichia coli* cultures were obtained by streaking for single colonies on LB Amp plates and incubating overnight at 37°C. Single colonies were selected and maintained on LB plates (appropriately supplemented). These cultures were stored at 4°C and routinely subcultured.

A single colony (obtained from a recently streaked culture) was used to inoculate 3-5 mL of liquid LB (section 2.3.1), by means of a sterile inoculation loop. The culture was then incubated overnight at 37°C, with shaking at 250 rpm. The following day, a small scale plasmid DNA preparation (section 2.5.1) could be carried out, or the culture could be used for further inoculations eg. for larger scale plasmid DNA preparations (section 2.5.2) or for the preparation of electroporation-competent cells (section 2.13.1).

LONG-TERM STORAGE

For long-term storage of *E. coli* strains, 3 mL of LB medium (supplemented as required) was inoculated with a loopful of culture and incubated overnight at 37°C, with shaking at 250 rpm. An aliquot of the culture was then mixed with 100% glycerol (0.85 mL and 0.15 ml, respectively) and frozen at -80°C. To revive a culture, the frozen stock was removed and placed on ice. A scraping of the culture was obtained using a sterile inoculation loop, streaked onto an LB plate, and the plate was then incubated at 37°C overnight. Following this, a single colony could be selected, and streaking for single colonies repeated, so as to ensure a homogeneous culture.

2.4.2 FUNGAL CULTURES

Aspergillus nidulans cultures were grown at 37°C on appropriately supplemented MYG or AMM plates. Alternatively, siliconised flasks containing liquid MYG or AMM media (supplemented as required) were inoculated with a spore suspension to give approximately 10^6 spores per mL. Incubation was carried out at 37°C, with shaking at 250 rpm.

Pure *Saccharomyces cerevisiae* cultures were obtained by streaking the sample on YPD plates (appropriately supplemented) and incubating for 1 - 2 days at 30°C. Single colonies were selected and maintained on YPD plates as previously. These cultures were stored at 4°C and routinely subcultured.

PURIFICATION OF *A. NIDULANS* STRAINS

Spores from a pure colony were suspended in sterile dH_2O and transferred to a MYG plate (appropriately supplemented) by means of a sterile loop, whereupon the suspension was then streaked for single colonies. The plate was incubated at 37°C for 1 - 2 days. This process was repeated twice to ensure a homogenous culture.

PREPARATION OF *A. NIDULANS* SPORE SUSPENSIONS

As detailed above (section 2.4.2), a sterile inoculation loop was used to transport the spores from a single colony, suspended in sterile dH_2O, to a fresh MYG plate (supplemented as required). The suspension was dispersed evenly on the plate by use of a sterile glass spreader. The plate was incubated at 37°C until a substantial amount of sporulation was observed (approximately 3 to 4 days). In a biohazard cabinet, the spores were resuspended in a Tween 80/Saline Solution (contained (g/L) NaCl, 8.0; 25 mL of Tween 80 stock solution (10 mL of Tween 80/L dissolved at 60°C); and dH_2O added to 1 L) by gentle scraping with a sterile inoculation loop. The spores were then harvested by centrifugation (5 min at 6 000 g), washed twice with sterile dH_2O, and resuspended in Saline Solution (contained (g/L) NaCl, 8.0). The concentration of the spore suspension was estimated by use of a haemocytometer, and aliquots were then stored at 4°C, -20°C and -80°C.

LONG-TERM STORAGE OF *A. NIDULANS*

Storage of fungal cultures in glycerol at -80°C can be carried out by homogenising mycelia in sterile dH_2O and adding an equal volume of 100% glycerol.

For storage on silica, *A. nidulans* spore suspensions were resuspended in a 5% solution of non-fat milk powder. The solutions were pipetted into cooled glass jars approximately 2/3 full of silica gel (previously dry sterilised at 180°C for at least 90 minutes) and stood on ice for 15 minutes. The glass jars were then placed at room temperature until the crystals readily separated upon shaking (approximately one week). The cap was then securely fastened and the jars stored at 4°C.

SHORT-TERM STORAGE OF *A. NIDULANS*

Strains of *A. nidulans* were streaked onto plates of MYG or MM media (supplemented as required) and incubated at 37°C for 2 - 3 days prior to being stored at 4°C. Subculturing of the strains was carried out approximately every 6 months.

LONG-TERM STORAGE OF *S. CEREVISIAE*

For long-term storage of *S. cerevisiae* strains, 3 mL of YPD medium (supplemented as required) was inoculated with a loopful of culture and incubated at 30°C overnight, with shaking at 250 rpm. An aliquot of 100% glycerol was then mixed with the culture (0.15 mL and 0.85 ml, respectively) and frozen at -80°C. To revive a culture, the frozen stock was removed and placed on ice. A scraping of the culture was obtained using a sterile inoculation loop, streaked onto an YPD plate, and the plate was then incubated at 30°C for 2 – 3 days. Following this, a single colony could be selected, and streaking for single colonies repeated, to ensure a pure culture.

SHORT-TERM STORAGE OF *S. CEREVISIAE*

Strains of *S. cerevisiae* were streaked onto plates of YCM media (supplemented as required) and incubated at 30°C for 2 - 3 days prior to being stored at 4°C. Subculturing of the strains was carried out approximately every one month.

2.5 PLASMID DNA ISOLATION

2.5.1 SMALL SCALE PLASMID DNA ISOLATION

Unless otherwise indicated, all centrifugation steps for the small scale plasmid preparations were carried out at 13 000 g in a microcentrifuge, using 1.5 mL microcentrifuge tubes, at room temperature.

SMALL SCALE PLASMID DNA ISOLATION BY THE RAPID BOIL METHOD

This method was based on that of Holmes & Quigley (1981). 3 mL of LB medium (supplemented with appropriate antibiotics) was inoculated with a single colony and incubated overnight at 37°C, with shaking at 250 rpm. The cells were pelleted by centrifugation for 1 minute, and the supernatant removed. The pellet was resuspended in 0.35 mL of STET Buffer (contained 50 mM Na_2EDTA (pH 8.0), 50 mM Tris-HCl (pH 8.0), 8% (w/v) sucrose and 5% (v/v) Triton X-100) and lysozyme was added (25 µL of a 10 mg/mL solution (in 10 mM Tris-HCl, pH 8.0). The contents of the tube were thoroughly mixed, and the tube was placed in a boiling waterbath for 40 seconds. The tube was immediately centrifuged for 10 minutes and the gelatinous pellet removed with a sterile toothpick. An equal volume (0.3 mL) of isopropanol was then added to precipitate the DNA, and the contents of the tube mixed by inversion. The tube was stood on ice for 5 minutes, and the DNA then pelleted by centrifugation for 10 minutes. The pellet was washed with 70% ethanol, dried under vacuum, and then resuspended in 30 µL of sterile dH_2O.

SMALL SCALE ALKALINE LYSIS PLASMID PREPARATION

This method was modified from that of Sambrook *et al* (1989). 3 mL of LB medium (supplemented as required) was inoculated with a single colony and incubation carried out overnight at 37°C, with shaking at 250 rpm. Following pelleting of the cells by centrifugation for 1 minute, the supernatant was removed and the pellet resuspended in 0.2 mL of alkaline lysis solution I (50 mM glucose, 25 mM Tris-HCl (pH 8.0) and 10 mM EDTA (pH 8.0)). 0.3 mL of freshly prepared alkaline lysis solution II (0.2 M NaOH and 1% SDS) was then added, the contents of the tube mixed by inversion, and the tube placed

on ice for 5 minutes. 0.3 mL of alkaline lysis solution III ((mL/100 mL): 5M potassium acetate, 60; glacial acetic acid, 11.5; and dH_2O, 28.5) was added, the tube thoroughly mixed, and incubated on ice for 5 minutes. Following centrifugation for 10 minutes, the supernatant was transferred to a fresh tube. RNase A was added (20 μg/mL) and the tube incubated at 37°C for 20 minutes. The supernatant was then extracted twice with an equal volume of chloroform, with the layers being mixed by vortexing for 30 seconds after each extraction. Centrifugation was carried out after each extraction for 1 minute to separate the layers. The aqueous layer was transferred to a fresh tube, and the DNA precipitated by the addition of an equal volume of isopropanol. Following centrifugation for 10 minutes, the pellet was washed with 70% ethanol, dried under vacuum, and resuspended in 30 μL of sterile dH_2O.

SMALL SCALE PLASMID DNA ISOLATION USING THE CONCERT KIT

The Concert Mini Plasmid Preparation kit manufactured by Invitrogen was used to isolate plasmid DNA, according to the manufacturers instructions. This kit is based upon the alkaline lysis method of plasmid DNA preparation.

2.5.2 PURIFICATION OF SMALL SCALE PLASMID DNA BY PEG PRECIPITATION

The pellet obtained by the alkaline lysis plasmid preparation or by the rapid boil method (section 2.5.1) was resuspended in 33.6 μL of dH_2O. The DNA was then precipitated by the addition of 6.4 μL of 5 M NaCl and 40 μL of 13% PEG 8000. After thorough mixing, the tube was incubated on ice for 20 minutes, then centrifuged for 15 minutes at 4°C using a fixed-angle rotor. The supernatant was removed and the pellet rinsed with 70% ethanol. The pellet was then dried under vacuum and resuspended in 30 μL of sterile dH_2O.

2.5.3 LARGE SCALE PLASMID DNA PREPARATION

This protocol is based on that by Ish-Horowicz & Burke (1981). Large scale plasmid preparations were carried out with 15/30 mL Corex tubes, using a Sorvall centrifuge with a SS34 rotor, set at 4°C.

LARGE SCALE ALKALINE LYSIS PLASMID PREPARATION

5 mL of LB media (supplemented as required) was inoculated with a single colony and incubation carried out overnight at 37°C, with shaking at 250 rpm. This culture was used to inoculate 250 mL of LB media (supplemented as previously), 1/100 (ie. 2.5 mL of culture added). The flask was incubated overnight at 37°C, with shaking at 250 rpm. The cells were harvested by centrifugation for 10 minutes at 6 000 g. The pellet was resuspended in 4 mL of alkaline lysis solution I (50 mM glucose, 25 mM Tris-HCl (pH 8.0) and 10 mM EDTA (pH 8.0)), 1 mL of lysozyme solution (25 mg/mL in alkaline lysis solution I) added and the contents of the tube well mixed. Following incubation for 10 minutes at room temperature, 10 mL of freshly prepared alkaline lysis solution II (0.2 M NaOH and 1% SDS) was added. The contents of the tube were mixed by inversion and the tube placed on ice for 10 minutes, after which 7.5 mL of alkaline lysis solution III ((mL/100 mL): 5M potassium acetate, 60; glacial acetic acid, 11.5; and dH_2O, 28.5) was added and thoroughly mixed. The precipitate was then pelleted by centrifugation (10 min at 20 000 g), then the supernatant (containing the DNA) was transferred to a fresh tube. Isopropanol (0.6 volumes) was added to precipitate the DNA, and the tube incubated at room temperature for 10 minutes. Centrifugation was then carried out (10 min at 16 000 g) to pellet the DNA. After washing with 70% ethanol, the pellet was airdried and resuspended in sterile dH_2O.

LARGE SCALE PLASMID ISOLATION USING THE QIAGEN MIDI PLASMID PREPARATION KIT

The Midi Plasmid Preparation kit manufactured by QIAGEN was used to isolate plasmid DNA according to the manufacturers instructions. This kit is based upon the alkaline lysis method of plasmid DNA preparation.

2.6 FUNGAL DNA EXTRACTION

A. nidulans genomic DNA was extracted from mycelia. A spore suspension was spread onto sterile cellophane discs overlaid on appropriately supplemented MYG plates (approximately 2 x 10^7 spores per plate). After the plate had been incubated at 37°C for approximately 20 hours, the mycelia were scraped off the disc and freeze-dried.

Alternatively, a siliconised flask containing liquid MYG media (supplemented as required) was inoculated with 10^6 spores/mL. The flask was incubated at 37°C with shaking (250 rpm) for approximately 20 hours. The mycelia were then harvested by filtration through sterile Mira cloth (Calbiochem), washed with sterile dH_2O, and freeze-dried.

Unless otherwise stated, all centrifugation steps for the small scale DNA preparation procedure (section 2.6.1) were carried out at 13 000 g in a microcentrifuge, using 1.5 mL microcentrifuge tubes. The large scale DNA preparation procedure (section 2.6.2) was carried out with 15/30 mL Corex tubes, using a Sorvall centrifuge with a SS34 rotor. All centrifugation steps were carried out at 4°C.

2.6.1 SMALL SCALE DNA PREPARATION

This method is based on that of Al-Samarrai & Schmid (2000). Approximately 30 mg of freeze-dried mycelia were ground in a 1.5 mL microcentrifuge tube using liquid nitrogen. Vigorous pipetting with 0.5 mL of freshly prepared lysis buffer (40 mM tris acetate buffer (pH 7.8), 20 mM sodium acetate, 1 mM EDTA and 1% SDS) was carried out to ensure the mycelia were resuspended. 0.165 mL of 5 M NaCl was added, the contents of the tube mixed by inversion, and the tube centrifuged at 4°C for 20 minutes. The supernatant was transferred to a fresh tube and an equal amount of chloroform was added. Following thorough mixing of the sample, centrifugation was carried out (20 min), after which the aqueous phase was transferred to a fresh tube. To precipitate the DNA, 2 volumes of cold 95% ethanol was added and the sample well mixed by inversion. The tube was immediately centrifuged for 5 minutes. The resulting DNA pellet was washed 5 - 6 times with 70% ethanol, then resuspended in 0.5 mL of lysis buffer. To further purify, 0.165 mL of 5 M NaCl was added and the contents of the tube thoroughly mixed. An equal volume of chloroform was then added, the contents of the tube mixed by inversion and then centrifuged for 20 minutes. The supernatant was transferred to a fresh tube, 2 volumes of cold 95% ethanol added and centrifugation carried out (5 min). The DNA pellet was then washed 5 - 6 times with 70% ethanol, airdried, and resuspended in 50 µL of sterile dH_2O.

2.6.2 LARGE SCALE DNA PREPARATION

This method is based on that of Byrd *et al* (1990). Approximately 100 mg of freeze-dried

mycelia was ground to a powder in liquid nitrogen, using a mortar and pestle. The mycelia was resuspended in 10 mL of extraction buffer (100 mM LiCl, 10 mM EDTA, 10 mM Tris-HCl (pH 7.4) and 0.5% SDS) and Proteinase K added to a final concentration of 2 mg/mL. The sample was transferred to a Corex tube and incubated at 37°C for 20 minutes prior to centrifugation (10 min at 13 000 g). The supernatant was transferred to a fresh tube and an equal volume of phenol added. The contents of the tube were then mixed by inversion and the centrifugation carried out for 15 minutes at 13 000 g. The aqueous phase was removed to a fresh tube, and an equal volume of phenol:chloroform was added. After thorough mixing, the tube was centrifuged (15 min at 13 000 g) and the supernatant retained. An equal volume of isopropanol was added to the supernatant, after which the tube was incubated at -20°C for 15 minutes. The DNA was then pelleted by centrifugation for 30 minutes at 13 000 g. To remove polysaccharides, the pellet was resuspended in 5 mL of 1 M NaCl and the sample centrifuged (5 min at 13 000 g). The supernatant was then transferred to a fresh tube, 5 mL of isopropanol added, and the sample incubated at -20°C for 15 minutes. Following centrifugation (30 min at 13 000 g), the pellet was washed with 70% ethanol, airdried, then resuspended in 0.5 mL of sterile dH_2O.

2.7 DNA MANIPULATION PROCEDURES

DNA samples, genomic or plasmid, could be further purified by phenol/chloroform extraction. The extracted DNA was then precipitated with ethanol or isopropanol (Sambrook *et al.*, 1989).

2.7.1 ENZYMATIC DIGESTION OF DNA

This method is obtained from Sambrook *et al* (1989). To check that digestion had gone to completion, the samples were observed following electrophoresis (section 2.9.2).

2.7.2 ELECTROPHORESIS

This method is obtained from Sambrook *et al* (1989). For routine electrophoresis, e.g. of digested DNA samples or ligations, a 1-1.5% gel was prepared with Seakem agarose (Invitrogen) or agarose (BRL) and 1 x TBE Buffer (10x Stock (g/L): tris, 108; boric acid, 55; and Na_2EDTA, 9.3)). Following electrophoresis, the ethidium bromide-stained gel was

placed on a UV transilluminator and photographed under ultraviolet using the Gel Documentation System (Alpha Innotech).

2.7.3 DETERMINATION OF MOLECULAR WEIGHTS

The sizes of the DNA fragments (kilobases, kb) were estimated by comparison to a set of standard fragments of known size which was loaded onto the same agarose gel. The distances travelled by the standard fragments could be plotted against their corresponding molecular weights, and the resulting graph used to estimate the sizes of the DNA fragments based on their migration distances. Alternatively, the sizes of the fragments could be estimated by the Gel Documentation System (Alpha Innotech).

Molecular weight ladders used routinely were the 1 kb ladder (Roche Biochemicals Ltd), 100 bp ladder (Roche Biochemicals Ltd), and λ DNA (Roche Biochemicals Ltd) digested with *Hind* III or *Eco* RI/*Hind* III.

2.7.4 ISOLATION OF DNA FRAGMENTS

DNA fragments were recovered from agarose gels following electrophoresis by a variety of methods (section 2.7.4). Generally, the digested DNA sample (previously checked by electrophoresis) (section 2.7.2) was loaded onto a gel prepared with 2.5% Seaplaque agarose (low melting point, Invitrogen), in 1 x TAE Buffer (10x Stock (g/L) Tris, 48.4; Na_2EDTA, 7.5; and 11.5 mL of glacial acetic acid). Electrophoresis was generally carried out at 95 V for approximately 1 hour. Following staining of the gel in ethidium bromide, bands were visualised using a long wave ultraviolet lamp, and the required fragments sliced out of the gel using a sterile scalpel blade.

EXTRACTION OF DNA FROM AGAROSE

The extraction of DNA fragments from Seaplaque agarose was generally carried out with the QIAGEN Gel Extraction kit, according to the manufacturers instructions. The GeneClean kit (BIO 101 inc.) was also used, according to the manufacturers instructions. The 'phenol freeze' method, based on that by Thuring *et al* (1975) was also used. In this method, the excised DNA fragment was placed in a 1.5 mL microcentrifuge tube. The

agarose was melted by heating in 65°C waterbath, covered with phenol, thoroughly mixed and placed at -20°C for at least 2 hours. Following centrifugation for 10 minutes, the aqueous phase was extracted with phenol/chloroform and purified by ethanol/isopropanol precipitation.

2.7.5 QUANTIFICATION OF DNA CONCENTRATION

ESTIMATION OF DNA CONCENTRATION USING ELECTROPHORESIS

Electrophoresis of the DNA sample (section 2.7.2) was carried out alongside appropriate DNA concentration standards, prepared with either λ (Roche Biochemicals) or pUC118 DNA. Following staining of the gel in ethidium bromide, the gel was photographed. The concentration of the DNA sample was estimated by comparing the intensity of fluoresence with that of the DNA standards.

ESTIMATION OF DNA CONCENTRATION BY FLUOROMETRY

A Hoefer Scientific TKO 100 Fluorometer was used for the quantification of DNA samples, especially fungal genomic DNA, prepared as described in section 2.6. The fluorometer was initially calibrated to a concentration of 100 ng/μL, with 2μL samples of calf thymus DNA, resuspended in fluorometry solution 'A' (0.1 μg/mL Hoechst 33258 dye, and 1 x TNE Buffer (10x Stock (g/L): tris, 12.1; Na_2EDTA, 3.7; and NaCl, 58.4. The pH was adjusted to 7.4). 2 μL samples of the DNA sample were assayed for concentration as for the control DNA sample, until three consistent readings were obtained.

2.8 POLYMERASE CHAIN REACTION (PCR)

The Polymerase Chain Reaction (PCR) amplifies specific DNA fragments from plasmid or genomic DNA samples. A standard reaction set-up and thermal cycling programme were utilised (section 2.8.1), with specific primers as listed in Table 2.2.

2.8.1 STANDARD PCR PROTOCOL

Generally, a master mix of n+1 reactions was prepared, which contained (per 25 μL

sample):

2.5 µL of 10x *Taq* Buffer

0.75 µL of 25 mM $MgCl_2$

2.5 µL of 1.0 mM dNTP's

2.5 µL of each 2 mM/mL primer

0.25 µL of *Taq* DNA polymerase or Expand High Fidelity (1 U per µL)

sterile dH_2O to a final volume of 25 µL

To the required reactions were added 10 ng or 50 ng of plasmid or genomic DNA, respectively. Appropriate negative (including 'no template') and positive controls were always included. The *Taq* DNA polymerase, and Expand High Fidelity enzyme, were sourced from Invitrogen and Roche Biochemicals (respectively).

The thermal cycling programme used (unless stated otherwise) was:

Denaturation	95°C for 2 minutes	
Denaturation	95°C for 30 seconds	
Annealing	60°C for 30 seconds	(25 cycles)
Elongation	72°C for 1 minute	
Elongation	72°C for 5 minutes	
Cooling	4°C for 5 minutes	

2.8.2 GEL-STAB PCR

Gel-stab PCR was carried out to obtain a particular PCR product from a sample which produced more than one product. A band resulting from electrophoresis (corresponding to a particular PCR product) was pierced with a pipette tip (20-200 µL), and the tip then placed into a microcentrifuge tube containing 200 µL of sterile dH_2O. The solution was mixed thoroughly, and a 5 µL aliquot used per 25 µL PCR reaction (section 2.8.1).

Table 2.2 PCR and Sequencing Primers

Primer	Size	Tm (°C)	Sequence (5' to 3')	Source
CPD1	24	77	AGGCCTCGAGGCATCAAGGCACAA	This study
CPD2	22	79	GCCACTCGAGATTAGGGCCGGG	This study
CPD3	24	73	AAAACTCGAGATGACTAGGCGAATG	This study
CPD4	22	74	ATTCCTCGAGACAGACAGTGCAGA	This study
CPD5	23	74	CTATCTCGAGCTGCATAACTGAGC	This study
CYCP1	22	75	ACCAGGATCCTAGCTGTCACCG	This study
CYCP2	24	74	CAGACTGCAGTTTGCGGGTAAATG	This study
YP1	24	74	GTTGCTCGAGATGTTAACACACCC	This study
YP2	25	77	CACCCTCGAGCTGGGAATTGACCAA	This study
AOX1	23	76	GTYGCYGGYGTICCIGGIATGGT	This study
AOX2	23	70	GARGCYTAYAAYGARMGIATGCA	This study
AOX3	23	71	GCYTCYTCYTCIARRTAICCIAC	This study
AOX4	23	74	TCICKRTGCTTIGCYTCRTCIGC	This study
AnidAOX1	20	65	GTGGTTCATGCGCTTAATGG	This study
AnidAOX2	18	60	CTTCATGGTCCGGTTACC	This study
BenA1	21	64	GCACTCCGATGAGACTTTCTG	This study
BenA2	21	66	AACATCTGCTGGGTCAACTCG	This study
Lacz1	18	64	TCGTGACTGGGAAAACCC	This study
Lacz2	18	59	CCGTCTGAATTTGACCTG	This study
Lacz3	18	65	ACGGCACGCTGATTGAAG	This study
Lacz4	18	59	CGCTGGATCAAATCTGTC	This study
Lacz5	18	64	CGGAAGCAAAACACCAGC	This study
Lacz6	18	64	CACAGATGTGGATTGGCG	This study
Lacz7	18	62	CTGGGATCTGCCATTGTC	This study
LM6	25	76	CAGGAATAGGATTGCTCGTGCTGAG	McGlynn (1997)
pUC/M13 Forward	22	70	GCCAGGGTTTTCCCAGTCACGA	Perkin Elmer
pUC/M13 Reverse	24	70	GAGCGGATAACAATTTCACACAGG	Perkin Elmer

*Tm (°C) was calculated by the formula: 2 x (A+T) + 4 x (G+C)

Key to symbols: R=A+G, Y=C+T, M=A+C, K=G+T, I=Inosine.

2.8.3 PURIFICATION OF PCR PRODUCTS

Products generated by PCR and required for cloning were purified using a QIAGEN PCR Purification Column Kit, according to the manufacturers instructions. This kit utilises spin cartridges which contain a silica gel membrane that specifically binds DNA and removes impurities.

2.9 CLONING

DNA fragments to be cloned were generated by enzymatic digestion (section 2.7.1) or PCR (section 2.8), and purified by gel extraction (section 2.7.4). When necessary, phosphatase treatment (section 2.9.1) was carried out to prevent re-ligation of the vector fragment, prior to the ligation reaction (section 2.9.2). The ligation mixes were then transformed by electroporation (section 2.10.1) into competent *E. coli* cells (section 2.10.1).

2.9.1 PHOSPHATASE TREATMENT OF VECTORS

Vector DNA (2-5 μg) was digested with the appropriate enzyme (section 2.7.1) and the cleaved fragment checked by electrophoresis (section 2.7.2). The sample was purified by phenol/chloroform extraction (section 2.6) prior to precipitation by ethanol/isopropanol, after which the pellet was resuspended in 35 μL of sterile dH_2O. Approximately 20 ng of vector was then set aside as a 'before treatment' control; the rest of the digested vector underwent phosphatase treatment. The phosphatase used was shrimp alkaline phosphatase (Invitrogen) and treatment was carried out according to the manufacturers instructions.

2.9.2 DNA LIGATION

GENERAL LIGATION REACTIONS

In a 1.5 mL microcentrifuge tube, a reaction mixture was set up containing 2 μL of 10x ligation buffer, x ng of insert DNA, 20 ng of vector DNA (SAP treated) (section 2.9.1) and sterile dH_2O to a final volume of 19 μL. A 3 μL 'before ligation' sample was removed prior to addition of 40 U of T4-Ligase (Invitrogen). The samples were incubated at 4°C overnight. Gel electrophoresis was carried out the following day, with a comparison made

between a 3 μL sample of the 'after ligation' mixture, and a 3 μL 'before ligation' sample.

The following equation was used to estimate the amount of insert required:

$$\frac{\text{Amount of vector (ng) x Size of insert (kb)}}{\text{Size of vector (kb)}} = \text{Amount of insert (ng)}$$

The ratio of vector: insert could be increased from 1:1 (eg. 1:2, 1:4), thereby enhancing the possibility of a successful outcome.

LIGATIONS WITH THE pGEM SYSTEM

For ligations involving the pGEM vector, the procedure was carried out according to the manufacturers instructions (Promega).

2.10 TRANSFORMATION OF GENETIC MATERIAL INTO CELLS

Unless otherwise stated, all centrifugation steps for the transformation procedures were carried out at 4°C with 15/30 mL Corex tubes, using a Sorvall centrifuge with a SS34/GSA rotor. Care was taken throughout to ensure that all solutions and equipment used were sterile and ice-cold. A Shimadzu UV-160A UV-visible recording spectrophotometer was used for the determination of cell densities.

2.10.1 BACTERIAL TRANSFORMATION

The protocol was obtained from Dower *et al* (1988). All centrifugation steps were carried out with a GSA rotor and 100 ml plastic centrifuge tubes.

PREPARATION OF COMPETENT CELLS

An overnight 10 mL culture of the appropriate *E.coli* strain (XL-1, Table 2.1) was used to inoculate 1 L of liquid LB (appropriately supplemented). The culture was grown at 37°C with shaking at 250 rpm for approximately 3 hours (until the cells had reached mid-

exponential phase, with an OD_{600} of 0.5 – 1.0). The cells were placed on ice for 20 minutes, then harvested by centrifugation at 5 000 rpm for 10 minutes. The cells were washed sequentially with the following solutions (with subsequent centrifugation at 5 000 rpm for 10 minutes after each washing, and resuspension in the next solution): 1 L of sterile dH_2O; 0.5 L of sterile dH_2O; 20 mL of 10% glycerol. The cells were finally resuspended in 4 mL of 10% glycerol, aliquoted into 200 µL volumes and stored at -80°C until required. Immediately prior to electroporation, aliquots of the cells were thawed quickly by gentle flicking of the tube and the tube was then placed on ice.

ELECTROPORATION OF COMPETENT CELLS

A 40 µl aliquot of electroporation competent *E. coli* cells and the DNA sample (2 – 4 µL) were placed in a 1.5 mL microcentrifuge tube, gently mixed, and the sample then transferred to an electroporation cuvette (Biorad). A Biorad gene pulser, set at 25 µF, 2.5 kV and 200 Ω, was used to electroporate the sample, with a time constant of 4 – 5 msec indicating a successful outcome. The cells were immediately transferred to a 1.5 mL microcentrifuge tube, 0.2 mL of liquid LB was added, and the sample then incubated at 37° C for 1 hour, with shaking at 200 rpm. Appropriate dilutions of the mixture were spread onto LB plates (appropriately supplemented) and incubated at 37°C overnight.

2.10.2 *ASPERGILLUS* TRANSFORMATION

Transformation of *A. nidulans* strains was carried out by PEG precipitation, with competent cells (protoplasts) prepared by the incubation of mycelia in a hydrolytic solution (Fincham, 1989). From liquid media, protoplasts were prepared by the method of Vollmer & Yanofsky (1986), as modified by Itoh *et al* (1994). All centrifugation steps were carried out with a Sorvall centrifuge and a SS34 rotor.

PREPARATION OF FUNGAL PROTOPLASTS FROM LIQUID CULTURE

A 1 L flask containing 100 mL of liquid MYG (section 2.3.2) (supplemented appropriately) was inoculated with 1 x 10^6 spores/mL. The flask was then incubated at 37°C, shaking at 250 rpm, for approximately 18 hours. The mycelia were harvested by filtration through sterile Mira cloth (Calbiochem), and washed thoroughly with sterile dH_2O and OM Buffer

(1.2 M $MgSO_4.7H_2O$ and 10 mM Na_2HPO_4. The pH was adjusted to 5.8 by addition of stock $NaH_2PO_4.2H_2O$). The mycelia were then placed in a sterile glass petri dish with approximately 20 mL of filter-sterilised (0.45 μM filters, Gelman Sciences) Glucanex solution (Novo Nordisk) (10 mg/mL, prepared in OM Buffer) and incubated at 37°C for 2 - 2.5 hours with gentle shaking.

PREPARATION OF FUNGAL PROTOPLASTS FROM SOLID CULTURE

Appropriately supplemented MYG plates (section 2.3.2) were overlaid with sterile discs of cellophane, and inoculated with approximately 2 x 10^7 spores per plate. The plates were incubated at 37°C for 18 hours. The discs (covered with mycelial growth) were then incubated in a sterile glass petri dish with approximately 20 mL of filter-sterilised Glucanex solution (10 mg/mL, prepared in OM Buffer) and incubated at 37°C for 2 - 2.5 hours with gentle shaking.

PROTOPLAST FLOTATION

5 mL aliquots of the protoplast isolation suspensions (section 2.10.2), previously filtered through sterile Mira cloth (Calbiochem), were transferred to sterile 15 mL Corex tubes. The samples were carefully overlaid with 2 mL ST buffer (0.6 M sorbitol and 100 mM Tris-HCl; pH 8.0) and centrifuged for 5 minutes at 5000 rpm, using an SS34 rotor. The protoplasts formed a white 'floating' layer between the Glucanex solution and the ST buffer; this was removed and all such samples pooled in a fresh corex tube. The protoplasts were mixed gently with 5 mL of STC buffer (1 M sorbitol, 50 mM Tris-HCl (pH 8.0) and 50 mM $CaCl_2.2H_2O$) and centrifuged for 5 minutes at 8000 g. This step was repeated twice, and the protoplast pellet was then resuspended in 0.5 mL of STC buffer. A sample was diluted in STC buffer and the concentration estimated using a haemocytometer. If necessary, the samples was diluted in STC buffer to 1.25 x 10^8 protoplasts/mL prior to transformation.

TRANSFORMATION OF COMPETENT CELLS BY PEG PRECIPITATION

For each reaction, 80 μL of protoplast suspension (1 x 10^7 protoplasts) was placed in a sterile Corex tube. To this was added: 20 μL of PEG transformation solution 1 (40%

polyethylene glycol 4000, 1 M sorbitol, 50 mM $CaCl_2$ and 50 mM Tris-HCl (pH 8.0)); 1 µL spermidine (50 mg/mL) and 5 µg of DNA. The suspension was mixed thoroughly and incubated on ice for 30 minutes. Approximately 900 µL (9 x volume) of PEG transformation solution 1 was then added, the suspension mixed and incubated at room temperature for 15 - 20 minutes. The suspension was then diluted 10-fold in STC buffer, and incubated on ice for a further 15 minutes. Centrifugation of the suspension (5 min at 8000 rpm) was followed by resuspension of the pellet in 0.5 mL of STC buffer. The suspension was diluted as appropriate, and 100 µL aliquots added to 3 mL of molten selective media (0.8% agar) at 50°C, mixed, and overlaid onto selective plates.

2.10.3 YEAST TRANSFORMATION

Procedures were obtained from Rose *et al* (1990). All centrifugation steps were carried out with a Sorvall centrifuge and a SS34 rotor.

PREPARATION OF COMPETENT CELLS

A culture was inoculated into appropriately supplemented YPD (section 2.3.2) and incubated at 30°C overnight, with shaking at 250 rpm. The culture was then re-inoculated (1/100) into a 1 L flask containing 100 mL of the same media, and incubation carried out at 30°C (with shaking at 250 rpm) until the OD_{600} was estimated to be in the range of 0.5 – 1.0. The cells were then pelleted by centrifugation (5 000 rpm, 5 minutes), and resuspended in 20 mL of TE buffer (10:0.1) (10 mM Tris-HCl (pH 8.0) and 0.1 mM Na_2EDTA (pH 8.0)). The cells were again pelleted, resuspended in 10 mL of LiAc buffer (100 mM LiOAc), and incubated for 1 hour at 30°C with gentle agitation. Centrifugation of the samples was carried out again, the pellet resuspended in 2 mL of LiAc buffer, and dispensed into 0.3 mL aliquots. The samples could then be used immediately for transformation, or alternatively frozen with liquid nitrogen and stored at -80°C for up to 2 months.

LITHIUM ACETATE TRANSFORMATION

For each transformation sample, an aliquot of the competent cells was placed in a sterile Corex tube with 10 µL of DNA (0.1 – 10 µg) and 0.7 mL of PEG transformation solution 2 (a sterile solution of 1 M LiOAc, 0.1 M Tris-HCl (pH 7.5) and 10 mM Na_2EDTA was prepared. This was added (1/10 volume) to a stock solution of 44% polyethylene glycol 3300 and mixed thoroughly). Following incubation for one hour at 30°C, the sample was then heat-shocked at 42°C for 5 minutes. Two volumes of liquid YPD (section 2.3.2) were immediately added, the sample thoroughly mixed, and incubated at 30°C for 1 hour. The sample was then centrifugated (5 000 rpm, 5 minutes), and resuspended in 0.5 mL of liquid YPD. Appropriate dilutions of the sample were plated onto selective X-gal indicating media (section 2.3.2).

2.11 SEQUENCING

The sequencing of DNA samples was carried out with either an automated sequencer, or manually. Both methods were based on the Sanger method of chain termination (Sanger *et al.*, 1977).

2.11.1 AUTOMATED SEQUENCING

DNA samples were sequenced using an automated DNA sequencer (ABI377, Applied Biosystems). The dye terminator programme was used, with each sample containing 3.2 pmol of primer and approximately 250 ng of plasmid DNA.

2.11.2 MANUAL SEQUENCING

PREPARATION OF THE DNA SAMPLES

A master mix of n+1 reactions was prepared with the Amplicycle Sequencing kit, according to the manufacturers instructions (Applied Biosystems). This mixture contained (per reaction):

10x Cycling Mix	4.0 µL
Specific primer (20 µM)	1.0 µL
[α-^{33}P] - dATP (12.5 µCi/µL)	1.0 µL

Plasmid DNA (1 µg) and sterile dH_2O were added to a final volume of 30 µL. Each reaction was aliquoted into four separate samples, to which 2 µL of a termination mixture (ie. G, A, T or C) was then added.

The thermal cycler programme used for the sequencing reactions is described below:

Denaturation	95°C for 2 minutes	
Denaturation	95°C for 45 seconds	(35 cycles)
Annealing	60°C for 45 seconds	
Elongation	72°C for 1 minute	
Elongation	72°C for 5 minutes	
Cooling	4°C for 5 minutes	

4 µL of stop solution was then added to each of the tubes, and the reactions stored at -20° C for up to one week.

PREPARATION OF THE SEQUENCING GEL

A polyacrylamide sequencing gel was prepared by adding 42 µL of TEMED (N, N, N^1, N^1 - tetramethylethylenediamine) (BDH) and 420 µL of 10% ammonium persulfate (BDH) to 70 mL of Long Ranger Acrylamide Mix (Invitrogen). The solution was mixed thoroughly before being carefully poured between two glass plates, the combs inserted, and the gel was left to set overnight.

ELECTROPHORESIS OF THE SEQUENCING SAMPLES

The sequencing gel was assembled in the electrophoresis apparatus, covered with 1 x TBE sequencing buffer (10x Stock (g/L): tris, 162.0; boric acid, 27.5; and Na_2EDTA, 9.4. The

pH was adjusted to 8.8), and pre-run at approximately 1500 V (60 mA) for 30 minutes. The samples were heated at 75°C for 2 minutes prior to being loaded into the wells, and the gel then run at 1500 V (60 mA) for the necessary time.

The electrophoresis apparatus was then disassembled, and the gel placed in fixing solution (10% acetic acid and 10% ethanol (95%)) (while still on a glass plate) for approximately 30 minutes. The gel was carefully transferred to blotting paper and dried under vacuum at 80° C for 45 minutes. Exposure of the gel to X-ray film at -80°C for 2-3 days was then carried out.

2.11.3 ANALYSIS OF DNA OR AMINO ACID SEQUENCE

These analyses were carried out using NCBI's sequence similarity search tools, located at http://www.ncbi.nlm.nih.gov:80/BLAST/

A BLAST search of the nr database (using the BLOSUM62 matrix) determined sequences of high similarity (amino acid or nucleotide). Generally, the programs used were blastn and blastp for nucleotide and amino acid sequences, respectively.

To determine the similarity between 2 specific amino acid or nucleotide sequences, a BLAST analysis (BLAST 2 sequences) was carried out with the blastp and blastn programs for amino acid and nucleotide sequences, respectively. The BLOSUM62 matrix was used for the analysis of amino acid sequences, while nucleotide sequence analysis did not require a matrix.

2.12 SOUTHERN BLOTTING & PROBE HYBRIDISATION

Southern blotting was carried out as described by Southern (1975). The DNA samples (2 μ g) were digested with the appropriate enzyme (section 2.7.1) prior to being loaded on a 0.7% Seakem agarose/TBE gel, and separated by electrophoresis (section 2.7.2) overnight at 30 V (room temperature). Hybond-N nylon membrane (Amersham) was used. Following blotting, the membrane was washed in 2 x SSC for 5 minutes, prior to being dried for 2 hours in a 80°C vaccum oven.

DNA fragments to be used as probes were prepared by digestion of the DNA with the appropriate enzyme (section 2.7.1), electrophoresis (section 2.7.2) and gel extraction (section 2.7.4). The probe (25 ng - 100 ng) was labelled with the 'Ready to Go' DNA labelling kit (-dCTP), according to the manufacturers instructions (Amersham Pharmacia Biotech). Alternatively, the procedure was carried out with the 'High Prime' kit, according to the manufacturers instructions (Roche Biochemicals). For each probe, 5 μL of [α-^{32}P]dCTP (3000 Ci/mmol) was used. Excess unlabelled probe was removed with a ProbeQuant™ G-50 Micro-Column, according to the manufacturers instructions (Pharmacia Biotech). Following this, the efficiency of labelling was estimated by use of a Geiger counter. The probe could be stored at -80°C for approximately one week. Immediately prior to hybridisation, the probe was heated in a boiling waterbath for 2 - 3 minutes before being placed quickly on ice.

For hybridisation, the membrane was incubated in approximately 30 mL of Denhardts solution ((g/L): ficoll, 2.0; bovine serum albumin, 2.0; and polyvinylpyrolidone, 2.0. Also contained (mL/L) 1 M hepes (pH 7.0), 50.0; 20 x SSC, 50.0; herring DNA (3 mg/mL), 6.0; *E. coli* tRNA (10 mg/mL), 2.0; and 20% SDS, 5.0 (not sterilised)) at 65°C for ½ - 1 hour prior to the addition of the probe. About 25 mL of the solution was then removed, the denatured probe was added and the filter incubated overnight at 65°C. The filter was gently agitated in washing solution (2 x SSC and 0.2% SDS (not sterilised), warmed to 65°C prior to use) for 15 minutes (repeat 3 times) and blotted with 3MM paper. The filter was then enclosed in a plastic bag, and exposed to X-ray film overnight at -70°C.

2.13 QUANTITATIVE ASSAYS

2.13.1 β-GALACTOSIDASE ASSAYS

β-galactosidase is an enzyme which hydrolyzes β-D-galactosidases, eg. σ-nitrophenyl-β-D-galactoside (ONPG), which is converted to galactose and σ-nitrophenyl. The absorbance of the latter compound can be measured at 420 nm. If the concentration of ONPG is high enough, the amount of σ-nitrophenyl produced is considered to be correlated to the amount of β-galactosidase present and the duration of the reaction (Miller, 1972).

The samples were assayed on a Shimadzu UV-160A UV-visible recording spectrophotometer, using quartz cuvettes. All centrifugation steps were carried out with a SS34 rotor in a Sorvall centrifuge, at 4°C. The ONPG (Sigma) solution was prepared at the start of each experiment. Assays were carried out in triplicate.

Initial screening of *A. nidulans* colonies was carried out on MM (section 2.3.2) containing 1 % glucose. A toothpick was used to transfer a sample of each colony to a plate, which was then incubated overnight at 37°C. Approximately 10 µl of X-gal (60 mg/mL) was pipetted onto each colony, and the plate left at 25°C for blue colour to develop.

ASPERGILLUS β-GALACTOSIDASE ASSAYS

Fresh mycelia were always used for the *A. nidulans* assays, as storage at -80°C (after freezing in liquid nitrogen) has been reported to decrease β-galactosidase activity by up to 50% (van Gorcom *et al.*, 1985). The protocol was based on that of Davis *et al* (1988).

Mycelia were harvested from 1 L flasks containing 100 mL of appropriately supplemented MM (section 2.3.2) which had been inoculated with 1 x 10^6 spores/mL and incubated at 37 °C, shaking at 250 rpm, for approximately 18 hours. The mycelia were filtered through sterile Mira cloth (Calbiochem) and washed throughly with sterile dH_2O. Approximately 0.2 g of fresh mycelia were then powdered with liquid nitrogen in a mortar and pestle. The ground mycelial sample was resuspended in 2 mL of Z buffer ((g/L): NaH_2PO_4, 5.5; Na_2HPO_4, 16.1; $MgSO_4.7H_2O$, 0.246; KCl, 0.75. The pH was adjusted to 7.0). The Z buffer was stored at 4°C, and 2.7 mL of mercaptoethanol was added prior to use. The mycelial sample resuspended in Z buffer was centrifuged for 15 min, 13 000 rpm at 4°C. The supernatant was transferred to a fresh tube, and 0.1 mL of this solution (already diluted by 100-fold in Z buffer) was then added to 0.9 mL of Z buffer. Three drops of chloroform and 2 of 0.1% SDS were then added and the sample vortexed vigorously for 15 seconds. Following incubation at 28°C for 5 minutes, 0.2 mL of ONPG (4 mg/mL, prepared in Z buffer) was added and the sample mixed thoroughly. Incubation at 28°C was then carried out for 30 minutes, after which time the reaction was stopped by adding 0.5 mL of 1 M Na_2CO_3 and the A_{420} reading then taken.

The samples were compared against a 'blank' sample which contained all the reagents, with Z buffer in place of the mycelial sample. The β-galactosidase activity of the sample was estimated as follows:

β-galactosidase activity (units/min/mL) = A_{420} x 10^3/min/mg protein

YEAST β-GALACTOSIDASE ASSAYS

Five individual colonies of each type of transformant were inoculated into liquid YPD (section 2.3.2), and incubated overnight at 30°C with shaking at 250 rpm. The five cultures were then pooled, and the A_{600} readings of this pooled sample (diluted 10-fold) obtained. The cells were pelleted by centrifugation at 4°C (15 min, 13 000 rpm), and the supernatant removed. The cells were resuspended in 1 mL of Z buffer (as for *Aspergillus*). For each pooled sample, 0.1 mL of sample (already diluted 100-fold) was placed in a Corex tube with 0.9 mL of Z buffer. Three drops of chloroform and 2 of 0.1% SDS were then added and the sample vortexed vigorously for 15 seconds. Following incubation at 28°C for 5 minutes, 0.2 mL of ONPG (4 mg/mL, prepared in Z buffer) was then added and the sample mixed thoroughly. Incubation at 28°C was then carried out for 20 minutes, after which time the reaction was stopped by adding 0.5 mL of 1M Na_2CO_3, and the A_{420} reading then taken.

The samples were compared against a 'blank' sample which contained all the reagents, with Z buffer in place of the mycelial sample. The β-galactosidase activity of the sample was estimated as follows:

β-galactosidase activity (units/min/mL) = A_{420} x 10^3/ A_{600} x min x mL

2.13.2 PROTEIN ASSAYS

The protein assays were carried out using the Bradford method (Sambrook *et al.*, 1989), simultaneously with the β-galactosidase assays (section 2.13.1). Alternatively, the protein assays were carried out using the Biorad protein assay kit, according to the manufacturers instructions. Both methods used bovine serum albumin (BSA) as a standard control, with

the assays carried out in triplicate.

2.14 GROWTH MEASUREMENTS OF THE FUNGAL STRAINS

2.14.1 GROWTH ON VARIOUS CARBON SOURCES

The strains were plated onto AMM plates (section 2.3.2) and incubated at 37°C until sporulation was observed. Three squares of mycelial growth (approximately 5 mm in circumference) from a strain were then cut and placed onto a fresh plate of AMM media containing 1% v/v glucose, glycerol or lactic acid. Incubation at 37°C was carried out for 3 and 5 days, for strains A67 and A68, respectively.

2.14.2 RADIAL GROWTH MEASUREMENTS

Conidiospores were spread onto AMM plates (section 2.3.2) supplemented with 1 mg/L para-aminobenzoic acid, 1 mg/L pyridoxin.HCl and various carbon sources (1% v/v glucose, glycerol or lactic acid). Due to differences in germination rate, approximately 8● spores of A67 and 1 x 10^4 spores of A68 were spread to achieve 6-30 measurable colonies per plate. Growth of the strains was estimated by measuring colony diameters after 3 or 5 days at 37°C for A67 and A68 respectively.

2.15 RESPIRATORY MEASUREMENTS OF THE FUNGAL STRAINS

2.15.1 GROWTH OF CULTURES

One litre flasks containing 100 mL of liquid MYG (section 2.3.2) were inoculated with 10^6 spores/mL for *gcA*$^+$ strains A57 and A67, or 1 g of homogenised mycelia for *gcA*$^-$ strains A58 and A68, and incubated at 37°C with shaking (25● rpm). Strains A57 and A58 also required the addition of uracil (section 2.3.2). For the oxygen consumption assays and spectral analysis, 1 L baffled flasks were used, and additional aeration was provided.

For the growth curves, 5 mL samples were removed, freeze-dried and the dry weight recorded. Triplicate flasks were sampled for each strain.

The cultures were grown to mid-exponential and stationary time-points for the oxygen consumption assays. A 5 mL sample was removed immediately prior to the mycelia being harvested; this sample was freeze-dried and its dry weight recorded to confirm that the culture followed the pre-determined growth curve for that strain. The oxygen consumption assays were carried out in triplicate.

2.15.2 PREPARATION OF MYCELIAL SAMPLES

The mycelia were harvested by filtration through sterile Mira cloth (Calbiochem) and washed with sterile dH_2O and isolation buffer (0.5 M mannitol, 1 mM EDTA and 10 mM tris-HCl; pH was adjusted to 7.0 prior to autoclaving). Excess moisture was then removed by squeezing, and the samples were homogenised to 0.125 g/mL or 0.0625 g/mL in isolation buffer.

2.15.3 OXYGEN CONSUMPTION ASSAYS

The respiratory measurements were carried out at 37°C, using an oxygen electrode (Ganstech, UK). The system was initially calibrated with a sample of dH_2O (warmed to 37 °C), to which approximately 10 mg of sodium dithionite (BDH) was added. A 1.0 mL mycelial sample (grown and prepared as described in sections 2.15.1 and 2.15.2) was used for each assay (carried out in triplicate). Succinate (10 μM) was added to each assay to obtain a measurable rate of respiration.

The respiratory rate (RR) (nmol O_2 consumed/min/g wet weight) was calculated as follows:

$$RR = \frac{\text{Slope x volume (L) x } (245 \times 10^{-6})}{\text{Calibration}} \times \frac{60 \text{ sec}}{\text{min}}$$

This value was then divided by: [the volume of cells used (L)] x [wet weight (g/L)]

2.16 ETHANOL ASSAYS

Strains were grown in liquid MYG (section 2.3.2), as described in section 2.15.1, to late-exponential and stationary phase. Ethanol assays were carried out on the extracellular

culture filtrate with an Ethanol Enzymatic BioAnalysis kit (Roche Biochemicals) according to the manufacturers instructions. Assays were carried out in triplicate.

2.17 CYTOCHROME SPECTRA & CO BINDING ASSAY

The cultures were grown and harvested as detailed previously (section 2.15.1). To ensure a high degree of oxygenation, baffled flasks were used, and the cultures were supplied with additional oxygen by means of a fish-tank bubbler. The mycelia was homogenised to 0.125 g/mL in isolation buffer (0.5 M mannitol, 1 mM EDTA and 10 mM tris-HCl. The pH was adjusted to 7.0 prior to autoclaving). Spectra were obtained at ambient temperature in a lab-built single beam spectrophotometer with a slit width of 1 nm. The cuvette (path length 10 mm) contained 3.0 mL of suspension. Sodium dithionite (BDH) was added to obtain the fully reduced spectrum.

For the carbon monoxide binding assays, the mycelial sample for which spectra were recorded was then bubbled with carbon monoxide. This was carried out in a enclosed glass vial, with constant mechanical stirring. The sample was assayed for spectra before and after the addition of carbon monoxide.

2.18 RNA WORK

All glassware used was soaked in chromic acid overnight, prior to being thoroughly rinsed and sterilised at 180°C for 2 hours. Pipette tips and microcentrifuge tubes utilised were sourced from previously unopened bags, and placed in sterilised glassware before being autoclaved. Fresh disposable rubber gloves were replaced regularly. All solutions were prepared from RNA-only stocks, and dissolved in DEPC-treated dH_2O. The DEPC-treated sterile dH_2O was prepared by adding DEPC (0.01%) to an appropriate volume of dH_2O and incubating the solution overnight at 37°C. The solution was then autoclaved twice. All surfaces used were first cleaned thoroughly with 95% ethanol.

2.18.1 RNA EXTRACTION

The cultures were grown as described in section 2.15.1. The mycelia were harvested by filtration through sterile Mira cloth (Calbiochem) and washed with DEPC-treated dH_2O.

Frozen (not freeze-dried) mycelia were ground in a mortar and pestle with liquid nitrogen. The appropriate volume of Trizol reagent (Invitrogen) was added as per the manufacturers instructions (1 mL per 100 mg mycelia) and the mycelia mixed to a fine paste. Additional Trizol was then added if required (1 mL per 100 mg mycelia). The sample was transferred to a Corex tube and incubated at room temperature for 5 minutes, after which centrifugation was carried out at 9 000 rpm for 10 minutes. The cleared homogenate was then transferred to a fresh tube, and chloroform added (0.2 mL per 100 mg of mycelia). The sample was mixed thoroughly prior to incubation at room temperature for 3 minutes. Centrifugation was carried out at 9 000 rpm for 15 minutes, and the aqueous phase then transferred to a fresh tube. An appropriate volume of isopropanol was then added (0.5 mL per 100 mg of mycelia), and the sample incubated at room temperature for 10 minutes. Following centrifugation at 9 000 rpm for 15 minutes, the supernatant was carefully removed and the pellet was washed with 70% ethanol (1 mL per 100 mg of mycelia). The sample was centrifuged at 6 700 rpm for 5 minutes, and the supernatant decanted. The pellet was briefly air-dried prior to resuspension in 200 µL of DEPC-treated sterile dH_2O.

2.18.2 ESTIMATION OF RNA CONCENTRATION

Appropriate dilutions of the RNA samples were prepared with DEPC-treated dH_2O, to a total of 1 mL. Two 'blanks' were also prepared of DEPC-treated dH_2O only. Using a Shimadzu UV-160A UV-visible recording spectrophotometer, the absorbances of the RNA samples were recorded at A_{260} and A_{280}.

The concentration and purity of the RNA samples were estimated as follows:

$A_1 = 260$, $A_2 = 280$

A_1 / A_2 indicates the purity of the RNA sample (a value of 1.8 indicates contamination by DNA or protein)

A_1 x dilution factor (100) x 40 = concentration of the RNA sample (µg/mL).

2.18.3 DNase TREATMENT

This was carried out with the AmpliGrade DNase kit or an alternative DNase treatment.

AMPLIGRADE DNase KIT

This was carried out according to the manufacturers instructions (Invitrogen). The 1 µg RNA samples (prepared in duplicate for 'with reverse transcriptase (RT)' and 'without RT' samples) could then be used immediately for cDNA synthesis, without transferring the sample to a fresh tube.

DNase TREATMENT

This protocol was obtained from Bradshaw & Pillar (1992). All centrifugation steps were carried out at 4°C, using a Sorvall centrifuge with a SS34 rotor.

In a Corex tube was placed:

120 µg of RNA

6 µL of DNase I

3.75 µL of RNase Inhibitor

12 µL of 100 mM DDT

DNase Buffer (100 mM NaOAc and 5 mM $MgSO_4$. The pH was adjusted to 5 prior to autoclaving) to a final volume of 2.4 mL.

Incubation was carried out at 37°C for 30 minutes, after which time the sample was re-extracted with phenol/chloroform (1:1). Following vigorous mixing, the sample was centrifuged for 10 minutes at 11 500 rpm. The aqueous phase was transferred to a fresh tube, and 2 volumes of chloroform added. The sample was again thoroughly mixed, and then centrifuged at 11 500 rpm for 10 minutes. In a fresh tube, the aqueous phase was then added to 240 µL of 3 M NaOAc and 6 mL of 95% ethanol. Incubation of the sample on ice was then carried out for 30 minutes, followed by centrifugation at 9 000 rpm for 15 minutes. The pellet was washed with 6 mL of 70% ethanol, air-dried and then resuspended in 100 µL of DEPC-treated sterile dH_2O.

2.18.4 cDNA SYNTHESIS

This was carried out using either: the Expand Reverse Transcriptase (RT) kit, or the GeneAmp Gold RNA PCR Reagent kit (One-step).

THE EXPAND REVERSE TRANSCRIPTASE (RT) KIT

This kit was used according to the manufacturers instructions (Roche Biochemicals).

In a PCR tube was placed:
11.0 µL of DNased RNA (1 µg)
0.6 µL of Random Primer (3 µg/µL) (Invitrogen)
1.6 µL of DEPC-treated sterile dH_2O

A control of 'no RNA' was also prepared, which contained DEPC-treated sterile dH_2O in place of an RNA sample. The samples were denatured at 90°C for 5 minutes before being placed immediately on ice.

A master mix of n+1 reactions was prepared, which contained for each sample:
4.0 µL of 5x Expand RT Buffer
2.0 µL of 100 mM DTT
0.8 µL of 1.25 mM dNTP's

6.8 µL of the master mix was aliquoted into each sample, with 1 µL of Expand RT then being added to the 'plus RT' RNA samples, as well as the 'no RNA' control sample. The corresponding 'without RT' RNA samples had the 1 µL of DEPC-treated sterile dH_2O. The samples were incubated at room temperature for 10 minutes, then 42°C for 45 minutes. The samples were used immediately or stored at -20°C until required.

THE GENEAMP GOLD RNA PCR REAGENT KIT (ONE-STEP):

This kit was used for one-step RNA PCR, according to the manufacturers instructions (PE Biosystems). The 1 µg RNA sample previously DNase-treated (section 2.18.3) was used for this process.

The thermal cycling parameters utilised for the one-step RNA PCR are detailed below:

Reverse Transcription	42°C for 12 minutes	
AmpliTaq Gold Activation	95°C for 10 minutes	
Denaturation Annealing/Elongation	94°C for 20 seconds 62°C for 1 minute	] (43 cycles)
Annealing/Elongation	72°C for 7 minutes	
Cooling	4°C for 5 minutes	

2.18.5 RT-PCR

The cDNA sample synthesised with the Expand RT kit (section 2.18.4) then underwent PCR amplification by either of the following two methods: a standard PCR protocol, or the GeneAmp Gold RNA PCR Reagent kit (Two-step).

Generally, dilutions of the cDNA samples (eg. 10-fold, 50-fold, 100-fold) were prepared for use in the RT-PCR process. The same volume of the corresponding 'no RT' samples were used undiluted. All equipment and pipette tips, etc used were those prepared for RNA work (section 2.18).

STANDARD PCR PROTOCOL

This involved the use of normal PCR conditions, with standard *Taq* DNA polymerase and gene-specific primers, and a standard thermal cycler programme. The reactions were generally prepared as 25 µL or 12.5 µL volumes.

A cocktail mixture was prepared, each 20.0 µL aliquot (for a 25.0 µL reaction) containing:

15.4 µL of DEPC-treated sterile dH_2O

2.5 µL of 10x *Taq* Buffer containing $MgCl_2$

1.0 µL of 1.25 mM dNTP's

0.5 µL of 10 pM primer/µL (of each primer)

0.1 µL of *Taq* DNA polymerase (1 U per µL)

Following the allocation of aliquots for each sample, 5.0 µL of the cDNA sample was added to the tube. For the 'no template' control, 5.0 µL of DEPC-treated sterile dH_2O was added.

The thermal cycler programme utilised is detailed below:

Denaturation	95°C for 2 minutes		
Denaturation	95°C for 45 seconds	⎫	
Annealing	60°C for 45 seconds	⎬	(35 cycles)
Elongation	72°C for 1 minute	⎭	
Elongation	72°C for 5 minutes		
Cooling	4°C for 5 minutes		

THE GENEAMP GOLD RNA PCR REAGENT KIT (TWO-STEP)

The GeneAmp Gold RNA PCR Reagent kit was utilised for the PCR amplification of cDNA samples as recommended by the manufacturers (PE Biosystems). The use of the two-step reaction mix enables a portion of the cDNA sample to be archived.

Ten-fold dilutions of the cDNA mixes were prepared with DEPC-treated sterile dH_2O, and 5 µL aliquots of this were added to the reaction mix, resulting in a final volume of 50 µL. Although the kit recommended the preparation of 50 µL samples, the reactions were downscaled to 25 µL samples successfully.

The thermal cycler conditions utilised for the two-step RNA PCR were as follows:

AmpliTaq Gold Activation	95°C for 10 minutes		
Denaturation	94°C for 20 seconds	⎫	
Annealing/Elongation	62°C for 1 minute	⎭	(43 cycles)
Annealing/Elongation	72°C for 7 minutes		
Cooling	4°C for 5 minutes		

CHAPTER 3

CHARACTERISATION OF THE CYTOCHROME *C* DEFICIENT STRAINS OF *A. NIDULANS*

Characterisation of *A. nidulans* strains deficient in cytochrome *c* was carried out. Initial studies compared the growth of a *cycA*$^-$ strain to its corresponding *cycA*$^+$ strain, while subsequent experiments sought to determine the alternative means of energy transduction employed by the *cycA*$^-$ strains.

3.1 INTRODUCTION

The cytochrome *c* deficient strain was created at Massey University by direct gene replacement (Bird, 1996). The vector used for the gene disruption (Appendix 1.1) contained the 5' and 3' regions of the *cyc*A gene (ie the sequences upstream and downstream of the coding region), flanking the *pyr*4 gene sequence from *N. crassa*. Integration of the vector at the *cyc*A locus in a *cyc*A$^+$ *pyr*4$^-$ strain would therefore result in *cyc*A$^-$ *pyr*4$^+$ genotype. The experiment was initially carried out in a diploid strain, as it was thought the disruption of the cytochrome *c* gene in an obligate aerobe would be lethal. However, the (*cyc*A$^-$ *pyr*4$^+$) haploid segregants from this strain were surprisingly observed to be viable. Thus targeted gene replacement was carried out in a haploid strain also.

Preliminary studies (Bird, 1996) determined the *cycA*$^-$ strains were capable of growth, albeit less strongly than for the corresponding wildtype strains. Growth of the mutant strains was observed to be slightly stronger on media containing fermentable carbon sources than on media containing non-fermentable carbon sources. This observation led to speculation that fermentation may be occuring in these strains.

An alternative respiratory pathway was also postulated to be present in the mutant strain. As mentioned previously (section 1.4), the alternative respiratory pathway has been discovered to be present in many plants and an increasing number of fungi (McIntosh, 1994). The alternative respiratory pathway generally branches off the cytochrome *c* dependent pathway at the ubiquinone pool (Figure 1.4). Electron partitioning between the

two pathways has traditionally been determined by the use of specific inhibitors to the oxidases in either pathway; SHAM and KCN for the AOX and COX, respectively (section 1.4.1).

3.2 GROWTH STUDIES OF THE MUTANT STRAIN

Initial studies involved growth of the *cycA*⁻ strain on a number of different carbon sources, and subsequent measurement of its radial growth. A growth study was also carried out for the *cycA*⁻ strain, thus allowing a growth curve to be plotted, and a comparison was made with the corresponding *cycA*⁺ strain.

3.2.1 GROWTH ON VARIOUS CARBON SOURCES

The growth of the *cycA*⁻ *pyr*4⁺ strain (A68), and its corresponding *cycA*⁺ *pyr*4⁺ strain (A67), was investigated on non-fermentable (glycerol, lactic acid) and fermentable (glucose) carbon sources, as described in section 2.14.1. As can be observed in Figure 3.1, the *cycA*⁻ strain grew most strongly on glucose, and less strongly on the non-fermentable carbon sources. The amount of growth observed for strain A68 on all types of media was considerably less than for strain A67. These results concur with those obtained from a previous study (Bird, 1996).

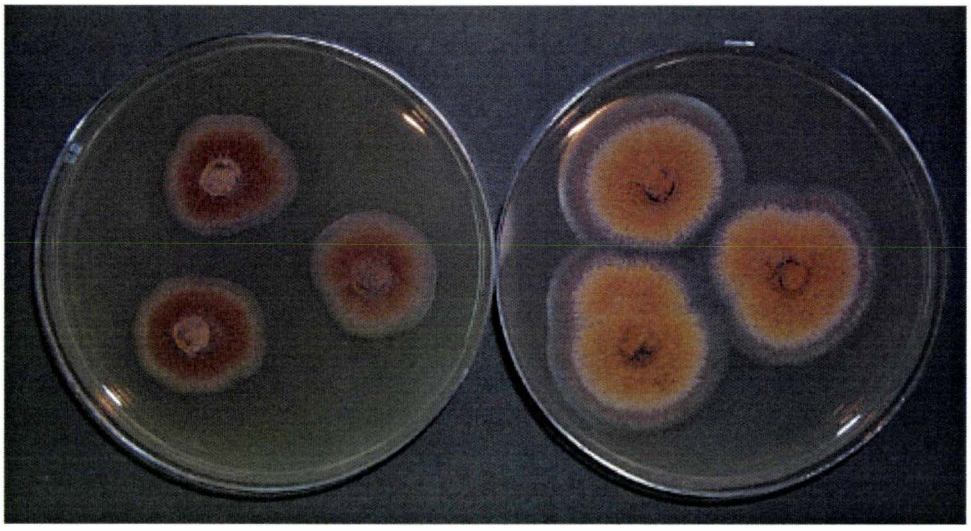
3.2.2 RADIAL GROWTH AND SPORE GERMINATION

Measurement of the growth rates of strains A67 and A68 was carried out to confirm the results obtained in section 3.2.1. The strains were plated as described in section 2.14.2, and subsequently analysed for radial growth and % viability of the spores (Table 3.1) after 3 (*cycA*⁺ strain) and 5 (*cycA*⁻ strain) days growth at 37°C.

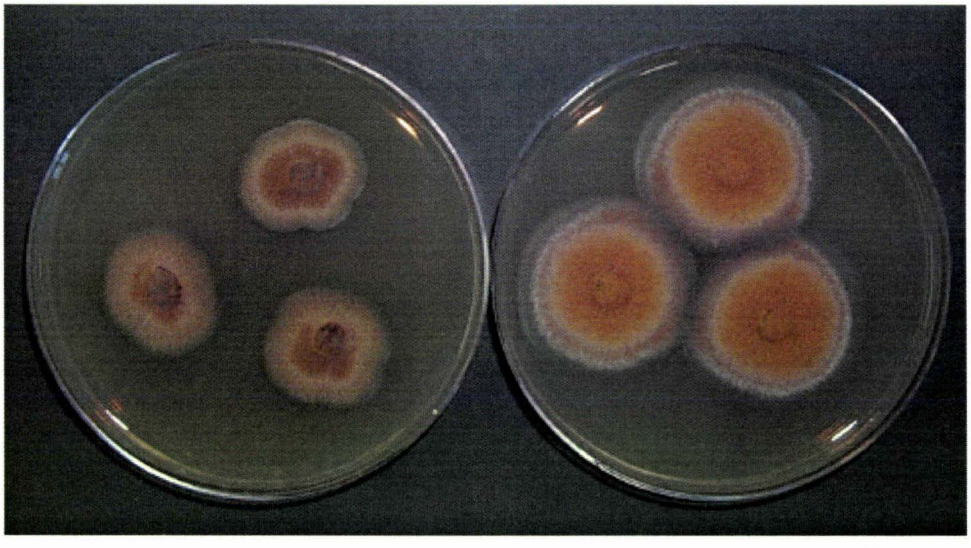
As shown in Table 3.1, growth of strain A68 (*cycA*⁻) on media containing glucose was diminished compared with the strain A67 (*cycA*⁺). Strain A68 was also observed to grow more slowly than strain A67 on non-fermentable carbon sources, with fewer colonies of lesser diameter. Germination (as determined by % spore viability) of A68 *cycA*⁻ conidiospores on the different carbon sources was diminished, compared to the A67 *cycA*⁺

Figure 3.1 **Growth of the *cyc*A$^+$ and *cyc*A$^-$ strains on different carbon sources**

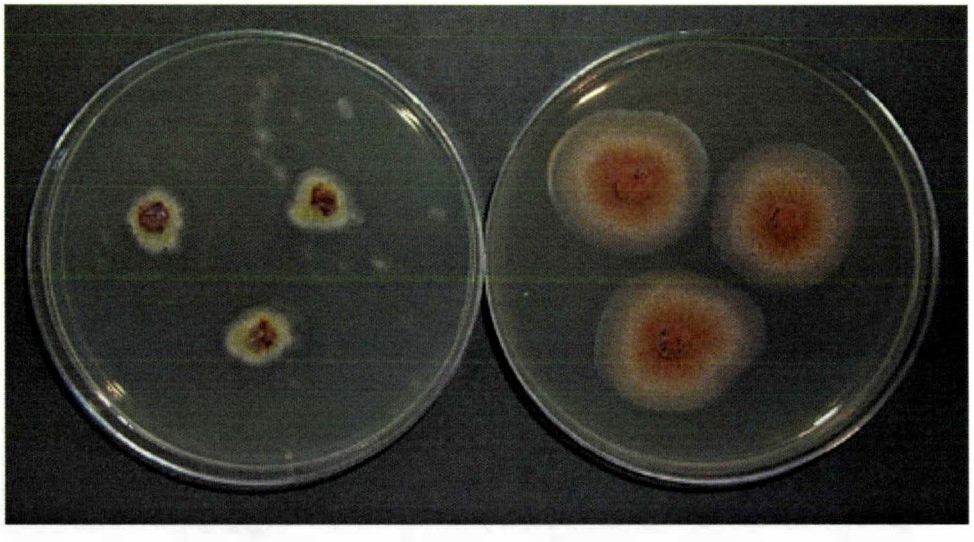
The *cyc*A$^+$ and *cyc*A$^-$ strains A67 and A68 (respectively) were plated onto AMM media containing 1% glucose (**A**), glycerol (**B**) or lactic acid (**C**). Incubation at 37°C was carried out for 3 and 5 days, for strains A67 and A68, respectively (section 2.16.1). The *cyc*A$^+$ and *cyc*A$^-$ strains are positioned on the right and left of the plates, respectively.



A



B



C

strain. Microscopic examination revealed very low levels of germ tube formation from the *cycA*⁻ spores (results not shown).

Table 3.1 Radial Growth and % Spore Viability for Strains A67 (*cycA*⁺) and A68 (*cycA*⁻)

Strain	Media	Colony Count	Diameter of Colonies (average) (mm) ± SEM	% Spore Viability
A67	MM with glucose	15	22.7 ± 1.6	9.4
	MM with glycerol	19	17.2 ± 1.1	11.9
	MM with lactic acid	13	12.8 ± 1.8	6.9
A68	MM with glucose	32	3.0 ± 0.3	0.16
	MM with glycerol	19	< 1	0.1
	MM with lactic acid	1	< 1	0.001

3.2.3 GROWTH CURVE FOR THE *cycA*- STRAIN

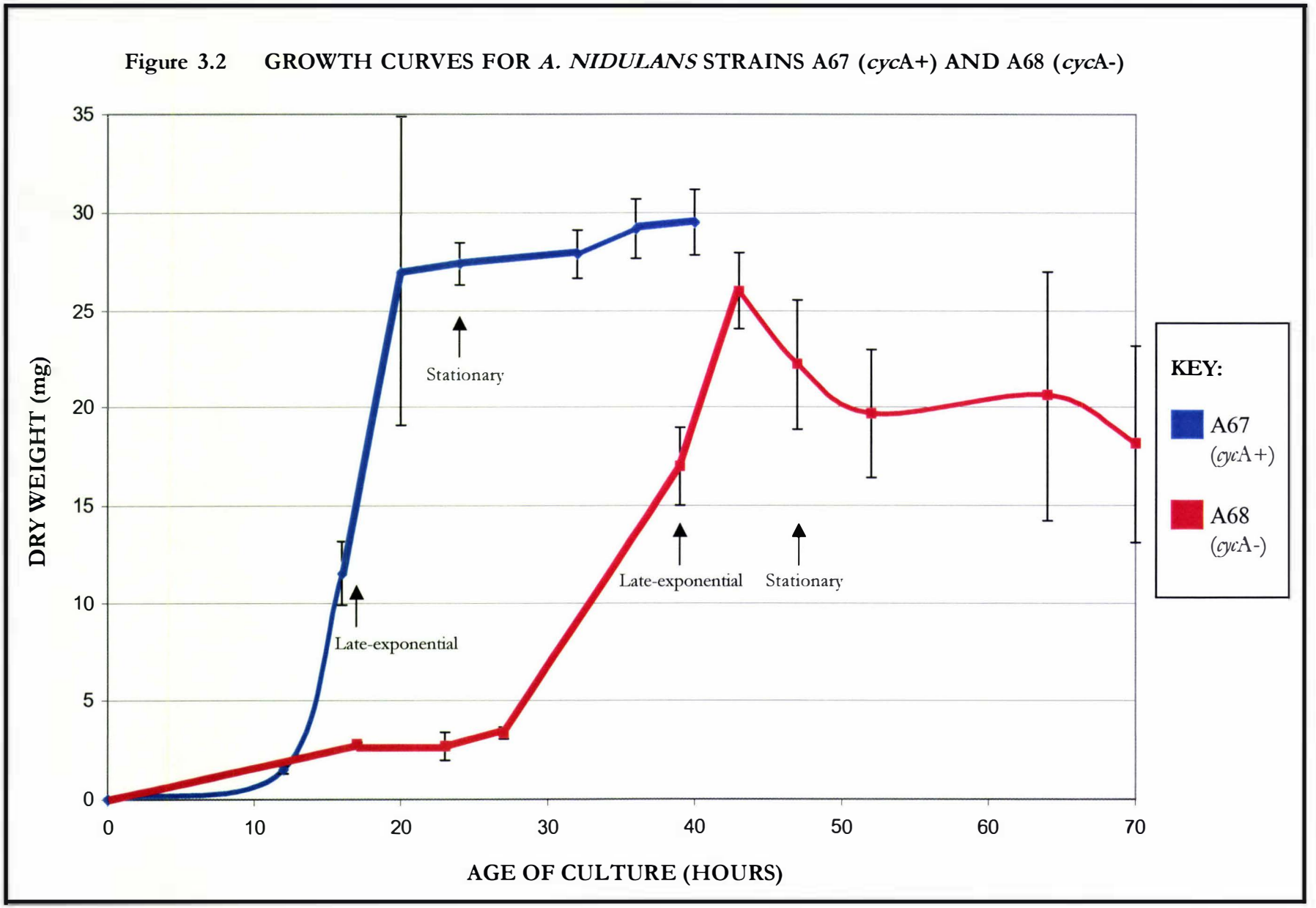
A growth study was carried out for strains A67 and A68, to obtain a better estimation of the growth rate of the *cycA*⁻ strain, compared to the *cycA*⁺ strain. The growth curve (Figure 3.2) was generated as described in section 2.15.1, with MYG containing 1% glucose, at 37° C. The *cycA*⁻ strain has a considerably slower rate of growth than the *cycA*⁺ strain (Figure 3.2). The lag phases of strains A67 (*cycA*⁺) and A68 (*cycA*⁻) were approximately 10 and 27 hours, respectively, with stationary phase estimated to commence at approximately 24 and 43 hours, respectively. The exponential growth rate of the *cycA*⁻ strain also appears to be less steep than for the *cycA*⁺ strain (although accurate quantification is not possible), possibly indicating that the growth rate of the *cycA*⁻ strain during this time is less than optimal (Figure 3.2).

Figure 3.2 **Growth curves for *cycA*$^+$ and *cycA*$^-$ strains of *A. nidulans***

One litre flasks containing 100 mL of liquid MYG (1% glucose) were inoculated with 10^6 spores/mL for *cyc*A$^+$ strain A67, and 1 g of homogenised mycelia for *cyc*A$^-$ strain A68, and incubated overnight at 37°C with shaking (250 rpm).

At the indicated timepoints, 5 mL samples were removed, freeze-dried, and the dry weight recorded to plot the growth curve. The arrows indicate the timepoints at which samples were taken which were representative of the mid-exponential and stationary phases for each strain. Triplicate flasks were sampled for each strain. The error bars at each timepoint on the growth curves indicate the standard error for the triplicate samples. Raw data for the curves are in Appendix 1.2.

Figure 3.2 GROWTH CURVES FOR *A. NIDULANS* STRAINS A67 (*cycA+*) AND A68 (*cycA-*)



3.2.4 DISCUSSION

The results from the growth studies were consistent with those expected if strain A68 is deficient in cytochrome *c*. The cytochrome *c*-deficient mutants of the yeasts *Kluyveromyces lactis* and *Pichia stipitis* also have been shown to grow slowly compared to wildtype controls and were unable to grow on non-fermentable carbon sources (Chen & Clark-Walker 1993, Shi *et al.*, 1999).

Metabolism of the simple monosaccharide glucose by the glycolytic pathway (Figure 1.1) produces pyruvate, which feeds into the fermentation pathway (Figure 1.3). Lactic acid is not utilised by these pathways, and is therefore classified as a non-fermentable carbon source. Fermentation of glycerol does occur in some organisms, however the process is dependent upon the activity of the glycerol-3-phosphatase enzyme, and does not yield energy (Van Laere, 1995). Therefore, diminished growth of the *cycA* strains upon lactic acid or glycerol, compared to glucose, suggest that fermentation is occurring in these strains.

However, these methods of measuring growth rate have limitations. The spore viability of the *cycA* strain was decreased, compared to the *cycA*$^+$ strain. This is a possible source of data variation, for the subsequent measurement of colony diameter and number. However, this is unlikely, as the diminished growth capacity of the *cycA* strain (in comparison to the *cycA*$^+$ strain) was also shown by Bird (1996).

For the ethanol production and oxygen consumption assays (sections 3.4 & 3.5, respectively), sampling timepoints for strains A67 and A68 were chosen at mid-exponential and stationary phases, as determined by the growth curve (section 3.2.3). However, the oxygen consumption assays indicated KCN-resistant respiration (and therefore activity of the alternative respiratory pathway) at the mid-exponential timepoint for the *cycA*$^+$ strains, as well as at the stationary timepoint. These results are contrary to a previous study of *A. nidulans* respiratory mutants (Turner & Rowlands, 1976), in which it was reported a wildtype (*cycA*$^+$) strain exhibited KCN-resistant respiration only at late exponential/early stationary phase, and not during the exponential phase of growth.

It was speculated that this discrepancy could be due to the 'mid-exponential' samples in fact being mistakenly sampled from the 'late-exponential/early stationary' phase of growth. As stated in section 2.17.1, samples of mycelium were taken from the cultures prior to harvesting, and their dry weights checked to ensure concurrence with the growth curve. However, as can be seen in the growth curve for strain A67 (Figure 3.2), the standard error bar for the 20 hour sample is extremely large. Thus it is possible that this timepoint corresponds to the 'late-exponential/early stationary' growth phase, rather than to the presumed mid-exponential growth phase. The standard error bars for the *cycA*⁻ strain (after 40 hours growth) are also quite large (Figure 3.2), which does not facilitate a reliable estimation of the mid-exponential timepoint. The variations in dry weight, contributing to the large standard errors, could have been due to small sample size, contamination or differences in pellet morphology of the mycelial samples. Therefore, the 'mid-exponential' samples for both strains were not considered to be reliable representations of that growth phase, and were referred to as 'late-exponential' samples. Earlier timepoints could have been selected as a better representative of the mid-exponential phases for the *cycA*⁺ and *cycA*⁻ strains. Alternatively, the growth curves should have been repeated to ensure correct estimation of the growth phases.

3.3 LEVELS OF CYTOCHROME *C* IN THE *cycA*⁻ STRAINS

Spectral analysis was carried out to determine the level of cytochrome *c* in strains A68 (*cycA*⁻) and A67 (*cycA*⁺), as described in section 2.17. These results were confirmed with another *cycA*⁻ strain, A58. Although strains A58 and A68 are both deficient in cytochrome *c*, the strains were generated by direct gene replacement in a haploid strain, and haploidisation following gene replacement in a diploid strain, respectively (Table 2.1).

3.3.1 REDOX SPECTRA RESULTS

The reduced-oxidised (redox) spectra are shown in Figure 3.3. Redox spectra for *cycA*⁻ mutant strains A58 and A68 suggested the lack of detectable levels of cytochrome *c* (at 552 nm) compared to the wildtype strain A67 (Figure 3.3). The concentration of cytochrome *c* in the *cycA*⁺ strain A67 was estimated to be 5.5 nmoles/g wet weight of mycelium (0.71 nmoles/mg protein), whilst the *cycA*⁻ mutant strains contained less than 0.6 nmoles/g wet weight of mycelium (0.22 nmoles/mg protein) (Appendix 1.3). The latter value represents

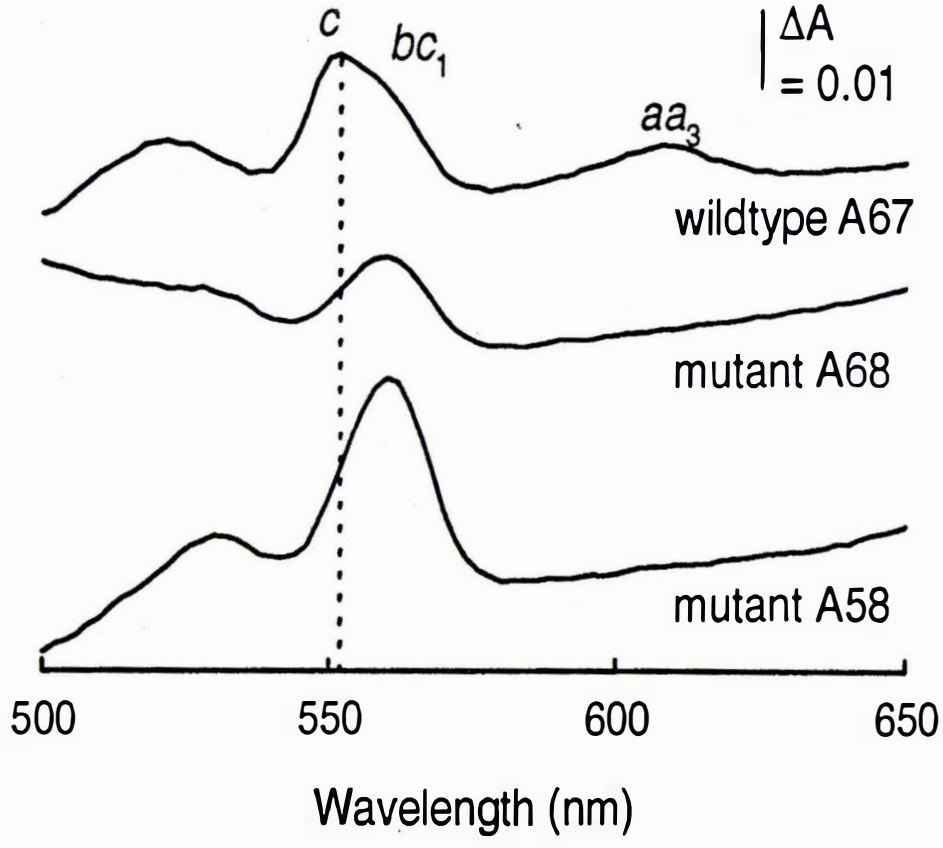

c
bc1
aa3
ΔA
= 0.01
wildtype A67
mutant A68
mutant A58
500
550
600
650
Wavelength (nm)

Figure 3.3 Spectral analysis of *A. nidulans* strains

The reduced-oxidised spectra of strains A67 (*cycA*$^+$), A68 (*cycA*$^-$) and A58 (*cycA*$^-$) are shown.

To obtain the mycelial samples used for the spectra, one litre baffled flasks containing 100 mL of liquid MYG (1% glucose) were inoculated with 10^6 spores/mL for *cycA*$^+$ strain A67, or 1 g of homogenised mycelia for *cycA*$^-$ strain A68, and incubated at 37°C with shaking (250 rpm) overnight. Additional aeration was also supplied to the cultures. The cultures were grown to late-exponential timepoints, harvested and homogenised to 0.125 g/mL in isolation buffer (0.5 M mannitol, 1 mM EDTA and 10 mM tris-HCl; pH 7.0).

Spectra were obtained at ambient temperature in a lab-built single beam spectrophotometer with a slit width of 1 nm. The cuvette (path length 10 mm) contained 3.0 ml of suspension. The reduced and oxidised spectra were obtained by addition of sodium dithionite (BDH) and aeration (respectively).

the limit of resolution of the system used, as determined by experiments with *cycA* mycelium samples spiked with horse heart cytochrome *c*.

The amount of cytochrome *c* oxidase (heme aa_3) present in the visible region of the spectrum (at 608 nm) for the *cycA* strains was not measurable, while the level of COX in the $cycA^+$ strain was estimated as being approximately 0.16 nmoles/mg protein (Appendix 1.3).

The *cycA* strains contained elevated levels of *b*-type heme (at 560 nm) in comparison to the $cycA^+$ strain A67. The levels of *b*-type heme were estimated as being 1.01 and 0.89 nmoles/mg protein (*cycA* strains A58 and A68, respectively) and 0.34 nmoles/mg protein ($cycA^+$ strain A67) (Appendix 1.3).

3.3.2 DISCUSSION

It is notable that the band of cytochrome *c* (at 552 nm) in a study of *A. nidulans* by Rowlands & Turner (1974) is approximately 4-fold larger than the peak observed in this study for the $cycA^+$ strain. There are a number of possible explanations for the decreased amount of cytochrome *c* observed for $cycA^+$ strains A57 (results not shown) and A67, compared to the previous *A. nidulans* study. Although most parameters (eg. temperature used for growth and spectral analysis, isolation buffer) were the same, some conditions varied between this experiment and the study by Rowlands & Turner (1974). Firstly, growth conditions between the two studies were slightly different. The study by Rowlands & Turner (1974) used 20 L aspirators containing 16 L of liquid MM (1% glucose). By comparison, 1 L baffled flasks containing 100 ml of liquid MYG (1% glucose), with additional aeration (section 2.17.1), were used in this experiment.

The strains used in the two studies were wildtype with respect to the *cycA* locus, yet also contained other differing genetic markers. The $cycA^+$ strain R21 (Rowlands & Turner, 1974) was a *p*-aminobenzoic acid auxotroph with yellow conidia (ie. *pabaA1*; *yA2*). The oligomycin-resistant strains derived from this strain contained the markers *pabaA1*; *yA2*; *oliA1*. The $cycA^+$ strains utilised in this experiment had the markers *pabaA1* and *pyroA4*. The mutant strains derived from these strains contained these markers in addition to a deficiency of cytochrome *c* (*cycA*). It is possible that integration of the gene replacement

vector pR54 (Appendix 1.1) could have altered other genetic material in the *cycA*$^+$ strains A57 and A67. Therefore, it is possible that the reduced levels of cytochrome *c* observed for the *cycA*$^+$ strains in this experiment may be due to changes in strain genotype, growth media or oxygen supply.

A reduction in cytochrome *c* oxidase (COX) levels in the *cyc*A$^-$ strains, compared to wild type strains, has also been reported in *N. crassa* mutants with low cytochrome *c* (Bottorff *et al.*, 1994) and in cytochrome *c*-deficient mutants of the yeasts *K. lactis* (Chen & Clark-Walker, 1993) and *P. stipitis* (Shi *et al.*, 1999). There is evidence in *N. crassa* that the levels of cytochromes *c*, *b* and aa_3 are responsive to each other (Bottorff *et al.*, 1994). Studies on *S. cerevisiae* have indicated the presence of a regulatory mechanism which balances the respiratory pathway stoichoimetry (Boumans *et al.*, 1998). Thus, a diminished level of one component of the ETC would lead to lowered levels of the other constituents, especially for respiratory complexes which interact (eg. the bc_1 complex and COX) (Brown *et al.*, 1993; Boumans *et al.*, 1998). This phenomenon has been suggested to occur via a regulatory mechanism which monitors mitochondrial activity and controls nuclear gene expression accordingly (Forsburg & Guarente, 1989; Bottorff *et al.*, 1994). Reduced levels of ETC components would lead to the non-functioning of the cytochrome *c*-dependent pathway, and the employment of other ATP-producing processes, such as fermentation (Boumans *et al.*, 1998).

Therefore, it may be expected that the *A. nidulans* cytochrome *c* deficient strains would have lowered levels of cytochrome *b*. However, the strains had elevated levels of *b*-type heme, compared to the *cycA*$^+$ strain. The cytochrome *c* deficient strains of *N. crassa* did not have significantly different levels of *b*-type heme, compared to the *cycA*$^+$ strains. A recent study on the pathogenic filamentous fungus *Gaeumannomyces graminis* (var. *tritici*) reported high levels of *b*-type heme (Joseph-Horne *et al.*, 1998). This heme was found to be antimycin A and myxothiazol insensitive, and not associated with the bc_1 complex. Therefore, similar to *G. graminis tritici* (Joseph-Horne *et al.*, 1998), it is postulated that elevated levels of *b*-type heme in the *cyc*A$^-$ strains of *A. nidulans* may be associated with a putative third terminal oxidase (in addition to COX and the AOX) (section 3.5.3).

3.4 FERMENTATION IN *A. NIDULANS*

As mentioned previously (section 1.1.4), alcoholic fermentation has long been speculated to occur in *A. nidulans* due to the presence of many enzymes involved in this process. Alcohol dehydrogenases, which catalyse the conversion of acetylaldehyde to ethanol (Figure 1.3), have been found in wildtype strains of *A. nidulans* (Kelly *et al.*, 1990; Martinelli *et al.*, 1994). Pyruvate decarboxylase, which converts pyruvate to acetylaldehyde and carbon dioxide (Figure 1.3), is also present in *A. nidulans* (Lockington *et al.*, 1997). The observation that the *cycA*⁻ strain A68 grows preferentially on fermentable carbon sources supports the possibility that fermentative metabolism is occurring in the fungus.

3.4.1 RESULTS

An ethanol production assay was carried out (section 2.16) for strains A67 and A68 (Table 3.2). The sampling timepoints during late-exponential phase and stationary phase were 17 and 24 hours (respectively) for the *cyc*A⁺ strain, in comparison to 40 and 48 hours (respectively) for the *cycA*⁻ strain A68 (section 3.2.4) (Figure 3.2).

Table 3.2 Ethanol Production in Strains A67 (*cycA*⁺) and A68 (*cycA*⁻)

Strain	Ethanol Production			
	(mg ethanol/L)		(mg ethanol/mg dry weight of mycelium)	
	Late-exponential phase	Stationary phase	Late-exponential phase	Stationary phase
A67 (*cyc*A⁺)	≤ 5.0*	≤ 5.0	≤ 0.912	≤ 0.912
A68 (*cyc*A⁻)	307 ± 69.4	224 ± 31.8	69.8 ± 15.8	50.5 ± 7.23

* Note: The values represent the mean ± standard error (n=3).

Strain A67 (*cyc*A⁺) produced negligible amounts of ethanol for both late-exponential and stationary phase samples (Table 3.2). However, the cytochrome *c* mutant (A68) produced

considerably more ethanol than the *cycA*$^+$ strain, from an equivalent amount of mycelium (Table 3.2).

3.4.2 DISCUSSION

Fermentative metabolism produces less ATP than aerobic respiration (section 1.2.3) Figures 1.1 and 1.3), thus explaining the slower growth rate of the *cycA*$^-$ strain. The *cyc*A$^+$ strain produces neglible amounts of ethanol at both timepoints measured, probably because aerobic respiration is preferentially carried out, due to its efficient ATP production (section 1.2.2). The amount of ethanol production reported here for the *A. nidulans cycA*$^-$ strain is comparable to that reported for an *aldA67* (aldehyde dehydrogenase) mutant of *A. nidulans*, which produced 320 mg/L (Lockington *et al.*, 1997). This mutation in the *aldA* gene prevents the consumption of ethanol, therefore the ethanol assay generated a true reflection of the amount of ethanol produced (Lockington *et al.*, 1997). It is therefore possible that ethanol production in strain A67 (*cyc*A$^+$) is higher than indicated by this assay. An increase in ethanol production compared to the wildtype was also observed for a cytochrome *c* mutant of the yeast *Pichia stipitis* (Shi *et al.*, 1999), although this was only a 21% increase. The observed increase in ethanol production by the *cycA*$^-$ mutant, compared to the wild-type, is contrary to studies with the *N. crassa*, *mi-1* (*poky*) mutant, which has low levels of cytochromes *b* and aa_3 but produced ethanol at the same level as wild-type strains (Colvin *et al.*, 1973). However, the *mi-1* mutant also had a residual cytochrome *c*-dependent electron transfer chain, which appears to be absent in the *cycA* mutant. This could account for differences in the need for alcoholic fermentation.

3.5 OXYGEN CONSUMPTION ASSAYS AND INHIBITOR STUDIES

3.5.1 ACTIVITY OF THE ALTERNATIVE RESPIRATORY PATHWAY

Oxygen consumption assays (section 2.15.3) were carried out to determine the presence of an alternative respiratory pathway in the *cyc*A$^-$ strains A58 and A68, and the respective wildtype strains. Specific inhibitors (Figure 1.4) to the oxidases within the alternative and

cytochrome *c*-dependent respiratory pathways (SHAM and KCN, respectively) were used. antimycinA, an inhibitor of the bc_1 complex, was also utilised.

For many fungi, the activity of the alternative pathway appears to change over time. For example, in *A. niger* the SHAM-sensitivity of the culture (and therefore activity of the AOX) was observed at all timepoints studied, and increased as the age of the culture increased, especially in the late-log phase (Kirimura *et al.*, 1987). Simultaneously, the KCN-sensitivity of the culture (and thus activity of COX) was observed to decrease (Kirimura *et al.*, 1987). Thus it was suggested that stress factors resulting from environmental changes which occur during the stationary growth phase may induce the alternative respiratory pathway's increased activity.

However, this particular pattern of activity for the two pathways over time is not observed in all organisms. While the activity of the alternative respiratory pathway is also constitutive over time in *Candida albicans* and *Histoplasma capsulatum*, the extent to which it is utilised remains at a constant level. Activity of the classical respiratory pathway in these organisms is known to decrease as the age of the culture reaches stationary phase, however this occurs with a simultaneous increased rate of fermentation, so that ATP production is unaffected.

The activity of the alternative respiratory pathway has also been found to be constitutive in all plants investigated to date (Vanlerberghe & McIntosh, 1997). However, recent studies have indicated that activity of the pathway in plants is more complex than previously anticipated. For example, soybean (Glycine max) has 3 *AOX* genes (*GmAOX1*, *GmAOX2* and *GmAOX3*), the expression of which is regulated in a developmental and tissue-specific manner (section 1.4.3) (Finnegan *et al.*, 1997; McCabe *et al.*, 1998). Developmental expression of multiple *AOX* genes has also been observed during the ripening of mangoes and tomatoes (McCabe *et al.*, 1998).

In other organisms, such as *N. crassa*, the activity of the alternative respiratory pathway is induced only by inhibitors or mutations affecting mitochondrial protein synthesis (Li *et al.*, 1996). However, it was expected that the activity of the alternative respiratory pathway in *A. nidulans* may be similar to that of other fungi, especially *A. niger*. Thus two timepoints were investigated for *A. nidulans*; located within late-exponential and stationary phases

(section 3.2.4) (Figure 3.2), to investigate whether a difference in the activity of the alternative respiratory pathway or the cytochrome *c*-dependent pathway could be determined between these two timepoints.

3.5.2 RESULTS

The change in respiration rate (RR) was estimated following the addition of specific inhibitors (section 2.15.3). The results are expressed as a % of original oxygen consumption (ie 'relative activity') or as a % inhibition of that original respiration (1 - relative activity). The concentration of inhibitor was increased until a maximal amount of inhibition (MAI) was observed (ie. the inhibitor concentration at which no further inhibition of RR is possible).

Samples from late-exponential and stationary growth phases were analysed for strains A67 (*cyc*A$^+$) and A68 (*cyc*A$^-$). Late-exponential samples from strains A57 (*cyc*A$^+$) and A58 (*cyc*A$^-$) were also analysed. The results are presented in Table 3.3, with raw data in Appendices 1.4 (actual respiratory rates) & 1.5 (relative activities). To determine significant differences between the stationary and late-exponential phase results, ANOVA analysis (single factor, Microsoft Excel program) was carried out. The null hypothesis (Ho) was that the samples are equal (ie drawn from populations with the same mean). Ho was rejected if the calculated F value was outside the F critical value (4.60).

STRAIN A67 (*cycA*$^+$)

Respiration of late-exponential samples of strain A67 (*cycA*$^+$) was found to be sensitive to both SHAM and KCN (Figure 3.4 A - D). The addition of 0.25 mM SHAM resulted in a MAI of approximately 11.0% (Figure 3.4 C, Table 3.3 A). A MAI of approximately 47.4% (Figure 3.4 A, Table 3.3 A) was also observed upon the addition of 0.25 mM KCN. The addition of both inhibitors resulted in a MAI of approximately 60%, and thus a residual activity of approximately 40% (Figure 3.4 A & C , Table 3.3 A). A residual oxygen consumption of about 40% was also observed upon the addition of 10 μM antimycin A + 0.5 mM SHAM (Table 3.3 A, Figure 3.5 A).

Table 3.3 Respiratory Measurements Of *A. nidulans*

The % of respiration inhibited upon the addition of a specific inhibitor, and the respiratory rate (RR) before and after addition are shown for *cycA*$^+$ and *cycA*$^-$ strains of *A. nidulans*. Oxygen consumption (RR) is expressed as nmol O_2 consumed/min/g wet weight.
Samples were from **A** Late-exponential phase and **B** Stationary phase.

The concentrations of KCN (potassium cyanide) or SHAM (salicyl hydroxamic acid) added were 0.25mM for the *cycA*$^+$ strains, 2mM for the *cycA*$^-$ strains. Antimycin A (10μM) was added with 0.5mM or 1mM SHAM for the *cycA*$^+$ and *cycA*$^-$ strains, respectively.

Values are mean (n=3) ± standard error. Control RR (without addition of inhibitors, but subsequent to succinate addition) were between 107-475 and 145-706 for the *cycA*$^+$ and *cycA*$^-$ strains, respectively. Raw data for the tables are in Appendices 1.4 & 1.5 for the late-exponential and stationary growth phases (respectively).

A Late-exponential Phase Samples

Strain	Inhibitor	Respiratory Rate		% Inhibition
		Before addition	After addition	
A57	KCN	305	146	52.5 ± 4.4
	SHAM	289	210	25.5 ± 7.7
	SHAM + KCN	305	101	71.8 ± 6.6
	KCN + SHAM	289	81	71.8 ± 3.8
	Antimycin A + SHAM	292	92	68.0 ± 7.8
A58	KCN	338	338	0
	SHAM	338	24	93.8 ± 3.2
	Antimycin A + SHAM	318	59	82.5 ± 5.2
A67	KCN	138	71	47.4 ± 0.07
	SHAM	198	175	11.0 ± 0.01
	SHAM + KCN	164	63	62.8 ± 2.44
	KCN + SHAM	234	88	62.8 ± 0.01
	Antimycin A + SHAM	376	145	58.7 ± 19.37
A68	KCN	336	336	0
	SHAM	373	39	89.8 ± 0.02
	Antimycin A + SHAM	304	37	87.7 ± 0.38

B Stationary Phase Samples

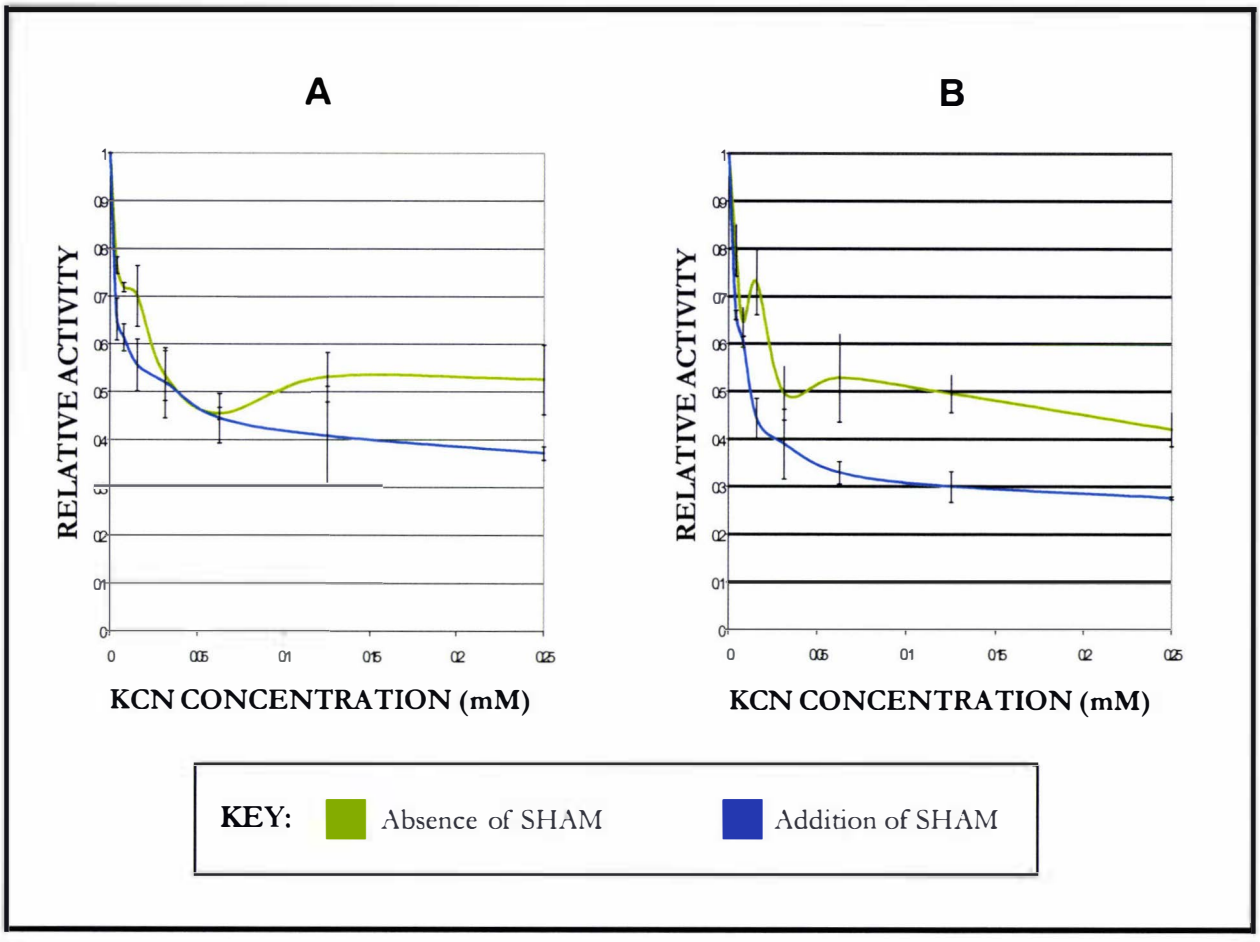
Strain	Inhibitor	Respiratory Rate		% Inhibition
		Before addition	After addition	
A67	KCN	127	51	58.0 ± 0.04
	SHAM	206	177	12.9 ± 0.01
	SHAM + KCN	235	61	72.2 ± 0.02
	KCN + SHAM	257	72	72.4 ± 0.003
	Antimycin A + SHAM	177	46	74.3 ± 4.3
A68	KCN	293	293	0
	SHAM	306	33	88.8 ± 0.01
	Antimycin A + SHAM	247	36	85.1 ± 0.5

Figure 3.4 **Relative Activity of strain A67 (*cycA*$^+$) following the addition of inhibitors, mid-exponential and stationary phase samples**

The relative activity of the samples upon the addition of specific inhibitors are shown for strain A67 (*cycA*$^+$). KCN (potassium cyanide) was added in the presence or absence of SHAM (salicyl hydroxamic acid) for late-exponential phase (**A**) and stationary phase (**B**) samples. SHAM was added in the presence or absence of KCN for late-exponential phase (**C**) and stationary phase (**D**) samples.

To obtain the mycelial samples used for the spectra, one litre baffled flasks containing 100 mL of liquid MYG (1% glucose) were inoculated with 10^6 spores/mL and incubated at 37° C with shaking (250 rpm) overnight. The cultures were harvested and homogenised to 0.125 g/mL in isolation buffer (0.5 M mannitol, 1 mM EDTA and 10 mM tris-HCl; pH 7.0).

The rate at which oxygen was consumed (i.e. the respiration rate; RR) was measured over time, before and after the addition of an inhibitor. The results were plotted as a % of original oxygen consumption (i.e. 'relative activity') (RR after addition of inhibitor/RR before addition of inhibitor). For the graphs indicating "Addition of...(KCN or SHAM)", 0.5 mM of the appropriate inhibitor was added. The oxygen consumption assays were carried out in triplicate, with the error bars on the graphs representing the standard error of the samples. Raw data for the assays can be found in Appendices 1.4 & 1.5.


A
B
RELATIVE ACTIVITY
KCN CONCENTRATION (mM)
KEY:
Absence of SHAM
Addition of SHAM

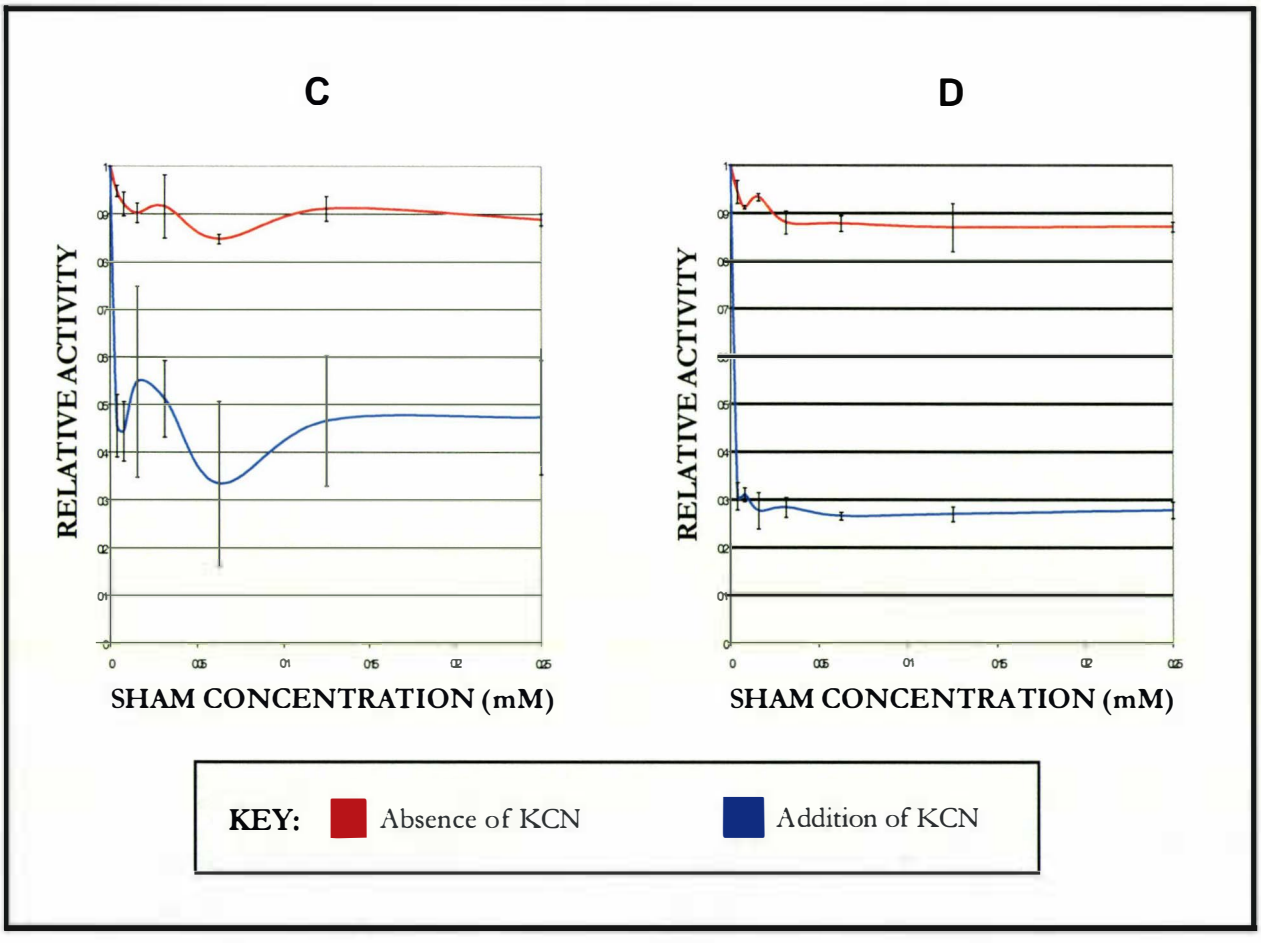
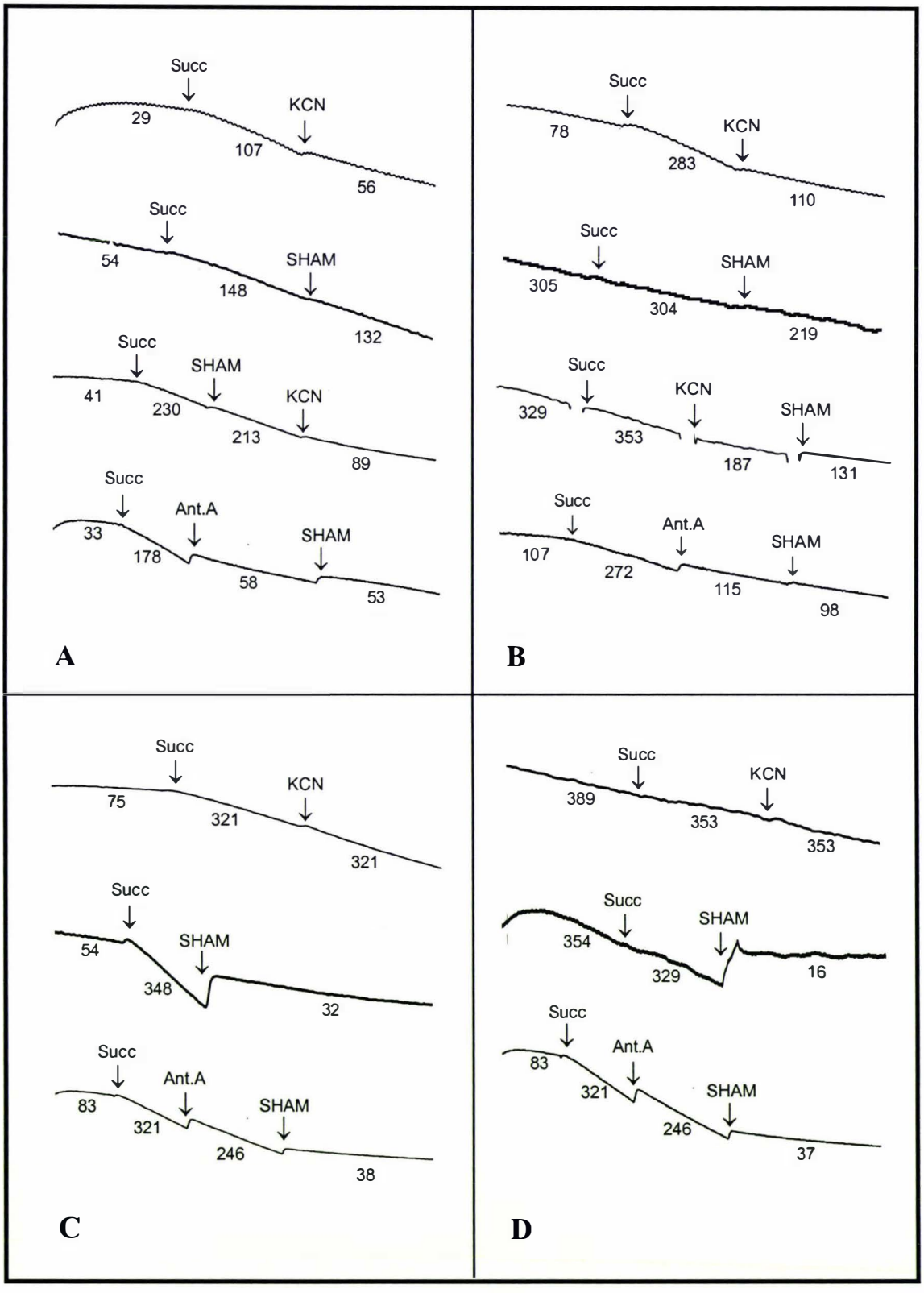

C
D
RELATIVE ACTIVITY
SHAM CONCENTRATION (mM)
KEY:
Absence of KCN
Addition of KCN

Figure 3.5 **Representative Oxygen Consumption Assay Traces of the *A. nidulans* strains**

Oxygen Consumption Assay Traces of the *cyc*A$^+$ (strains A67, **A**; and A57, **B**) and *cyc*A$^-$ (strains A68, **C**; and A58, **D**) are shown.

To obtain the mycelial samples used for the spectra, one litre baffled flasks containing 100 mL of liquid MYG (1% glucose) were inoculated with 10^6 spores/mL for *cyc*A$^+$ strain A67, and 1 g of homogenised mycelia for *cyc*A$^-$ strain A68, and incubated at 37°C with shaking (250 rpm) overnight. Additional aeration was also supplied to the cultures. The cultures were grown to late-exponential timepoints, harvested and homogenised to 0.125 g/mL in isolation buffer (0.5 M mannitol, 1 mM EDTA and 10 mM tris-HCl; pH 7.0).

The rate at which oxygen was consumed (i.e. the respiration rate; RR) is indicated by the numbers underneath the traces. The gradient of the trace (oxygen consumption) was measured over time, thus RR is expressed as nmol O_2 consumed/min/g wet weight. The arrows indicate the addition of succinate (Succ) (10μM) or specific inhibitors. The concentrations of KCN (potassium cyanide) or SHAM (salicyl hydroxamic acid) added were 0.25mM for the *cycA*$^+$ strains and 2mM for the *cycA*$^-$ strains. Antimycin A (10μM) was added with 0.5mM and 1mM SHAM for the *cycA*$^+$ and *cycA*$^-$ strains, respectively. The oxygen consumption assays were carried out in triplicate, and traces representative of these assays are shown in this figure. Raw data for the assays can be found in Appendix 1.4.



Oxygen Consumed (nmol)

Time (minutes)

* The concentration of inhibitor used was the same for both samples unless otherwise stated.

Stationary phase samples from strain A67 had a higher maximal amount of inhibition (MAI)* than late-exponential samples upon the addition of KCN (58% ± 0.04 and 47.4% ± 0.07, respectively) (Figure 3.4 B, Table 3.3 B). The addition of SHAM to the stationary phase samples inhibited the respiratory rate by approximately 13% (Figure 3.4 D, Table 3.3 B). No significant difference was found between the late-exponential and stationary phase samples, upon the addition of SHAM or KCN (F values of 0.07 and 0.03, respectively).

The addition of one inhibitor (KCN or SHAM) in the presence of the other resulted in a residual oxygen consumption of approximately 28% for the stationary phase samples (Figure 3.4 B & D). This residual activity is approximately 10 - 20% lower than observed for the late-exponential phase samples (Table 3.3 B), however the difference was not found to be significant (F values of 0.40 and 1.85 for the addition of KCN + SHAM, and SHAM + KCN, respectively). No significant difference was observed between the late-exponential and stationary phase samples upon the addition of antimycin A + SHAM (Table 3.3 B) (F value of 0.56).

STRAIN A68 (*cycA*$^-$)

Late-exponential and stationary samples of strain A68 (*cycA*$^-$) were insensitive to KCN, as expected (Figure 3.5 C, Table 3.3). For late-exponential samples, a MAI of approximately 89.8% resulted from the addition of 2 mM SHAM (Figure 3.6, Table 3.3 A). A residual oxygen consumption (approximately 10.2%) was also observed following the addition of 10 μM antimycin A + 1 mM SHAM (Figure 3.5 C, Table 3.3 A) to these samples.

The amount of MAI* after the addition of SHAM was not significantly different for late-exponential and stationary phase samples (Figure 3.6, Table 3.3 B) (F value of 0.05). However, the SHAM concentrations required to obtain a MAI for the late-exponential and stationary phase samples were 2 mM and 3 mM, respectively. There was also no significant difference in oxygen consumption between these samples following the addition of antimycin A and antimycin A + SHAM (Table 3.3 B) (F value of 1.2).

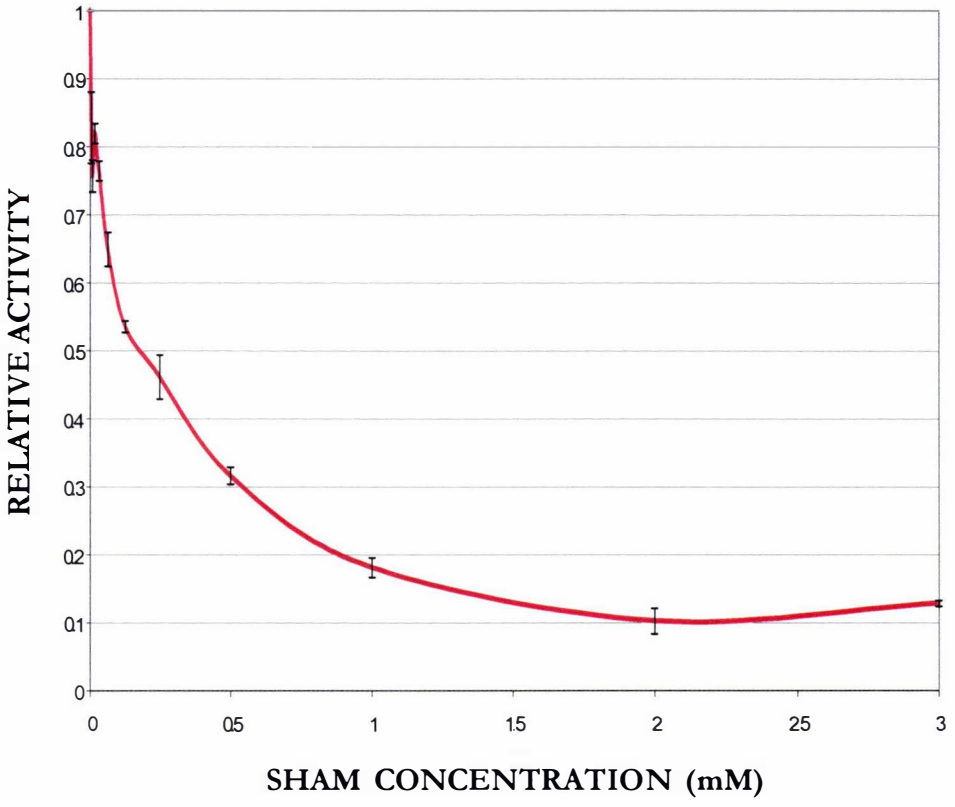
Figure 3.6 **Relative Activity of strain A68 (*cycA*$^-$) following the addition of SHAM, mid-exponential and stationary phase samples**

The relative activity of the samples upon the addition of SHAM are shown for strain A68 (*cycA*). Late-exponential phase (**A**) and stationary phase (**B**) samples.

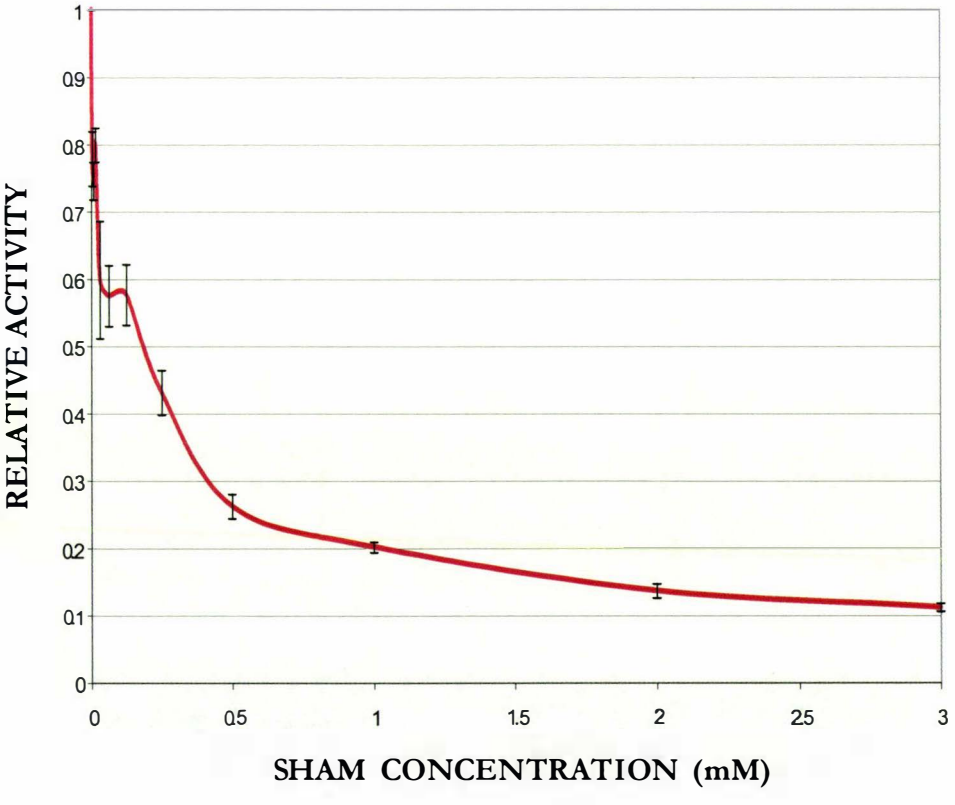
To obtain the mycelial samples used for the spectra, one litre baffled flasks containing 100 mL of liquid MYG (1% glucose) were inoculated with 1 g of homogenised mycelia, and incubated at 37°C with shaking (250 rpm) overnight. The cultures were harvested and homogenised to 0.125 g/mL in isolation buffer (0.5 M mannitol, 1 mM EDTA and 10 mM tris-HCl; pH 7.0).

The rate at which oxygen was consumed (i.e. the respiration rate; RR) was measured over time, before and after the addition of an inhibitor. The results were plotted as a % of original oxygen consumption (i.e. 'relative activity') (RR after addition of inhibitor/RR before addition of inhibitor). The oxygen consumption assays were carried out in triplicate, with the error bars on the graphs representing the standard error of the samples. Raw data for the assays can be found in Appendices 1.4 & 1.5.

A


RELATIVE ACTIVITY
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.5
1
1.5
2
2.5
3
SHAM CONCENTRATION (mM)

B


RELATIVE ACTIVITY
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
0.5
1
1.5
2
2.5
3
SHAM CONCENTRATION (mM)

STRAINS A57 (*cycA*$^+$) AND A58 (*cycA*$^-$)

The results observed for the strains A67 and A68 were confirmed with strains A57 (*cycA*$^+$) and A58 (*cycA*$^-$), with samples obtained from a late-exponential growth phase. As expected, strain A58 also exhibited respiration which was sensitive to SHAM, yet insensitive to KCN (Figure 3.5 D, Table 3.3 A). Residual activities of approximately 5 – 10%, and 12 – 23%, were observed for strain A58 following the addition of SHAM, and SHAM + antimycin A (respectively) (Table 3.3 A, Figure 3.5 D). These results are similar to those observed for strain A68 (Table 3.3 A). Strain A58 also exhibited a residual oxygen consumption of approximately 12 – 17% upon the addition of SHAM + antimycin A, similar to strain A68 (Table 3.3 A).

Strain A57 was observed to have SHAM- and KCN-sensitive respiration (Figure 3.5 B). The % of respiration inhibited following the addition of 0.25 mM SHAM or KCN was observed to be 25.5% (± 7.7) and 52.5% (± 4.4), respectively (Table 3.3 A, Figure 3.5 B). The relative activity observed following the addition of both SHAM + KCN, or antimycin A + SHAM, was approximately 30% (Table 3.3 A, Figure 3.5 B). These results are similar to those observed for strain A67 (Table 3.3 A).

3.5.3 DISCUSSION

STRAINS A57 AND A67 (*cycA*$^+$)

The presence of respiration in the *cycA*$^+$ strains A57 and A67 which were sensitive to SHAM and KCN indicates the presence of the alternative and cytochrome *c*-dependent respiratory pathways, respectively. Respiration sensitive to antimycin A was also observed in these strains, indicating inhibition of the bc_1 complex (Figure 1.2). A residual oxygen consumption observed for both strains following the addition of SHAM + KCN, or SHAM + antimycin A, possibly indicates the presence of a third terminal oxidase (section 3.5.2).

These results were observed for late-exponential and stationary phase samples of strain A67 (section 3.5.2). ANOVA analysis revealed no significant differences between samples

from the two timepoints, for levels of RR inhibition following the addition of any inhibitors.

STRAINS A58 AND A68 (*cycA*$^-$)

As expected, the *cycA*$^-$ strains A58 and A68 did not exhibit KCN-sensitive respiration, due to a non-functional cytochrome *c*-dependent pathway. However, respiration in both strains was sensitive to SHAM, indicating the presence of the alternative respiratory pathway. Oxygen consumption observed following the addition of SHAM, or SHAM + antimycin A, suggests the presence of a third terminal oxidase, as for the *cycA*$^+$ strains (section 3.5.2). The presence of antimycin A-sensitive respiration in these strains may be due to (partial) inhibition of the putative third terminal oxidase, as elevated *b*-type heme levels observed in the *cycA*$^-$ strains were suggested to be associated with this oxidase (section 3.3.2). Results for late-exponential and stationary phase samples were not significantly different for strain A68, although a higher concentration of SHAM was required to obtain the MAI for the latter samples (section 3.5.2).

PUTATIVE THIRD TERMINAL OXIDASE

A third terminal oxidase was postulated to be present in the *cycA*$^+$ and *cycA*$^-$ strains of *A. nidulans*, due to residual oxygen consumption observed in the presence of inhibitors (section 3.5.3). An elevated level of *b*-type heme was observed for the *cycA*$^-$ strains during the spectral analysis (section 3.3). Thus it was postulated that the increased level of *b*-type heme in these strains could be correlated with an increased activity of the putative third terminal oxidase.

Residual oxygen consumption, suggesting the presence of a putative third terminal oxidase, has been observed in other organisms (Robinson *et al.*, 1995). Oxygen consumption in the presence of both KCN and SHAM has been reported for *A. niger* and soybean. In *A. niger*, the effect has been seen with isolated mitochondria, but not intact tissue (Kirimura *et al.*, 1987), while the reverse has been reported for soybean (Ribas-Carbo *et al.*, 1997). The residual oxygen consumption observed for *A. niger* after addition of KCN (2.5 mM) and SHAM (2 mM) was approximately 40% (Kirimura *et al.*, 1987), higher than reported for *A. nidulans* in this study (section 3.5.2). However, the results are not comparable, as the assay

material and the inhibitor concentrations differed between the studies. It is possible that the observation of residual oxygen consumption in *A. nidulans* may be due to incomplete inhibition of the two respiratory pathways (section 3.5.3), or an artefact from using intact tissue.

THE VALIDITY OF INHIBITOR STUDIES

Inhibitor studies were carried out to determine the presence of the alternative pathway in the *cyaA*$^-$ and *cyaA*$^+$ strains, and the absence of the cytochrome *c*-dependent pathway in the *cyaA*$^-$ strains. Early studies (Baher & Bonner, 1973) suggested that it was also possible to determine the 'capacity' of either respiratory pathway using this technique. The capacity of a pathway refers to the amount of oxygen consumed in the presence of the inhibitor for the other pathway (Baher & Bonner, 1973). For example, the capacity of the alternative pathway is measured as the amount of oxygen consumed in the presence of KCN. The amount of remaining respiration that is inhibited by cyanide is considered to be the capacity of the cytochrome *c*-dependent pathway (Kirimura *et al.*, 1987; McIntosh, 1994).

The use of this technique to measure the capacity and steady-state activity of the pathways requires the following assumptions: that the inhibitors are specific and reach the target site, and that inhibition of the electron flow to one pathway does not affect the flow to the other pathway (Robinson *et al.*, 1995). It was also initially thought that the alternative respiratory pathway merely existed as an 'overflow' mechanism for the cytochrome *c*-dependent pathway, and thus was kinetically unfavoured (section 1.4.1). The alternative pathway was suggested to become active only when the cytoplasm contained excess reducing equivalents and/or had a high adenylate charge (ie ATP:ADP ratio) (Day *et al.*, 1996).

However, the validity of using specific inhibitors to ascertain electron partitioning between the two pathways has since been questioned (Millar *et al.*, 1995; Ribas-Carbo *et al.*, 1995; Ribas-Carbo *et al.*, 1997). Firstly, the alternative pathway has been shown to be able to compete for electrons (Day *et al.*, 1996). Many factors have also been discovered which regulate AOX activity (section 1.4.4), thus modulating the electron flow through the pathway (Sluse & Jarmuszkiewicz, 1998). That the inhibition of one pathway invariably affects the redox status of the ubiquinone pool, and therefore modifies the electron flux

through the other pathway, was also overlooked (Millar *et al.*, 1995; Sluse & Jarmuszkiewicz, 1998). Many recent studies have suggested possible functions for the alternative pathway (section 1.4.2), although its role in controlling energy overflow has not been ruled out (Day *et al.*, 1996).

The results obtained from inhibitor studies were also often misleading. Samples which exhibited activity of the alternative respiratory pathway upon the inhibition of the cytochrome *c*-dependent pathway, sometimes displayed no inhibition of RR upon the addition of SHAM (Day *et al.*, 1996). These results apparently suggest the inactivity of the alternative pathway in the absence of an inhibitor affecting the cytochrome *c* dependent pathway. This could be true for organisms in which the activity of the alternative pathway has to be induced by certain conditions (section 1.4.1). However, the pathways could be actively competing for electrons, and inhibition by SHAM could result in the diversion of electron flow to the cytochrome *c*-dependent pathway, so that no effect on electron flow is observed. It has also been shown that saturation of the cytochrome *c*-dependent pathway does not occur, even at high levels of ubiquinone. Thus there will always be some diversion of electron flow to this pathway upon inhibition of the alternative pathway (Day *et al.*, 1996). Therefore, an underestimation of the alternative respiratory pathway's activity is a possibility even when SHAM-sensitive respiration is observed. It has also been reported that low concentrations of SHAM can stimulate the oxygen consumption rate of plant tissues and high SHAM concentrations can inhibit the cytochrome pathway (Robinson *et al.*, 1995). Thus, inhibitor studies are highly unsuitable for estimating the capacities of the cytochrome *c*-dependent and alternative respiratory pathways. It therefore follows that the use of inhibitors to estimate a maximal amount of inhibition is unreliable (Day *et al.*, 1996).

Quantitative assessment of the electron partitioning between the two pathways is only possible with a technique that measures the actual contribution of each oxidase, when both are active (Sluse & Jarmuszkiewicz, 1998). These determinations are hampered by the fact that both oxidative pathways have the same substrate and product.

TECHNIQUES FOR ASCERTAINING ELECTRON PARTITIONING

Several techniques have been developed to investigate the electron partitioning between the alternative respiratory pathway and the classical respiratory pathway. The most commonly used technique is oxygen isotope fractionation (OF) (Ribos-Carbo *et al.*, 1997; Gonzalez-Meler *et al.*, 1999; Ribos-Carbo *et al.*, 2000). This method is considered to be the only method that exists at present for making quantitative measurements of AOX activity, even *in vivo* (Jarmuszkiewicz *et al.*, 1998). Changes in the isotope composition of oxygen within a closed system are determined; ie. the buildup of O^{18} O^{16} relative to O^{16} O^{16} after respiration has occurred (Sluse & Jarmuszkiewicz, 1998). The observation that AOX and COX have different oxygen isotope discriminations, with AOX having a higher fractionation factor, led to the development of this mass spectrophotometric technique (Guy *et al.*, 1989; Robinson *et al.*, 1995; Ribos-Carbo *et al.*, 1995). Both oxidases contribute to the oxygen isotope fractionation results, which can be interpreted as the relative electron partitioning between the two pathways. OF can be used with intact tissue, isolated mitochondria (Ribos-Carbo *et al.*, 1997), and whole plants (Gonzalez-Meler *et al.*, 1999). However, the technique does have some disadvantages, including the use of inhibitors, which determine the 'discrimination factor' for each pathway, thus representing the limits within which the overall respiratory discrimination factor must fall. The representative discrimination factors for the two pathways are often similar, another disadvantage of this time-consuming method (Sluse & Jarmuszkiewicz, 1998). The method also assumes that a change in the total respiration rate does not change the discrimination factors (Jarmuszkiewicz *et al.*, 1998).

The ADP/PO method is also used to determine electron partitioning between the two respiratory pathways. The method, based on the non-phosphorylating property of alternative oxidase, was first proposed by Bahr & Bonner in 1973. However, it has not been widely used, despite its advantages; the method does not measure the rates of electron transport of both pathways in the presence of inhibitors, nor does it assume the homogeneity of the quinone pool (Sluse & Jarmuszkiewicz, 1998). The technique was developed recently, and successfully used to determine the contribution of both respiratory pathways in isolated amoeba mitochondria. Pair measurements of ADP/O ratios were taken in the presence or absence of an inhibitor of the alternative oxidase (Jarmuszkiewicz *et al.*, 1998). Measurement of the overall respiration was measured in the presence of GMP

(an activator of the AOX), with succinate as an oxidisable substrate (plus rotenone, an inhibitor of NADH dehydrogenase). Because the alternative pathway does not contribute to ATP synthesis with succinate (in the presence of rotenone) as a substrate, a comparison of the ADP/O ratio can be made (with and without an inhibitor of AOX) which allows the estimation of the contribution of this pathway to the total respiration (Jarmuszkiewicz *et al.*, 1998). However, this method has a limited range of application; it cannot be applied under certain 'states' of respiration, nor can it be used with intact tissues. A number of requirements must also be met to assure the validity of the method (Jarmuszkiewicz *et al.*, 1998).

A kinetic approach has also been developed to predict the contribution of each respiratory pathway at a given respiration rate (Van den Bergen *et al.*, 1994). This technique takes into account the interaction between quinol-oxidizing and quinone-reducing steady-state electron fluxes, and the redox poise of the quinone pool; the homogenous quinone pool is considered to be the common link between the oxidising and reducing components of the system (Sluse & Jarmuszkiewicz, 1998). However, the presumption of a homogeneous quinone pool is one of the disadvantages of this method. Another disadvantage of the kinetic approach is the use of specific inhibitors to the oxidases, and NADH dehydrogenase; the activity of one pathway is measured, and the redox staus of the ubiquinone pool determined, while the other pathway is inhibited (Sluse & Jarmuszkiewicz, 1998). For a more detailed description of this complex technique, see Van den Bergen *et al* (1994).

While all of these techniques have their disadvantages, they provide a more accurate estimation of electron partitioning between respiratory pathways than the traditional inhibitor studies.

3.5.4 CONCLUSION

The capacities of the respiratory pathways in the *cyc*A$^+$ and *cyc*A$^-$ strains of *A. nidulans* can not be deduced from this study. The alternative respiratory pathway was shown to be present in all strains prior to the addition of SHAM. In the *cyc*A$^+$ strains, the cytochrome *c*-dependent pathway was present prior to the addition of KCN. Residual oxygen consumption observed upon the addition of inhibitors suggests the presence of a putative

third terminal oxidase in all strains. As discussed in section 3.5.3, it is possible that the inhibitor studies give an underestimation of the activities of the two respiratory pathways. However, the possibility of a third terminal oxidase cannot be excluded.

Differing amounts of inhibition observed upon the addition of a particular inhibitor(s), either between strains or between different growth phase samples from the same strain, cannot be presumed to be significant, for reasons discussed in section 3.5.3. Therefore, no reliable conclusions can be drawn regarding the differences observed between late-exponential and stationary samples of strains A67 (*cycA*$^+$) and A68 (*cycA*$^-$). The study on *A. niger*, which reported an increase in the activity of the alternative respiratory pathway over time in a wildtype (*cycA*$^+$) strain, was also carried out with inhibitor studies (Kirimura *et al.*, 1987). Determination of the contribution of each respiratory pathway to the total respiration in *A. nidulans* would need to be carried out with one of the techniques discussed in section 3.5.3.

3.6 CARBON MONOXIDE BINDING ASSAY

A carbon monoxide (CO) binding assay was carried out to ascertain the presence of a heme type which may possibly be associated with a third terminal oxidase. CO binding was followed by dissociation from the bound molecules, caused by flash photolysis. The subsequent recombination of molecules with CO generates spectral patterns that are often characteristic of particular compounds. Spectrum contain three bands in the visible region (δ,α and β bands); the wavelength of the β band indicates the identity of a particular compound (Voet & Voet, 1990). For example, the recombination of CO to COX generates an identifiable spectral pattern, with a β band at a characteristic wavelength of approximately 650 nm (Figure 3.7 A; *i* & *iii*). The CO binding assay was carried out as described in section 2.17.

3.6.1 RESULTS

The spectral pattern characteristic of COX was observed for the *cycA*$^+$ strains following CO recombination, as expected (Figure 3.7 A; *i* & *iii*). However, these peaks were absent from the spectra obtained from the *cycA*$^-$ strains (Figure 3.7 A; *ii* & *iv*). This result concurs with the reduced-oxidised redox spectra obtained from the *cycA*$^-$ strains, in which it was

Figure 3.7 **Carbon Monoxide Binding Spectra of *A. nidulans* and *S. cerevisiae* strains**

A The CO difference spectrum (reduced + CO) – (reduced), of *cyc*A$^+$ strains A67 (***i***) & A57 (***iii***), and *cyc*A$^-$ strains A68 (***ii***) and A58 (***iv***) are shown.

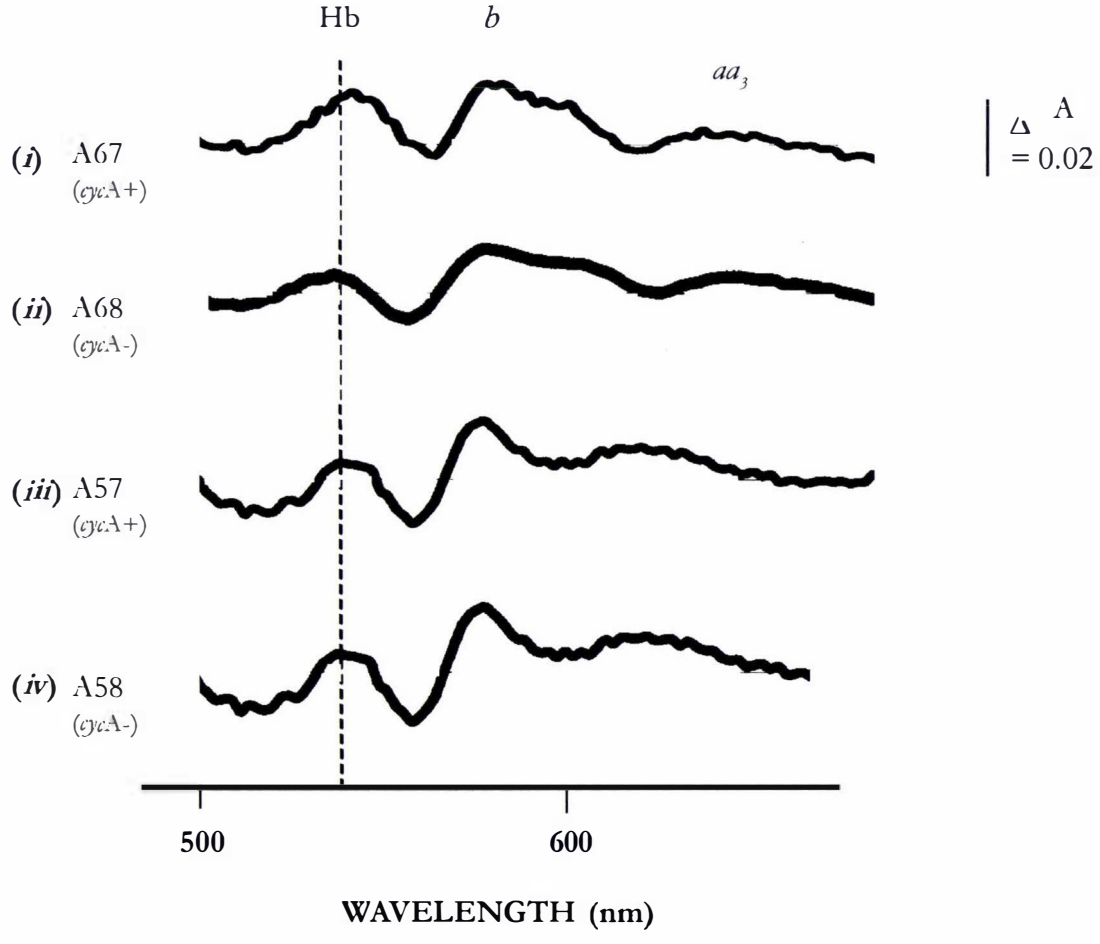
To obtain the mycelial samples used for the spectra, one litre baffled flasks containing 100 mL of liquid MYG (1% glucose, supplemented as required) were inoculated with 10^6 spores/mL for the *cyc*A$^+$ strains, and 1 g of homogenised mycelia for the *cyc*A$^-$ strains, and incubated at 37°C with shaking (250 rpm) overnight. Additional aeration was also supplied to the cultures. The cultures were grown to late-exponential timepoints, harvested and homogenised to 0.125 g/mL in isolation buffer (0.5 M mannitol, 1 mM EDTA and 10 mM tris-HCl; pH 7.0).

Spectra were obtained at ambient temperature in a lab-built single beam spectrophotometer with a slit width of 1 nm. The cuvette (path length 10 mm) contained 3.0 mL of suspension. The samples were assayed for spectra before and after the addition of carbon monoxide. The reduced spectra were obtained by addition of sodium dithionite (BDH).

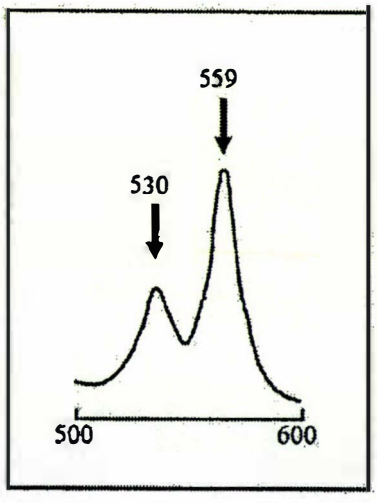
Peaks characteristic of hemoglobin are present in traces *i* – *iv* at approximately 540 and 570 nm. The presence of *b* and aa_3 type heme are indicated by peaks at approximately 570 and 650 nm (respectively).

B The CO difference spectrum (reduced + CO – reduced) of the purified Ynl234wp (hemoglobin) protein from *S. cerevisiae*. The characteristic peaks of hemoglobin can be observed at 530 and 559 nm.

A


Hb
b
aa_3
(i) A67
(cycA+)
(ii) A68
(cycA-)
(iii) A57
(cycA+)
(iv) A58
(cycA-)
Δ A
= 0.02
500
600
WAVELENGTH (nm)

B


559
530
500
600
WAVELENGTH (nm)

observed that the levels of heme aa_3 (associated with COX) were exceedingly low (section 3.3).

The elevated levels of *b*-type heme in the *cyoA* strains which were seen in the redox spectra can also be observed in Figure 3.7 (A; *ii* & *iv*). The presence of *b*-type heme was determined because of the observed β bands at the expected wavelength of approximately 570 nm. As mentioned previously (section 3.3.2), it has been postulated that the increased level of *b*-type heme may be associated with a putative third terminal oxidase in these strains. CO flash photolysis was also used in the study by Joseph-Horne *et al* (1998), to determine elevated levels of *b*-type heme in the fungus *G. graminis tritici*, which was also suggested to be associated with a third terminal oxidase.

All *cyoA*$^+$ and *cyoA1* strains of *A. nidulans* also generated a spectral pattern which was highly analogous to that observed for hemoglobin in several species, including yeast and the bacteria *Vitreoscilla* (Dikshit *et al.*, 1992) (Figure 3.7). Hemoglobin produces a characteristic spectra at approximately 530 and 559 nm in yeast (Figure 3.7 B) (Satori *et al.*, 1999). These spectra are analogous to the peaks observed for the *cyoA*$^+$ and *cyoA* strains of *A. nidulans* at approximately 540 and 570 nm (Figure 3.7 A).

3.6.2 DISCUSSION

The putative presence of hemoglobin in *A. nidulans*, as suggested by the spectral determination of hemoglobin-like molecules, is an extremely interesting finding. To our knowledge, this is the first time this observation has been reported for *A. nidulans*.

THE ROLE OF HEMOGLOBIN IN OTHER ORGANISMS

Hemoglobins have been discovered in a whole range of organisms, including mammals, plants, protozoa, fungi and bacteria. Their role as facilitators in the diffusion of oxygen/carbon dioxide is well known, especially in mammals (Crawford *et al.*, 1995). However, recent studies have also revealed the involvement of hemoglobin in nitric oxide (NO)-related functions (Liu *et al.*, 2000). NO is a free-radical produced by the oxidation of reduced nitrogen compounds and the reduction of nitrogen oxides, and has a number of divergent functions. At low cellular levels, NO has been found to function as a signalling

molecule in processes controlling blood pressure and memory. NO has also been reported to be produced in response to cellular invasion by foreign species, as a means of containing infection. However, excess levels of NO and NO-derived reactive nitrogen species are extremely toxic to cells. Mechanisms involved in cellular protection against these compounds have been suggested to be similar to those systems which have evolved to guard against damage by ROS (section 1.4.2) (Gardner *et al.*, 1998). Recent studies have reported bacterial and yeast flavohemoglobins (hemoglobin with binding sites for both heme and flavin) to have roles in protection against nitrosative stress (Crawford *et al.*, 1998; Liu *et al.*, 2000).

Flavohemoglobins from *S. cerevisiae* and *E. coli* have been found to confer protection against NO (Nitric Oxide) and S-nitrosothiols. It has been suggested that the proteins contain two NADH-dependent activities: that of a NO dioxygenase, and a NO reductase, thus generating NO_3 and N_2O (respectively) (Liu *et al.*, 2000). The hemoglobin (VtHb) from the bacteria *Vitreoscilla* is a single domain protein, whilst the (flavo)hemoglobins from other organisms, including *S. cerevisiae* and *E. coli*, have been found to be two-domain proteins. One domain shares sequence similarity with VtHb, while the second domain functions as a reductase and contains binding sites for flavin (FAD) and NADPH. VtHb is thought to interact with a separate reductase, thus forming a two-polypeptide analog of the flavohemoglobins (Crawford *et al.*, 1995).

EXPRESSION OF HEMOGLOBIN

Expression of VtHb gene in *Vitreoscilla* is regulated by oxygen; transcriptional activation of the gene is observed under hypoxic environmental conditions (Dikshit *et al.*, 1992). Expression of the *E. coli hmp* gene is upregulated by NO itself, or compounds that promote NO formation (Membrillo-Hernandez *et al.*, 1999). By contrast, expression and activity of flavohemoglobin (YHB1) in *S. cerevisiae* appears to be constitutive. However, expression of the *YHB1* gene is lower in anaerobic conditions (compared with aerobic conditions in bacteria), and in stationary phase cells (compared with logarithmic phase in bacteria) (Liu *et al.*, 2000). *YHB1* gene expression has also been suggested to be regulated by HAP1 and HAP 2/3/4/5 - yet peculiarly, activation by the latter is not observed during growth on non-fermentable carbon sources (Crawford *et al.*, 1995). Interestingly, the gene encoding a second hemoprotein in *S. cerevisiae* (Sartori *et al.*, 1999) is expressed under conditions of

oxygen depletion, as well as other stress conditions such as glucose repression, nitrogen starvation and heat shock.

POSSIBLE ROLES OF HEMOGLOBIN IN *A. NIDULANS*

Although there are differences in gene expression and structure, the basic function of the bacterial and yeast flavohemoglobins are similar; protection against nitrosative stress. A phylogenetic study carried out by Liu *et al.* (2000) showed that the interaction of hemoglobin with NO is an evolutionary conserved function (more so than O_2/CO_2 transport and storage), although the specifics of this interaction are highly divergent amongst organisms (Gardner *et al.*, 1998).

Overexpression of VtHb has been shown to rescue terminal oxidase mutants in *E. coli*, therefore indicating that the globin is capable of transporting molecular oxygen, and can also facilitate its reduction *in vivo* (Dikshit *et al.*, 1992). Spectral analysis indicated the putative presence of hemoglobin (Hb)–like molecules in both *cycA*+ and *cycA*- strains of *A. nidulans*. However, it would be interesting to determine whether an increased level of Hb gene expression is present in the *cycA*- strains, to compensate for the non-functioning cytochrome *c*-dependent pathway.

Expression and function of the putative *A. nidulans* hemoglobin is postulated to be most analogous to *S. cerevisiae*. The *A. nidulans* strains examined were obtained from a mid/late-exponential phase of growth; therefore it would be interesting to investigate the pattern of Hb gene expression over time.

3.7 CONCLUSION

The results of the growth studies are consistent with those expected if the strain A68 is deficient in cytochrome *c*. In comparison to the corresponding wildtype strain, a slower growth rate and decreased spore viability of the *cycA*⁻ strain was observed. Growth of the cytochrome *c*-deficient strain was increased on a fermentable carbon source, compared to growth on a non-fermentable carbon source. Fermentative metabolism in the *cycA*⁻ strain was determined by an ethanol production assay.

Spectral analysis revealed the lack of detectable levels of cytochrome *c* in the mutant strains (less than 0.22 nmoles/mg protein). A diminished level of cytochrome *c* oxidase was also observed in these strains.

Oxygen consumption assays revealed the presence of an alternative respiratory pathway in *cycA* and *cycA*$^+$ strains of *A. nidulans*, prior to the addition of SHAM. The cytochrome *c*-dependent pathway was suggested to be absent from the *cycA* strains (yet present in the *cycA*$^+$ strains), due to the lack of KCN-sensitive respiration in the former. Residual oxygen consumption observed following the addition of inhibitors could suggest the presence of a putative third terminal oxidase in the *cycA*$^+$ and *cycA* strains. Elevated levels of *b*-type heme observed in the redox spectra of *cycA* strains possibly suggest an association of the putative third terminal oxidase with this heme type.

CO binding assays revealed the presence of a hemoglobin-like molecule in the *cycA* and *cycA*$^+$ strains. It was suggested the putative hemoglobin molecule in the *cycA* strains could act as a terminal oxidase, to compensate for the non-functioning cytochrome *c*-dependent pathway. Another possible function for the putative hemoglobin molecule in the *cycA* and *cycA*$^+$ strains could be a role in NO detoxification, similar to that in yeast (section 3.6.2).

Thus it can be concluded that the *cycA*$^+$ strains of *A. nidulans* contain an alternative means of energy transduction, in addition to the classical respiratory pathway; the alternative respiratory pathway. The strains also possibly contain hemoglobin, which may function as a terminal oxidase. The possibility of alternative processes of energy production in this fungus was first speculated upon when putative regulatory elements were identified within the cytochrome *c* (*cycA*) gene promoter (Chapter 5). This observation raised the question as to why regulation of a seemingly essential gene would be necessary in an obligate aerobe. The subsequent creation and observed viability of cytochrome *c*-deficient strains supported the possibility of alternative energy-producing processes in this fungus.

CHAPTER 4

THE ALTERNATIVE OXIDASE GENE OF *A. NIDULANS*

PCR amplification with degenerate primers was carried out to obtain fragments of the *A. nidulans AOX* gene. A preliminary study of the putative regulatory elements contained within the *A. nidulans AOX* gene promoter was then carried out, and a comparison made between fungal and plant AOX protein sequences.

4.1 INTRODUCTION

The alternative respiratory pathway is utilised in many plants, as well as in a variety of fungal and yeast species (McIntosh, 1994). As noted previously (section 1.4), in these organisms the alternative pathway has been found to consist of a single enzyme, the alternative oxidase (AOX). The observation of respiration in *cyc*A$^+$ and *cyc*A$^-$ strains of *A. nidulans* which was resistant to cyanide, yet sensitive to SHAM, suggested the presence of an alternative respiratory pathway in this fungus (section 3.5). PCR Amplification with degenerate primers was carried out to verify the presence of an alternative oxidase gene in *A. nidulans*. The protein encoded by that gene may be involved in the activity of the alternative respiratory pathway. A preliminary study was carried out into the molecular mechanisms which may regulate the expression of the *A. nidulans AOX* gene, and a comparison made with the possible regulation of other fungal *AOX* genes. A comparison was also carried out between fungal and plant AOX protein sequences.

4.1.1 THE ALTERNATIVE OXIDASE PROTEIN

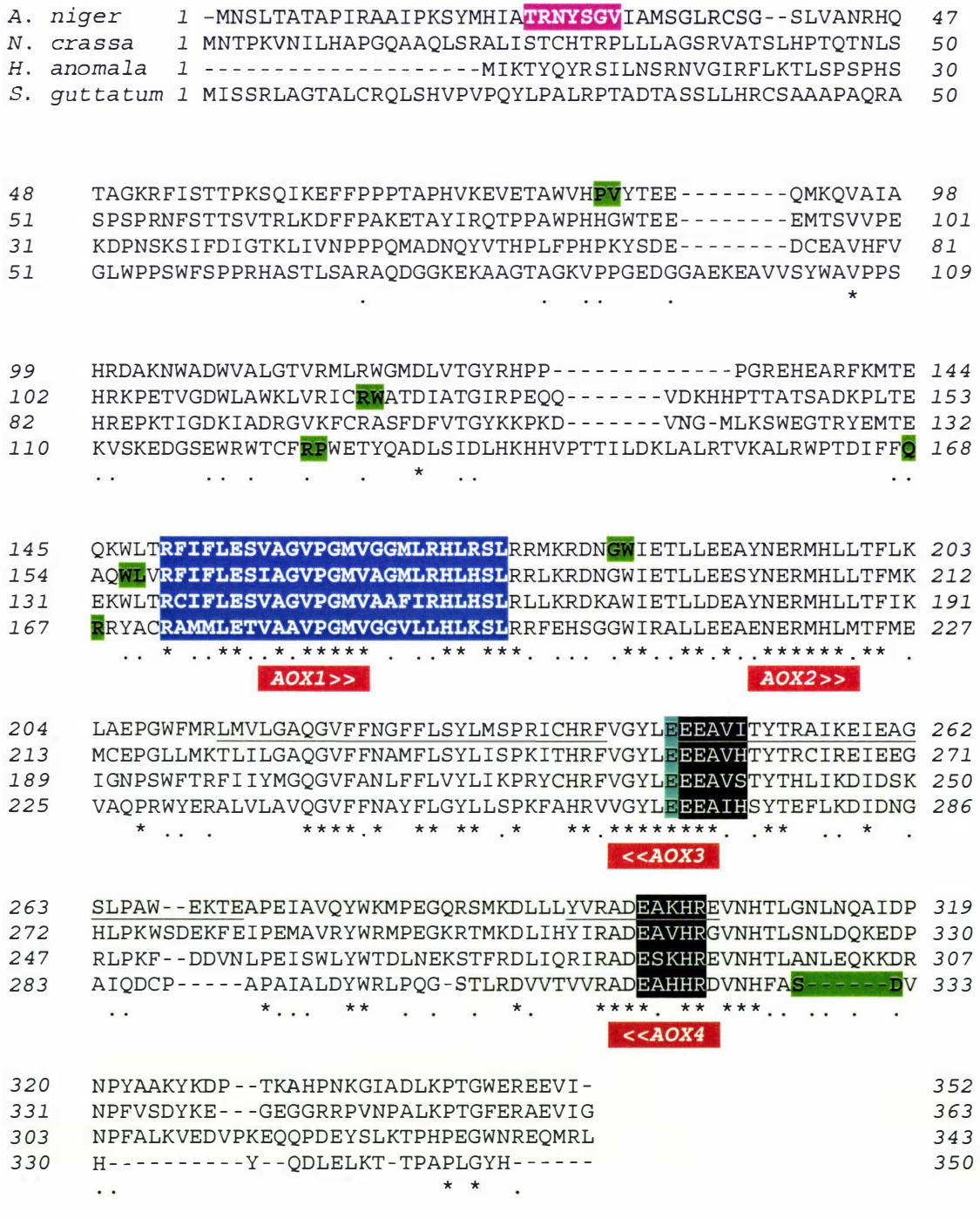
The AOX enzyme, encoded by the *AOX* gene, is highly conserved at the amino acid level, with specific residues required for correct functioning (Albury *et al.*, 1998). These residues correspond to regions such as putative transmembrane domains and iron-binding motifs (section 1.4.3) (Figure 4.1). Other highly conserved amino acid residues include Glu-270, which has been suggested to be essential for the protein's activity, as it is situated within the active site of the enzyme (section 1.4.3). This residue is present in the aligned fungal

Figure 4.1 Alignment of Alternative Oxidase Amino Acid Sequences.

An alignment (Kirimura *et al.*, 1999) has been created with the AOX sequences from the following organisms: *Aspergillus niger* (Ac. No. AB016540); *Neurospora crassa* (Ac. No. L46869); *Hansenula anomala* (Ac. No. D00741) and *Sauromatum guttatum* (Ac. No. Z15117 S57369).

The annealing sites of the four degenerate primers (AOX 1 - 4) designed for the PCR amplification of the *AOX* gene from *A. nidulans* are indicated. Amino acid residues conserved over three or four of the species are indicated by a dot or an asterisk, respectively. A potential membrane-spanning domain is indicated by dark blue highlighting, while a thin line underneath the first line of the alignment indicates suggested helical domains. The amino acids in black boxes represent the putative iron-binding motifs. The conserved Glu-270 residue, postulated to be involved in the active site of the enzyme, is indicated by light blue highlighting. Intron boundaries present in the sequences are shown in green boxes (the intron being between the two residues highlighted). The proposed start of the mature *A. niger* AOX protein (Kirimura *et al.*, 1999) is indicated by pink highlighting.

Alignment of the sequences was carried out using ClustalW 1.8, located on the BCM Search Launcher (http://searchlauncher.bcm.tmc.edu/).
The multiple sequence alignment output was created using BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html).


AOX1>>
AOX2>>
<<AOX3
<<AOX4

AOX gene sequences (Figure 4.1). Introns are also present in many *AOX* sequences, including *Aspergillus niger* (Kirimura *et al.*, 1999), *Neurospora crassa* (Li *et al.*, 1996) and *Sauromatum guttatum* (Rhoads & McIntosh, 1993) (Figure 4.1). However, the position, length and sequence of the introns vary between organisms.

4.1.2 PUTATIVE REGULATORY ELEMENTS INVOLVED IN *AOX* TRANSCRIPTIONAL REGULATION

Transcriptional regulation is one of the mechanisms controlling the expression of *AOX*, and therefore the activity of the alternative respiratory pathway (section 1.4.4). A number of possible regulatory elements have been suggested to play a role in the transcriptional regulation of *AOX* genes. TATA and CCAAT boxes have been identified in *AOX* gene promoters from various organisms, including *N. crassa* and *S. guttatum* (Vanlerberghe & McIntosh, 1997). A recent study (Sakajo *et al.*, 1999) identified a putative CCAAT element in the promoter region of the *AOX* gene from *Pichia anomala*. Electrophoretic mobility shift assays demonstrated the CCAAT element was bound by a transcription factor of a size similar to the HAP2/3/4/5 complex found in *Saccharomyces cerevisiae*. As mentioned previously (section 1.6.1), a homologue of the HAP2/3/4/5 complex has been found in *A. nidulans*, generally referred to as the AnCF. The activity of the AnCF complex is induced by non-fermentable carbon-sources (section 1.6.1). The *AOX* gene expression in many organisms has been reported to be regulated in some way by carbon source (section 1.4.1), thus it is possible that mediation of this regulation occurs via CCAAT-binding complexes.

CCAAT-binding complexes can regulate gene expression by recruiting other transcriptional activators. For example, the *A. nidulans amdS* and *gatA* genes (required for the efficient utilisation of acetamide as a nitrogen and carbon source) are transcriptionally activated by the $Zn(II)_2Cys_6$-finger protein AMDR (Brakhage *et al.*, 1999). Binding of this protein to its target site was dependent on a functional AnCF binding to an adjacent CCAAT element. Thus a number of transcription factors, including CCAAT-binding complexes, could be involved in the regulation of *AOX* genes.

Other regulatory binding sites which have been implicated in the transcriptional regulation of *AOX* gene expression include the cAMP-responsive element (CREA). This element has been identified in the promoter region of the *N. crassa Aod1* gene (Vanlerberghe & McIntosh, 1997). Carbon catabolite repression can be mediated through the binding of the

CREA repressor protein to the CREA element, thus repressing gene expression (section 1.6.2). Homologues of the CREA protein have been found in many fungi, including *A. nidulans* (Davis & Hynes, 1991), *A. niger* (Drysdale *et al.*, 1993) and *N.crassa* (de la Serna *et al.*, 1999).

Stress-responsive elements (STRE) have also been implicated in carbon source regulation in yeast (Flattery-O'Brien *et al.*, 1997). As *AOX* gene expression in many organisms has been reported to be regulated by carbon source, and induced under stress conditions (Vanlerberghe & McIntosh, 1997), it is possible that this regulation occurs via sites similar to STRE's. Interestingly, STRE sites have been identified within the *SOD2* gene (encoding Manganese Superoxide Dismutase, MnSOD) in *S. cerevisiae* (Flattery-O'Brien *et al.*, 1997). During the stationary phase of growth, the utilisation of non-fermentable carbon sources results in a higher level of respiration. Simultaneously, cellular cAMP levels decrease and expression of *SOD2* is elevated. Increased transcription of *SOD2* is thought to be mediated through the additive, positive effects of the STRE and HAP2/3/4/5 sites present in the *SOD2* promoter. Expression of the *SOD2* gene is also activated by the HAP1 protein, which mediates oxygen regulation (section 1.8) (Pinkham *et al.*, 1997). As mentioned in section 1.4.2, the MnSOD enzyme encoded by the *SOD2* gene guards against cellular damage by ROS. Another mechanism suggested to have evolved for cellular protection against ROS is the alternative respiratory pathway (section 1.4.2). Thus it is possible that the factors which regulate the expression of the *SOD2* gene (and other genes encoding ROS-scavenging enzymes) are also involved in the regulation of *AOX* expression, in a coordinated manner.

4.2 PCR AMPLIFICATION OF THE ALTERNATIVE OXIDASE GENE FROM *A. NIDULANS*

Note: All steps were carried out with both *cyc*A$^+$ and *cyc*A$^-$ DNA, although the sequence was ultimately obtained from the *cyc*A$^-$ strain. However, it was assumed that the gene is the same in both strains.

4.2.1 DESIGN OF THE DEGENERATE PCR PRIMERS

The *AOX* sequences obtained from the fungi *A. niger*, *N. crassa* and *H. anomala* were presented in an alignment in a recent paper by Kirimura *et al* (1999), along with the *AOX*

sequence from *S. guttatum*. This sequence alignment was used to facilitate the design of degenerate primers for the PCR Amplification of the *AOX* from *A. nidulans*. The four degenerate primers (AOX1, AOX2, AOX3 and AOX4) were designed to conserved regions of the gene sequences (Figure 4.1; Appendix 2.1). A codon useage table for *A. nidulans* (Appendix 2.2) was also used in the design of the primers.

4.2.2 PCR AMPLIFICATION OF FRAGMENTS OF THE *A. NIDULANS* ALTERNATIVE OXIDASE GENE

Fragments of the *A. nidulans AOX* gene were PCR amplified using different combinations of the degenerate primers; AOX1 & AOX3, AOX1 & AOX4, AOX2 & AOX3, as well as AOX2 & AOX4 (Figures 4.1 & 4.2). The PCR was carried out with genomic DNA from strains A67 (*cycA*$^+$) and A68 (*cycA*$^-$), using a standard PCR set-up and thermal cycling programme (section 2.8). The initial annealing temperature of 45°C resulted in a number of non-specific PCR products for the reactions containing primers AOX1 & AOX3 or AOX1 & AOX4, in addition to the expected PCR products (results not shown). Upon increasing the annealing temperature to 47°C, non-specific PCR products were still observed (Figure 4.2), although fewer than at 45°C. However, electrophoresis (section 2.7.2) with a 3% TBE/Seakem Agarose gel separated the PCR products sufficiently. Thus, all further PCR amplifications were carried out at an annealing temperature of 47°C.

All of the reactions produced bands indicating fragments of approximately the expected sizes for the different primer combinations: primers AOX1 & AOX4, 500bp (PCR product 'A'); AOX1 & AOX3, 330bp (PCR product 'B'); AOX2 & AOX3, 170bp (PCR product 'C'); and AOX2 & AOX4, 350bp (PCR product 'D') (Figure 4.2). Re-amplification of each PCR product by gel-stab PCR was then carried out, to facilitate subsequent isolaton of the specific fragments. For each primer combination, a 'gel-stab' was taken of the PCR product of the expected size (Figure 4.2), and the sample was then re-amplified (section 2.8.2) with the primer combination originally used to generate the fragment. To determine the authenticity of each fragment, nested PCR was carried out. For example, the PCR product 'A' generated by primers AOX1 and AOX4 was also re-amplified using the primer combinations AOX1 & AOX3, AOX2 & AOX3 and AOX2 & AOX4. PCR products of approximately the expected size were observed for all reactions, although some

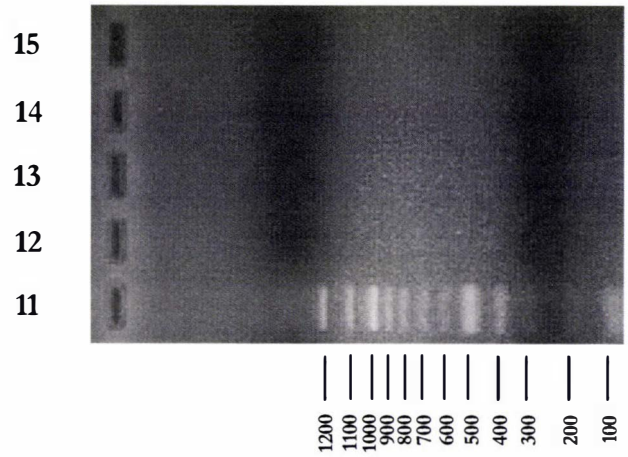
Figure 4.2 Fragments of the *A. nidulans* Alternative Oxidase gene, generated by PCR Amplification using degenerate primers (annealing temperature of 47°C).

The PCR products were separated by electrophoresis on a 1% gel prepared with agarose (BRL) and 1 x TBE Buffer (10x Stock (g/L): tris, 108; boric acid, 55; and Na_2EDTA, 9.3)). Following electrophoresis, the ethidium bromide-stained gel was placed on a UV transilluminator and photographed under ultraviolet using the Gel Documentation System (Alpha Innotech).

Lanes 1, 10 and 11 contain the 100 bp ladder (Roche Biochemicals Ltd). The sizes of the bands indicated are in bp.

PCR reactions containing the indicated primer pair are loaded on the gel as follows:
Template of A67 (*cycA*$^+$) DNA: Lane 2: AOX1 & AOX3. Lane 3: AOX1 & AOX4. Lane 4: AOX2 & AOX3. Lane 5: AOX2 & AOX4.
Template of A68 (*cycA*$^-$) DNA: Lane 6: AOX1 & AOX3. Lane 7: AOX1 & AOX4. Lane 8: AOX2 & AOX3. Lane 9: AOX2 & AOX4.
dH_2O Controls: Lane 12: AOX1 & AOX3. Lane 13: AOX1 & AOX4. Lane 14: AOX2 & AOX3. Lane 15: AOX2 & AOX4.

The PCR products which were generated by the different primer combinations, and re-amplified by gel-stab PCR, are indicated on the gel:
AOX1 &AOX4, 500 bp (PCR product 'A')
AOX1 & AOX3, 330 bp (PCR product 'B')
AOX2 & AOX3, 170 bp (PCR product 'C')
AOX2 & AOX4, 350 bp (PCR product 'D')


15
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13
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11
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1100
1000
900
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500
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300
200
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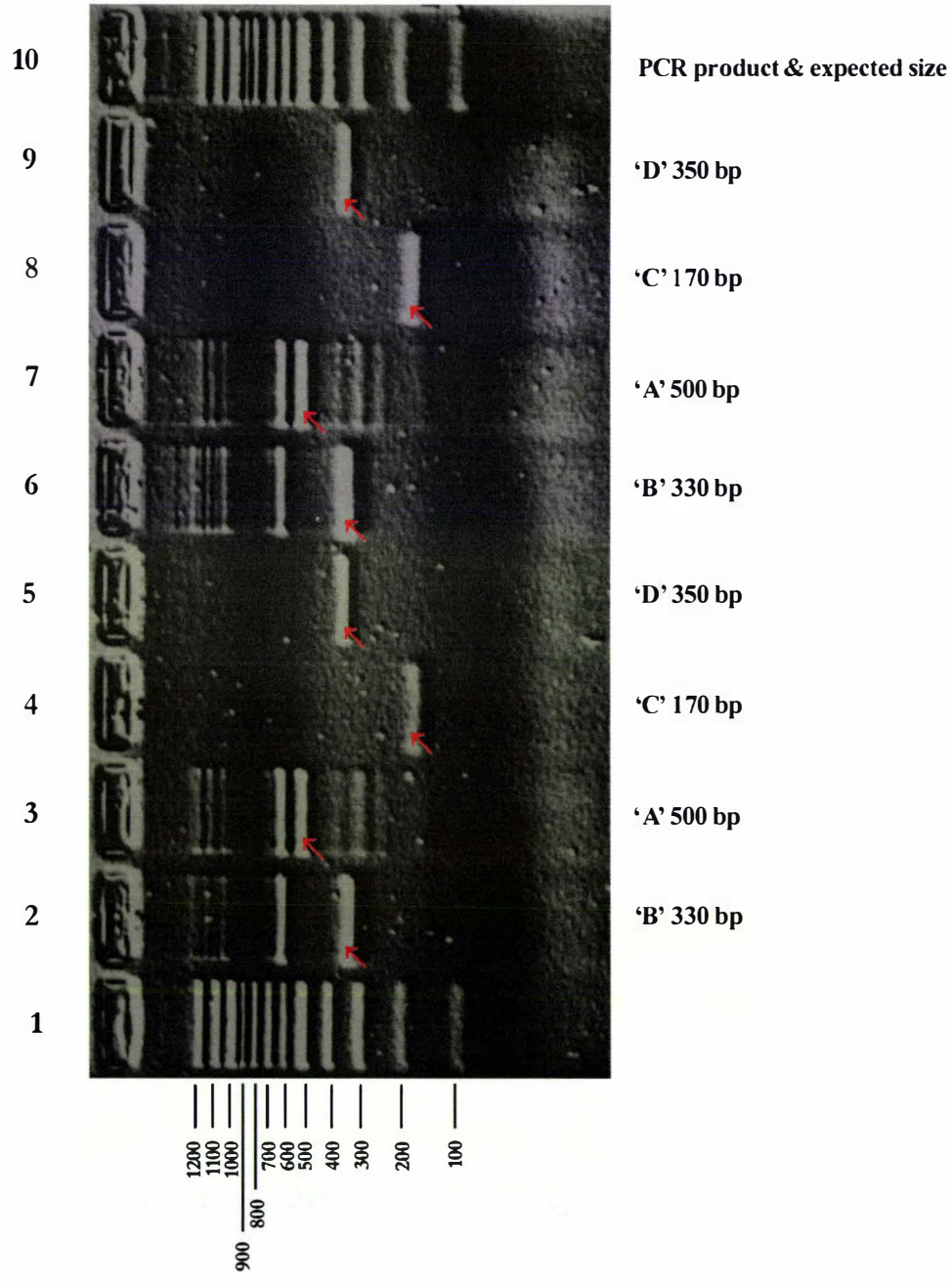

10
9
8
7
6
5
4
3
2
1
PCR product & expected size
'D' 350 bp
'C' 170 bp
'A' 500 bp
'B' 330 bp
'D' 350 bp
'C' 170 bp
'A' 500 bp
'B' 330 bp
1200
1100
1000
900
800
700
600
500
400
300
200
100

of the reactions containing primers AOX1 & AOX3 again generated a small number of non-specific PCR products (Figure 4.3).

4.3 CLONING AND SEQUENCING OF THE *A. NIDULANS AOX* FRAGMENTS

The PCR products 'C', 'D' and 'A' (approximately 170 bp, 350 bp and 500 bp respectively), generated by the appropriate primer pairs (Figure 4.3), were purified using a QIAGEN PCR Purification Column (section 2.8.3). The fragments were then ligated (section 2.12) into the pGEM vector (Appendix 2.3), thus forming plasmids R187, R188 and R189 (respectively) (Table 2.1).

The presence of PCR product 'A' in plasmid R189 was verified by PCR amplification with the lacZ forward and reverse primers, prior to automated sequencing (section 2.11.1) of R189 from two clones, with the lacZ forward primer. A 'BLASTN' search (section 2.11.3) revealed no similarity to any other published *AOX* gene, and weak homology to other sequences in the database.

PCR amplification with the lacZ forward and reverse primers was also used to determine the presence of PCR products 'C' and 'D' in plasmids R187 and R188 (respectively). Bands corresponding to PCR products of the expected sizes were observed for plasmids containing DNA from the *cycA*$^+$ and *cycA*$^-$ strains (results not shown). To ensure the specificity of these PCR products, PCR amplification was also carried out on selected clones with the primers AOX2 and AOX3. PCR products of the expected size (170 bp) were observed for plasmids containing DNA from strain A68 (*cycA*$^-$) and A67 (*cycA*$^+$) (results not shown).

PCR amplification with primers AOX2 and AOX3 was also carried out on clones containing the R189 plasmid, to determine the fidelity of the 500 bp PCR fragments (results not shown). Only one clone out of six generated the expected 170 bp PCR product. Thus, PCR product 'A' (Figure 4.3, Lanes 3 and 11) was probably a pool of PCR products. It was expected that the specific PCR product would be found by sequencing of a number of clones, as its presence was determined previously by nested PCR (section 4.2.2). However, in retrospect, nested PCR should also have been carried out on the PCR products once cloned into pGEM (prior to sequencing).

Figure 4.3 Re-Amplification of PCR products, using degenerate primers to the *A. nidulans* Alternative Oxidase gene.

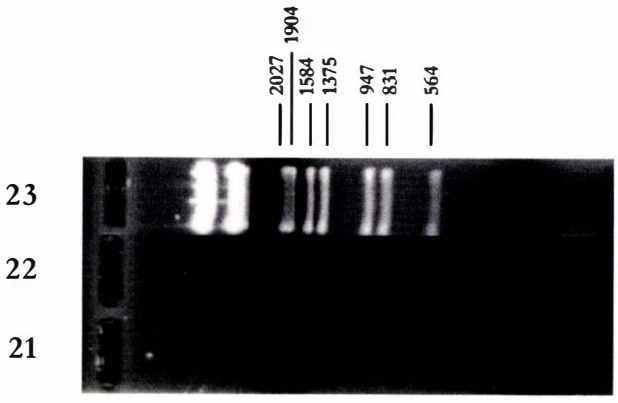
The PCR products were separated on by electrophoresis on a 1% gel prepared with agarose (BRL) and 1 x TBE Buffer (10x Stock (g/L): tris, 108; boric acid, 55; and Na_2EDTA, 9.3)). Following electrophoresis, the ethidium bromide-stained gel was placed on a UV transilluminator and photographed under ultraviolet using the Gel Documentation System (Alpha Innotech).

Lanes 1 and 18 contain the 100 bp ladder (Roche Biochemicals Ltd). The sizes of the bands indicated are in bp.

PCR reactions containing the indicated primer pair are loaded on the gel as follows:
Template of PCR product 'A', A67 (*cycA*+) DNA: Lane 2: AOX1 & AOX3. Lane 3: AOX1 & AOX4. Lane 4: AOX2 & AOX4. Lane 5: AOX2 & AOX3. Template of PCR product 'B', A67 (*cycA*+) DNA: Lane 6: AOX2 & AOX3. Lane 7: AOX1 & AOX3. Template of PCR product 'D', A67 (*cycA*+) DNA: Lane 8: AOX2 & AOX3. Lane 9: AOX2 & AOX4.
Template of PCR product 'A', A68 (*cycA*-) DNA: Lane 10: AOX1 & AOX3. Lane 11: AOX1 & AOX4. Lane 12: AOX2 & AOX4. Lane 13: AOX2 & AOX3. Template of PCR product 'B', A68 (*cycA*-) DNA: Lane 14: AOX2 & AOX3. Lane 15: AOX1 & AOX3. Template of PCR product 'D', A68 (*cycA*-) DNA: Lane 16: AOX2 & AOX3. Lane 17: AOX2 & AOX4.
dH_2O Controls: Lane 19: AOX1 & AOX3. Lane 20: AOX1 & AOX4. Lane 21: AOX2 & AOX3. Lane 22: AOX2 & AOX4.

The sizes of the PCR products generated by the different primer combinations are indicated on the gel, as well as the templates used for the PCR reactions (in brackets):
AOX1 &AOX4, 500 bp (= PCR product 'A')
AOX1 & AOX3, 330 bp (= PCR product 'B')
AOX2 & AOX3, 170 bp (= PCR product 'C')
AOX2 & AOX4, 350 bp (= PCR product 'D')

The PCR products which were sequenced are indicated by red arrows.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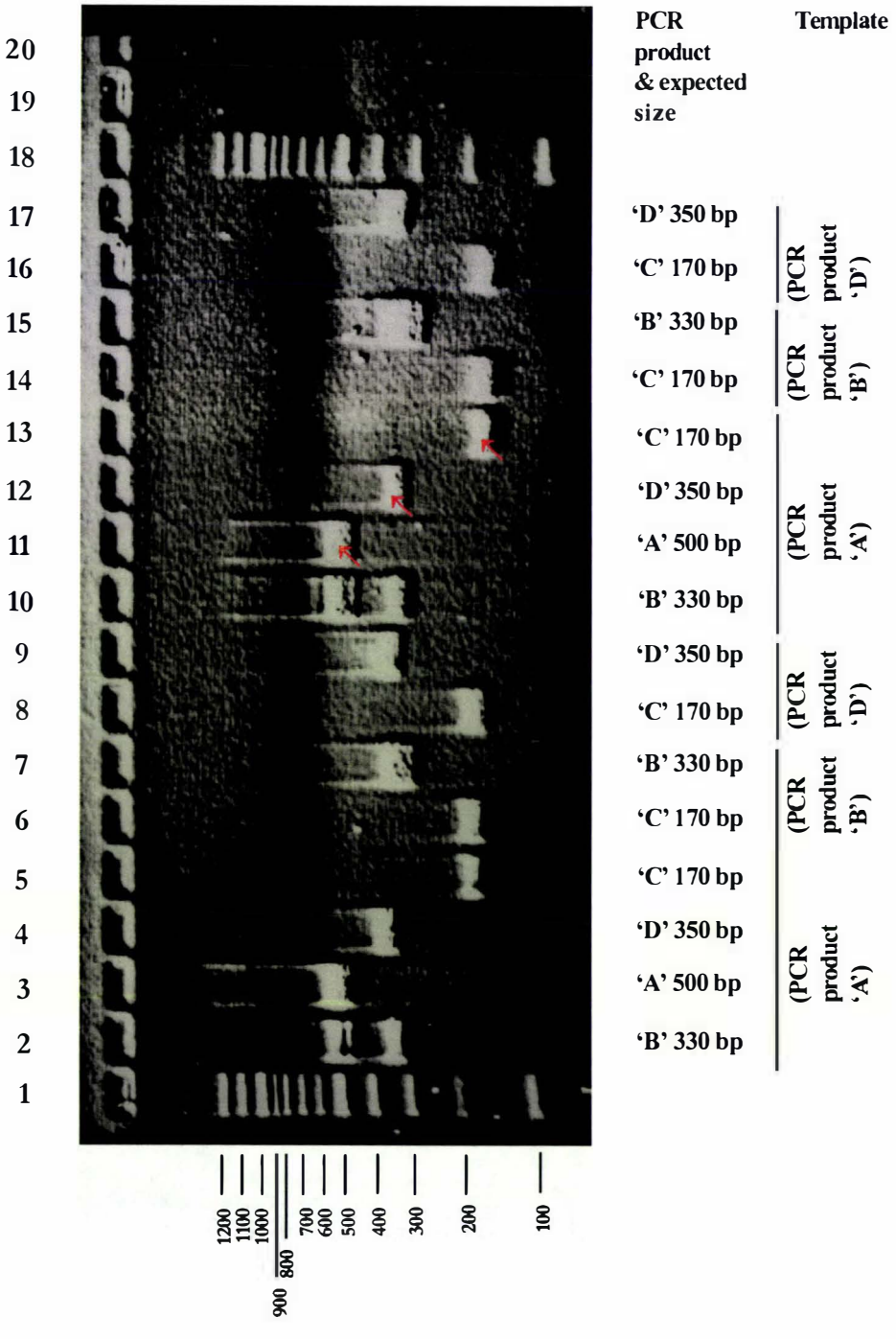
1584
1375
947
831
564
23
22
21


20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1
1200
1100
1000
900
800
700
600
500
400
300
200
100
PCR product & expected size
Template
'D' 350 bp
'C' 170 bp
'B' 330 bp
'C' 170 bp
'C' 170 bp
'D' 350 bp
'A' 500 bp
'B' 330 bp
'D' 350 bp
'C' 170 bp
'B' 330 bp
'C' 170 bp
'C' 170 bp
'D' 350 bp
'A' 500 bp
'B' 330 bp
(PCR product 'D')
(PCR product 'B')
(PCR product 'A')
(PCR product 'D')
(PCR product 'B')
(PCR product 'A')

4.3.1 SEQUENCE RESULTS

Plasmids R187 and R188 were sequenced with the lacZ forward primer. Sequencing of R188 with the lacZ reverse primer was later carried out to obtain both strands of sequence (Figure 4.4 B). The *AOX* gene sequences contained in plasmids R187 and R188 were aligned to check the fidelity of the 170 bp fragment. 'BLASTN' search analysis (section 2.11.3) of both of these *AOX* fragments confirmed high similarity to *AOX* nucleotide sequences from other organisms.

The deduced amino acid sequence of the 347 bp fragment (denoted the 'partial *A. nidulans AOX* fragment') (Figure 4.4 B) exhibited the following % identity to the corresponding regions of other fungal AOX sequences: *A. niger* (91%); *N. crassa* (74%); *Candida albicans* (60%); *Pichia stipitis* (59%); *H. anomala* (61%) (results not shown). The partial *A. nidulans AOX* fragment was observed to share 82% and 79% nucleotide sequence identity with the analogous regions of the *AOX* genes from *A. niger* and *N. crassa*, respectively (Figure 4.5). However, the partial *A. nidulans AOX* fragment had no significant similarity to the *AOX* sequences from *C. albicans*, *P. stipitis*, *H. anomala* or *S. guttatum* (results not shown).

4.4 DISCUSSION OF SEQUENCE RESULTS

4.4.1 ALIGNMENT OF THE COMPLETE *A. NIDULANS AOX* NUCLEOTIDE SEQUENCE WITH THE PARTIAL FRAGMENT

The complete *A. nidulans AOX* nucleotide sequence was entered into the NCBI database (Ac. No. AB039832) (Sakajo *et al.*, not yet published) while this thesis was being completed. The partial *A. nidulans AOX* fragment obtained from this study was aligned with the complete *A. nidulans AOX* sequence and found to have 100% similarity (Figure 4.4 B). The deduced amino acid sequence of the partial *A. nidulans* AOX fragment was found to share 100% identity with the complete *A. nidulans* AOX sequence.

Figure 4.4 A *A. nidulans AOX* gene promoter

The region of the *A. nidulans AOX* gene (Ac. No. AB039832) upstream of the translational start site. The putative transcriptional start site (in bold) is underlined; the sequence upstream of this is denoted the *A. nidulans AOX* gene promoter. Putative TATA and CCAAT boxes are highlighted in red and blue, respectively. Blue and red font indicate pyrimidine-rich sequences and putative HAP1 binding sites (respectively). Possible *cre*A and STRE sites are indicated by green and pink highlighting, respectively.


-2168 GAATTCCTGGAGCGAAGTGGCGGATCTGGCGAGTCTCGAAAGGCTCCTTCAGCACGCTAA -2109
-2108 ACCCGCCGCCAGCATCGTCATCTCCTCGCTGTACTAGTTATGGAGGTAGTGCAGTTGTTT -2049
-2048 TGAATCTTGACACAGTTGTATCTCTGGAATAATATAGTAGATAAGCGGAATCTTTGGAAT -1989
-1988 TGCGTCGGAAAGGCCGGGTGCTTTAAGGGTAACACCTCTTACGGTCTA AGATGC -1929
-1928 CAGGTTCGAGTCCCAGGTAATTTTTTTGCCATTTTTCTCCATAATTCATGACCGGATTAG -1869
-1868 ATCCCACACCTAGTTAGAAATATTTGTTTTTCAGAAGTCAGCTGCTAAAACTCGATTGAT -1809
-1808 AATTTTGTGTTTCTGAGCCCATTCAAGTCAGCAAATTTGCCGCCCAAAACCTTGCAATTC -1749
-1748 TCGGTGGAAGTCTCTGCAATGTGATTTAGATCACAT AATGAAGTATCCAACTGT -1689
-1688 CGAAAGCAAGAGAATATTAGGAACGAGATGTTAATCCCAAATTCAAGGTTGAGGCGGGTT -1629
-1628 ATATGCAGCTCGCTTGAAGCCCCGCAGTGGGCCCTGGTCAACCTCAGCTTCAACCGCTCT -1569
-1568 AGACTCCTTCGGATCCTACACCTACCGACCAGGATGACATGTTAACACTCCTCTGCTTGA -1509
-1508 TCGTCGGCGAGAATATCACAAAATAAACATGGCAACTCAAAGGGCATGTTTGGCTGTCAG -1449
-1448 ATTTATTGCTACAAGCTTATACTTACGACTGTTGTCGCTGACTTTTCTCAGATTGCGTTC -1389
-1388 AAAGTCCATGGCACCGTTCAGGGTACGTCTTCAGTTCTAACTAGTTTCATTCTTAGCCAG -1329
-1328 CTATGTTTAACAAGTACCTCTACAGGTGTGGGTTTCCGGTAGGCACAGTTTCTACAGGTC -1269
-1268 AACATTAGACATTTCTGATCATGTCGTTACACCAGTGATTTCACCATGAAGCGTGCTGCG -1209
-1208 GAATACGGTCTCAGCGGCTGGGTGAAGAACACTGATTGCGGAAGGGTACGTTTGTAGTCC -1149
-1148 CTATTAATGCTAACAATCTTGCAATGTCTTGCATCGCTCGTCAGAGTCACTAAATGCATA -1089
-1088 TGAAGGTCGAAGGCGAAGCACAAGGCTCAGAAGAGGGTATCCAAAAATTTCTCAAGGATA -1029
-1028 TCAATAATGGCCCTCGACTTGCACATGTAGTCAAG AAGAAGACACTCGATCCGA -969
-968 AAGACGGAGAGAGTCATTTTGGGCTTAGGAAGACATCGGCGACTGTTTTTGATTCACTTT -909
-908 GATGGTCGACTATACAGTGTATACTCTGGTTCAAGCAGAATAAAGTCCATCCGCTCGGCC -849
-848 TCCCAGTTTCCATGAAAAGTAGTCTCAGCGTAGCCTCTTCATCCGGTCCTCTGCTTACGT -789
-788 GGTAGCCAATTTCT CAAGCAGGAACGCATTGCCCCAGAATCTTTCAGCTTCGGG -729
-728 AATATTTCCAGTGACGTCGATAGGGCTACGCAAACGTCGTTATTGAATCCCGATTTATTC -689
-688 TCGGCGTTTGTCTGAGTCATGGCCCAGTTAGTGGTCCCGTCCCGGCTTCGTCCGTGATCA -629
-628 AGTGACAGAGAGCTGGATCCATAATACCGTAATCTGATAACACAGGTCAATCTTGCATCT -569
-568 CAGCAAGTAGGACTCCCAACAGACTGAAAAATCAATGGGAAGTAAGTATAATCTGATTTC -509
-508 GCTTCTGCTAAAGCTAAATATTTCAAACACACCTGTATATGATTGAACTATATTTGTTTT -449
-448 ATGCCCCTACTCAATGCTAAATGGAGTATCCAGATTTGTAACGCTCCGTAAGGTAGGGCG -389
-388 CCCTAAATACATGGCGTTTTGCCAGGTACGACAAGTCAGGCTCATAATTAGAAATACTCA -329
-328 GGCACTCTTCCTTTACAGCCACCATCTAGAATGGGCGTCAGAT CCTTCTTTCGG -269
-268 TGAATGAAAAGTTCTGTTCG AGCGTGTGACTCCTGTGT ACTCAGCCCT -209
-208 GAGAGTTCCGATTCCTGCCTCAGATTGCCTCTGGTTGAATTTTTTTTATAGGTCTTTGTA -149
-148 TTTAGTTGGCATATATAAATACAAAAAAAGTTGCAGTCCCAGTCAACTCCTTATGTATGT -89
-88 CAAAGCAACATCTTATCTTGACTAAGTACAGTTCGTCAGAATTGACAGCACCTTTTCAAC -29
-28 AATAAACAATATCAGCATTATGAATTCG -1

Figure 4.4 B *A. nidulans AOX* gene coding region

The coding region of the *A. nidulans AOX* gene (Ac. No. AB039832). The amino acid residues encoded are positioned beneath the corresponding nucleotide sequence, initiating at the translational start site (in bold, underlined). Blue lettering indicates the introns present. The annealing sites for the primers AOX1 – 4 (degenerate PCR) and AnidAOX1 & 2 (RT-PCR) are indicated. The partial fragment of the *A. nidulans AOX* gene generated in this study was PCR amplified with the primers AOX2 & AOX4.

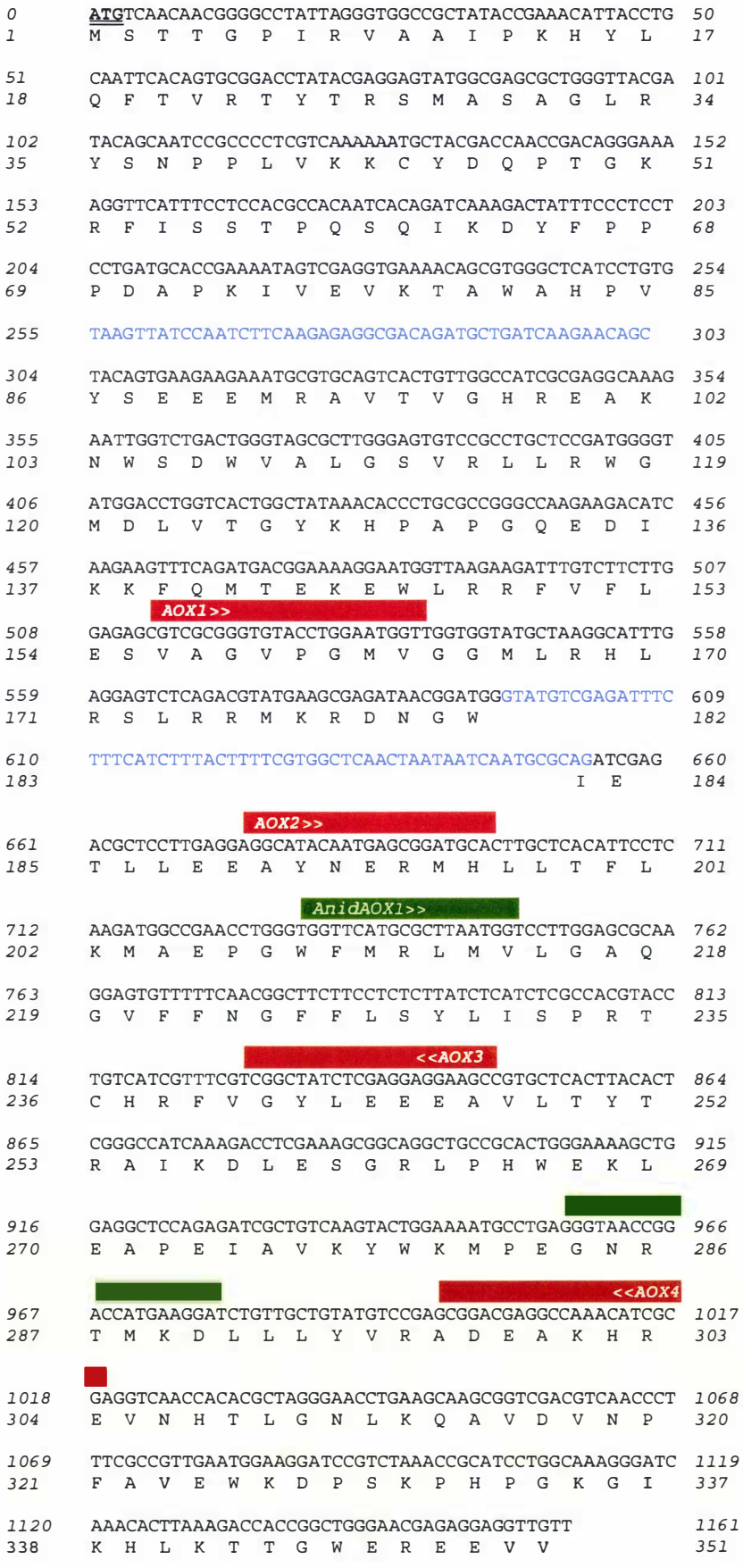
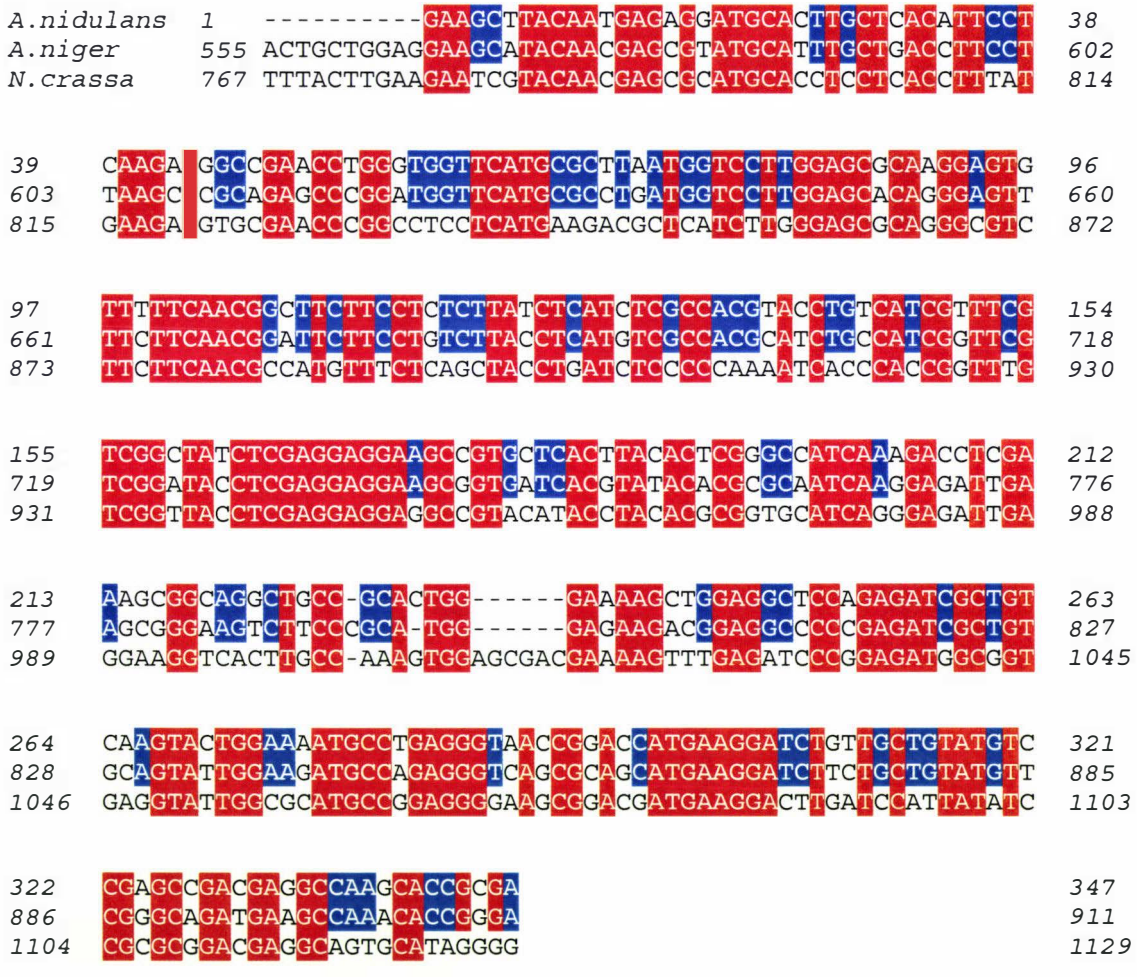

AOX1>>
AOX2>>
AnidAOX1>>
<<AOX3
<<AOX4

Figure 4.5 **Alignment of the Nucleotide Sequence of the Partial *A. nidulans AOX* fragment with *AOX* Sequences from *A. niger* and *N. crassa*.**

The nucleotide sequence of the partial *A. nidulans AOX* fragment was aligned with the *AOX* sequences from *A. niger* (Ac. No. AB016540) and *N. crassa* (Ac. No. L46869), to which it exhibited 82% and 79% identity, respectively. Red highlighting indicates those bases which are conserved over all three organisms, while bases conserved only between *A. nidulans* and *A. niger* are highlighted in blue.

Alignment of the sequences was carried out using ClustalW 1.8, located on the BCM Search Launcher (http://searchlauncher.bcm.tmc.edu/). The multiple sequence alignment output was created using BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html).

Predicted amino acid sequence of the partial *A. nidulans AOX* fragment was determined using the Sequence Utilities on the BCM Search Launcer (http://searchlauncher.bcm.tmc.edu/).


A.nidulans 1 ---------- GAAGCTTACAATGAGAGGATGCACTTGCTCACATTCCT 38
A.niger 555 ACTGCTGGAG GAAGCATACAACGAGCGTATGCATTTGCTGACCTTCCT 602
N.crassa 767 TTTACTTGAA GAATCGTACAACGAGCGCATGCACCTCCTCACCTTTAT 814
39 CAAGA GGCCGAACCTGGGTGGTTCATGCGCTTAATGGTCCTTGGAGCGCAAGGAGTG 96
603 TAAGC CGCAGAGCCCGGATGGTTCATGCGCCTGATGGTCCTTGGAGCACAGGGAGTT 660
815 GAAGA GTGCGAACCCGGCCTCCTCATGAAGACGCTCATCTTGGGAGCGCAGGCGTC 872
97 TTTTTCAACGGCTTCTTCCTCTCTTATCTCATCTCGCCACGTACCTGTCATCCTTTCG 154
661 TTCTTCAACGGATTCTTCCTGTCTTACCTCATGTCGCCACGCATCTGCCATCCGTTCG 718
873 TTCTTCAACGCCATGTTTCTCAGCTACCTGATCTCCCCCAAAATCACCCACCCGTTTG 930
155 TCGGCTATCTCGAGGAGGAAGCCGTGCTCACTTACACTCGGGCCATCAAAGACCTCGA 212
719 TCGGATACCTCGAGGAGGAAGCGGTGATCACGTATACACGCGCAATCAAGGAGATTGA 776
931 TCGGTTACCTCGAGGAGGAGGCCGTACATACCTACACGCGGTGCATCAGGGAGATTGA 988
213 AAGCGGCAGGCTGCC-GCACTGG------GAAAAGCTGGAGGCTCCAGAGATCGCTGT 263
777 AGCGGGAAGTCTTCCCGCA-TGG------GAGAAGACGGAGGCCCCCGAGATCGCTGT 827
989 GGAAGGTCACTTGCC-AAAGTGGAGCGACGAAAAGTTTGAGATCCCGGAGATGGCGGT 1045
264 CAAGTACTGGAAAATGCCTGAGGGTAACCGGACCATGAAGGATCTGTTGCTGTATGTC 321
828 GCAGTATTGGAAGATGCCAGAGGGTCAGCGCAGCATGAAGGATCTTCTGCTGTATGTT 885
1046 GAGGTATTGGCGCATGCCGGAGGGGAAGCGGACGATGAAGGACTTGATCCATTATATC 1103
322 CGAGCCGACGAGGCCAAGCACCGCGA 347
886 CGGGCAGATGAAGCCAAACACCGGGA 911
1104 CGCGCGGACGAGGCAGTGCATAGGGG 1129

4.4.2 ALIGNMENT OF THE COMPLETE *A. NIDULANS AOX* NUCLEOTIDE SEQUENCE WITH OTHER FUNGAL *AOX* GENES

The 1054 bp open reading frame of the complete *A. nidulans AOX* gene encodes a protein of 351 amino acids (Figure 4.4 A & B). 'BLASTN' analysis (section 2.11.3) revealed the complete *A. nidulans AOX* nucleotide sequence exhibited 83% and 79% similarity to the complete *AOX* sequences from *A. niger* and *N. crassa*, respectively (results not shown), similar to the results obtained for the partial *AOX* fragment. Surprisingly, no significant similarity of the complete *A. nidulans AOX* sequence was observed to the *AOX* sequences from *C. albicans*, *P. stipitis* or *H. anomala*. However, 74% and 75% similarity was observed between the complete *A. nidulans AOX* sequence and the *AOX* sequences from *Magnaporthe grisea* and *Histoplasma capsulatum* (respectively) (results not shown).

THE PRESENCE OF INTRONS

Interestingly, two introns can be observed in the complete *A. nidulans AOX* sequence. These are located at 255 and 593 bp (downstream of the translational start site), and are of 49 and 59 bp in size, respectively (Figure 4.4 B). The locations and lengths of these introns are similar to those of the introns observed within the *A. niger AOX* gene, which are 50 bp and 51 bp in size and are located at 254 and 595 bp (downstream of the translational start site), respectively (Figure 4.6). The location and lengths of the introns within the *N. crassa AOX* gene bear no similarity to the introns within the *A. nidulans* or the *A. niger AOX* sequences (results not shown). Whilst the intron sequences from *A. nidulans*, *A. niger* and *N. crassa* contain some similarities, a BLAST search (section 2.11.3) revealed no significant similarity (results not shown). It is not yet known whether the *AOX* nucleotide sequences from *C. albicans*, *P. stipitis* and *H. anomala* contain introns, as these sequences were obtained from cDNA.

4.4.3 CONSERVED SEQUENCES WITHIN THE CODING REGION

The annealing sites for the degenerate primers AOX2, AOX3 and AOX4 were observed in the partial and complete *A. nidulans AOX* sequences (Figures 4.4 B & 4.6). The annealing site for primer AOX1 is also present in the complete *A. nidulans AOX* sequence (Figures 4.4 B & 4.6). As mentioned previously (Figure 4.1), the primer annealing sites correspond

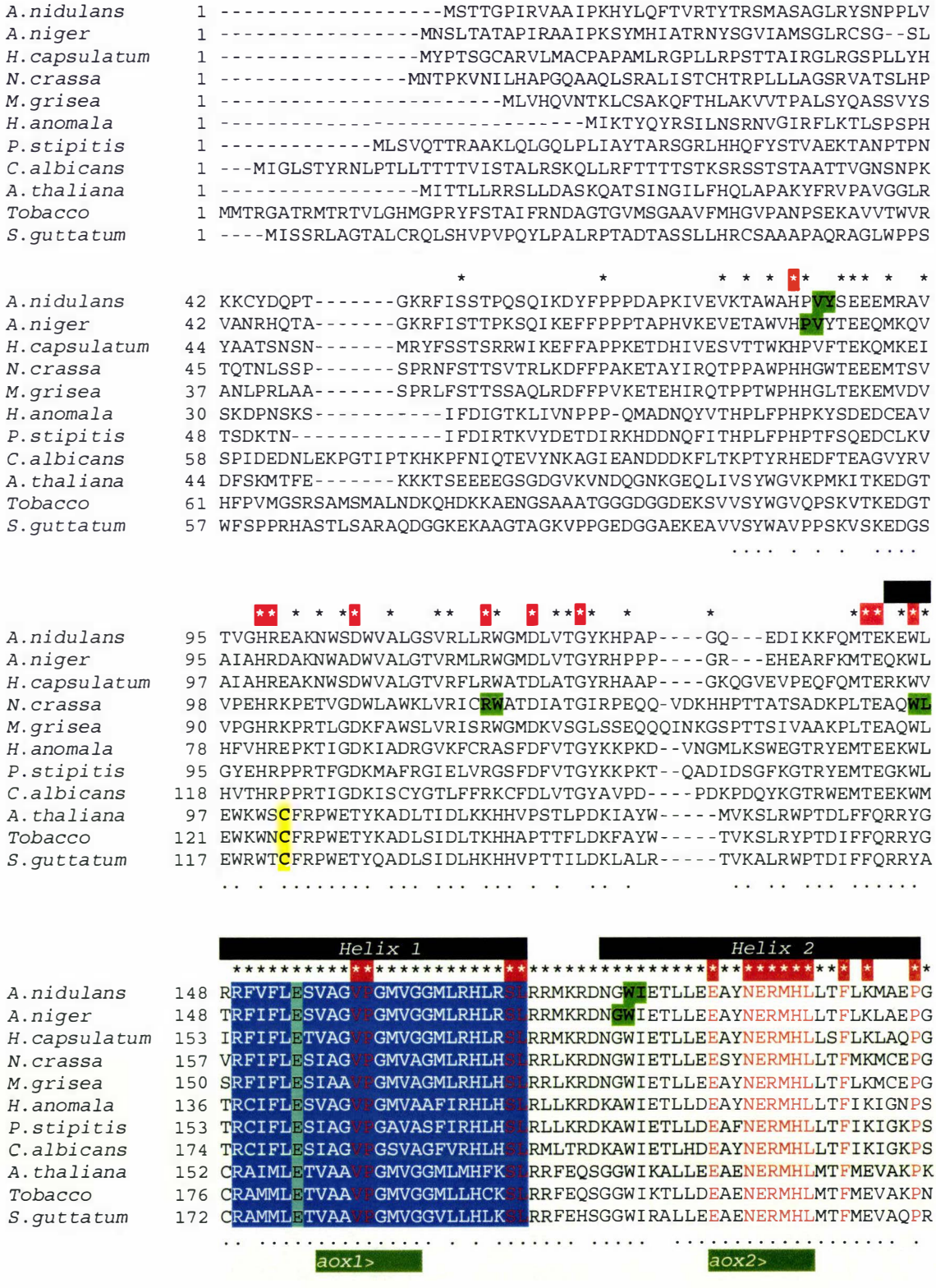
Figure 4.6 Alignment of fungal and plant AOX protein sequences

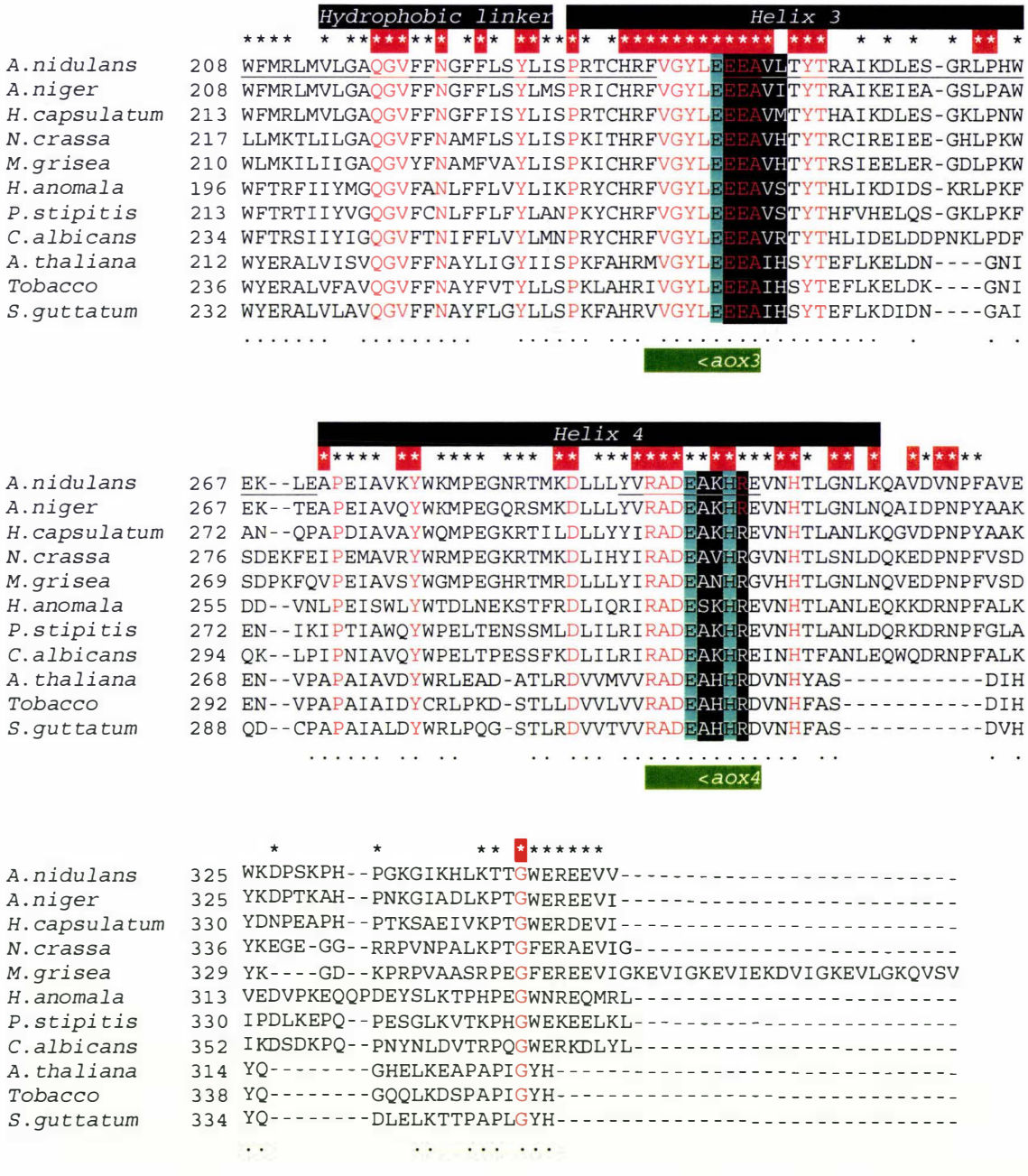
The AOX sequences from *A. nidulans* (Ac. No. AB039832), *A. niger* (Ac. No. AB016540), *H. capsulatum* (Ac. No. AF133236), *N. crassa* (Ac. No. L46869), *M. grisea* (Ac. No. AB005144), *H. anomala* (Ac. No. D00741), *P. stipitis* (Ac. No. AY004212) and *C. albicans* (Ac. No. AF031229) are presented in an alignment with plant AOX sequences from *A. thaliana* (Ac. No. BAB02686), Tobacco (Ac. No. T04094) and *S. guttatum* (Ac. No. Z15117 S57369).

Amino acid residues conserved over all fungal and plant AOX sequences are indicated by red font. Residues conserved over at least five of the fungal species are indicated by an asterisk over the sequence. Those residues which are conserved over all fungal species are indicated by a red highlighted asterisk over the sequence. Residues which are present in all three plant species are indicated by a dot beneath the sequence. Black boxes over the sequence alignment represent suggested helical domains and a hydrophobic linker (as indicated).

Within the sequence alignment, the amino acids in black boxes represent the putative iron-binding motifs, while a potential membrane-spanning domain is indicated by a dark blue box. Residues highlighted in light blue indicate the suggested metal ligands, and include the conserved Glu-270 residue. The Cysteine residues indicated with yellow highlighting have been implicated in dimer formation and pyruvate regulation in the plant AOX. Intron boundaries are shown in green boxes (the intron being between the two residues highlighted).

Alignment of the sequences was carried out using ClustalW 1.8, located on the BCM Search Launcher (http://searchlauncher.bcm.tmc.edu/).
The multiple sequence alignment output was created using BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html).


A.nidulans
A.niger
H.capsulatum
N.crassa
M.grisea
H.anomala
P.stipitis
C.albicans
A.thaliana
Tobacco
S.guttatum
Helix 1
Helix 2
aox1>
aox2>


Hydrophobic linker
Helix 3
A.nidulans 208 WFMRLMVLGAQGVFFNGFFLSYLISPRTCHRFVGYLEEEAVLTYTRAIKDLES-GRLPHW
A.niger 208 WFMRLMVLGAQGVFFNGFFLSYLMSPRICHRFVGYLEEEAVITYTRAIKEIEA-GSLPAW
H.capsulatum 213 WFMRLMVLGAQGVFFNGFFISYLISPRTCHRFVGYLEEEAVMTYTHAIKDLES-GKLPNW
N.crassa 217 LLMKTLILGAQGVFFNAMFLSYLISPKITHRFVGYLEEEAVHTYTRCIREIEE-GHLPKW
M.grisea 210 WLMKILIIGAQGVYFNAMFVAYLISPKICHRFVGYLEEEAVHTYTRSIEELER-GDLPKW
H.anomala 196 WFTRFIIYMGQGVFANLFFLVYLIKPRYCHRFVGYLEEEAVSTYTHLIKDIDS-KRLPKF
P.stipitis 213 WFTRTIIYVGQGVFCNLFFLFYLANPKYCHRFVGYLEEEAVSTYTHFVHELQS-GKLPKF
C.albicans 234 WFTRSIIYIGQGVFTNIFFLVYLMNPRYCHRFVGYLEEEAVRTYTHLIDELDDPNKLPDF
A.thaliana 212 WYERALVISVQGVFFNAYLIGYIISPKFAHRMVGYLEEEAIHSYTEFLKELDN----GNI
Tobacco 236 WYERALVFAVQGVFFNAYFVTYLLSPKLAHRIVGYLEEEAIHSYTEFLKELDK----GNI
S.guttatum 232 WYERALVLAVQGVFFNAYFLGYLLSPKFAHRVVGYLEEEAIHSYTEFLKDIDN----GAI
<aox3
Helix 4
A.nidulans 267 EK--LEAPEIAVKYWKMPEGNRTMKDLLLYVRADEAKHREVNHTLGNLKQAVDVNPFAVE
A.niger 267 EK--TEAPEIAVQYWKMPEGQRSMKDLLLYVRADEAKHREVNHTLGNLNQAIDPNPYAAK
H.capsulatum 272 AN--QPAPDIAVAYWQMPEGKRTILDLLYYIRADEAKHREVNHTLANLKQGVDPNPYAAK
N.crassa 276 SDEKFEIPEMAVRYWRMPEGKRTMKDLIHYIRADEAVHRGVNHTLSNLDQKEDPNPFVSD
M.grisea 269 SDPKFQVPEIAVSYWGMPEGHRTMRDLLLYIRADEANHRGVHHTLGNLNQVEDPNPFVSD
H.anomala 255 DD--VNLPEISWLYWTDLNEKSTFRDLIQRIRADESKHREVNHTLANLEQKKDRNPFALK
P.stipitis 272 EN--IKIPTIAWQYWPELTENSSMLDLILRIRADEAKHREVNHTLANLDQRKDRNPFGLA
C.albicans 294 QK--LPIPNIAVQYWPELTPESSFKDLILRIRADEAKHREINHTFANLEQWQDRNPFALK
A.thaliana 268 EN--VPAPAIAVDYWRLEAD-ATLRDVVMVVRADEAHHRDVNHYAS----------DIH
Tobacco 292 EN--VPAPAIAIDYCRLPKD-STLLDVVLVVRADEAHHRDVNHFAS----------DIH
S.guttatum 288 QD--CPAPAIALDYWRLPQG-STLRDVVTVVRADEAHHRDVNHFAS----------DVH
<aox4
A.nidulans 325 WKDPSKPH--PGKGIKHLKTTGWEREEVV----------
A.niger 325 YKDPTKAH--PNKGIADLKPTGWEREEVI----------
H.capsulatum 330 YDNPEAPH--PTKSAEIVKPTGWERDEVI----------
N.crassa 336 YKEGE-GG--RRPVNPALKPTGFERAEVIG----------
M.grisea 329 YK----GD--KPRPVAASRPEGFEREEVIGKEVIGKEVIEKDVIGKEVLGKQVSV
H.anomala 313 VEDVPKEQQPDEYSLKTPHPEGWNREQMRL----------
P.stipitis 330 IPDLKEPQ--PESGLKVTKPHGWEKEELKL----------
C.albicans 352 IKDSDKPQ--PNYNLDVTRPQGWERKDLYL----------
A.thaliana 314 YQ--------GHELKEAPAPIGYH----------
Tobacco 338 YQ--------GQQLKDSPAPIGYH----------
S.guttatum 334 YQ--------DLELKTTPAPLGYH----------

to a possible transmembrane domain (AOX1), a highly conserved sequence region (AOX2) and putative iron-binding motifs (AOX3 and AOX4). These conserved regions were found in all of the fungal AOX sequences analysed (Figure 4.6). Amino acid residues which have been suggested to act as metal ligands in the AOX active site are present within all the fungal AOX sequences analysed, including the complete *A. nidulans* AOX (Figure 4.6). This includes the highly conserved Glu-270 residue, which has been implicated in the correct functioning of the enzyme's active site (section 1.4.3; Figure 4.6). The suggested helices (and hydrophobic linker) which form the AOX active site are also illustrated in Figure 4.6 (Joseph-Horne *et al.*, 2001).

BLAST analysis (section 2.11.3) determined the complete *A. nidulans* AOX amino acid sequence to have quite a high degree of identity to other AOX sequences: *A. niger* (74%); *Histoplasma capsulatum* (67%), *Magnaporthe grisea* (55%), *N. crassa* (52%); *C. albicans* (52%); *P. stipitis* (52%); *H. anomala* (48%) (Figure 4.6). The complete *A. nidulans* AOX sequence also exhibited some degree of identity to AOX sequences from various plant species: *S. guttatum* (48%); *Nicotiana tabacum* (Tobacco) (54%), and *Arabidopsis thaliana* (45%) (Figure 4.6).

Although the plant AOX sequences also contained the conserved regions (corresponding to the annealing sites for primers AOX1-4) and the proposed metal ligands (Figure 4.6), one important difference was noted between the fungal and plant AOX sequences. Plant AOX sequences contain a highly conserved cysteine residue which is the likely binding site of pyruvate, and has been implicated in the dimerisation process (Joseph-Horne *et al.*, 2001; Joseph-Horne *et al.*, 2000). As mentioned previously, fungal AOX activity is unaffected by pyruvate regulation, and the proteins are considered to function as monomers (section 1.4.4). Thus, it is unsurprising that the conserved cysteine residue is present within all of the plant AOX sequences in the alignment, yet absent from the fungal AOX sequences (Figure 4.6). It has been suggested that the lack of pyruvate regulation in fungal AOX is in keeping with the constitutive activity of the alternative respiratory pathway observed in many fungi (Joseph-Horne *et al.*, 2001).

4.4.4 ANALYSIS OF THE *A. NIDULANS AOX* GENE PROMOTER

The 1.99 kb region upstream of the *A. nidulans AOX* coding sequence (i.e. the promoter) (Figure 4.4 A) was examined to determine the presence of any putative regulatory elements. Possible TATA and CCAAT boxes were identified at -137 and -783 bp, respectively (i.e.

upstream of the translational start site) (Figure 4.4 A). As mentioned previously (section 1.5.1), the TATA element may possibly be involved in determining the transcriptional efficiency of this gene. The putative CCAAT element could be the target site for the AnCF complex, thus contributing to the possible carbon source-controlled regulation of the AOX gene (section 4.1.2). A number of pyrimidine-rich sequences are also present within the *A. nidulans AOX* gene promoter, which may be involved in transcription initiation (section 1.5.1) (Figure 4.4 A). Alternatively, the presence of these sequences could suggest the *A. nidulans AOX* gene is strongly transcribed (section 1.5.1; Jacobs & Stahl, 1995). The putative transcriptional start site was determined by the consensus sequence (A/GCTGTTC) (Elder, 1992).

A number of putative CREA sites can also be observed in the promoter region of the complete *A. nidulans AOX* sequence (Figure 4.4 A). Those sequences located at -1941 and -1713 bp fit the consensus sequence for the CREA site (section 1.6.2). Other putative CREA sites located at -994, -774, -286, -248 and -224 bp contain one nucleotide alteration of the consensus sequence. Another sequence, homologous to the STRE (stress response element) is also located within the *A. nidulans AOX* promoter, at -445 bp (Figure 4.4 A) (sections 4.1.2 & 1.6.2).

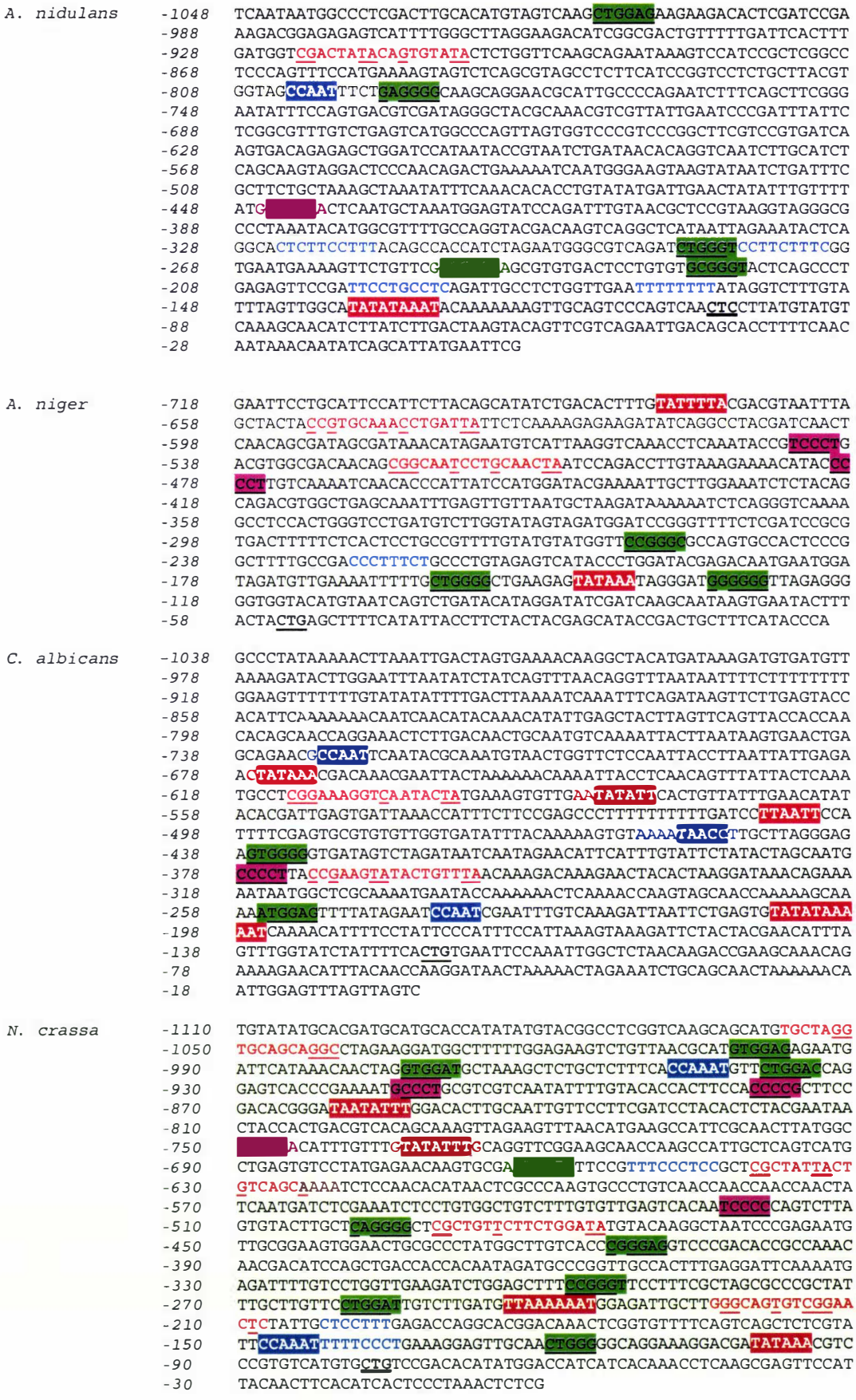
A putative binding site for a HAP1-like transcription factor was also identified within the *A. nidulans AOX* promoter sequence. This sequence, located at -902 bp, contains some similarity to the consensus HAP1 binding site (Figures 4.4 A, 1.5). As mentioned previously, HAP1 mediates oxygen and heme regulation, and is involved in the regulation of many respiratory-related genes in *S. cerevisiae* (section 1.8).

An investigation of the promoter regions (i.e. sequence upstream of the translational start site) of other fungal *AOX* genes was carried out, to determine the presence of putative regulatory elements. Similar to the promoter region of the *A. nidulans AOX* gene, the *AOX* gene promoter regions from *A. niger*, *N. crassa* and *C. albicans* were all found to contain putative CCAAT and TATA boxes, in addition to potential *cre*A and HAP1 binding sites, STRE elements, and pyrimidine-rich sequences (Figure 4.7). Therefore, it is possible that similar molecular mechanisms may control the expression of *AOX* genes from *A. nidulans*, *A. niger*, *N. crassa* and *C. albicans*.

Figure 4.7 Fungal *AOX* gene promoter sequences

The *AOX* gene sequences (upstream of the translational start site) from *A. niger* (Ac. No. AB016540), *N. crassa* (Ac. No. L46869) and *C. albicans* (Ac. No. AF031229) are shown.

The putative transcriptional start sites (in bold) are underlined; the sequence upstream of this is denoted the *AOX* gene promoter. Putative TATA and CCAAT boxes are highlighted in red and blue, respectively. Blue and red font indicate pyrimidine-rich sequences and putative HAP1 binding sites (respectively). Possible *cre*A and STRE sites are indicated by green and pink highlighting, respectively.


A. nidulans
-1048 TCAATAATGGCCCTCGACTTGCACATGTAGTCAAGCTGGAGAAGAAGACACTCGATCCGA
-988 AAGACGGAGAGAGTCATTTTGGGCTTAGGAAGACATCGGCGACTGTTTTTGATTCACTTT
-928 GATGGTCGACTATACAGTGTATACTCTGGTTCAAGCAGAATAAAGTCCATCCGCTCGGCC
-868 TCCCAGTTTCCATGAAAAGTAGTCTCAGCGTAGCCTCTTCATCCGGTCCTCTGCTTACGT
-808 GGTAGCCAATTTCTGAGGGGCAAGCAGGAACGCATTGCCCCAGAATCTTTCAGCTTCGGG
-748 AATATTTCCAGTGACGTCGATAGGGCTACGCAAACGTCGTTATTGAATCCCGATTTATTC
-688 TCGGCGTTTGTCTGAGTCATGGCCCAGTTAGTGGTCCCGTCCCGGCTTCGTCCGTGATCA
-628 AGTGACAGAGAGCTGGATCCATAATACCGTAATCTGATAACACAGGTCAATCTTGCATCT
-568 CAGCAAGTAGGACTCCCAACAGACTGAAAAATCAATGGGAAGTAAGTATAATCTGATTTC
-508 GCTTCTGCTAAAGCTAAATATTTCAAACACACCTGTATATGATTGAACTATATTTGTTTT
-448 ATGNNNNNACTCAATGCTAAATGGAGTATCCAGATTTGTAACGCTCCGTAAGGTAGGGCG
-388 CCCTAAATACATGGCGTTTTGCCAGGTACGACAAGTCAGGCTCATAATTAGAAATACTCA
-328 GGCACTCTTCCTTTACAGCCACCATCTAGAATGGGCGTCAGATCTGGGTCCTTCTTTCGG
-268 TGAATGAAAAGTTCTGTTCGNNNNNNNAGCGTGTGACTCCTGTGTGCGGGGTACTCAGCCCT
-208 GAGAGTTCCGATTCCTGCCTCAGATTGCCTCTGGTTGAATTTTTTTTATAGGTCTTTGTA
-148 TTTAGTTGGCATATATAAATACAAAAAAAGTTGCAGTCCCAGTCAACTCCTTATGTATGT
-88 CAAAGCAACATCTTATCTTGACTAAGTACAGTTCGTCAGAATTGACAGCACCTTTTCAAC
-28 AATAAACAATATCAGCATTATGAATTCG
A. niger
-718 GAATTCCTGCATTCCATTCTTACAGCATATCTGACACTTTGTATTTTACGACGTAATTTA
-658 GCTACTACCGTGCAAACCTGATTATTCTCAAAAGAGAAGATATCAGGCCTACGATCAACT
-598 CAACAGCGATAGCGATAAACATAGAATGTCATTAAGGTCAAACCTCAAATACCGTCCCTG
-538 ACGTGGCGACAACAGCGGCAATCCTGCAACTAATCCAGACCTTGTAAAGAAAACATACCC
-478 CCTTGTCAAAATCAACACCCATTATCCATGGATACGAAAATTGCTTGGAAATCTCTACAG
-418 CAGACGTGGCTGAGCAAATTTGAGTTGTTAATGCTAAGATAAAAAATCTCAGGGTCAAAA
-358 GCCTCCACTGGGTCCTGATGTCTTGGTATAGTAGATGGATCCGGGTTTTCTCGATCCGCG
-298 TGACTTTTTCTCACTCCTGCCGTTTTGTATGTATGGTTCCGGGCGCCAGTGCCACTCCCG
-238 GCTTTTGCCGACCCTTTCTGCCCTGTAGAGTCATACCCTGGATACGAGACAATGAATGGA
-178 TAGATGTTGAAAATTTTTGCTGGGGCTGAAGAGTATAAATAGGGATGGGGGGTTAGAGGG
-118 GGTGGTACATGTAATCAGTCTGATACATAGGATATCGATCAAGCAATAAGTGAATACTTT
-58 ACTACTGAGCTTTTCATATTACCTTCTACTACGAGCATACCGACTGCTTTCATACCCA
C. albicans
-1038 GCCCTATAAAAACTTAAATTGACTAGTGAAAACAAGGCTACATGATAAAGATGTGATGTT
-978 AAAAGATACTTGGAATTTAATATCTATCAGTTTAACAGGTTTAATAATTTTCTTTTTTTT
-918 GGAAGTTTTTTTGTATATATTTTGACTTAAAATCAAATTTCAGATAAGTTCTTGAGTACC
-858 ACATTCAAAAAAACAATCAACATACAAACATATTGAGCTACTTAGTTCAGTTACCACCAA
-798 CACAGCAACCAGGAAACTCTTGACAACTGCAATGTCAAAATTACTTAATAAGTGAACTGA
-738 GCAGAACCCCAATTCAATACGCAAATGTAACTGGTTCTCCAATTACCTTAATTATTGAGA
-678 ACTATAAACGACAAACGAATTACTAAAAAACAAAATTACCTCAACAGTTTATTACTCAAA
-618 TGCCTCGGAAAGGTCAATACTATGAAAGTGTTGAATATATTCACTGTTATTTGAACATAT
-558 ACACGATTGAGTGATTAAACCATTTCTTCCGAGCCCTTTTTTTTTTGATCCTTAATTCCA
-498 TTTTCGAGTGCGTGTGTTGGTGATATTTACAAAAAGTGTAAAATAACCTTGCTTAGGGAG
-438 AGTGGGGGTGATAGTCTAGATAATCAATAGAACATTCATTTGTATTCTATACTAGCAATG
-378 CCCCTTACCGAAGTATACTGTTTAACAAAGACAAAGAACTACACTAAGGATAAACAGAAA
-318 AATAATGGCTCGCAAAATGAATACCAAAAAACTCAAAACCAAGTAGCAACCAAAAAGCAA
-258 AAATGGAGTTTTATAGAATCCAATCGAATTTGTCAAAGATTAATTCTGAGTGTATATAAA
-198 AATCAAAACATTTTCCTATTCCCATTTCCATTAAAGTAAAGATTCTACTACGAACATTTA
-138 GTTTGGTATCTATTTTCACTGTGAATTCCAAATTGGCTCTAACAAGACCGAAGCAAACAG
-78 AAAAGAACATTTACAACCAAGGATAACTAAAAACTAGAAATCTGCAGCAACTAAAAAACA
-18 ATTGGAGTTTAGTTAGTC
N. crassa
-1110 TGTATATGCACGATGCATGCACCATATATGTACGGCCTCGGTCAAGCAGCATGTGCTAGG
-1050 TGCAGCAGGCCTAGAAGGATGGCTTTTTGGAGAAGTCTGTTAACGCATGTGGAGAGAATG
-990 ATTCATAAACAACTAGGTGGATGCTAAAGCTCTGCTCTTTCACCAAATGTTCTGGACCAG
-930 GAGTCACCCGAAAATGCCCTGCGTCGTCAATATTTTGTACACCACTTCCACCCCGCTTCC
-870 GACACGGGATAATATTTGGACACTTGCAATTGTTCCTTCGATCCTACACTCTACGAATAA
-810 CTACCACTGACGTCACAGCAAAGTTAGAAGTTTAACATGAAGCCATTCGCAACTTATGGC
-750 NNNNNACATTTGTTTGTATATTTGCAGGTTCGGAAGCAACCAAGCCATTGCTCAGTCATG
-690 CTGAGTGTCCTATGAGAACAAGTGCGANNNNNTTCCGTTTCCCTCCGCTCGCTATTACT
-630 GTCAGCAAAATCTCCAACACATAACTCGCCCAAGTGCCCTGTCAACCAACCAACCAACTA
-570 TCAATGATCTCGAAATCTCCTGTGGCTGTCTTTGTGTTGAGTCACAATCCCCCAGTCTTA
-510 GTGTACTTGCTCAGGGGCTCGCTGTTCTTCTGGATATGTACAAGGCTAATCCCGAGAATG
-450 TTGCGGAAGTGGAACTGCGCCCTATGGCTTGTCACCCGGGAGGTCCCGACACCGCCAAAC
-390 AACGACATCCAGCTGACCACCACAATAGATGCCCGGTTGCCACTTTGAGGATTCAAAATG
-330 AGATTTTGTCCTGGTTGAAGATCTGGAGCTTTCCGGGTTCCTTTCGCTAGCGCCCGCTAT
-270 TTGCTTGTTCCTGGATTGTCTTGATGTTAAAAAAATGGAGATTGCTTGGGCAGTGTCGGAA
-210 CTCTATTGCTCCTTTGAGACCAGGCACGGACAAACTCGGTGTTTTCAGTCAGCTCTCGTA
-150 TTCCAAATTTTTCCCTGAAAGGAGTTGCAACTGGGGGCAGGAAAGGACGATATAAACGTC
-90 CCGTGTCATGTGCTGTCCGACACATATGGACCATCATCACAAACCTCAAGCGAGTTCCAT
-30 TACAACTTCACATCACTCCCTAAACTCTCG

The promoter regions of the catalase (*catA-C*) and superoxide dismutase (*sod1*) genes of *A. nidulans* were also investigated, to determine the presence of putative regulatory elements. These genes encode proteins which are involved in detoxification of ROS. The promoter regions of these genes were found to contain putative regulatory elements which were analogous to those identified within the fungal *AOX* genes (see above) (Figure 4.8). The alternative respiratory pathway has been recently suggested to have evolved as a mechanism to reduce ROS (section 1.4.2). Therefore, these results suggest that coordinate regulation of the genes involved in ROS detoxification may be occurring in *A. nidulans*.

4.5 EXPRESSION STUDY OF THE *A. NIDULANS* ALTERNATIVE OXIDASE GENE

The expression of the *AOX* gene in *A. nidulans* was investigated by RT-PCR, using primers designed to the 347 bp fragment described in section 4.3.

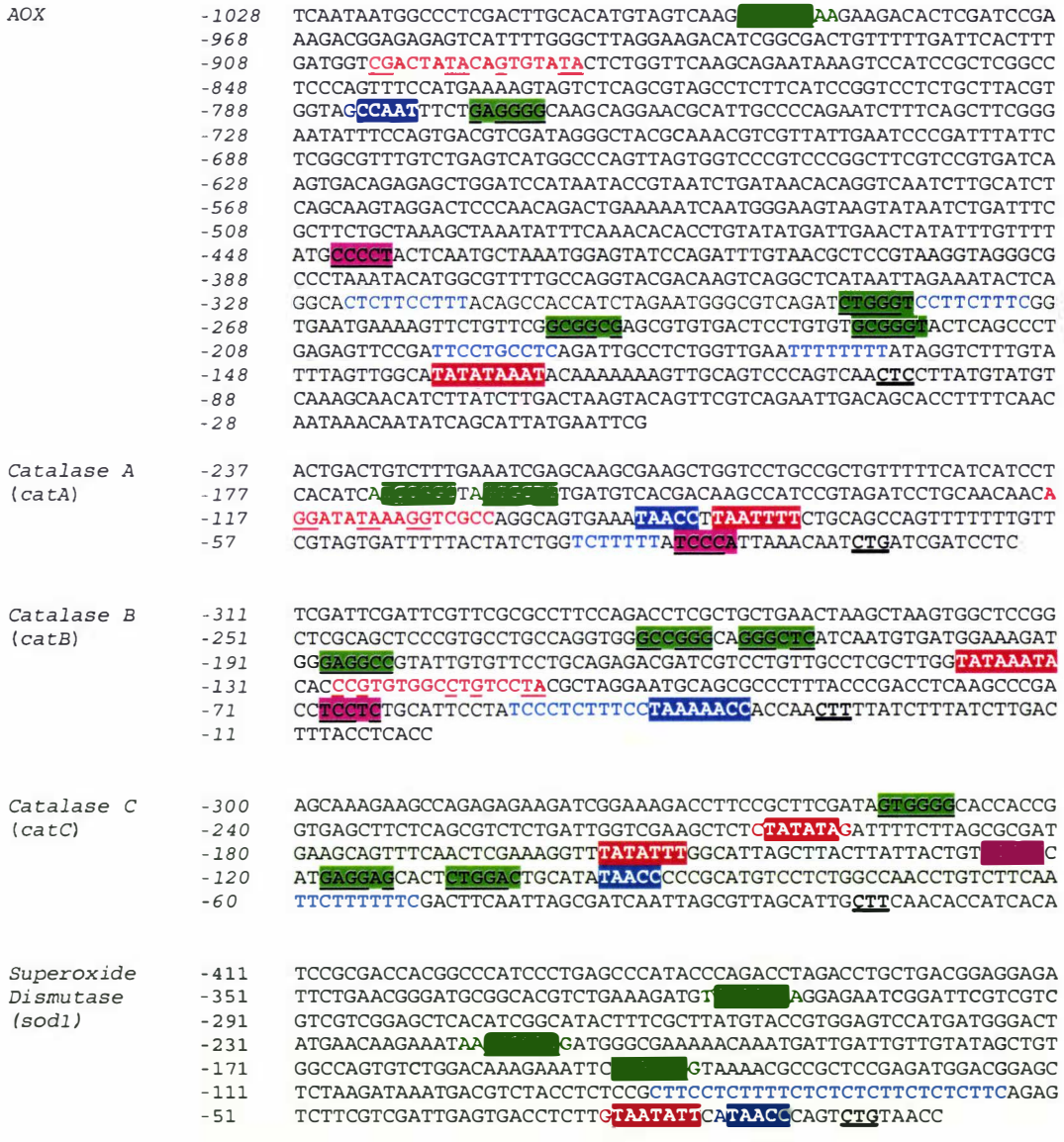
Previous studies had reported that an increase in mRNA transcripts was typically accompanied by an increase in AOX protein (McCabe *et al.*, 1998). The amount of AOX protein can sometimes be higher than the activity of the alternative respiratory pathway (due to regulatory factors discussed in section 1.4.4), although the two do generally correlate. For example, in *N. crassa* induction of the pathway's activity appears to be regulated by variations in the electron flux through the cytochrome pathway (Li *et al.*, 1996). Mutants were obtained which had differing degrees of impaired electron flow through the cytochrome pathway. The levels of *AOX* mRNA transcript from these different mutants were compared, and an increased level of *AOX* gene expression was observed in the mutant with the most highly defective cytochrome pathway.

It was therefore postulated that the activity of the alternative pathway may be greater in the *cyc*A$^-$ strain, to compensate for the non-functioning cytochrome-*c* dependent pathway (section 3.5.2), and that this may be reflected in the different levels of *AOX* gene expression, detectable by RT-PCR. Such an estimation could not be determined from the oxygen consumption assays, due to the limitations of that procedure (section 3.5.3).

Figure 4.8 ***A. nidulans* gene promoter sequences**

The promoter gene sequences (upstream of the translational start site) from the *A. nidulans* catalase genes: *catA* (Ac. No. U37803); *catB* (Ac. No. U80672; and *catC* (Ac. No. AF316033) are shown, along with the promoter gene sequences from the *A. nidulans* superoxide dismutase (*sod1*; Ac. No. AF062654) and *AOX* (Ac. No. AB039832) genes.

The putative transcriptional start sites (in bold) are underlined; the sequence upstream of this is denoted the *AOX* gene promoter. Putative TATA and CCAAT boxes are highlighted in red and blue, respectively. Blue and red font indicate pyrimidine-rich sequences and putative HAP1 binding sites (respectively). Possible *cre*A and STRE sites are indicated by green and pink highlighting, respectively.


AOX
-1028 TCAATAATGGCCCTCGACTTGCACATGTAGTCAAG AAGAAGACACTCGATCCGA
-968 AAGACGGAGAGAGTCATTTTGGGCTTAGGAAGACATCGGCGACTGTTTTTGATTCACTTT
-908 GATGGTCGACTATACAGTGTATACTCTGGTTCAAGCAGAATAAAGTCCATCCGCTCGGCC
-848 TCCCAGTTTCCATGAAAAGTAGTCTCAGCGTAGCCTCTTCATCCGGTCCTCTGCTTACGT
-788 GGTAGCCAATTTCTGAGGGGCAAGCAGGAACGCATTGCCCCAGAATCTTTCAGCTTCGGG
-728 AATATTTCCAGTGACGTCGATAGGGCTACGCAAACGTCGTTATTGAATCCCGATTTATTC
-688 TCGGCGTTTGTCTGAGTCATGGCCCAGTTAGTGGTCCCGTCCCGGCTTCGTCCGTGATCA
-628 AGTGACAGAGAGCTGGATCCATAATACCGTAATCTGATAACACAGGTCAATCTTGCATCT
-568 CAGCAAGTAGGACTCCCAACAGACTGAAAAATCAATGGGAAGTAAGTATAATCTGATTTC
-508 GCTTCTGCTAAAGCTAAATATTTCAAACACACCTGTATATGATTGAACTATATTTGTTTT
-448 ATGCCCCTACTCAATGCTAAATGGAGTATCCAGATTTGTAACGCTCCGTAAGGTAGGGCG
-388 CCCTAAATACATGGCGTTTTGCCAGGTACGACAAGTCAGGCTCATAATTAGAAATACTCA
-328 GGCACTCTTCCTTTACAGCCACCATCTAGAATGGGCGTCAGATCTGGGTCCTTCTTTCGG
-268 TGAATGAAAAGTTCTGTTCGGCGGCGAGCGTGTGACTCCTGTGTGCGGGTACTCAGCCCT
-208 GAGAGTTCCGATTCCTGCCTCAGATTGCCTCTGGTTGAATTTTTTTTATAGGTCTTTGTA
-148 TTTAGTTGGCATATATAAATACAAAAAAAGTTGCAGTCCCAGTCAACTCCTTATGTATGT
-88 CAAAGCAACATCTTATCTTGACTAAGTACAGTTCGTCAGAATTGACAGCACCTTTTCAAC
-28 AATAAACAATATCAGCATTATGAATTCG
Catalase A
(catA)
-237 ACTGACTGTCTTTGAAATCGAGCAAGCGAAGCTGGTCCTGCCGCTGTTTTTCATCATCCT
-177 CACATCA TGATGTCACGACAAGCCATCCGTAGATCCTGCAACAACA
-117 GGATATAAAGGTCGCCAGGCAGTGAAATAACCTTAATTTTCTGCAGCCAGTTTTTTTGTT
-57 CGTAGTGATTTTTACTATCTGGTCTTTTTTATCCCATTAAACAATCTGATCGATCCTC
Catalase B
(catB)
-311 TCGATTCGATTCGTTCGCGCCTTCCAGACCTCGCTGCTGAACTAAGCTAAGTGGCTCCGG
-251 CTCGCAGCTCCCGTGCCTGCCAGGTGGGCCGGGCAGGGCTCATCAATGTGATGGAAAGAT
-191 GGGAGGCCGTATTGTGTTCCTGCAGAGACGATCGTCCTGTTGCCTCGCTTGGTATAAATA
-131 CACCCGTGTGGCCTGTCCTACGCTAGGAATGCAGCGCCCTTTACCCGACCTCAAGCCCGA
-71 CCTCCTCTGCATTCCTATCCCTCTTTCCTAAAAACCACCAACTTTTATCTTTATCTTGAC
-11 TTTACCTCACC
Catalase C
(catC)
-300 AGCAAAGAAGCCAGAGAGAAGATCGGAAAGACCTTCCGCTTCGATAGTGGGGCACCACCG
-240 GTGAGCTTCTCAGCGTCTCTGATTGGTCGAAGCTCTCTATATAGATTTTCTTAGCGCGAT
-180 GAAGCAGTTTCAACTCGAAAGGTTTATATTTGGCATTAGCTTACTTATTACTGT C
-120 ATGAGGAGCACTCTGGACTGCATATAACCCCCGCATGTCCTCTGGCCAACCTGTCTTCAA
-60 TTCTTTTTTCGACTTCAATTAGCGATCAATTAGCGTTAGCATTGCTTCAACACCATCACA
Superoxide
Dismutase
(sod1)
-411 TCCGCGACCACGGCCCATCCCTGAGCCCATACCCAGACCTAGACCTGCTGACGGAGGAGA
-351 TTCTGAACGGGATGCGGCACGTCTGAAAGATGT AGGAGAATCGGATTCGTCGTC
-291 GTCGTCGGAGCTCACATCGGCATACTTTCGCTTATGTACCGTGGAGTCCATGATGGGACT
-231 ATGAACAAGAAATAA GATGGGCGAAAAACAAATGATTGATTGTTGTATAGCTGT
-171 GGCCAGTGTCTGGACAAAGAAATTC GTAAAACGCCGCTCCGAGATGGACGGAGC
-111 TCTAAGATAAATGACGTCTACCTCTCCGCTTCCTCTTTTCTCTCTCTTCTCTCTTCAGAG
-51 TCTTCGTCGATTGAGTGACCTCTTGTAATATTCATAACCCAGTCTGTAACC

4.5.1 PREPARATION OF RNA SAMPLES

RNA samples were obtained from *cycA*$^+$ (A67) and *cycA*$^-$ (A68) strains, grown to late-exponential and stationary timepoints (the former in the presence/absence of SHAM/Cyanide). The growth of the cultures and confirmation of the time-points were carried out as outlined in section 2.15.1. RNA extractions were carried out (section 2.18.1), and the concentration of the samples estimated (section 2.18.2) prior to DNase treatment (section 2.18.3). The synthesis of cDNA was carried out as detailed in section 2.18.4., prior to amplification of the *AOX* fragment by RT-PCR (section 2.18.5).

4.5.2 PRIMERS FOR RT-PCR AMPLIFICATION

For the RT-PCR procedure, specific primers AnidAOX1 and AnidAOX2 (Table 2.2) were designed which annealed within a 347 bp fragment of the *A. nidulans AOX* gene (Figure 4.4 B), which did not contain an intron. Thus, the size of the PCR product generated from cDNA (approximately 250 bp) was expected to be the same as that produced from genomic DNA. As a positive control, primers were designed which annealed to one of the *A. nidulans* β-tubulin genes (*ben*A) (May *et al.*, 1987).

4.5.3 RESULTS FROM RT-PCR AMPLIFICATION

Most of the samples from both strains generated a PCR product of the expected size with the primers AnidAOX1 and AnidAOX2, as well as with the positive control primers benA1 and benA2 (results not shown). However, PCR products were repeatedly observed in the 'no RT' (negative control) lane for some samples, indicating possible DNA contamination. This was a continuing problem, despite attempts at removing contamination, e.g. using fresh template and additional purification steps (phenol/chloroform extractions). Therefore, the expression of the alternative oxidase in the strains could not be reliably determined from this experiment.

For future expression studies, the amount of mycelia from the *cycA*$^-$ strain used in the RNA extraction could be increased, to obtain a larger amount of RNA, as yield was extremely low. Additional phenol/chloroform extractions and DNase treatments (section 2.18.3) could be carried out to ensure the purity of the samples. It has been suggested that the

reduced quality of the RNA samples obtained from strain A68 could be due to the diminished growth capacity of this strain.

RNA samples could be divided into numerous aliquots, and extreme care would have to be taken to ensure contamination of the samples did not occur. The number of cycles used in the PCR amplification of the cDNA should be decreased (protocols used included 35 and 43 cycles; section 2.18.5) to approximately 25, to ensure non-specific sequences and contaminants are not amplified. Primers which amplify a larger fragment of the *AOX* gene, containing an intron, should also be used, to facilitate easy recognition of DNA contamination.

4.6 CONCLUSION

PCR amplification with degenerate primers was carried out to generate *AOX* fragments from *A. nidulans*. The 347 bp *AOX* fragment exhibited high similarity (98% and 82%) to the complete *A. nidulans* and *A. niger AOX* nucleotide sequences, respectively. The (complete) *A. nidulans AOX* demonstrated high homology to *AOX* sequences from other organisms, particularly *A. niger* (83%). These results confirm the presence of an alternative oxidase gene in *cycA*$^+$ and *cycA*$^-$ strains of *A. nidulans*. The protein encoded by this gene may be located within the alternative respiratory pathway, shown to be present in these strains by oxygen consumption assays (section 3.5.3).

An alignment of fungal and plant AOX sequences indicated that fungal AOX proteins, including the *A. nidulans* AOX, lack the conserved cysteine residue which has been implicated in pyruvate regulation and dimer formation.

Analysis of the *A. nidulans AOX* gene promoter revealed the presence of a number of putative regulatory elements, including a TATA box, pyrimidine-rich sequences and a possible STRE element. Putative CCAAT and *creA* elements identified within the *A. nidulans AOX* gene promoter may be involved in carbon source regulation of the gene. Possible oxygen regulation of the *A. nidulans AOX* gene could be mediated through putative HAP1 binding sites located within the promoter. A functional analysis of the *A. nidulans AOX* gene promoter would determine whether any of these putative regulatory elements are functionally important.

A preliminary investigation of the *AOX* gene promoters from *A. niger*, *N. crassa* and *C. albicans* revealed similar putative regulatory elements to those identified within the *A. nidulans AOX* gene promoter. It is therefore possible that similar mechanisms may regulate *AOX* expression in these fungal species. The mechanisms suggested to control the expression of the *A. nidulans AOX* gene may also be similar to the mechanisms which regulate the expression of other genes involved in ROS detoxification, such as the *A. nidulans catA-C* and *sod-1* genes. It is therefore possible that there is coordinate regulation of the genes involved in guarding against cellular damage by ROS.

An expression study of the *A. nidulans AOX* was attempted using RT-PCR. However, due to contamination problems, it was not possible to quantifiably determine any differences in *AOX* expression between the samples. For future expression studies, it would be interesting to see if changes in the physiological conditions (eg. carbon source, oxygen supply and temperature) have an effect on *AOX* mRNA transcript levels. It is likely that control of *AOX* gene expression by some of these parameters (eg. carbon source) is occuring, given the presence of putative regulatory elements within the *AOX* gene promoter which mediate these regulatory mechanisms.

Determining the presence of the alternative respiratory pathway in *A. nidulans* could have important medical applications. The AOX could be a target for antifungal agents, if it is shown to be important for the virulence and survival of the fungus during infection of a mammalian host (eg. in *Invasive Aspergillosis*, section 1.1.2). Studies are currently underway investigating the role of the alternative respiratory pathway in the pathogenic fungus *Histoplasma capsulatum* (McEwen & Johnson, 1999). Preliminary results in this study have indicated that the alternative respiratory pathway in the fungus may be induced when its cytochrome *c*-dependent pathway is inhibited by oxidative stresses, triggered by the host defenses. Other studies support this suggestion, as the alternative respiratory pathway has been shown to reduce ROS (section 1.4.4).

CHAPTER 5

FUNCTIONAL ANALYSIS OF THE CYTOCHROME *C* GENE (*cycA*) PROMOTER IN *A. NIDULANS*

5.1 INTRODUCTION

A functional analysis of the *A. nidulans* cytochrome *c* (*cycA*) gene promoter was carried out to ascertain the regulatory mechanisms governing expression of the gene. This type of *in vivo* analysis is far more powerful than *in vitro* methods, such as gel mobility shift assays and DnaseI footprinting, as it allows gene expression to be investigated under the true environmental conditions of the organism. A number of studies on filamentous fungal gene expression have been carried out using this technique (Punt *et al.*, 1990; Streatfield *et al.*, 1992; Brakhage *et al.*, 1992).

A system for the analysis of expression signals in *A. nidulans* has been established, which involves the use of integration vectors containing the *lacZ* reporter gene (van Gorcom *et al.*, 1986). Vectors are available which allow the in-phase translational or transcriptional fusion of any gene to *lacZ*. The expression signals can be quantitatively analysed, due to the vectors integrating at a specific chromosomal locus in single copy, eg. the *argB* locus. The transformation efficiency is greatly diminished with this method, eg. studies have reported an efficiency of approximately 1 transformant per μg of DNA (Punt *et al.*, 1990; Brakhage *et al.*, 1992). However, a large proportion of the transformants contain the fusion constructs integrated in single copy at the precise locus. The influence of the chromosomal environment on gene expression is therefore constant, which is of particular importance when a comparison of expression from differently modified promoters is to be carried out (van Gorcom *et al.*, 1986; Streatfield *et al.*, 1992). This was demonstrated in a recent study on *A. parasiticus*, using the GUS (β-glucuronidase) reporter system, where single copy integration into a homologous genomic environment resulted in a higher GUS expression (500- to 1000- fold) than integration into an ectopic site. This positional effect was proposed to be associated with topological structure of the chromatin, regional cis-acting elements, or location-specific trans-acting mechanisms (Liang *et al.*, 1997).

As mentioned previously (section 1.6.2), preliminary studies by Raitt *et al* (1994) indicated that regulation of expression of the *cyc*A gene may be analogous to the well-characterised system in *S. cerevisiae*. A number of putative regulatory elements have been located within the *cyc*A gene promoter (Johnson & Bradshaw, 1998), with particular focus on possible binding sites for the yeast HAP1 transcription factor (section 1.6.1), which is known to mediate oxygen regulation (section 1.8) (Forsburg & Guarente, 1989).

To identify functional regulatory elements, a 1.3 kb fragment of the *cyc*A gene promoter, and progressive deletion fragments, were cloned into a reporter vector, thus forming a translational fusion with the *lacZ* reporter gene. Following single copy integration of the constructs into *A. nidulans* at a site-specific locus, quantitative analysis of the transformants was carried out. Thus it was expected, given the results of previous studies using this system (Streatfield *et al*., 1992; Brakhage *et al*., 1992; Litzha *et al*., 1995), that the *in vivo* function and relative importance of the putative regulatory elements would be indicated by differences in reporter gene expression.

5.2 THE COMPONENTS OF THE REPORTER CONSTRUCTS

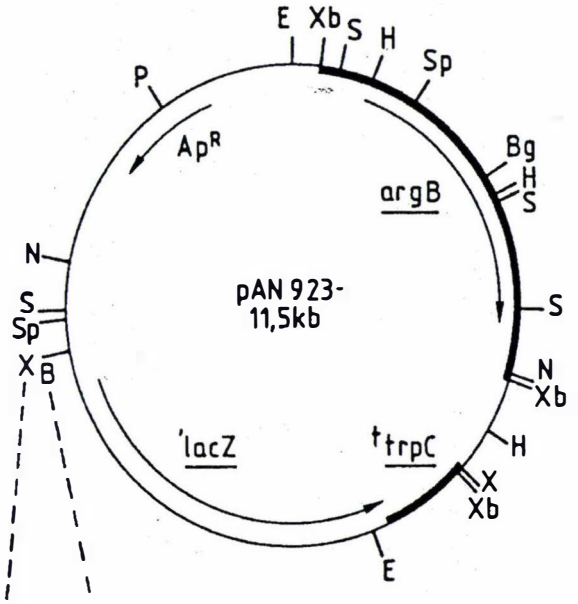
5.2.1 THE REPORTER VECTOR

The reporter vector used in this study, pAN923-42B$_{BglI}$ (R117; Table 2.1 and Figure 5.1), was constructed by Van Gorcom *et al* (1986). The 11.5 kb vector contains a unique *Bam* HI site at the 5' end of the *E.coli lacZ* gene (from which the first 8 codons have been removed). The vector was created as a set of 3; with the *Bam* HI site in three possible translational reading frames (41B, 42B and 43B), thus allowing the in-phase translational fusion of promoter fragments to the *lacZ* gene. The vector also contains an *A. nidulans argB* gene, in which the *Bgl* II site was deleted by Punt *et al* (1990) to create a non-functional gene. The presence of the *argB* gene in R117 should facilitate integration of the reporter constructs at the *argB* locus of an *A. nidulans argB2* strain. Homologous recombination is expected to occur between the two *argB* genes (within R117 and the *A. nidulans argB2* strain) which contain different mutations, thus forming a functional *argB* gene (Figure 5.4 A & B). Subsequent selection of transformant colonies would then be possible, given their *argB*$^+$ phenotype.

Figure 5.1 **The pAN923-42B$_{BglII}$ plasmid (R117), containing the *lacZ* reporter gene**

The whole *cyc*A promoter fragment, and *cyc*A promoter deletion fragments, were cloned into the *Bam* HI (B) site of pAN923-42B$_{BglII}$. Thick lines indicate *A. nidulans* DNA, thin lines indicate *E. coli* DNA. The restriction sites shown are: B, *Bam* HI; Bg, *Bgl* II; E, *Eco* RI; H, *Hind* III; N, *Nru* I; P, *Pst* I; S, *Sal* I; Sp, *Sph* I; Ss, *Sst* I; X, *Xho* I; Xb, *Xba* I.

Reference: Punt *et al* (1990)



X B S P H 'lacZ

41B CT.CGA.GGA.TCC.GTC.GAC.CTG.CAG.CCA.AGC.TTG.GCA.CTG.GCC.GTC.

X B

42B CTC.GAG.GAT.CCC.GTC.

X B E

43B C.TCG.AGG.ATC.CCC.GGG.AAT.TCA.CTG.GCC.GTC.

The control reporter vector (R116; Table 2.1) used in this study contains 2 kb of the *A. nidulans* glyceraldehyde-3-phosphate dehydrogenase (*gpdA*) gene promoter translationally fused to the *lacZ* reporter gene contained in the pAN923-42B$_{BglI}$ vector. The *gpdA* gene encodes an enzyme essential for the glycolytic and gluconeogenic pathways, and is highly expressed (Punt *et al.*, 1990). The *gpdA* gene promoter has been used previously as a control in fungal functional analyses (e.g. Streatfield *et al.*, 1992).

5.2.2 THE *A. NIDULANS cycA* GENE PROMOTER FRAGMENTS

A 1.3 kb fragment selected as the *A. nidulans cycA* gene promoter contains approximately 1257 bp of sequence upstream of the transcriptional start site, and 30 bp of coding sequence (Figure 5.2). A fragment of this size was selected for analysis, as putative regulatory elements had already been identified which were contained within this fragment (Johnson & Bradshaw, 1998). In addition, other functional analysis studies on fungal gene promoters (Streatfield *et al.*, 1992; Brakhage *et al.*, 1992; Litzha *et al.*, 1995) have found similarily-sized promoter fragments to contain most of the functional regulatory elements.

PCR AMPLIFICATION OF THE *cycA* PROMOTER FRAGMENTS

PCR amplification was used to generate the 1.3 kb fragment of the *cycA* gene (hereafter denoted the 'whole *cycA* promoter fragment'), and the progressive deletions of this fragment. The whole *cycA* promoter fragment was PCR amplified using the primers CYCP1 and CYCP2 (Figure 5.2; Table 2.2). For amplification of the five *cycA* promoter deletion fragments (denoted 'CPD1 - 5 fragments'), the primer CYCP1 was used in concert with the appropriate nested primer, CPD1 - 5 (Figure 5.2; Table 2.2). Other commonly used methods for the generation of promoter deletion fragments include digestion with the exonuclease *Bal* 31 (Sambrook *et al.*, 1989). Specific putative regulatory elements are also routinely removed by site-directed mutagenesis.

Each progressive deletion of the whole *cycA* promoter fragment removed a putative regulatory site from the preceding *cycA* fragment, due to the annealing site of the CPD-primer used (Figures 5.2, 5.3). The particular focus of this study was on the three reputed HAP1 binding sites, known as UAS elements in yeast (section 1.7.1). In the *cycA* gene promoter, these putative HAP1 binding sites are located at -634, -669 and -905 bp (ie.

Figure 5.2 The promoter region of the *A. nidulans cycA* gene

The 1.3 kb 'whole *cycA* promoter fragment' used in the study consists of approximately 1257 bp of sequence upstream of the transcriptional start site, and 30 bp of coding sequence. The postulated transcriptional and translational start sites are indicated in bold, with thick and double underlining (respectively). Putative regulatory elements located within the *cycA* gene are represented by the indicated highlighting: CREA elements, green; STRE sites, pink; CCAAT box, blue; TATA boxes, red. Possible HAP1 sites and pyrimidine-rich regions are indicated by red and blue lettering, respectively. Similarities of the putative regulatory elements to the relevant consensus sequences are indicated by underlining. The annealing sites of the primers used for amplification of the *cycA* promoter fragments are indicated. The *Sph* I site contained within the *cycA* promoter is shown by wave underlining.

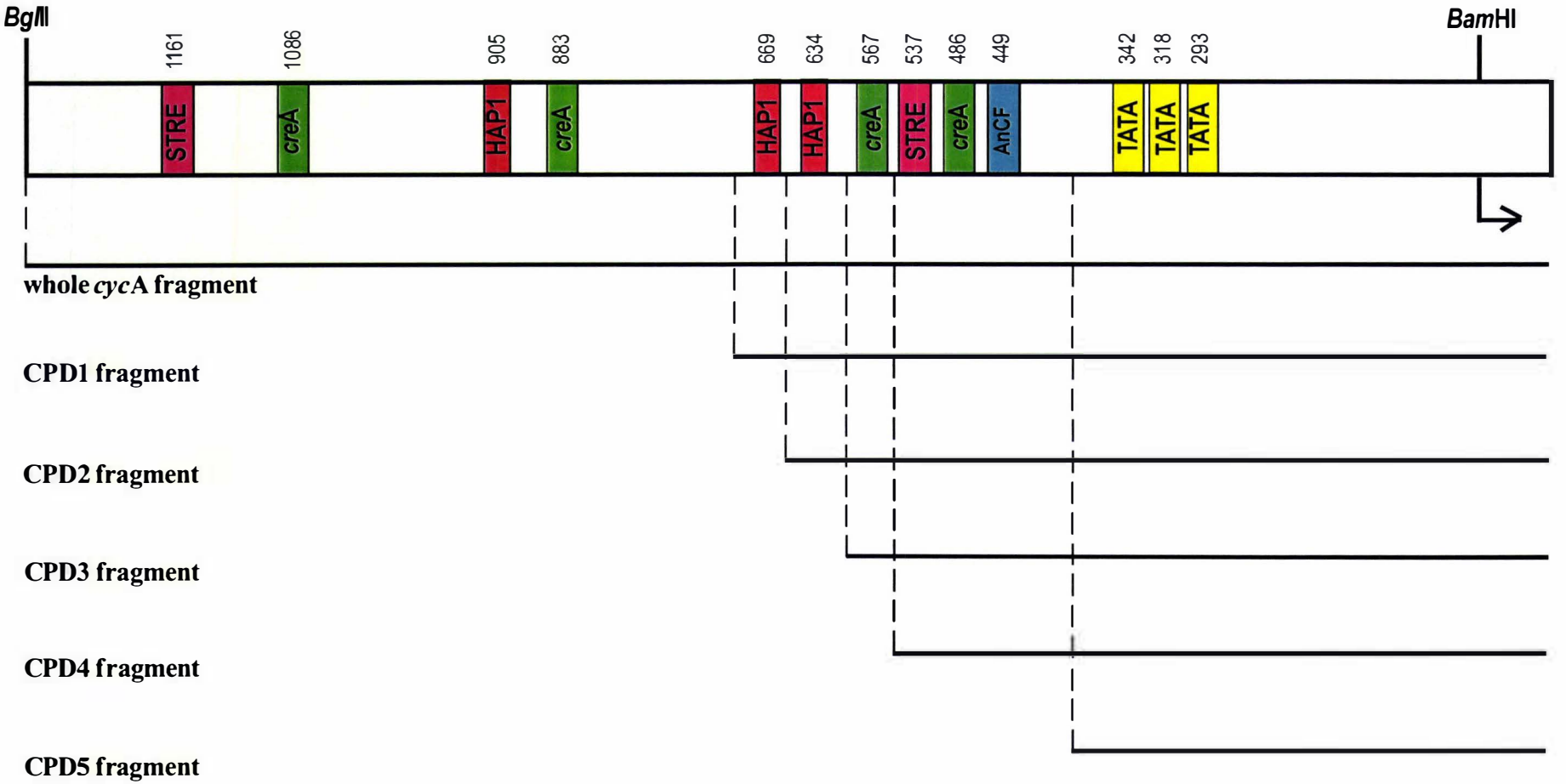
Reference: McGlynn (1997)


YP1>>
-1322 AGCTAAATGTTGCAAGAGATGTTAACACACCCTGCCAACTCACATATTAG -1272
CYCP2>>
-1271 TCACATAGCTCCCAACCCAGAAAGCAGTTTGCGGGTAAATGAGTACGCAC -1221
-1220 AAAAGCAATCCAGACATGAATCCACCGACTCGTCAAAAACCGAAACATGA -1170
-1169 CCGTCGGGCGGGAACATATTCGGGTACTTCTTTTTTGCCCGCTCC -1119
-1118 GCCTCTTCTTTCTCAGAGAACTTGGGATAGTTAACGACTTTACC -1068
-1067 ATTGCGTGTTGGAACGACGCGGCGCGCAGGATGTGAGGGCGGGCGAAATT -1017
-1016 TATCTGGTTGCGCGAGGACGCGCGGATTTTGGCTGTTGTCACTTGAGGAA -966
-965 TTTGTTGATGCGCAGCGGTGCGTCGGTGGGAGCCTGCCGATGCGGGAGGA -915
-914 CCGGGACAGGAATAGGATTGCTCGTGGAACACCTCGGGGAAGGA -864
-863 GGAATGGGGGAATGGCTGATGGGGGCATTCTGTACGAATTGCTGGTTCTT -813
-812 GGCTGCGATTTCTCTATATGCTAGCTTCTGGTCGCCGGCATAACATTTTG -762
CPD1
-761 GTGTTGATATAATCATGTGACTTCTGCCGCCGGGAATAAGGCATCAAGGC -711
>>
CPD2>
-710 ATCAAGGCACAAACACATTTTTTCTAATGGCCGCTAAGGCATCAGGCCAC -660
>
CPD3>>
-659 TTCGGATTAGGGCCGGGGAGAGCGGGAAAAACTCGCCATGACTAGGCGAA -609
-608 TGAAAGGATGCAGATTTGTTATTACGGGGAGGGCTACCTCCGTA -558
CPD4>>
-557 GCCCACCGTTGCCCATTCCCCGAGACAGACAGTGCAGAGCTCCAAGTAAC -507
-506 CAGCGTCTCTATGCGCTGGAATGAGGTCATGCCATGACGGCATGCAGAAT -456
-455 CACCAATCATCTTTTACAGTATAGTAGTTAGGCTCTATGATAGATAGATG -405
CPD5>>
-404 TCATAGAAGGTGCATTGTTGCTATCTAGAGCTGCATAACTGAGCCCTTAG -354
-353 ACGTAGTATATAGGATTACAATAGTCTCTAAATAAAGCTTCATCCAGCCA -303
-302 GGCCTTATTTGCAGTGACCGATTCCTGACCTCAGACCCGGCAGCGCCCGT -252
-251 TACTCTGAGCACAGTGCTGAATCATCCTACCTCTGATTGGTCAATTCCCA -201
<<YP2
-200 GATCACGGGTGTCGTCGGGGGCGCGACCAAGAAACCAGCTCTACAAATTC -150
-149 CCTCCAAGTTTTTTTTCTTCCCTTTGGCCAGTCCGCTTGACTTGAATTCTG -99
-98 TCTTTCATCTTCTCTGCTACATACAACTCTTTGTACTATACCACTTACCT -48
-47 CTTTACATAACCCTTTCTCTCTACCTCTTTATTTTTTATCACTCACAATG +2
<<CYCP1
+3 GCTAAGGGCGGTGACAGCTACTCTCCTGGTAAGTAGTTTGAATTCATCTC +53

Figure 5.3 **Putative regulatory elements contained within the *cyc*A promoter fragments**

The schematic diagram indicates the putative regulatory elements contained with the whole *cyc*A promoter fragment, and the promoter deletion fragments (CPD1 - 5 fragments). The locations of the putative regulatory sites are indicated (in bp), upstream of the transcriptional start site.

Note:
Whole *cyc*A fragment: 1.3 kb *cyc*A promoter cloned in plasmid R117 (plasmid R159)
CPD1 fragment: 0.74 kb *cyc*A promoter cloned in plasmid R117 (plasmid R169)
CPD2 fragment: 0.68 kb kb *cyc*A promoter cloned in plasmid R117 (plasmid R170)
CPD3 fragment: 0.65 kb kb *cyc*A promoter cloned in plasmid R117 (plasmid R171)
CPD4 fragment: 0.56 kb kb *cyc*A promoter cloned in plasmid R117 (plasmid R172)
CPD5 fragment: 0.4 kb *cyc*A promoter cloned in plasmid R117 (plasmid R173)


BglII
BamHI
1161
1086
905
883
669
634
567
537
486
449
342
318
293
STRE
creA
HAP1
creA
HAP1
HAP1
creA
STRE
creA
AnCF
TATA
TATA
TATA
whole cycA fragment
CPD1 fragment
CPD2 fragment
CPD3 fragment
CPD4 fragment
CPD5 fragment

upstream of the transcriptional start site) (McGlynn, 1997). The *cyc*A promoter fragments containing these, and other possible regulatory sites, are displayed in Figures 5.2 and 5.3.

The CPD1 – 3 fragments all contain sequence which bear considerable homology to the yeast 'consensus' binding site (CGGNNNTANCGGNNNTA) for the HAP1 protein (Figures 5.2 & 1.5). As mentioned previously, this consensus sequence was derived from an alignment of various yeast UAS1 elements (Figure 1.7 B). However, the putative HAP1 binding site positioned at -634 bp is considered to display the best match to the yeast consensus sequence (Figure 1.5). As discussed in section 1.8.1, the conservation of the CGG triplets has been found to be extremely important for the correct binding of the HAP1 protein. Similarly, the TA repeats, especially the second nucleotide pair, are highly important for binding and subsequent activation by the transcription factor (Zhang & Guarente, 1996). The putative UAS1 element (located at -634 bp) possesses both CGG triplets, in addition to both adenine bases in each of the two nucleotide pairs. Therefore, this sequence is the most likely candidate for a putative HAP1 binding site.

5.3 PREPARATION OF THE REPORTER CONSTRUCTS

5.3.1 OVERVIEW OF THE CLONING STRATEGY

The restriction endonuclease sites contained within the PCR primers (CYCP1: *Bam* HI; CYCP2: *Pst* I; CPD1 – 5: *Xho* I) (Table 2.2) facilitated the cloning of the PCR-amplified *cyc*A promoter fragments.

The initial cloning strategy involved the ligation of the *Xho* I-*Bam* HI PCR products into *Xho* I-*Bam* HI digested R117. The *Xho* I and *Bam* HI restriction sites in R117 actually overlap by 1 bp (Figure 5.1). Control experiments determined that the *Bam* HI and *Xho* I sites in R117 were too proximal to allow both sites to be intact, and suitable for cloning, following cleavage with both enzymes.

Thus, an alternative cloning strategy was devised. The PCR amplified *cyc*A promoter fragments were cloned into R12 (Appendix 3.1), then cleaved with *Bam* HI and *Bgl* II, prior to being ligated into *Bam* HI-linearised R117 (Figure 5.1). Once ligated into R117, the *cyc*A promoter fragments were checked for correct orientation and translational fusion.

5.3.2 METHODOLOGY

Plasmid DNA was generally prepared with a small scale alkaline lysis protocol (section 2.5.2) for routine digestions (2.7.1), ligations (section 2.9.2), etc. However, for fungal transformations, large scale preparations of plasmid DNA were carried out with a QIAGEN Midi plasmid preparation kit (section 2.5.3). For PCR amplification of DNA, a standard PCR set-up and thermal cycling programme (section 2.8) was used. The purification of PCR products was generally carried out with the QIAGEN PCR Purification kit (section 2.8.3). Digestion products were routinely purified by gel extraction, using a QIAGEN gel extraction kit (section 2.7.4). The ligation procedure used is described in section 2.9.2.

5.3.3 THE REPORTER CONSTRUCT

Plasmid R117 was linearised with *Bam* HI, then purified by gel extraction; this was required prior to SAP treatment (section 2.9.1), which was carried out to prevent re-ligation of the vector.

5.3.4 THE *A. NIDULANS cycA* GENE PROMOTER FRAGMENTS

The amplification of the whole *cycA* promoter fragment was carried out (section 5.2.2), with genomic DNA (from strain 1-85) or overlapping plasmids R41 and R133 (Table 2.1) as templates. The latter were required as there was no plasmid available which contained all of the necessary sequence. Once amplified, the PCR product was purified and re-digested with *Bam* HI and *Pst* I. The digested fragment was purified by gel extraction, then ligated into the R12 plasmid to form the plasmid R155 (Table 2.1). Automated sequencing (section 2.13.1) of the whole promoter fragment in both directions was carried out with the LM6, lacZ forward and lacZ reverse primers (Table 2.2).

The promoter deletion fragments were amplified (section 5.2.2) using R155 as a template (Table 2.1), thus generating '*cycA* promoter fragments 1 – 5' (Figure 5.3). The fragments were purified by gel extraction and digested with *Bam* HI and *Xho* I. The fragments were then ligated into the *Bam* HI-*Xho* I cleaved R12 plasmid, thus forming plasmids R164 - R168 (Table 2.1).

All *cycA* promoter fragments were cleaved from R12 with the restriction enzymes *Bam* HI and *Bgl* II, and purified by gel extraction. The fragments were ligated into the *Bam* HI linearised R117 reporter vector (section 5.3.3), forming the plasmids R159 and R169 - R173 (Table 2.1). The correct orientations of the fragments were checked by *Xho* I-*Bam* HI digestion. Correct translational fusion was confirmed by automated sequencing with the CPD5 and lacZ forward primers (Table 2.2) (Appendix 3.2).

5.4 TRANSFORMATION OF THE REPORTER CONSTRUCTS INTO *A. NIDULANS*

The *A. nidulans* mutant *argB2* strain (A71; Table 2.1), was transformed with the reporter constructs as described in section 2.10.2.

Homologous recombination of the constructs at the chromosomal *argB* locus was expected to result in the growth of colonies on media lacking arginine. Controls for the transformation experiments included the plasmid R108, which contains an intact *argB* gene, and R117 (without *cycA* promoter fragment) (Table 2.1). The transformation efficiency observed for a reaction involving R108 would be expected to be higher than for a reaction involving one of the reporter constructs (Table 5.1), as an integration event is more likely due to the presence of the intact *argB* gene contained within R108. Ectopic integration will also possibly occur for transformations with R108, while the reporter constructs are expected to only undergo targeted integration at the *argB* locus.

Table 5.1 Transformation of the *argB2* strain of *A. nidulans*

Transformed Construct	Promoter Fragment	*argB* gene	Transformants per μg DNA	Transformants per viable protoplast
R108	n/a*	Whole	2.4	6.64×10^{-5}
R159	1.3 kb *cycA*	Mutant (*Bgl* II)	0.4	2.45×10^{-6}
R169	0.74 kb *cycA*	Mutant (*Bgl* II)	0.5	1.54×10^{-5}
R170	0.68 kb *cycA*	Mutant (*Bgl* II)	0.3	7.45×10^{-6}
R171	0.65 kb *cycA*	Mutant (*Bgl* II)	0.15	7.17×10^{-7}
R172	0.56 kb *cycA*	Mutant (*Bgl* II)	0.3	9.09×10^{-7}
R173	0.4 kb *cycA*	Mutant (*Bgl* II)	0.4	1.31×10^{-6}
R116	2 kb *gpdA*	Mutant (*Bgl* II)	0.5	8.2×10^{-7}
R117	n/a	Mutant (*Bgl* II)	0.5	8.2×10^{-7}

Note: The results given in the table above are averages (* n/a = not applicable). Raw data for the table can be found in Appendix 3.3.

As expected, the transformation efficiency with R108 was higher than for the reporter constructs (2.4 and 0.15 – 0.5 transformants per μg DNA, respectively) (Table 5.1). All transformants were spore purified twice, prior to being plated out for spore suspensions (section 2.4.2).

5.5 ANALYSIS BY SOUTHERN BLOTTING

5.5.1 STRATEGY FOR SOUTHERN BLOTTING ANALYSIS

DNA extractions were generally carried out using the Byrd method (section 2.6.2), although the small scale DNA extraction method was also used at times (section 2.6.1).

The concentration of the samples was estimated using a fluorometer (section 2.7.5), prior to Southern blotting (section 2.12). Preparation of the radioactive probe and subsequent hybridisation were carried out as detailed in section 2.12.

The restriction pattern around the *arg*B locus of *A. nidulans* has been characterised (Upshall, 1986), so the sizes of the restriction fragments resulting from integrated plasmids can be estimated. Homologous recombination of the constructs into the *arg*B locus of the strain was therefore anticipated to occur as illustrated in Figure 5.4.

To determine integration of the reporter constructs at the *arg*B locus, and the presence of the plasmid, genomic DNA from *arg*B$^+$ transformants was cleaved with *Bam* HI and subsequently analysed by Southern blotting as described above. The Southern blots were probed with a 1 kb *Sal*I fragment of the *A. nidulans arg*B gene, which was obtained by digestion of the R117 vector and purified by gel extraction (section 2.7.4). An untransformed *A. nidulans* strain was expected to produce a single band of approximately 8 kb (Brakhage *et al.*, 1992). Homologous recombination of the constructs into the *arg*B locus of the strain was predicted to occur as shown in Figure 5.4 A. *Bam* HI linearises the reporter constructs at the 5' end of the *lacZ* reporter gene (Figure 5.1). Single copy integration of the construct (with the 1.3 kb *cyc*A fragment) was estimated to result in two *Bam* HI hybridisation fragments of approximately 7 and 14 kb in size (Figure 5.4 A). Double and multiple copy integration events at the *arg*B locus were predicted to result in the two *Bam* HI hybridisation fragments of approximately 7 and 14 kb, as well as another

Figure 5.4 **Diagrammatic Representation of Homologous Recombination of the Reporter Constructs at the *arg*B Locus**

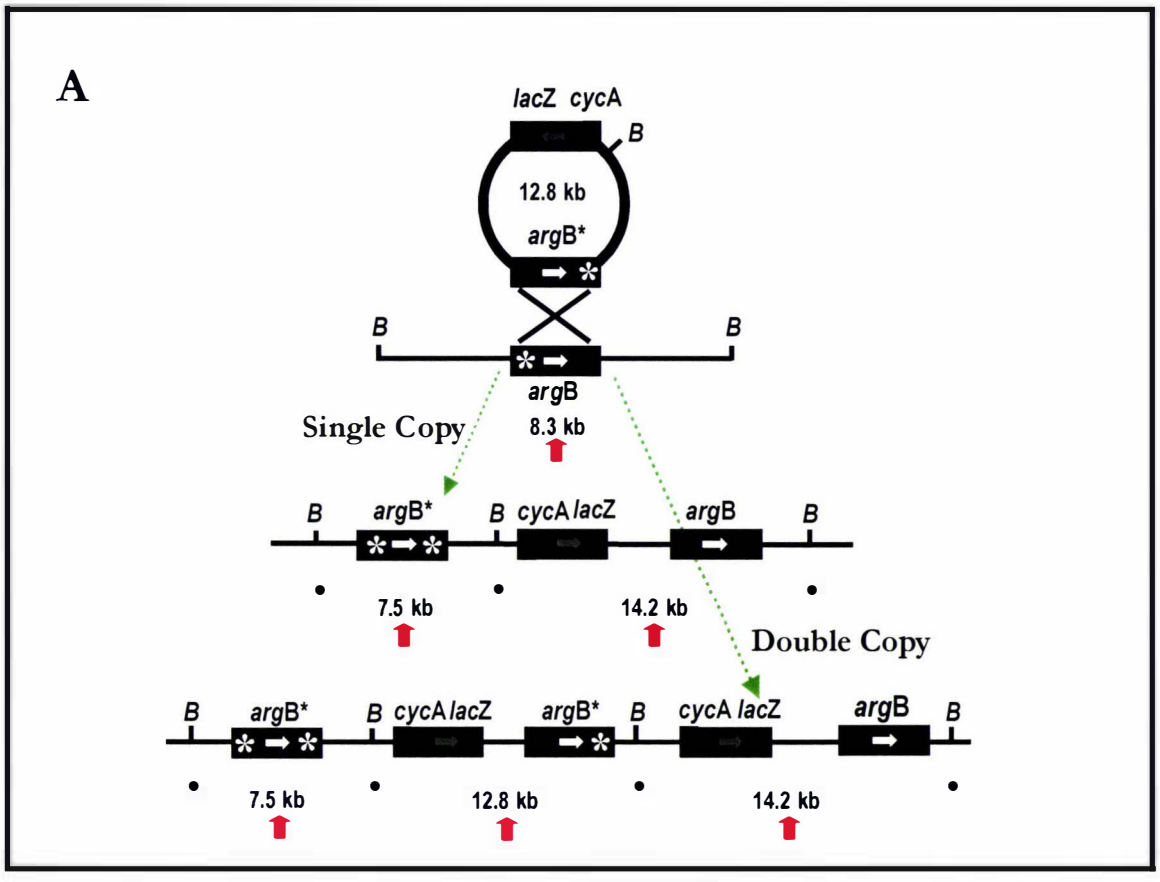
The possible homologous recombination event of a reporter construct (containing the 1.3 kb *cyc*A promoter fragment) at the *arg*B locus of *A. nidulans* is shown. The restriction map (*Bam*HI and *Sph*I sites) around the *arg*B locus, with and without an integrated construct, is shown. The approximate sizes of the digest fragments are given in kb. Mutated *arg*B loci are indicated by *. Arrows indicate the orientation of the *arg*B and *lac*Z genes.

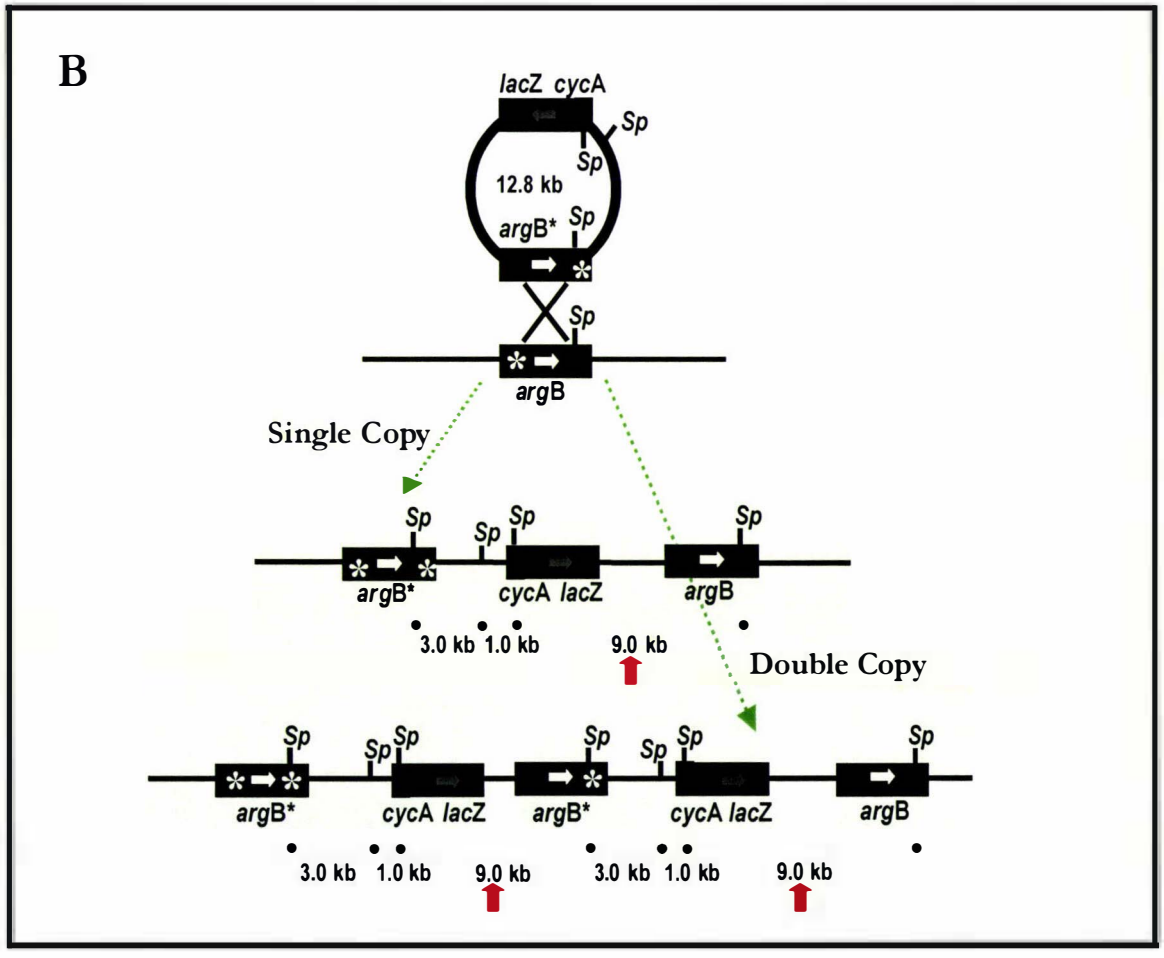
A ***Bam*HI digested samples, probed with *arg*B**

Chromosomal and construct *Bam*HI sites are represented by **B**. The *Bam*HI fragments expected to hybridise with the *arg*B probe, upon single/multiple copy integration at the *arg*B locus, are indicated by a red arrow.

B ***Sph*I digested samples, probed with *cyc*A**

Chromosomal and construct *Sph*HI sites are represented by **Sp**. The *Sph*HI fragments expected to hybridise with the *cyc*A probe, upon single/multiple copy integration at the *arg*B locus, are indicated a red arrow.


A
lacZ cycA
B
12.8 kb
argB*
B
B
argB
Single Copy
8.3 kb
B
argB*
B
cycA lacZ
argB
B
7.5 kb
14.2 kb
Double Copy
B
argB*
B
cycA lacZ
argB*
B
cycA lacZ
argB
B
7.5 kb
12.8 kb
14.2 kb


B
lacZ cycA
Sp
Sp
12.8 kb
argB*
Sp
Sp
argB
Single Copy
Sp
Sp
Sp
Sp
argB*
cycA lacZ
argB
3.0 kb
1.0 kb
9.0 kb
Double Copy
Sp
Sp
Sp
Sp
Sp
Sp
Sp
argB*
cycA lacZ
argB*
cycA lacZ
argB
3.0 kb
1.0 kb
9.0 kb
3.0 kb
1.0 kb
9.0 kb

fragment of approximately 12.8 kb (i.e. the vector) (Figure 5.4 A).

To determine the presence of the *cycA* promoter fragment, the DNA samples were digested with *Sph* I prior to Southern blotting. The blots were probed with a 0.4 kb *Bgl* II-*Bam* HI fragment of the *A. nidulans cycA* gene promoter (CPD5 fragment, generated by PCR with primers CYCP1 and CPD5). The fragment was prepared by digestion of plasmid R168 (Table 2.1) and purified by gel extraction (section 2.7.4). One *Sph* I restriction site is known to be present in the *A. nidulans argB* gene (Upshall, 1986). Homologous recombination of the constructs into the *argB* locus of the strain was predicted to occur as shown in Figure 5.4 B. Two *Sph* I restriction sites are known to be present in R117 (Figure 5.1), thus generating fragments of approximately 3 and 8.5 kb. A *Sph* I restriction site is also present within the *cycA* promoter fragment, at approximately -450 bp. Therefore, this site is not present within the CPD5 fragment used as the probe (Figure 5.2). Three *Sph* I restriction sites are therefore present within a construct containing the 1.3 kb *cycA* fragment, fragments of approximately 1.0, 3.0 and 9.0 kb (Figure 5.4 B). Single or multiple copy integration of the constructs at the *argB* locus was anticipated to result in hybridisation bands identical to the untransformed strain (due to the endogenous *cycA* gene). Those constructs containing a fragment of the *cycA* promoter were anticipated to generate an additional hybridisation band of approximately 9.0 kb (Figure 5.4 B).

5.5.2 SOUTHERN BLOTTING RESULTS

*Note: Several independent transformants were analysed for each type of reporter construct, eg. R159.1, R159.2, R159.3, R159.4 and R159.5 were all transformed with the R159 plasmid (Table 2.1).

DETERMINATION OF INTEGRATION AT THE *argB* LOCUS

The hybridisation patterns predicted in section 5.5.1 correspond to the fragments identified by Southern blot analysis (Table 5.2). For the blots with *Bam* HI-digested DNA, the *argB* probe hybridised to a band of approximately 8.3 kb for the untransformed strain A71 (Figures 5.5 A-D; Table 5.2). Of the 36 *argB+* transformants screened, only 6 had a hybridisation pattern characteristic of single copy integration of a reporter construct at the *argB* locus. The bands which hybridised to the *argB* probe for these transformants were approximately 7.5 and 14.2 kb in size (Figures 5.5 A-D; Table 5.2). The size of the 14.2 kb

Table 5.2 Southern Analysis Results

Strain (Promoter Fragment)	Type Of Integration Event at *arg*B	Digested with *Bam*HI, probed with *arg*B				Digested with *Sph*I, probed with *cyc*A			
		Size Of Bands Expected (kb)	Strain	Size Of Bands Observed (kb)	Figure (Lane #)	Size Of Bands Expected (kb)	Strain	Size Of Bands Observed (kb)	Figure (Lane #)
Strain A71	n/a*	8.0	Strain A71	8.3	5.6 A (10)	0.9	Strain A71	0.9	5.7 A (10)
R159 (1.3 kb *cyc*A)	single copy	14.2, 7.5	R159.3	14.2 and 7.5	5.6 C (2)	9.0, 0.9	R159.3	8.8, 0.9	5.7 B (2)
	multiple copy♣	14.2, 12.8, 7.5	R159.5	16.0 and 7.5	5.6 D (2)	9.0, 0.9	R159.5	0.9	5.7 B (3)
R170 (0.68 kb *cyc*A)	single copy	13.6, 7.5	ND	ND	ND	9.0, 0.9	ND	ND	ND
	multiple copy	13.6, 12.2, 7.5	R170.1	14.0, 13.0 and 7.5	5.6 A (4)	9.0, 0.9	R170.1	9.0, 0.9	5.7 A (4)
R172 (0.56 kb *cyc*A)	single copy	13.5, 7.5	ND	ND	ND	9.0, 0.9	ND	ND	ND
	multiple copy♣	13.5, 12.1, 7.5	R172.3	18.8, 16.4, 12.5 and 7.3	5.6 D (7)	9.0, 0.9	ND	ND	ND
R173 (0.4 kb *cyc*A)	single copy	13.3, 7.5	R173.2	13.3 and 7.5	5.6 B (7)	9.0, 0.9	R173.2	8.8, 0.9	5.7 B (4)
	multiple copy	13.3, 11.9, 7.5	ND	ND	ND	9.0, 0.9	ND	ND	ND
R116 (2.0 kb *gpd*A)	single copy	12.8, 7.5	R116.2	12.8 and 7.5	5.6 B (8)	0.9	R116.2	0.9	5.7 B (5)
	multiple copy	12.8, 11.5, 7.5	ND	ND	ND	0.9	ND	ND	ND
R117 (no promoter)	single copy	12.8, 7.5	R117.3	12.8 and 7.5	5.6 C (9)	0.9	R117.3	0.9	5.7 B (7)
	single copy	12.8, 7.5	R117.4	12.5 and 7.1	5.6 D (11)	0.9	R117.4	ND	ND
	multiple copy	12.8, 11.5, 7.5	R117.2	12.8, 11.5 and 7.5	5.6 B (9)	0.9	R117.2	0.9	5.7 B (6)
	multiple copy	12.8, 11.5, 7.5	R117.5	12.5, 11.0 and 7.1	5.6 D (12)	0.9	R117.5	ND	ND

Note:
*n/a = not applicable
♣ possible multiple integration at *arg*B locus, followed by rearrangement/deletion.
ND = not determined

Figure 5.5 **Southern Blot to Determine Integration of the Reporter Constructs at the *arg*B Locus**

Samples of genomic DNA were isolated from *A. nidulans* strains transformed with the various reporter constructs. The DNA samples were digested with *Bam* HI, Southern blotted, and the blot probed with a radioactively labelled 1 kb *Sal* I fragment of the *A. nidulans arg*B gene.

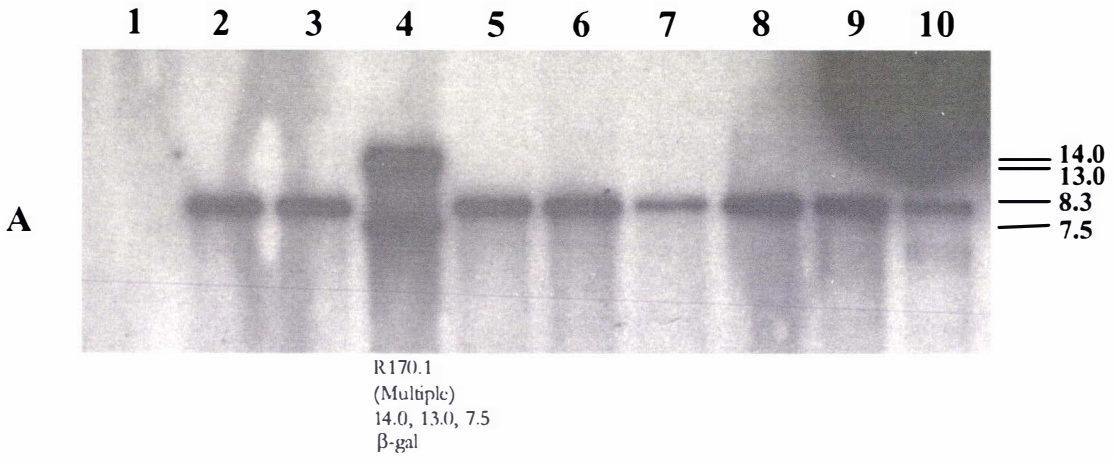
For the strains containing reporter constructs integrated at the *arg*B locus, the type of integration event (single or multiple integration) is indicated by labelling beneath the gels. The strains used for the β-galactosidase assays are also indicated (β-gal).

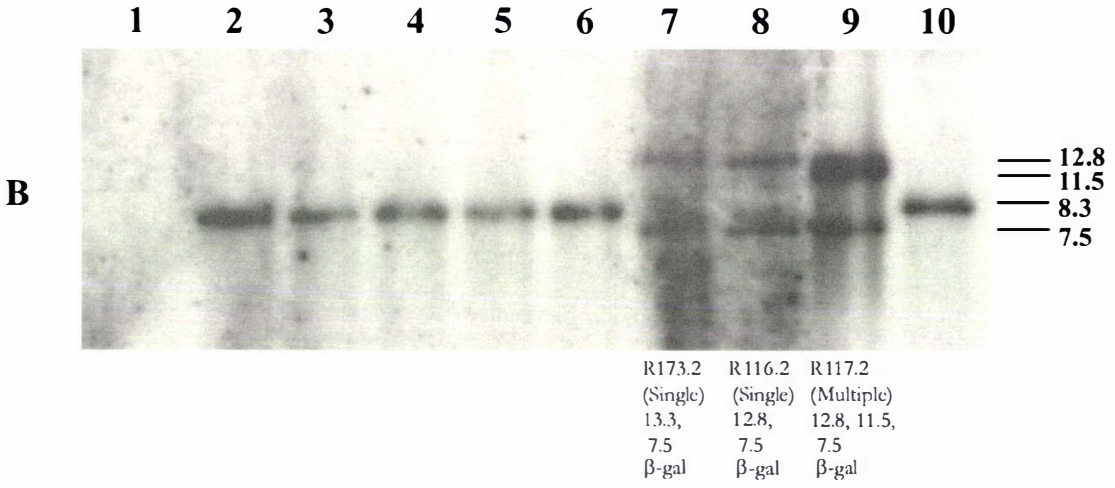
A Lane 1 contains the 1 kb ladder (Roche Biochemicals Ltd). Lanes 2 – 9 contain DNA from a fungal sample transformed with a particular construct (Table 2.1). Lane 2: R159.1; Lane 3: R169.1; Lane 4: R170.1; Lane 5: R171.1; Lane 6: R172.1; Lane 7: R173.1; Lane 8: R116.1; Lane 9: R117.1. Lane 10 contains A71 (untransformed) genomic DNA. The sizes of the bands generated for the wildtype strain and R170.1 are indicated in kb.

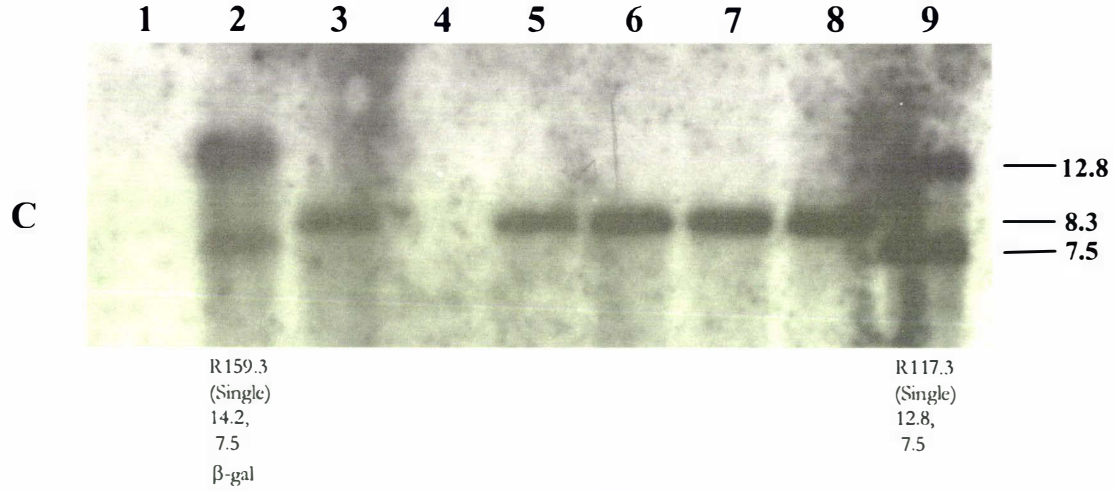
B Lane 1 contains λ DNA (Roche Biochemicals Ltd) digested with *Hind* III. Lanes 2 – 9 contain DNA from a fungal sample transformed with a particular construct (Table 2.1). Lane 2: R159.2; Lane 3: R169.2; Lane 4: R170.2; Lane 5: R171.2; Lane 6: R172.2; Lane 7: R173.2; Lane 8: R116.2; Lane 9: R117.2. Lane 10 contains A71 (untransformed) genomic DNA. The sizes of the bands generated for the wildtype strain and R117.2 are indicated in kb.

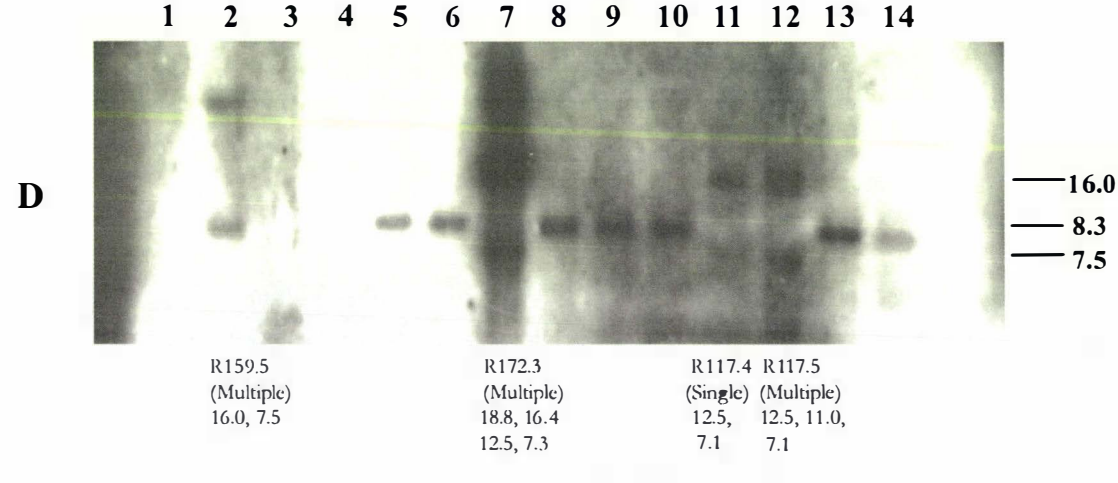
C Lane 1 contains λ DNA (Roche Biochemicals Ltd) digested with *Hind* III. Lanes 2 – 9 contain DNA from a fungal sample transformed with a particular construct (Table 2.1). Lane 2: R159.3; Lane 3: R159.4; Lane 4: R169.3; Lane 5: R170.3; Lane 6: R173.3; Lane 7: R116.3; Lane 8: R116.4; Lane 9: R117.3. The sizes of the bands generated for the wildtype strain and R117.3 are indicated in kb.

D Lane 1 contains λ DNA (Roche Biochemicals Ltd) digested with *Hind* III. Lanes 2 – 13 contain DNA from a fungal sample transformed with a particular construct (Table 2.1). Lane 2: R159.5; Lane 3: R169.3; Lane 4: R169.4; Lane 5: R170.4; Lane 6: R171.3; Lane 7: R172.3; Lane 8: R172.4; Lane 9: R173.4; Lane 10: R116.5; Lane 11: R117.4; Lane 12: R117.5; Lane 13: R117.6. Lane 14 contains A71 (untransformed) genomic DNA. The sizes of the bands generated for the wildtype strain and R159.5 are indicated in kb.


1 2 3 4 5 6 7 8 9 10
A
14.0
13.0
8.3
7.5
R170.1
(Multiple)
14.0, 13.0, 7.5
β-gal


1 2 3 4 5 6 7 8 9 10
B
12.8
11.5
8.3
7.5
R173.2
(Single)
13.3,
7.5
β-gal
R116.2
(Single)
12.8,
7.5
β-gal
R117.2
(Multiple)
12.8, 11.5,
7.5
β-gal


1 2 3 4 5 6 7 8 9
C
12.8
8.3
7.5
R159.3
(Single)
14.2,
7.5
β-gal
R117.3
(Single)
12.8,
7.5


1 2 3 4 5 6 7 8 9 10 11 12 13 14
D
16.0
8.3
7.5
R159.5
(Multiple)
16.0, 7.5
R172.3
(Multiple)
18.8, 16.4
12.5, 7.3
R117.4
(Single)
12.5,
7.1
R117.5
(Multiple)
12.5, 11.0,
7.1

band depended on the presence and size of the promoter fragment in the reporter construct (Table 5.2). The transformant strains which displayed this pattern are as follows: R159.3* (Figure 5.5 C, lane 2); R173.2 (Figure 5.5 B, lane 7); R116.2 (Figure 5.5 B, lane 8); R117.3 (Figure 5.5 C, lane 9) and R117.4 (Figure 5.5 D, lane 11). Transformant strain R159.5 also exhibited the banding pattern expected for single copy integration at the *arg*B locus, however the bigger band is larger than expected (16.0 kb rather than 14.2 kb) (Figure 5.5 D, lane 2; Table 5.2). It is possible that deletion of a portion of the vector may have occurred subsequent to multiple copy integration, although an erroneous estimation of band size is possible.

Three transformants (R170.1, Figure 5.5 A, lane 4; R117.2, Figure 5.5 B, lane 9; R117.5, Figure 5.5 D, lane 12) displayed patterns characteristic of multiple copy integration of a reporter construct at this locus. These transformants had a similar pattern to those with single copy integration at the *arg*B locus, with an additional band of approximately 12.8 or 11.5 kb (i.e. the size of the reporter construct) (Table 5.2).

The transformant strain R172.3 displayed a unique hybridisation pattern, with fragments of approximately 12.8 and 7.3 kb - similar to those transformants with single copy integration at the *arg*B locus. However, two additional bands were also observed, of approximately 16 and 19 kb (Figure 5.5 D; Table 5.2). This transformant was suggested to have undergone multiple integration at the *arg*B locus, with subsequent rearrangement of construct sequences.

The remaining *arg*B$^+$ transformants showed the identical hybridisation pattern as strain A71, indicating the reporter construct had not integrated (Figures 5.5 A-D). This result is despite the observation that all of these colonies displayed arginine independence.

DETERMINATION OF THE PRESENCE OF THE *A. NIDULANS cyc*A GENE PROMOTER FRAGMENT

For the blots with *Sph* I-digested DNA, the *cyc*A probe hybridised to a band of approximately 0.9 kb for the untransformed strain A71 (Figures 5.6 A & B; Table 5.2). The 2.5 kb hybridisation band observed has been reported previously for a wildtype strain of *A. nidulans*, and was thought to be due to partial digestion of the sample (Bird, 1996). The 0.4 kb *cyc*A probe should only hybridise once, to the 0.9 kb *Sph* I genomic fragment.

Most of the *arg*B$^+$ transformants shown to have integrated at the *arg*B locus in single copy (section 5.5.2) generated the expected pattern of bands upon hybridisation to the *cyc*A probe. In addition to the 0.9 kb band observed for the untransformed strain (due to the endogenous *cyc*A gene), these transformants generated a band of approximately 9.0 kb. For some transformants, a hybridisation band of approximately 2.5 kb was also observed. As mentioned for the wildtype strain above, this band is thought to be due to incomplete digestion of the samples. The transformant strains which displayed the additional 2.5 kb hybridisation band were R159.3 (Figure 5.6 B, lane 2) and R173.2 (Figure 5.6 B, lane 4) (Table 5.2).

Transformant R159.5, which possibly contains a reporter construct integrated in multiple copy integration at the *arg*B locus, generated only the 0.9 kb band (Figure 5.6 B, lane 3; Table 5.2). The reason for this is not known, however an unexpected banding pattern was also observed in Figure 5.5 (determination of integration at the *arg*B locus). It was suggested that deletion of a portion of the vector may have occurred subsequent to multiple copy integration, although an erroneous estimation of band size is possible.

The transformant strain R170.1, which contains a reporter construct integrated in mulitple copy at the *arg*B locus, also generated hybridisation bands of 9.0 and 0.9 kb bands with the *cyc*A probe (Figures 5.6 A, lane 4; Table 5.2). Other transformant strains, which did not contain a fragment of the *cyc*A promoter within the reporter construct (eg. R117), although integrated at the *arg*B locus, generated the expected band of 0.9 kb only (Figure 5.6 B; Table 5.2). Other bands observed in Figures 5.6 A & B (for strain A71 and the transformant strains) are due to partial digestion of the DNA samples.

The remaining *arg*B$^+$ transformants screened showed the identical hybridisation pattern as strain A71, indicating the reporter construct had not integrated (Figures 5.6 A & B).

5.6 BETA-GALACTOSIDASE ASSAYS

Transformants were initially screened on AMM containing X-gal (section 2.13.1) and 1% glucose as the carbon source to repress endogenous β-galactosidase activity of the strain (Van Gorcom *et al.*, 1986). An intense blue colour was observed for the transformant containing plasmid R116 (construct with the 2 kb *A. nidulans gpd*A promoter fragment,

integrated at the *arg*B locus in single copy) (Table 5.2). A very faint blue colour was observed for the remainder of the transformants, similar to that observed for strain A71. The intensity of colour observed on X-gal media has been reported to be predictive of (and proportional to) the number of *lacZ* fusion plasmids integrated at a specific locus (Davis *et al.*, 1988). The β-galactosidase assays were carried out using the protocol outlined in section 2.13.1.

For the β-galactosidase assays, a number of transformants were chosen which appeared to have the reporter constructs integrated at the *arg*B locus in single copy (R159.3, R173.2, R116.2) and multiple copy (R170.1, R117.2) (section 5.5.2). Previous studies had reported transformants containing the R116 construct to have a considerable β-galactosidase activity (9100 units/min/mg protein) on AMM containing 1% glucose. A transformed construct containing a severly truncated *gpd*A gene promoter (therefore, in essence a promoter-less construct) was reported to have a β-galactosidase activity of approximately 546 units/min/mg protein (6% the activity of the whole *gpd*A gene promoter) (Punt *et al.*, 1990).

It was therefore expected that the untransformed strain and the transformants containing the promoter-less construct R117 would have low *lacZ* expression, compared to the transformants containing the R116 construct. Transformants containing the constructs with *cyc*A promoter fragments were expected to have β-galactosidase activities intermediate to those determined for R116 and R117.

5.6.1 RESULTS

As expected, the transformant strain R116.2 (construct with the 2 kb *A. nidulans gpd*A promoter fragment) displayed a very high β-galactosidase activity upon AMM with 1% glucose (Table 5.3 A). In comparison to the transformant strain R116.2, the β-galactosidase activities of the transformant strains containing constructs with *cyc*A promoter fragments (R159.3, R170.1, R173.2) were very low (Table 5.3 A).

Table 5.3 Results For *A. nidulans* β-galactosidase Assays

The β-galactosidase assays were carried out on cultures grown in 1% glucose (**A**) and glycerol (**B**). One unit is defined as 1 nmol *ortho*-nitro-phenol produced per min at 37°C (Punt *et al.*, 1990).

A

Strain	Type of Integration	Promoter Fragment	β-galactosidase Activity (units/min/mg protein)
Untransformed strain A71	n/a*	n/a	21 ± 5.5
R159.3	Single	1.3 kb *cycA*	14 ± 0.9
R170.1	Multiple	0.68 kb *cycA*	22 ± 2.6
R173.2	Single	0.4 kb *cycA*	19 ± 4.0
R117.2	Multiple	n/a	22 ± 6.3
R116.2	Single	2.0 kb *gpdA*	82855 ± 52950.8

B

Strain	Type of Integration	Promoter Fragment	β-galactosidase Activity (units/min/mg protein)
Untransformed strain A71	n/a	n/a	46 ± 7.9
R159.3	Single	1.3 kb *cycA*	36 ± 6.1
R170.1	Multiple	0.68 kb *cycA*	71 ± 36.7
R173.2	Single	0.4 kb *cycA*	108 ± 42.9
R117.2	Multiple	n/a	38 ± 14.0
R116.2	Single	2.0 kb *gpdA*	18117 ± 5103.1

The values are given as the mean β-galactosidase activity ± standard error (n = 3) (n/a* = not applicable). Raw data for the table can be found in Appendix 3.4.

Assays were also carried out with 1% glycerol as a carbon source, in case carbon catabolite repression (CCR) was occuring. The β-galactosidase activities of the transformants containing constructs with *cycA* promoter fragments (R159.3, R170.1, R173.2) were again very low, in comparison to the transformant containing R116.2 (Table 5.3 B).

All of the transformants were screened on AMM containing X-gal and 1% glycerol as the carbon source (section 2.18.1). The same results were observed as for on X-gal media containing 1% glucose: an intense blue colour for the transformant containing plasmid R116.2, and a faint blue colour for the remaining transformants and strain A71.

5.6.2 DISCUSSION

All of the transformants displayed low β-galactosidase activity on AMM with 1% glucose or 1% glycerol, except for the transformant containing R116.2 (construct with the 2 kb *A. nidulans gpd*A promoter fragment) (Table 5.3 A & B). Although the β-galactosidase activities estimated for transformant strain R116.2 have high standard errors, the values are clearly dramatically increased compared to the other transformant strains and the untransformed strain (Table 5.3 A & B). These results indicate that the β-galactosidase assay appears to be working. Although *cyc*A$^+$ strains of *A. nidulans* have been shown to also contain an alternative respiratory pathway (section 3.5), the *cyc*A gene was expected to be highly expressed, due to its important role in an obligate aerobe. Thus an experiment was undertaken to determine the cause of these unexpected results.

5.7 EXPERIMENT TO DETERMINE THE CAUSE OF THE FAULTY EXPRESSION SYSTEM

5.7.1 THE *arg*B2 MUTANT STRAIN

To determine the cause of the high number of colonies which demonstrated arginine independence, yet did not contain a reporter construct, the growth of the *A. nidulans arg*B2 strain A71 was checked. It was postulated that reversion of the auxotrophic mutation may have been occurring, evidenced by leaky growth upon selective media. This problem had been experienced with the strain 2-124, originally selected for the experiment (Table 2.1). Strain A71 was checked for leaky growth on selective AMM (section 2.3.2) a number of times, and none was observed at any point. The strain, and all *arg*B$^+$ transformants, were maintained appropriately (section 2.4.2). Also, no *arg*B$^+$ colonies were observed for the control transformation samples (containing no DNA). Therefore, it is unlikely that the high number of *arg*B$^+$ transformants without reporter constructs is due to gene reversion, although this possibility cannot be dismissed.

5.7.2 TRANSLATIONAL FUSIONS

The sequences of the *cyc*A promoter fragments ligated to the *lac*Z reporter genes in each of the constructs were re-checked, by looking back over the original sequences. The results

confirmed that the translational fusions were correct (Appendix 3.2), and that the fragments were in the correct orientation.

5.7.3 SEQUENCING OF THE *lacZ* REPORTER GENE

The *E. coli lacZ* gene contained within the reporter vector was sequenced to ensure no mutations were present which would lessen *lacZ* expression levels. The sequential sequencing approach used the CPD5 primer, and also required the designing of primers (LacZ 1-7, Table 2.2) to the already published *lacZ* gene sequence (Kalnins *et al.*, 1983). Plasmid R172 (Table 2.1) was used as a template. Two base pair changes were observed in the R117 sequence, located at 271 bp (A → G) and 532 bp (T → C) (Appendix 3.5). However, these changes did not alter the amino acids encoded (Valine and Arginine, respectively). Also, the codons are already present in *A. nidulans*, and codon useage frequency was not predicted to be greatly affected by these changes (Appendix 3.2). Therefore, it is unlikely that the low levels of *lacZ* expression observed for the transformant strains R159.3, R170.1 and R173.2 are due to nucleotide changes within the *lacZ* gene sequence.

5.7.4 THE PROMOTER SWITCH EXPERIMENT

A 'promoter switch' experiment was carried out, to ascertain whether the *cycA* gene promoter sequence or the reporter vector sequence was responsible for the non-expression of the constructs containing *cycA-lacZ* gene fusions. The R116 construct, containing the 2 kb *A. nidulans gpdA* promoter fragment translationally fused to pAN923-42B$_{BglII}$ (R117; Table 2.1), was shown to have high β-galactosidase activity (section 5.6). By comparison, the *cycA* constructs, containing fragments of the *A. nidulans cycA* promoter which were also translationally fused to pAN923-42B$_{BglII}$ (R117; Table 2.1), had very low levels of *lacZ* expression. R116 was obtained from another laboratory (M.J.Hynes, Melbourne), while the *cycA* constructs were prepared during this study. Therefore, it was postulated that some part of the R117 vector sequence (other than the *lacZ* gene) contained within the *cycA* constructs may be hindering the expression of the *cycA-lacZ* fusion genes. It was therefore possible that the pAN923-42B$_{BglII}$ (R117) sequence from R116 may facilitate a measurable level of *lacZ* expression, when fused to the *A. nidulans cycA* promoter fragments.

The 1.3 kb whole *cyc*A promoter fragment (contained within R159) and the 2 kb *A. nidulans gpd*A promoter fragment (contained within R116) were cleaved out of their respective vectors by *Bam* HI/*Bgl* II and *Bam* HI digestions, respectively. The *Bam* HI/*Bgl* II *cyc*A promoter fragment was then ligated into the *Bam* HI R116 fragment (from which the *gpd*A promoter fragment had been removed), thus forming plasmids R183 and R184 (correct and reverse orientations, respectively) (Table 2.1). The presence and orientation of the insert was determined by PCR amplification (section 2.10) with the CYCP2 and lacZ forward primers (Table 2.1) (results not shown). The *Bam* HI *gpd*A promoter fragment was ligated into the *Bam* HI/*Bgl* II R117 fragment (from which the *cyc*A promoter fragment had been removed), thus forming plasmids R185 and R186 (correct and reverse orientations, respectively) (Table 2.1). Enzymatic digestion with *Xho*I was used to determine the presence and orientation of the insert (results not shown).

Plasmids R183 and R185 were sequenced with the CPD5 and lacZ forward primers (respectively) to determine correct translational fusion. All of the constructs were then transformed into the *arg*B2 strain A71 (section 5.4), and colonies demonstrating arginine independent growth were analysed as described in sections 5.5 and 5.6.

RESULTS

Preliminary screening of the *arg*B$^+$ colonies was carried out by plating onto AMM containing X-gal (section 2.13.1) and 1% glucose. The untransformed strain and transformant strain R116.2 (2 kb *A. nidulans gpd*A promoter fragment; section 5.6) were also included. As previously (section 5.6), an intense blue colour was observed for the strain transformed with R116.2. The remaining transformants generated a faint blue colour, which was indistinguishable from that observed for the untransformed strain.

All of the *arg*B$^+$ colonies obtained were also analysed by Southern blot, to check whether integration had occurred. Fourteen *arg*B$^+$ transformants were analysed: 6 transformed with R183 (1.3 kb *cyc*A in R116, correct orientation); 2 transformed with R184 (1.3 kb *cyc*A in R116, reverse orientation); 6 transformed with R185 (2.0 kb *gpd*A in R117, correct orientation); and 2 transformed with R186 (2.0 kb *gpd*A in R117, reverse orientation) (Table 2.1).

The *arg*B$^+$ transformants (Figure 5.7 A & B) displayed similar hybridisation patterns to the untransformed strain, therefore indicating that integration of the constructs had not taken place. When digested with *Bam* HI and probed with a fragment of the *arg*B gene (Figure 5.7 A & B), the *arg*B$^+$ transformants produced a single band of approximately 8.3 kb in size, similar to the untransformed strain. Upon probing the *Sph*I digested samples with the *cyc*A fragment, the *arg*B$^+$ transformants produced a fragment of approximately 0.9 kb (data not shown), similar to the untransformed strain.

Therefore, since integration of the constructs had not occurred, it could not be determined whether the *cyc*A promoter fragment or the vector sequence was the cause of the faulty *cyc*A-*lac*Z expression system. The experiment could not be continued, due to time constraints.

5.8 DISCUSSION

Constructs containing *cyc*A promoter fragments translationally fused to the *lac*Z reporter gene were transformed into an *arg*B2 strain of *A. nidulans*. Integration of the constructs was expected to occur by homologous recombination at the *arg*B locus. Despite the growth of numerous arginine-independent colonies, only a third of these (approximately) were determined by Southern blot analysis to have integrated in single/multiple copy at the desired (*arg*B) locus (section 5.5). This outcome has been reported previously in *A. nidulans* studies, e.g. Davis *et al* (1988) found that less than a third of *amd*S$^+$ transformants contained the *amd*S-*lac*Z fusion plasmid. This event has also been reported for other transformations involving *A. nidulans* strains with intact or mutant *arg*B genes (John & Peberdy, 1984; Hamer & Timberlake, 1987, respectively).

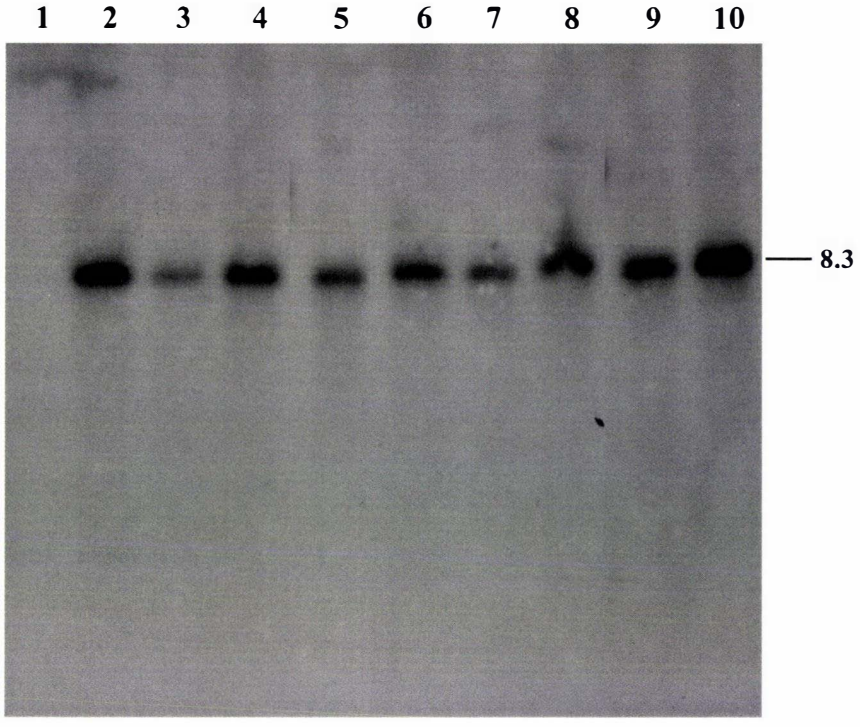
The generation of *arg*B$^+$ strains which do not contain the fusion plasmid, and are no longer auxotrophs, could have arisen a number of ways. Reversion of the auxotrophic mutation is a possibility, although no *arg*B$^+$ colonies were observed for the parental strain, or for the control transformation samples (containing no DNA). It is also possible that a homologous recombination event occurred within a small region of the *arg*B gene (type III transformation, Hinnen *et al.*, 1978). An alternative explanation is that integration of the whole reporter construct occurred, followed by the deletion of the *E. coli* and non-functional *A. nidulans* sequences (John & Peberdy, 1984).

Figure 5.7 Determination of Single Copy Integration at the *arg*B Locus

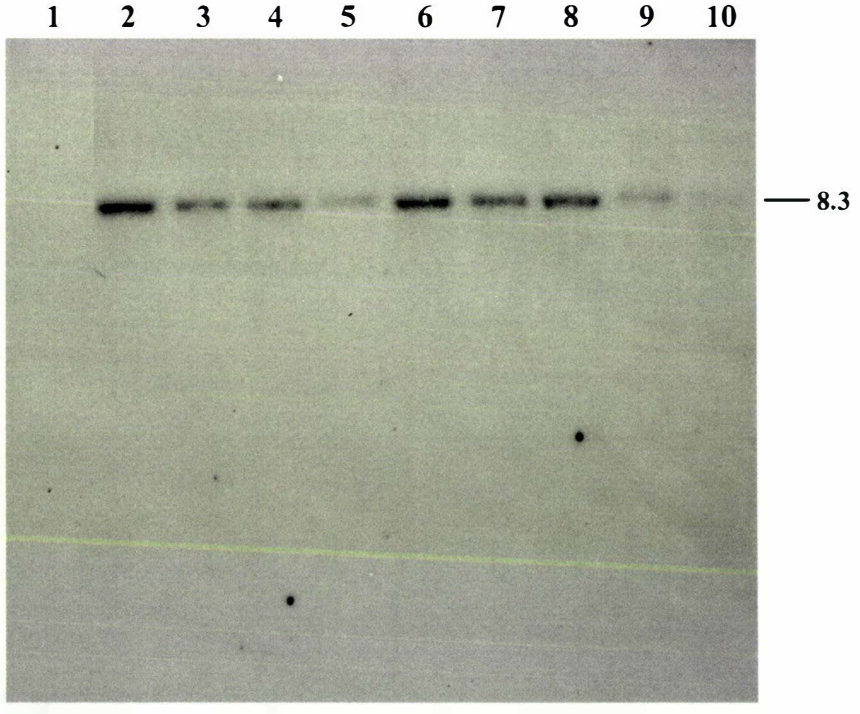
Samples of genomic DNA were isolated from *A. nidulans* strains transformed with the various reporter constructs. The DNA samples were digested with *Bam* HI, Southern blotted, and the blot probed with a radioactively labelled 1 kb *Sal* I fragment of the *A. nidulans arg*B gene. The sizes of the bands are indicated in bp.

A Lane 1 contains λ DNA (Roche Biochemicals Ltd) digested with *Hind* III. Lanes 2 – 12 contain DNA from a fungal sample transformed with a particular construct (Table 2.1). Lane 2: R183.1; Lane 3: R183.2; Lane 4: R183.3; Lane 5: R184.1; Lane 6: R185.1; Lane 7: R185.2; Lane 8: R185.3; Lane 9: R186.1. Lane 10 contains A71 (untransformed) genomic DNA.

B Lane 1 contains λ DNA (Roche Biochemicals Ltd) digested with *Hind* III. Lanes 2 – 12 contain DNA from a fungal sample transformed with a particular construct (Table 2.1). Lane 2: R183.4; Lane 3: R183.5; Lane 4: R183.6; Lane 5: R184.2; Lane 6: R185.4; Lane 7: R185.5; Lane 8: R185.6; Lane 9: R186.2. Lane 10 contains A71 (untransformed) genomic DNA.



A



B

Subsequent β-galactosidase assays revealed very low levels of *lacZ* expression from the *argB*$^+$ transformants containing constructs integrated at the *argB* locus, therefore the functional significance of the putative regulatory elements within the 1.3 kb *cycA* promoter fragment could not be reliably determined by this experiment. Although the conditions used for the β-galactosidase assays (AMM with 1% glucose) have been reported to repress endogenous β-galactosidase activity of *A. nidulans* (Van Gorcom *et al.*, 1986), the β-galactosidase activity of the reporter constructs is not expected to be affected. Therefore, the assay conditions are unlikely to be responsible for the low β-galactosidase activities of the reporter constructs, especially since low β-galactosidase activity was not observed for all of the transformants (ie. not R116.2). Other functional analysis studies have reported the used of *A. nidulans argB2* strains which lack significant β-galactosidase activity (Brakhage *et al.*, 1992; Litzha *et al.*, 1995). A better option may be to use one of these strains in the future.

Experiments were carried out to determine the cause of the faulty *cycA-lacZ* expression system (section 5.7). The translational fusion of the *cycA* promoter fragments to the *lacZ* reporter gene were confirmed to be correct. The *lacZ* gene in the R117 plasmid was sequenced; two nucleotide changes were observed (section 5.7.3), however this did not alter the encoded amino acids. It was estimated that these changes would have only a minimal effect on the *A. nidulans* codon frequency, although this cannot be assumed. The *lacZ* gene in the R116 plasmid should have been sequenced, to dismiss the possibility that the nucleotide changes in the R117 plasmid had affected *lacZ* expression. The 'promoter switch' experiment (section 5.7.4) failed to determine a fault within the promoter or vector sequences, as *argB*$^+$ transformants displayed similar hybridisation patterns to the untransformed strain, therefore indicating integration of the constructs had not taken place.

It is possible that the *cycA* promoter fragments used may lack an important regulatory element, or contain a strong repressor element, thus inhibiting transcriptional activation. Therefore, a larger sized fragment of the *cycA* gene promoter (eg. 2 kb) could be used for further investigations. Another possibility is that the estimated transcriptional start site (*tsp*) for the *cycA* gene is incorrect, therefore affecting the translational *cycA-lacZ* fusion. As mentioned in section 1.6, Raitt *et al* (1994) had used primer extension analysis to determine the *tsp* of the *cycA* gene (containing two introns), however McGlynn (1997) discovered a previously undetected (third) intron within the *A. nidulans cycA* gene sequence. The

amended *tsp* (Figure 5.2) was moved upstream by the length of the additional (third) intron, approximately 395 bp from the original *tsp*. The N-terminus of the amended *A. nidulans* cytochrome *c* protein sequence was found to be highly conserved in comparison to other fungal sequences. A strong Kozak sequence, important for the fidelity and efficiency of translation initiation, also surrounds the proposed translational start site. Therefore it is highly unlikely that the amended translational start site is incorrect. The correct *tsp* of the *cycA* gene must therefore be contained within the *cycA* gene promoter fragments used in this study.

It is possible that chromosomal positional effects (ie. of the *argB* locus) could be repressing expression from the *cycA* promoter fragments (present in the integrated constructs). However, repression of the integrated constructs containing the *gpdA* promoter did not occur (transformant R116.2), although the repression could be specific to the *cycA* promoter. Also, site-specific integration of reporter constructs at the *argB* locus has been used extensively in past studies, so this explanation is unlikely. However, the possibility cannot be dismissed.

5.9 CONCLUSION

An *argB2* strain of *A. nidulans* was transformed with reporter constructs containing *cycA* promoter fragments translationally fused to the *lacZ* reporter gene. The *argB*$^+$ transformants containing reporter constructs integrated at the *argB* locus were determined to have very low levels of expression, with the exception of the control construct (containing an *A. nidulans gpdA* promoter fragment). Possible explanations for the faulty *cycA-lacZ* expression system include: the presence/absence of important regulatory elements in the *cycA* promoter fragments; incorrect transcriptional start site; or chromosomal repression of the *cycA* promoter fragments.

CHAPTER 6

FUNCTIONAL EXPRESSION OF THE *A. NIDULANS cycA* GENE PROMOTER IN *S. CEREVISIAE*

6.1 INTRODUCTION

A fragment from the *A. nidulans* cytochrome *c* gene promoter (*cycA*) was cloned in front of a *CYC1-lacZ* fusion gene (bearing a minimal *CYC1* promoter) and transformed into wildtype and *hap1* mutant strains of *S. cerevisiae*. As described in sections 1.5 & 1.6, the regulatory mechanisms controlling the expression of cytochrome *c* in these two organisms are believed to be quite similar. Thus it was anticipated that *S. cerevisiae* may regulate the *cycA* gene in a similar manner to *CYC1* and *CYC7*. Functional expression of *A. nidulans* genes in *S. cerevisiae* has been carried out before. For example, Bonnefoy *et al* (1995) demonstrated that yeast proteins were able to regulate expression of the *A. nidulans amdS* gene.

Because of time constraints, the 'promoter switch' experiment carried out in section 5.7.4 failed to determine whether the cause of the faulty *cycA-lacZ* fusion expression system lay with the *cycA* promoter fragment, or the vector sequence. It was therefore hoped that expression of the *cycA* promoter fragment in *S. cerevisiae* would indicate whether the fragment was functional.

6.2 THE COMPONENTS OF THE REPORTER CONSTRUCTS

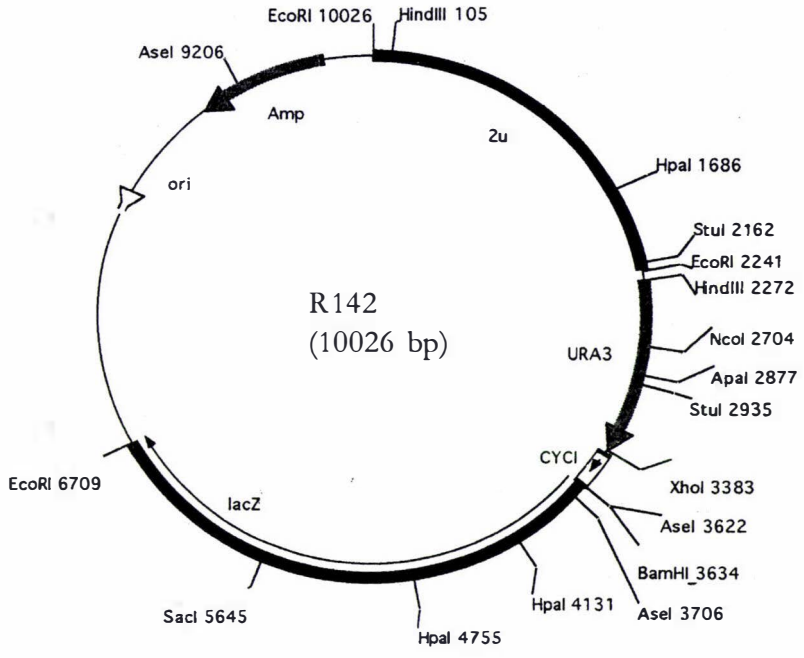
6.2.1 THE REPORTER CONSTRUCTS

Yeast vectors are available which allow the in-phase transcriptional or translational fusion of a promoter fragment to the *lacZ* reporter gene, similar to the *Aspergillus* expression vectors (section 4.1) (Altherr & Rodriguez, 1988). The reporter constructs R141 and R142 (Table 2.1) contain the *E. coli lacZ* reporter gene, translationally fused to 1.1 kb and 0.18 kb fragments of the *S. cerevisiae CYC1* gene promoter, respectively (Figure 6.1). Both vectors also contain the URA3 element and the *E. coli* ampicillin resistance gene (Amp^R), necessary

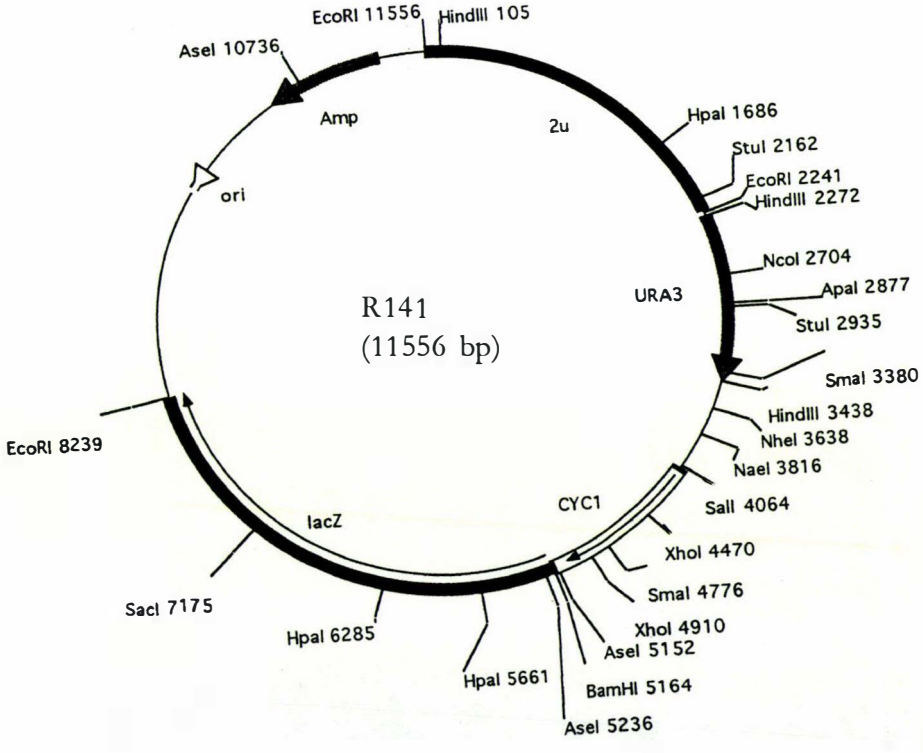
Figure 6.1 Reporter Constructs R141 And R142

The plasmids R142 (A) and R141 (B) both contain the *E. coli lacZ* reporter gene, translationally fused to a promoter fragment from the *S. cerevisiae CYC1* gene. The positions of restriction enzyme sites present within the plasmids are indicated.

A


EcoRI 10026
HindIII 105
AseI 9206
Amp
2u
ori
HpaI 1686
StuI 2162
EcoRI 2241
HindIII 2272
R142
(10026 bp)
NcoI 2704
URA3
ApaI 2877
StuI 2935
CYC1
XhoI 3383
AseI 3622
BamHI 3634
EcoRI 6709
lacZ
HpaI 4131
AseI 3706
SacI 5645
HpaI 4755

B


EcoRI 11556
HindIII 105
AseI 10736
Amp
2u
HpaI 1686
StuI 2162
ori
EcoRI 2241
HindIII 2272
NcoI 2704
R141
(11556 bp)
URA3
ApaI 2877
StuI 2935
SmaI 3380
HindIII 3438
NheI 3638
EcoRI 8239
NaeI 3816
CYC1
SalI 4064
lacZ
XhoI 4470
SmaI 4776
XhoI 4910
SacI 7175
AseI 5152
HpaI 6285
HpaI 5661
BamHI 5164
AseI 5236

for the selection of transformants in yeast and *E. coli* (respectively) (Guarente & Ptashne, 1981). The constructs also contain origins of replication from yeast and *E. coli* (2μ and Col E1, respectively), which facilitate autonomous replication of the vectors. Therefore, once transformed into the host strain, integration of the yeast reporter constructs does not occur.

Vector R141 was used as a positive control, as the 1.1 kb *CYC1* gene promoter fragment it contains is sufficient for a measurable level of *lacZ* expression (Bonnefoy *et al.*, 1995). Vector R142 contains only 0.18 kb of the *CYC1* gene promoter (the 'minimal promoter'), which generates a very low, unregulated level of *lacZ* expression (Guarente & Mason, 1991; Bonnefoy *et al.*, 1995). The *Xho* I site at the 5' end of the *CYC1* promoter fragment in R142 (Figure 6.1) facilitates the ligation of other promoter fragments to *CYC1* (Bonnefoy *et al.*, 1995). Transcription from the fusion gene is initiated from the transcriptional start site contained within the 0.18 kb *CYC1* promoter fragment.

6.2.2 THE *A. NIDULANS cycA* GENE PROMOTER FRAGMENT

A 1 kb fragment of the *A. nidulans cycA* gene promoter was PCR amplified with primers YP1 and YP2 (Figure 5.2; Table 2.2). These primers included *Xho* I sites at the ends of the *cycA* fragment, thus facilitating subsequent ligation steps. The 1 kb *cycA* promoter fragment contains most of the putative regulatory elements (Figure 5.2), with particular focus again on the putative HAP1-binding sites (section 5.2.2). The fragment also contains putative binding sites for the AnCF, and the CREA protein (Figure 5.2). The *cycA* fragment does not contain a translational start site, as the one contained within the minimal *CYC1* promoter fragment is used.

6.3 PREPARATION OF THE REPORTER CONSTRUCTS

6.3.1 METHODOLOGY

For routine PCR and enzymatic digestions (2.7.1), plasmid DNA was prepared with a small scale alkaline lysis protocol (section 2.6.2). A standard PCR set-up and thermal cycling programme (section 2.8) was used, and the purification of PCR products was carried out with the QIAGEN PCR Purification kit (section 2.8.3). The QIAGEN gel extraction kit

(section 2.7.4) was used to purify digestion products, and the routine ligation procedure carried out (section 2.9.2). For yeast transformations (section 2.10.3), plasmid DNA was prepared with the QIAGEN Midi plasmid preparation kit (section 2.6.3).

6.3.2 THE REPORTER CONSTRUCTS

Vector R142 (Figure 6.1) was linearised with *Xho* I, and the fragment purified by gel extraction. SAP treatment (section 2.9.1) of the fragment was then carried out, to prevent re-ligation of the vector.

The 1 kb fragment of the *A. nidulans cycA* gene promoter was PCR amplified with plasmid R155 as a template. The PCR product was purified and digested with *Xho* I. The digestion product was purified by gel extraction and ligated into the *Xho* I-digested R12 plasmid (Appendix 4.1), thus forming plasmid R160 (Table 2.1). Automated sequencing (section 2.11.1) of the *cycA* promoter fragment in R160 was carried out with the lacZ forward and reverse primers (Table 2.2). Site-directed mutagenesis of the putative HAP1 binding sites within the *cycA* promoter fragment (contained within plasmid R160) was going to be carried out, however time constraints prevented this.

The *Xho*I *cycA* promoter fragment was then ligated into the *Xho* I-digested R142 plasmid, forming plasmid R182. The correct orientations of the fragments were checked by *Xho* I-*Bam* HI digestion. Automated sequencing with the lacZ forward primer (Table 2.2) was carried out to ensure correct fusion of the fragments had taken place (Appendix 4.1).

6.4 TRANSFORMATION OF THE CONSTRUCTS INTO *S. CEREVISIAE*

The construct R182 was transformed into *S. cerevisiae*, as well as the control plasmids R141 and R142 (Table 2.1). Wildtype and *hap1* mutant strains of *S. cerevisiae* (BWG1-7A and LPY22, respectively; Table 2.1) were used for the transformations. The transformation mixes were plated onto YEP media containing X-gal (section 2.3.2), and transformants were selected on the basis of uracil-independent growth. All *ura*$^+$ colonies transformed with R141, R142 or R182 had a blue appearance, indicating *lacZ* expression. The intensity of colour upon X-gal media was similar for the different types of transformants.

6.5 β-GALACTOSIDASE ASSAYS

The transformants were analysed by β-galactosidase assays (section 2.13.1). To determine whether carbon catabolite repression (CCR) was occuring, the transformants were analysed after growth upon YPD media containing 1% glucose or glycerol (section 2.3.2).

6.5.1 RESULTS & DISCUSSION

The results of the β-galactosidase assays are presented in Table 6.1. Untransformed strains BWG1-7A (*hap1*$^+$) and LPY22 (*hap1*$^-$) displayed no measurable β-galactosidase activity upon either carbon source (Table 6.1).

The β-galactosidase activity of plasmid R141 (1.1 kb *CYC1* promoter) was significantly higher than for plasmid R142 (minimal *CYC1* promoter), when transformed into the *hap1*$^+$ strain, with growth on 1% glucose (Table 6.1, Appendix 4.5 [1]). The β-galactosidase activity of R141 was also significantly higher than R142, when transformed into the *hap1*$^+$ strain, with growth on 1% glycerol (Table 6.1, Appendix 4.5 [2]). This result was expected, as the *CYC1* promoter in R141 has been previously reported to express *lacZ* at a measurable level, higher than for R142 (Bonnefoy *et al.*, 1995). For example, Bonnefoy *et al* (1995) had reported activities of 101.8 ± 16.0 and 224.2 ± 36.4 for R141 when transformed into strain BWG1-7A, with growth upon 2% glucose and 0.1% glucose (respectively). The study by Bonnefoy *et al* (1995) also reported the β-galactosidase activities of the R142 plasmid to be 0.03 ± 0 and 0.04 ± 0 when transformed into the *hap1*$^+$ strain, with growth upon 2% glucose and 0.1% glucose (respectively). Therefore, the β-galactosidase activities obtained for R141 in this study were severely diminished, in comparison to the Bonnefoy *et al* (1995) study. For example, the β-galactosidase activities for R141 transformed into the *hap1*$^+$ strain were 10 ± 1.9, and 14 ± 1.2 upon 1% glucose and 1% glycerol, respectively (Table 6.1).

Table 6.1 Results For *S. cerevisiae* β-galactosidase Assays

The β-galactosidase assays were carried out on cultures grown in 1% glucose and glycerol. One unit of β-galactosidase activity is defined as 1 nmol *ortho*-nitro-phenol produced per min at 37°C (Punt *et al.*, 1990).

Strain + Plasmid	Promoter Fragment(s)	β-galactosidase Activity (units/min/ml)	
		1% Glucose	1% Glycerol
BWG1-7A only	n/aO	0	0
BWG1-7A + R142	0.18 kb *CYC1*	5 ± 1.6	7 ± 1.0
BWG1-7A + R141	1.1 kb *CYC1*	10 ± 1.9	14 ± 1.2
BWG1-7A + R182	0.18 kb *CYC1* + 1 kb *cycA*	7 ± 1.1	9 ± 1.0
LPY22 only	n/a	0	0
LPY22 + R142	0.18 kb *CYC1*	6 ± 0.9	12 ± 5.2
LPY22 + R141	1.1 kb *CYC1*	11 ± 3.2	27 ± 13.0
LPY22 + R182	0.18 kb *CYC1* + 1 kb *cycA*	9 ± 3.3	15 ± 1.5

O n/a = not applicable

BWG1-7A and LPY22 are wildtype and *hap1* strains of *S. cerevisiae*, respectively. The cytochrome *c* gene promoter fragments used in the constructs are from the *S. cerevisiae CYC1* gene and the *A. nidulans cycA* gene.

The values are given as the mean ± standard error. The assays were carried out in triplicate, on the 5 pooled transformants for each category (strain + plasmid).

Raw data for the table can be found in Appendix 4.4.

Data from the ANOVA and t-test analyses are presented in Appendices 4.5 & 4.6.

T-test analysis of R141 (1.1 kb *CYC1* promoter) β-galactosidase activities upon 1% glucose or glycerol (in either host strain) revealed no significant difference between these results, implying that carbon catabolite repression of the *CYC1* promoter was not occurring (Table 6.1; Appendix 6.5 [3]). By comparison, the results obtained for plasmid R141 by Bonnefoy *et al* (1995) indicated that carbon catabolite repression of expression from the *CYC1* promoter was occuring in strain BWG1-7A, as expression was derepressed upon a non-fermentable carbon source (see above).

No significant difference was observed between the β-galactosidase activities of R141, transformed into the *hap1*$^{+}$ or *hap1*$^{-}$ host strains (upon either carbon source) (Appendix 4.5 [4]). Transcription of the *CYC1* gene is activated by the HAP1 protein (Forsburg & Guarente, 1989), therefore it was expected that β-galactosidase activity would be decreased in a *hap1*$^{-}$ strain.

Therefore, the yeast expression system did not appear to be working properly, as β-galactosidase activity of the control plasmid (R141) was extremely low under all conditions, compared with the results obtained by Bonnefoy *et al* (1995). Possible explanations for this include a change in media (YMM used by Bonnefoy *et al*, YPD used in this study; section 2.3.2.1.2). YPD was used in this experiment as the growth of the cultures was enhanced in comparison to growth with YMM. The study by Bonnefoy *et al* (1995) also used ten pooled transformants for the β-galactosidase assays, in comparison to five pooled transformants in this experiment (section 2.15.1). However, it is not expected that this change would cause such a large decrease in β-galactosidase activity.

To ensure that the β-galactosidase assay conditions were correct, the *S. cerevisiae* assays included the positive control (transformant strain R116.2) from the *A. nidulans* assays. The two sets of assays (*A. nidulans* and *S. cerevisiae*) were also carried out at approximately the same time, using reagents prepared simultaneously. The restriction sites within R141 and R142 were checked prior to transformation, by enzymatic digestion. The sizes of the digestion products also confirmed the identities of the vectors. However, it is possible that the low β-galactosidase activities observed for R141 in this study could be due to the erroneous transformation of the strains with R142 (instead of R141). Therefore, the transformants could have been checked for the presence of a particular plasmid (eg. by PCR amplification).

No significant difference in β-galactosidase activity was found for R182 on 1% glucose or glycerol (transformed into either strain), indicating that CCR of the *cyc*A promoter was not occurring (Table 6.1, Appendix 4.5 [5]). A significant difference in β-galactosidase activity was observed for the R182 plasmid, transformed into the *hap1*$^{+}$ and *hap1* strains, with growth upon 1% glycerol (9 ± 1.0, and 15 ± 1.5, respectively) (Table 6.1, Appendix 4.5 [6]). The increased β-galactosidase activity of R182 in the *hap1* strain implies depression of the *cyc*A promoter may be occurring. This result was unexpected, as the HAP1 protein is an activatory transcription factor. However, no conclusions can be drawn from this data, as the strains (*hap1*$^{+}$ or *hap1*) transformed with R182 did not generate β-galactosidase activities which were significantly higher than the R142 plasmid (minimal *CYC1* promoter), on either carbon source (Table 6.1; Appendix 4.5 [7]).

Also, the β-galactosidase activity of R182 was only found to be significantly different to R141 (1.1 kb *CYC1* promoter) when transformed into the *hap1*$^{+}$ strain, with growth upon 1% glycerol (Table 6.1; Appendix 4.5 [8]). The activities of both plasmids were still very low, compared to results from a previous study. Bonnefoy *et al* (1995) had reported β-galactosidase activities of 1.4 ± 0.3 and 93.1 ± 14.1 for the 1.7 kb *A. nidulans amd*S gene promoter (ligated into R142), transformed into strain BWG1-7A, with growth upon 2% glucose and 0.1% glucose, respectively. These activities are significantly different to those reported for R141 and R142 under the same conditions (see above; Bonnefoy *et al.*, 1995). Mutagenised *amd*S gene promoter fragments investigated in this study also generated β-galactosidase activities which could be distinguished above background levels, and were still significantly lower than for R142.

Therefore, it was expected that in an efficient yeast expression system, R182 would generate a measurable β-galactosidase activity, which could be distinguished from the background and positive control levels, under all conditions. It is possible that R141 (1.1 kb *CYC1* promoter) and R182 (1.0 kb *cyc*A promoter) would have similar β-galactosidase activities, as expression of the *cyc*A gene has been suggested to be regulated by similar mechanisms as for the *CYC1* gene. It is possible that the low levels of *lac*Z expression from the *cyc*A-*lac*Z fusion gene in R182 is due to a problem with the *cyc*A promoter fragment. However, since neither R182 nor R141 consistently generated β-galactosidase

activities which were significantly higher than the background level (R142; minimal *CYC1* promoter), it is most likely that the expression system is not working correctly.

6.6 CONCLUSION

The β-galactosidase activity of R141 was significantly higher than for plasmids R142 and R182, transformed into the *hap1*$^+$ strain, upon 1% glycerol (Table 6.1, Appendix 4.5). The β-galactosidase activity of R141 was also significantly higher than R142 when transformed into the *hap1*$^+$ strain, upon 1% glucose (Table 6.1, Appendix 4.5). It was expected that the β-galactosidase activity of R141 would be consistently significantly higher than for plasmids R141 and R182.

Carbon catabolite repression was not observed to be occurring for any of the plasmids. This is contrary to previous reports for plasmid R141 (Bonnefoy *et al.*, 1995). The *CYC1* promoter is also known to be transcriptionally activated by HAP1 (section 1.6.1), yet the β-galactosidase activity of the R141 plasmid was not observed to be significantly decreased in the *hap1*$^-$ strain (Table 6.1). A significant increase in the β-galactosidase activity of the R182 plasmid was observed in the *hap1*$^-$ strain, with growth upon 1% glycerol (compared to the *hap1*$^+$ strain) (Table 6.1, Appendix 4.5), suggesting that depression of the *cycA* promoter may be occurring. However, due to the low β-galactosidase activity of R141, it is apparent that the expression system is not working. Therefore, the expression of the *A. nidulans cycA* gene promoter in *S. cerevisiae* cannot be reliably evaluated from this experiment.

CONCLUSION

Characterisation of the *A. nidulans* cytochrome *c*-deficient strains was carried out in this study. Growth parameters of strain A68 (*cycA*$^-$) were consistent with those expected for a cytochrome *c* mutant. Interestingly, alcoholic fermentation was suggested to be occuring in strain A68, as growth was preferential upon a fermentable carbon source, and the strain produced higher levels of ethanol than the corresponding wildtype strain. Spectral analysis confirmed the lack of detectable levels of cytochrome *c* in the *A. nidulans cycA* strains. This was an important finding, as it is the first report of a filamentous fungus shown to be viable after complete elimination of a functional cytochrome *c* gene. Spectral analysis also revealed diminished levels of cytochrome *c* oxidase in the *cycA*$^-$ strains, consistent with the non-functioning cytochrome *c*-dependent respiratory pathway.

Another intriguing finding was the presence of a hemoglobin-like molecule in the *cycA*$^-$ and *cycA*$^+$ strains, revealed by CO binding assays – the first known report of a hemoglobin-like molecule in *A. nidulans*. It was suggested the putative hemoglobin molecule in the *cycA*$^-$ strains could act as a terminal oxidase, to compensate for the non-functioning cytochrome *c*-dependent pathway. The putative hemoglobin molecules in the *cycA*$^-$ and *cycA*$^+$ strains could also possibly be involved in NO detoxification, similar to hemoglobin in yeast (Crawford *et al.*, 1998; Liu *et al.*, 2000).

The presence of an alternative respiratory pathway in *cycA*$^-$ and *cycA*$^+$ strains of *A. nidulans* was determined by inhibitor studies. The *cycA*$^-$ strains were postulated to have an increased activity of the alternative respiratory pathway, to compensate for the impaired cytochrome *c*-dependent pathway. However, this could not be determined in this study due to the limitations of the technique used (Day, 1996). As expected, an active cytochrome *c*-dependent pathway was found to be present in the *cycA*$^+$ strains, yet absent from the *cycA*$^-$ strains. Interestingly, the presence of a putative third terminal oxidase in both strains was also suggested. Elevated *b*-type heme levels observed in the redox spectra of the *cycA*$^-$ strains could possibly be associated with the putative third terminal oxidase. A putative third terminal oxidase has also been suggested to be present in the pathogenic filamentous fungus *Gaeumannomyces graminis* (var. *tritici*) (Joseph-Horne *et al.*, 1998).

Therefore, the results of this study indicated that the *A. nidulans cycA*$^-$ strains may compensate for the lack of the classical respiratory pathway by using fermentation and the alternative respiratory pathway. A putative hemoglobin molecule and putative terminal oxidase may also be involved in the respiration of these strains. The alternative respiratory pathway is generally less efficient at ATP production, therefore accounting for the slower growth of the *cycA*$^-$ strain. The presence of the alternative respiratory pathway in both the *cycA*$^-$ and *cycA*$^+$ strains of *A. nidulans* suggests a general role of the pathway. Recent studies have suggested the alternative respiratory pathway may have evolved as a cellular defense mechanism against damage by ROS (Wagner, 1995; Popov *et al.*, 1997; Maxwell *et al.*, 1999).

Fragments of the *A. nidulans AOX* gene were PCR amplified with degenerate primers, confirming the presence of an *AOX* gene in *cycA*$^+$ and *cycA*$^-$ strains of *A. nidulans*. The protein encoded by this gene may function as the terminal oxidase for the alternative respiratory pathway, shown to be present in these strains. A comparison of fungal and plant AOX sequences indicated the lack of pyruvate regulation and dimer formation for fungal AOX proteins, in contrast to plant AOX proteins, thus supporting results from previous studies (Joseph-Horne *et al.*, 2001). An expression study of the *A. nidulans AOX* gene was carried out by RT-PCR, however it was not possible to quantify any differences in *AOX* expression between the samples in this study.

A preliminary study of the *A. nidulans AOX* gene promoter was carried out, to elucidate the molecular mechanisms which may be governing the expression of the gene. Interestingly, the putative regulatory sites identified within the (published) *A. nidulans AOX* gene promoter are similar to the elements located within the promoters of other fungal *AOX* genes, thus suggesting similar mechanisms of gene regulation. The putative elements observed within the *A. nidulans AOX* gene promoter were also found to be similar to those located within the promoters of the *A. nidulans* superoxide dismutase (*sod1*) and catalase (*catA-C*) genes, which encode enzymes that reduce ROS. As mentioned above, the alternative respiratory pathway has been implicated in reducing ROS formation, and coordinate regulation of genes involved in this process is highly likely.

Many of the putative regulatory elements which have been identified within the *A. nidulans AOX* gene promoter are also located within the *A. nidulans cyc*A gene promoter, indicating

similar mechanisms of regulation. The presence of regulatory elements within the promoter of the *cyc*A gene was surprising, as it was expected that such an essential gene would be highly expressed at all times in an obligate aerobe. The discovery of an alternative respiratory pathway in *A. nidulans* explains the possible need for regulation of the cytochrome *c*-dependent pathway.

A functional analysis was carried out to determine the significance of the putative regulatory elements identified within the *A. nidulans cyc*A gene promoter. *A. nidulans* was transformed with reporter constructs containing *cyc*A-*lac*Z fusion genes. Although a number of *arg*B$^+$ transformants contained reporter constructs integrated at the *arg*B locus, these were determined to have very low levels of *lac*Z expression, with the exception of the control construct (containing an *A. nidulans gpd*A promoter fragment). This type of expression system has been used successfully many times before (e.g. Punt *et al.*, 1990; Brakhage *et al.*, 1992). Therefore, a number of parameters were investigated, and a 'promoter switch' experiment commenced. However, the cause of the faulty *cyc*A-*lac*Z expression system was not determined in this study.

Functional expression of the *A. nidulans cyc*A gene promoter in yeast was also attempted, as regulatory mechanisms controlling cytochrome *c* expression are believed to be analogous in *A. nidulans* and *S. cerevisiae* (McGlynn, 1997). *S. cerevisiae* was transformed with reporter constructs containing *cyc*A-*lac*Z fusion genes, with resulting low β-galactosidase activity, similar to the results in *A. nidulans*. The wildtype strain of *A. nidulans* from which the *cyc*A gene promoter fragment was amplified was shown by spectral analysis to have low levels of cytochrome *c*, in comparison to other wildtype strains of *A. nidulans*. Therefore, it is possible that the low levels of *lac*Z expression from the *cyc*A-*lac*Z fusion genes may be representative of the level of *cyc*A expression in that strain. However, low levels of *lac*Z expression were also observed for the *S. cerevisiae* positive control, indicating that the expression system was faulty. Therefore, the expression of the *A. nidulans cyc*A gene promoter in *S. cerevisiae* could not be assessed in this study.

FUTURE DIRECTIONS

Characterisation of the alternative respiratory pathway in *Aspergillus* species, including *A. nidulans*, could have important implications if it is shown to be important for the virulence and survival of the fungi. Although *AOX* genes have only been identified in *A. nidulans* and *A. niger* to date, a recent search of an *A. fumigatus* EST (expressed sequence tag) database revealed the presence of sequences with high similarity to the *A. nidulans AOX* gene. It is therefore very likely that other *Aspergilli* also contain *AOX* genes and thus an alternative respiratory pathway.

The confirmation and characterisation of the alternative respiratory pathway in *A. fumigatus* is an exciting area of possible future research, as the fungus is an extremely prevalent human pathogen. The AOX protein has been suggested as a potential target for antifungal agents, if the pathway is shown to be important for the virulence and survival of the fungus. Determining the presence of the alternative respiratory pathway in other *Aspergillus* species, especially those with industrial or pharmaceutical applications, is also an important area of further research. Knowledge attained regarding the regulation of the pathway's activity could be used to increase the fungus' metabolism e.g. to augment antibiotic production.

Recent studies have suggested that the alternative respiratory pathway could have an important role in enabling pathogenic fungi to survive defence mechanisms activated by the host upon invasion. For example, the activity of the alternative respiratory pathway in the pathogenic fungi *Histoplasma capsulatum* (McEwen & Johnson, 2001) and *Candida albicans* (Huh & Kang, 2001) has been reported to increase when the cytochrome *c*-dependent pathway is inhibited by ROS (simulating the oxidative burst generated by the host upon invasion). It is possible that the regulation of *AOX* expression in *A. nidulans* may be similar to *H. capsulatum* and *C. albicans*, as all of the *AOX* genes contain similar putative regulatory elements within their promoters. Determining the role of the alternative respiratory pathway in both non-pathogenic and pathogenic *Aspergillus* species will therefore be an interesting area of future investigation.

A functional analysis of the *A. nidulans AOX* gene promoter would determine whether the putative regulatory elements located within this region are functionally important. The

putative elements identified suggest that the mechanisms controlling expression of the *A. nidulans AOX* gene may be similar to the mechanims which regulate the expression of other respiratory-related genes (such as the *cyc*A gene). It is also possible that there is coordinate regulation of the *A. nidulans* genes involved in guarding against cellular damage by ROS (eg. *AOX*, *sod1*, *catA-C*). To determine whether such coordinate regulation of gene expression is occurring, a microarray analysis could be carried out. Microarray analysis is a very powerful technique that enables simultaneous and comparative analysis of the expression of many genes under a defined set of conditions (for a review, see Duggan *et al.*, 1999).

Before commencing further expression studies of the *A. nidulans AOX* gene, it would be advisable to carry out Southern analysis to ascertain the copy number of the gene. Recent studies have indicated that some fungi have multiple *AOX* genes, e.g. *N. crassa* (Li *et al.*, 1996) and *C. albicans* (Huh & Kang, 2001). Northern analysis could be used to investigate *AOX* expression, with RT-PCR being used only if expression levels are too low for easy detection. For either protocol, extreme care would have to be taken to ensure contamination does not occur. For future RT-PCR experiments, primers could be designed which amplify over an intron (for DNA templates), so that contamination by DNA is easily detected. The number of cycles used in the PCR programme should also be optimised, to minimise the possibility of amplifying non-specific targets.

For the expression studies, it would be interesting to see if changes in the physiological conditions (eg. carbon source, oxygen supply and temperature) have an effect on *AOX* mRNA transcript levels. This type of analysis could also be done with microarray analysis. It is likely that control of *AOX* gene expression by some of these parameters (e.g. carbon source) is occurring, given the presence of putative regulatory elements within the *AOX* gene promoter which mediate these regulatory mechanisms.

Electron partitioning between the alternative and cytochrome *c*-dependent pathways in *A. nidulans* could be determined using a technique such as oxygen fractionation (Jarmuszkiewicz *et al.*, 1998). A more accurate assessment of the activities of the pathways could then be made, as age of the culture increases, and under various physiological conditions. Importantly, a comparison of the activity of the alternative respiratory pathway could be made between the *cycA*$^{+}$ and *cycA*$^{-}$ strains of *A. nidulans*. These results may also

give an indication of the physiological parameters influencing expression of the *cyc*A and *AOX* genes.

The intriguing discovery of a hemoglobin-like molecule in the *cycA*$^+$ and *cycA*$^-$ strains of *A. nidulans* could also be explored further. Levels of the protein in the *cycA*$^+$ and *cycA*$^-$ strains could be analysed by heme staining following SDS-PAGE (Satori *et al.*, 1999). A recent search of an *A. nidulans* EST (expressed sequence tag) database revealed the presence of sequences with high similarity to flavohemoglobin from the bacterium *Deinococcus radiodurans* and the fungus *Fusarium oxysporum*. Expression of the gene encoding the (flavo)hemoglobin-like molecule could be investigated under different physiological conditions (e.g. carbon or nitrogen stress), to determine what the role of the encoded protein might be.

It would also be interesting to determine whether levels of the hemoglobin-like protein are higher in the *cycA*$^-$ strains, to compensate for the non-functioning cytochrome *c* dependent pathway. However, results from the inhibitor studies suggest that the hemoglobin-like molecule is not involved in electron transfer (at least in the *cycA*$^-$ strains); hemoglobin is inhibited by cyanide, yet the *cycA*$^-$ strains did not exhibit cyanide-sensitive respiration. Therefore, it may be more likely that the hemoglobin-like molecules in the *cycA*$^+$ and *cycA*$^-$ strains of *A. nidulans* have a general role, e.g. in response to nitrosative stress, similar to yeast hemoglobin (Satori *et al.*, 1999).

It is very likely that the cause of the faulty *cyc*A-*lacZ* expression system in *A. nidulans* could be determined, if the 'promoter switch' experiment had been successful (ie. if *arg*B$^+$ transformants were obtained which contained reporter constructs, integrated at the *arg*B locus). It is probable that these transformants would be obtained, given time. Analysis of these transformants would hopefully indicate whether the defective *cyc*A-*lacZ* expression system was due to a fault within the promoter or vector sequences.

Alterations to the *cyc*A-*lacZ* reporter constructs could also be made. Although the translational fusions within the reporter constructs are believed to be correct (given the position of the translational start site), determination of the transcriptional start site would confirm that it is present within the *cyc*A promoter fragments. Generally, two protocols are carried out to confirm the position of the transcriptional start site, e.g. primer extension

analysis and S1 nuclease mapping (Li *et al.*, 1996). A larger portion of the *cyc*A promoter (e.g. 2 kb) could also be used for further analysis, in case important regulatory elements were omitted from the 1.3 kb fragment. A different reporter gene could also be used, e.g. GUS (β-glucuronidase) or GFP (green fluorescent protein), both of which have been used extensively in *A. nidulans* (Redkar *et al.*, 1998 and Tavoularis *et al.*, 2001).

To ensure that chromosomal repression of the *cyc*A-*lac*Z reporter constructs at the *arg*B locus was not occurring, the constructs could be analysed using an alternative *A. nidulans* expression system, e.g. the *amd*S expression system (van Gorcom *et al.*, 1985). Alternatively, the reporter constructs could be transformed into a cytochrome *c* deficient strain, either in an ectopic fashion or at the *cyc*A locus (if the *cyc*A coding region was included in the reporter constructs). The latter would have the additional benefit of complementing the *cyc*A mutation and thus providing further confirmation of the cytochrome *c* deficient status of the *cycA*⁻ strains.

Electrophoretic mobility shift assays (EMSA) or DNA footprinting could be carried out to analyse the *cyc*A gene promoter, simultaneously with the functional analysis. EMSA and DNA footprinting both detect the interaction of DNA binding proteins with their cognate DNA recognition sequences. EMSA also facilitates any interactions to be analysed in both a qualitative and quantitative manner. Site-directed mutagenesis could be utilised to mutate putative binding sites within the *cyc*A promoter (e.g. the most probable HAP1 binding site) to determine the effect on complex formation. The identification of the proteins involved in any observed interactions could then be determined by western analysis or supershift experiments, using an antibody to the protein of interest (Voet & Voet, 1990).

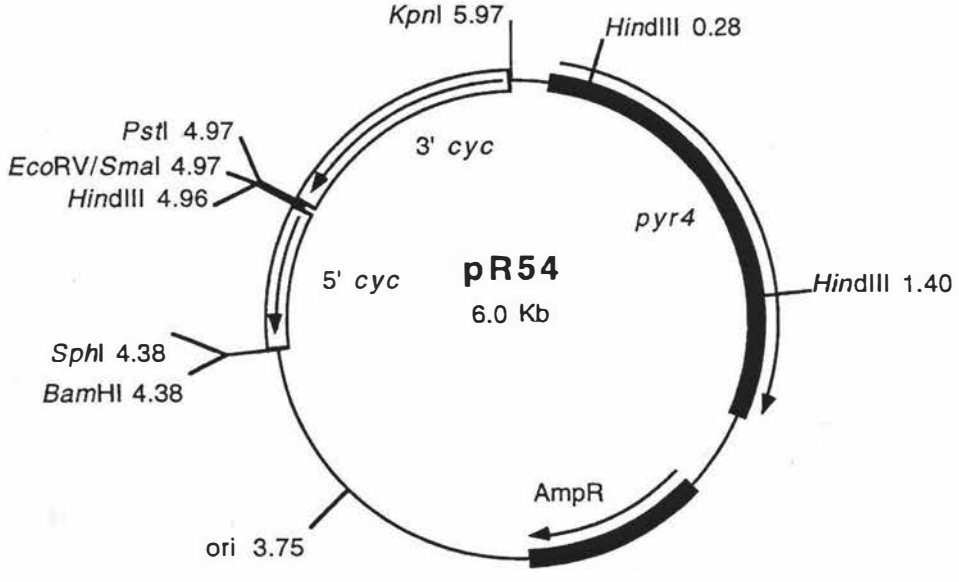
In conclusion, this study generated a number of novel and important findings; this is the first report of a filamentous fungus shown to be viable after complete elimination of a functional cytochrome *c* gene and also the first known report of a hemoglobin-like molecule in *A. nidulans*. The characterisation of the alternative mechanisms which these cytochrome *c* deficient strains utilise to generate energy were also intriguing findings. Further characterisation of the alternative respiratory pathway in *Aspergillus* species, including *A. nidulans*, could have important medical and commerical implications if it is shown to be important for the virulence and survival of the fungi, as the fungi are prevalent human pathogens, and have many industrial and pharmaceutical applications.

Despite the difficulties encountered with the functional analysis of the *A. nidulans cycA* gene promoter, this study has paved the way for exciting possible areas of future research concerning the possible coordinate expression of genes involved in interconnected respiratory pathways and ROS detoxification mechanisms in *A. nidulans*.

Appendix 1.1 Vector Map of pR54

The plasmid used for the disruption of the *cyc*A gene in wild type strains of *A. nidulans* is shown. The 5' and 3' regions of the cycA gene are indicated (ie. sequences upstream and downstream of the coding sequence, respectively). Restriction sites within the plasmid and the *N. crassa pyr4* selectable marker are also indicated.

Reference: Bird, 1996.



Appendix 1.2 Growth Curve Data For *A. nidulans* Strains A67 And A68

Strain A67 (*cycA*$^{+}$)

Age (hrs)	Dry Weights (mg)				Std error
	Average	Sample 1	Sample 2	Sample 3	
0	0	0	0	0	0
12	1.5	0.9	1.6	2.0	0.19
16	11.5	8.7	8.7	17.2	1.64
20	27.0	19.1	61.8	22.5	7.91
24	27.4	24.6	30.9	26.7	1.07
32	27.9	26.2	25.4	32.1	1.22
36	29.2	34.1	25.3	28.1	1.50
40	29.5	25.1	28.5	34.9	1.66

Strain A68 (*cycA*$^{-}$)

Age (hrs)	Dry Weights (mg)				Std error
	Average	Sample 1	Sample 2	Sample 3	
0	0	0	0	0	0
17	2.8	2.6	2.8	2.9	0.09
23	2.7	1.6	4.0	2.4	0.71
27	3.3	3.1	3.9	3.0	0.29
39	17.0	16.2	20.7	14.0	1.97
43	26.0	29.9	24.0	24.1	1.95
47	22.2	27.7	16.1	22.8	3.36
52	19.7	13.2	23.9	21.9	3.28
64	20.6	30.2	8.5	23.1	6.39
70	18.1	19.6	18.9	15.9	5.04

Appendix 1.3 Estimation Of Cytochrome *c* Amount

The amounts of cytochrome *c* in the *cyc*A$^+$ and *cyc*A$^-$ samples were estimated as follows:

$$[\text{cytochrome } c] = \frac{A \times (1.0 \times 10^{-3})/(18.7 \times 10^{3})}{[\text{protein}]}$$

A = Absorbance
1.0×10^{-3} = volume (L)
18.7 = extinction coefficient (mM x 10^{-1}) for cytochrome *c*
[protein] = concentration of protein (mg/L)

The extinction coefficient for cytochrome *c* was obtained from Margoliash & Frohwirt (1959).

The amounts of cytochromes *b* and aa_3 were estimated in the same manner, using the appropriate extinction coefficients (20.0 x 10^{-1} and 20.5 x 10^{-1} mM, respectively) (*West et al.*, 1988; Vanneste, 1966).

The protein concentrations of the samples were estimated using 0.5mg/ml Bovine Serum Albumin (BSA) used as a standard, and the Bradford method (Sambrook *et al.*, 1989). The protein concentrations (mg/ml) for the strains were as follows: A57, 1.173; A58, 0.435; A67, 0.766; A68, 0.248.

Appendix 1.4 A **Measurement Of Respiratory Rate Following The Addition Of Inhibitors (Late-Exponential Phase).**

Strain	Addition	ORR 1	ORR 2	ORR 3	FRR 1	FRR 2	FRR 3
A57	KCN	279.12	353.31	282.65	141.32	187.26	109.53
	Sham	303.85	339.18	222.57	218.95	211.99	197.84
	Ant + Sh	314.45	272.05	289.72	53.00	98.93	123.66
	KCN + Sh	279.12	353.31	282.65	88.33	130.73	84.79
	Sh + KCN	303.85	339.18	222.57	62.56	109.53	70.66
A58	KCN	282.65	706.62	353.31	282.65	706.62	353.31
	Sham	328.58	423.97	261.45	14.41	53.00	4.59
	Ant + Sh	144.86	635.96	173.12	36.22	127.19	12.89
A67	KCN	127.35	180.78	106.69	83.17	72.31	56.06
	Sham	177.90	266.85	147.94	161.89	231.28	132.27
	Ant + Sh	314.66	338.26	475.37	186.02	149.46	98.33
	KCN + Sh	39.57	226.09	225.65	28.26	77.25	82.74
	Sh + KCN	156.67	314.66	230.26	53.94	121.93	88.75
A68	KCN	321.41	321.41	363.91	321.41	321.41	363.91
	Sham	315.39	348.19	455.32	43.68	32.14	34.82
	Ant + Sh	241.29	348.19	321.41	31.54	42.85	37.50

ORR and FRR denote the original and final respiration rates (nM O_2 consumed/min/g wet weight), respectively. The inhibitors added were: KCN: Potassium Cyanide; Sh: salicyl hydroxamic acid (SHAM); Ant: Antimycin A.
The % Inhibition was calculated from the RR values: % I = 1 – [FRR (X)/ORR (X)]

Appendix 1.4 B **Measurement Of Respiratory Rate Following The Addition Of Inhibitors (Stationary Phase).**

Strain	Addition	ORR 1	ORR 2	ORR 3	FRR 1	FRR 2	FRR 3
A67	KCN	106.55	202.71	70.75	33.96	42.62	75.37
	SHAM	226.25	163.33	228.67	191.46	143.73	195.99
	ANT + SH	204.37	201.54	125.54	68.14	36.90	31.86
	KCN + SH	393.33	173.15	204.38	110.13	48.26	56.78
	SH + KCN	206.88	245.38	252.00	51.72	67.48	62.17
A68	KCN	224.48	218.24	436.48	224.48	218.24	436.48
	SHAM	227.59	221.36	467.66	27.13	25.88	46.77
	ANT + SH	374.13	162.12	205.77	53.01	25.87	30.25

ORR and FRR denote the original and final respiration rates (nM O_2 consumed/min/g wet weight), respectively. The inhibitors added were: KCN: Potassium Cyanide; SHAM: salicyl hydroxamic acid; Ant: Antimycin A.
The % Inhibition was calculated from the RR values: % I = 1 – [FRR (X)/ORR (X)]

Appendix 1.5 Raw Data For Inhibitor Studies

An example of the raw data generated by the inhibitor studies is shown (used for the estimation of % inhibition, shown in Table 3.3). The results are given as relative activities (RA). Standard error (Std error) is also indicated. The inhibitors added are: KCN, Potassium Cyanide; SHAM, salicyl hydroxamic acid; Ant. A, Antimycin A (+ indicates a stimulatory effect instead of the expected inhibitory result).

Strain A67 (*cycA*$^+$): Addition Of KCN In The Presence/Absence Of SHAM (Late-Exponential Phase)

[KCN] (mM)	Mean RA	Sample 1	Sample 2	Sample 3	Std error
In the absence of SHAM					
0	1	1	1	1	0
0.0039	0.765	0.797	0.760	0.737	0.02
0.0078	0.719	0.721	0.701	0.735	0.01
0.0156	0.701	0.812	0.592	0.698	0.06
0.0313	0.534	0.431	0.575	0.597	0.05
0.0625	0.455	0.436	0.448	0.480	0.01
0.125	0.531	0.430	0.570	0.598	0.05
0.25	0.526	0.653	0.525	0.400	0.07
In the presence of SHAM					
0	1	1	1	1	0
0.0039	0.652	0.684	0.707	0.565	0.04
0.0078	0.614	0.620	0.561	0.660	0.03
0.0156	0.556	0.615	0.447	0.604	0.05
0.0313	0.519	0.653	0.400	0.505	0.07
0.0625	0.445	0.528	0.350	0.457	0.05
0.125	0.408	0.615	0.293	0.315	0.10
0.25	0.372	0.344	0.385	0.388	0.01

Appendix 2.1 Design of Degenerate Primers for PCR amplification of *A. nidulans AOX* gene

Key to Sequence Symbols *(UNIX GCG program)*

IUB/GCG	Meaning	Complement	Staden/Sanger
A	A	T	A
C	C	G	C
G	G	C	G
T/U	T	A	T
M	A or C	K	M
R	A or G	Y	R
Y	C or T	R	Y
K	G or T	M	K

A. niger sequence is that of the coding strand for primers AOX1 & 2; and non-coding strand for primers AOX3 & 4.

Primer AOX1 Anneals to a potential membrane-spanning domain (located at ≈460bp downstream of the transcriptional start site)

Amino acid seq (5' to 3')	VAL (V)	ALA (A)	GLY (G)	VAL (V)	PRO (P)	GLY (G)	MET (M)	VAL (V)
Codon seq	GUA GUC GUG GUU	GCA GCC GCG GCU	GGA GGC GGG GGU	GUA GUC GUG GUU	CCA CCC CCG CCU	GGA GGC GGG GGU	AUG	GUA GUC GUG GUU
DNA seq (5' to 3')	GTI	GCI	GGI	GTI	CCI	GGI	ATG	GTI
A. niger seq (5' to 3')	GTC	GCT	GGC	GTA	CCA	GGC	ATG	GTG
PRIMER SEQ (5' to 3')	**GTY**	**GCY**	**GGY**	**GTI**	**CCI**	**GGY**	**ATG**	**GT**

Primer AOX 2 Anneals to a highly conserved sequence (located at ≈580bp downstream of the transcriptional start site)

Amino acid seq (5' to 3')	GLU (E)	ALA (A)	TYR (Y)	ASN (N)	GLU (E)	ARG (R)	MET (M)	HIS (H)
Codon seq	GAA GAG	GCA GCC GCG GCU	UAC UAU	AAC AAU	GAA GAG	AGA AGG CGA CGC CGG CGU	AUG	CAC CAU
DNA seq (5' to 3')	GAR	GCI	TAY	AAY	GAR	MGI	ATG	CAY
A. niger seq (5' to 3')	GAA	GCA	TAC	AAC	GAG	CTG	ATG	CAT
Primer seq (5' to 3')	**GAR**	**GCY**	**TAY**	**AAY**	**GAR**	**MGI**	**ATG**	**CA**

Primer AOX3 Anneals to a putative iron-binding motif (located at ≈720bp downstream of the transcriptional start site)

Amino acid seq: (5' to 3')	VAL (V)	GLY (G)	TYR (Y)	LEU (L)	GLU (E)	GLU (E)	GLU (E)	ALA (A)
Codon seq	GUA GUC GUG GUU	GGA GGC GGG GGU	UAC UAU	UUA UUG CUA CUC CUG CUU	GAA GAG	GAA GAG	GAA GAG	GCA GCC GCG GCU
DNA Seq (5' to 3')	GTI	GGI	TAY	YTI	GAR	GAR	GAR	GCI
Complementary Seq (3' to 5')	CAI	CCI	ATG	RAI	CTY	CTY	CTY	CGI
Complementary Seq (5' to 3')	IGC	YTC	YTC	YTC	IAR	GTA	ICC	IAC
A. niger seq (5' to 3')	CGC	TTC	CTC	CTC	GAG	GTA	TCC	GAC
Primer seq (5' to 3')	**GC**	**YTC**	**YTC**	**YTC**	**IAR**	**RTA**	**ICC**	**IAC**

Primer AOX4 Anneals to a putative iron-binding motif (located at ≈900bp downstream of the transcriptional start site)

Amino acid seq (5' to 3')	ALA (A)	ASP (D)	GLU (E)	ALA (A)	LYS (K)	HIS (H)	ARG (R)	GLU (E)
Codon seq	GCA GCC GCG GCU	GAC GAU	GAA GAG	GCA GCC GCG GCU	AAA AAG	CAC CAU	CGA CGC CGG CGU AGA AGG	GAA GAG
DNA seq (5' to 3')	GCI	GAY	GAR	GCI	AAR	CAY	MGI	GAR
Complementary Seq (3' to 5')	CGI	CTR	CTY	CGI	TTY	GTR	KCI	CTY
Complementary Seq (5' to 3')	YTC	ICK	RTG	YTT	IGC	YTC	RTC	IGC
A. niger (5' to 3')	CTC	CCG	GTG	TTT	GGC	TTC	ATC	TGC
Primer seq (5' to 3')	**TC**	**ICK**	**RTG**	**CTT**	**IGC**	**YTC**	**RTC**	**IGC**

Appendix 2.2 Codon Useage Table For *A. nidulans*

143 CDS's (90017 codons)

fields: [triplet] [frequency: per thousand] ([number])

UUU 14.4	(1300)	UCU 15.4	(1388)	UAU 13.1	(1175)	UGU 5.1	(455)
UUC 25.2	(2269)	UCC 15.4	(1382)	UAC 19.1	(1721)	UGC 7.8	(704)
UUA 5.5	(497)	UCA 12.0	(1076)	UAA 0.5	(48)	UGA 0.6	(54)
UUG 14.6	(1313)	UCG 14.1	(1267)	UAG 0.5	(41)	UGG 12.6	(1138)
CUU 19.5	(1759)	CCU 16.0	(1442)	CAU 11.6	(1042)	CGU 11.1	(997)
CUC 23.2	(2091)	CCC 14.0	(1258)	CAC 12.9	(1162)	CGC 16.2	(1457)
CUA 8.6	(774)	CCA 12.4	(1114)	CAA 15.2	(1366)	CGA 10.5	(944)
CUG 20.6	(1854)	CCG 13.8	(1238)	CAG 25.3	(2278)	CGG 11.0	(989)
AUU 18.5	(1661)	ACU 14.3	(1285)	AAU 14.7	(1319)	AGU 9.1	(815)
AUC 27.3	(2458)	ACC 19.1	(1722)	AAC 23.3	(2093)	AGC 15.3	(1379)
AUA 6.4	(580)	ACA 13.4	(1210)	AAA 15.7	(1411)	AGA 6.3	(569)
AUG 22.3	(2004)	ACG 12.5	(1127)	AAG 32.1	(2892)	AGG 6.1	(551)
GUU 17.7	(1589)	GCU 23.3	(2094)	GAU 26.0	(2340)	GGU 18.3	(1647)
GUC 23.3	(2100)	GCC 25.6	(2306)	GAC 29.3	(2640)	GGC 22.5	(2029)
GUA 6.1	(550)	GCA 16.5	(1488)	GAA 26.0	(2340)	GGA 13.8	(1241)
GUG 14.5	(1306)	GCG 16.9	(1521)	GAG 35.3	(3177)	GGG 10.9	(980)

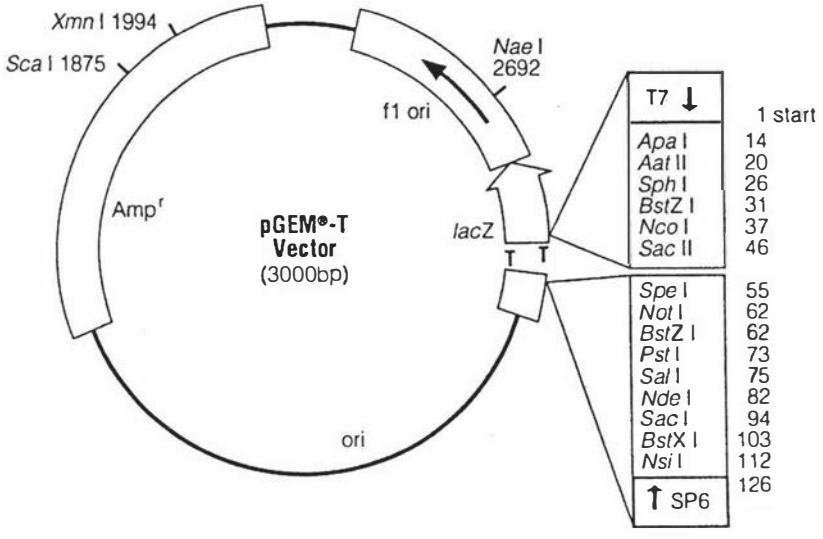
Coding GC 52.74% 1st letter GC 56.78% 2nd letter GC 43.18% 3rd letter GC 58.26%

Data obtained from http://www.kazusa.or.jp/codon/

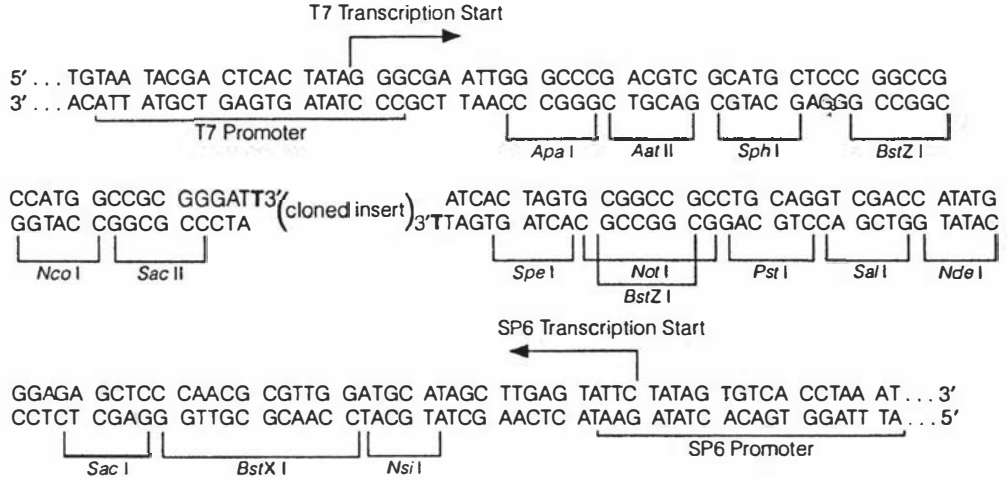
Appendix 2.3 Vector Map of pGEM-T

Restriction sites within the pGEM-T vector are shown.

Reference: Promega.



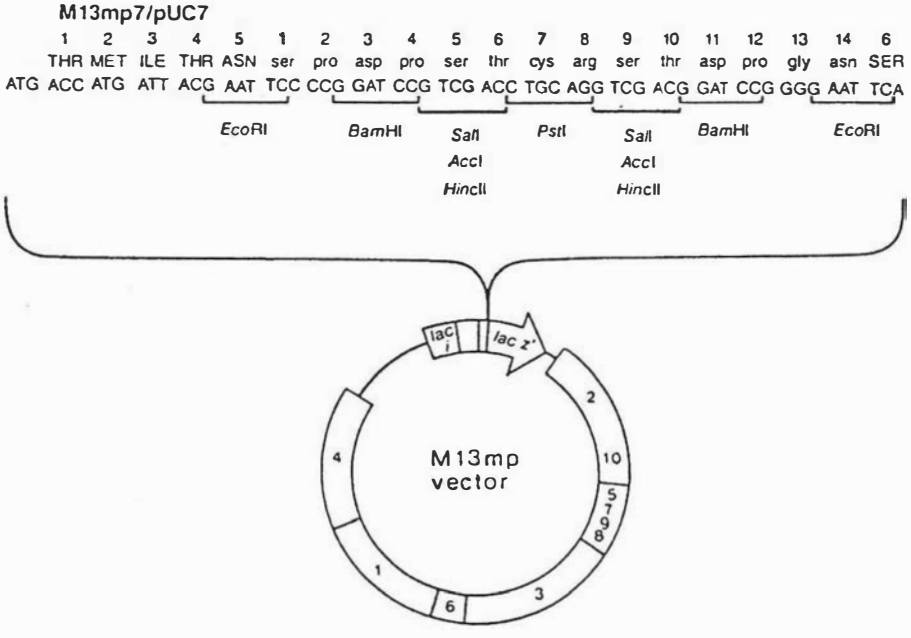
pGEM®-T Vector



Appendix 3.1 Vector Map Of R12

Restriction map of plasmid R12 (pIC-20R), showing all relevant restriction enzyme sites. The multiple cloning site of pUC7 was altered to form plasmid R12.

Reference: Sambrook *et al* (1989)



pIC-20R ACG AAT TCA TCG ATA TCT AGA TCT CGA GCT CGC GAA AGC TTG CAT GCC TGC
EcoRI *ClaI* *XbaI* *XhoI* *NruI* *HindIII* *SphI* *PstI*
EcoRV *BglII* *SacI*

AGG TCG ACT CTA GAG GAT CCC CGG GTA CCG AGC TCG AAT TCA
SalI *XbaI* *BamHI* *KpnI* *SacI* *EcoRI*
SmaI

Appendix 3.2 Sequence Of The Translational Fusion Of The *cyc*A Promoter Fragment With The *lac*Z Reporter Gene

The *Bam*HI site is indicated (in red), which forms the translational fusion between the *cyc*A promoter (black) and *lac*Z (blue) sequences. The position of the proposed transcriptional and translational start sites of the *cyc*A gene are indicated by thick and double underlining, respectively. The annealing sites of the primers (CPD5 and lacZ forward) used to check the *cyc*A-*lac*Z translational fusions are shown.

CPD5>>

CTATCTAGAGCTGCATAACTGAGCCCTTAGACGTAGTATATAGGATTACAATAGTCTCTAAATAAAGCTT

CATCCAGCCAGGCGTTATTTGCAGTGACCGATTCCTGACCTCAGACCCGGCAGCGCCCGTTACTCTGAGC

ACAGTGCTGAATCATCCTACCTCTGATTGGTCAATTCCCAGATCACGGGTGTCGTCGGGGGCGCGACCAA

GAAACCAGCTCTACAAATTCCCTCCAAGTTTTTTTCTTCCCTTTGGCCAGTCCGCTTGACTTGAATTCTG

TCTTTCATCTTCT**CTG**CTACATACAACTCTTTGTACTATACCACTTACCTCTTTACATAACCCTTTCTCT

CTACCTCTTTATTTTTTATCACTCACA**ATG**GCTAAGGGCGGTGACAGCTA**GGATCC**CGTCGTTTTACAAC

GTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTG

<<LacZ forward

Appendix 3.3 Transformation Of Strain A71 With Reporter Constructs

Construct	% Viability of pps	# of Viable pps in sample	# of *arg*B$^+$ trnsfmts	Amount of DNA (μg)	# of trnsfmts per μg DNA	# of trnsfmts per viable pp	# of trnsfmts per viable pp (average)	# of trnsfmts per μg DNA (average)
R108	7.75	775000	15	5	3	1.94E-05	6.64E-05	2.4
	0.39	39000	9	5	1.8	0.000231		
	18.75	1875000	14	5	2.8	7.47E-06		
	12.25	1225000	10	5	2	8.16E-06		
R159	7.75	775000	3	5	0.6	3.87E-06	2.45E-06	0.4
	9.75	975000	1	5	0.2	1.03E-06		
R169	7.75	775000	4	5	0.8	5.16E-06	1.54E-05	0.5
	0.39	39000	1	5	0.2	2.56E-05		
R170	7.75	775000	1	5	0.2	1.29E-06	7.45E-06	0.3
	0.39	39000	1	5	0.2	2.56E-05		
	18.75	1875000	2	5	0.4	1.07E-06		
	11	1100000	2	5	0.4	1.82E-06		
R171	12.25	2450000	1	10	0.1	4.08E-07	7.17E-07	0.15
	9.75	975000	1	5	0.2	1.03E-06		
R172	18.75	1875000	1	5	0.2	5.33E-07	9.09E-07	0.3
	30.5	3050000	2	5	0.4	6.56E-07		
	9.75	1950000	3	10	0.3	1.54E-06		
R173	18.75	1875000	3	5	0.6	1.6E-06	1.31E-06	0.4
	9.75	975000	1	5	0.2	1.03E-06		
R116	30.5	6100000	5	10	0.5	8.2E-07	8.2E-07	0.5
R117	30.5	6100000	5	10	0.5	8.2E-07	8.2E-07	0.5

The following abbreviations are used: pp, protoplast; trnsfmt, transformant.

Appendix 3.4 Raw Data For *A. Nidulans* β-Galactosidase Assays

The β-galactosidase assays were carried out on cultures grown in 1% glucose (**A**) and glycerol (**B**). One unit is defined as 1 nmol *ortho*-nitro-phenol produced per min at 37°C (Punt *et al.*, 1990).

A

Strain	β-galactosidase Activity (units/min/mg protein)				
	Sample 1	Sample 2	Sample 3	Average	± Standard Error
Strain A71	10.8	29.6	22.5	21.0	5.5
R159.3	11.9	13.9	14.9	13.5	0.9
R170.1	16.9	25.1	24.1	22.0	2.6
R173.2	23.7	21.3	10.7	18.5	4.0
R117.2	17.1	34.7	14.6	22.1	6.3
R116.2	29990.6	188756.7	29818.2	82855.2	52950.8

B

Strain	β-galactosidase Activity (units/min/mg protein)				
	Sample 1	Sample 2	Sample 3	Average	± Standard Error
Strain A71	41.8	61.7	35.6	46.4	7.9
R159.3	45.4	37.5	24.5	35.8	6.1
R170.1	28.6	143.7	39.4	70.5	36.7
R173.2	24.5	166.7	132.9	108.0	42.9
R117.2	24.9	66.2	23.6	38.2	14.0
R116.2	9277.8	26955.6	18116.7	18116.7	5103.1

Note: The promoter fragments contained within the constructs are as follows:
Strain A71, R117.2: none
R159.3: 1.3 kb *cycA*
R170.1: 0.68 kb *cycA*
R173.2: 0.4 kb *cycA*
R116.2: 2.0 kb *gpdA*

Appendix 3.5 Alignment Of The *lacZ* Reporter Gene Sequences

The nucleotide sequence of the *lacZ* gene from the plasmid R117 is shown aligned with the known *E. coli lacZ* gene sequence. The first eight codons (in bold) are not included in the R117 *lacZ* gene sequence. The *Bam*HI site which facilitated the cloning of fragments into R117 is indicated by thick underlining. The amino acid residues encoded are positioned beneath the *E. coli lacZ* nucleotide sequence, initiating at the translational start site (underlined). The amino acids residues encoded by R117 are indicated by blue lettering. Differences in the R117 *lacZ* nucleotide sequence (compared to the *E. coli lacZ* gene sequence) are indicated by red highlighting. The annealing sites of the primers used to sequence the *lacZ* reporter gene within plasmid R117 are indicated (LacZ1 – 7, Table 2.1).

```
                                                                lacZ1>>
LacZ          1  ACCATGATTACGGATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGC  60
R117          1              CTCGAGGATCCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGC  22
              1  T  M  I  T  D  S  L  A  V  V  L  Q  R  R  D  W  E  N  P  G   20

             61  GTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAA  120
             23  GTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAA   82
             21  V  T  Q  L  N  R  L  A  A  H  P  P  F  A  S  W  R  N  S  E   40

            121  GAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCTTT  180
             83  GAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGCGCTTT  142
             41  E  A  R  T  D  R  P  S  Q  Q  L  R  S  L  N  G  E  W  R  F   60

            181  GCCTGGTTTCCGGCACCAGAAGCGGTGCCGGAAAGCTGGCTGGAGTGCGATCTTCCTGAG  240
            143  GCCTGGTTTCCGGCACCAGAAGCGGTGCCGGAAAGCTGGCTGGAGTGCGATCTTCCTGAG  202
             61  A  W  F  P  A  P  E  A  V  P  E  S  W  L  E  C  D  L  P  E   80

            241  GCCGATACTGTCGTCGTCCCCTCAAACTGGCAGATGCACGGTTACGATGCGCCCATCTAC  300
            203  GCCGATACTGTCGTCGTCCCCTCAAACTGGCAGATGCACGGTTACGATGCGCCCATCTAC  262
             81  A  D  T  V  V  V  P  S  N  W  Q  M  H  G  Y  D  A  P  I  Y  100

            301  ACCAACGTAACCTATCCCATTACGGTCAATCCGCCGTTTGTTCCCACGGAGAATCCGACG  360
            263  ACCAACGTCACCTATCCCATTACGGTCAATCCGCCGTTTGTTCCCACGGAGAATCCGACG  322
            101  T  N  V  T  Y  P  I  T  V  N  P  P  F  V  P  T  E  N  P  T  120

            361  GGTTGTTACTCGCTCACATTTAATGTTGATGAAAGCTGGCTACAGGAAGGCCAGACGCGA  420
            323  GGTTGTTACTCGCTCACATTTAATGTTGATGAAAGCTGGCTACAGGAAGGCCAGACGCGA  382
            121  G  C  Y  S  L  T  F  N  V  D  E  S  W  L  Q  E  G  Q  T  R  140

            421  ATTATTTTTGATGGCGTTAACTCGGCGTTTCATCTGTGGTGCAACGGGCGCTGGGTCGGT  480
            383  ATTATTTTTGATGGCGTTAACTCGGCGTTTCATCTGTGGTGCAACGGGCGCTGGGTCGGT  442
            141  I  I  F  D  G  V  N  S  A  F  H  L  W  C  N  G  R  W  V  G  160

                                                 lacZ2>>
            481  TACGGCCAGGACAGTCGTTTGCCGTCTGAATTTGACCTGAGCGCATTTTTACGCGCCGGA  540
            443  TACGGCCAGGACAGTCGTTTGCCGTCTGAATTTGACCTGAGCGCATTTTTACGCGCCGGA  502
            161  Y  G  Q  D  S  R  L  P  S  E  F  D  L  S  A  F  L  R  A  G  180

            541  GAAAACCGCCTCGCGGTGATGGTGCTGCGTTGGAGTGACGGCAGTTATCTGGAAGATCAG  600
            503  GAAAACCGCCTCGCGGTGATGGTGCTGCGCTGGAGTGACGGCAGTTATCTGGAAGATCAG  562
            181  E  N  R  L  A  V  M  V  L  R  W  S  D  G  S  Y  L  E  D  Q  200

            601  GATATGTGGCGGATGAGCGGCATTTTCCGTGACGTCTCGTTGCTGCATAAACCGACTACA  660
            563  GATATGTGGCGGATGAGCGGCATTTTCCGTGACGTCTCGTTGCTGCATAAACCGACTACA  622
            201  D  M  W  R  M  S  G  I  F  R  D  V  S  L  L  H  K  P  T  T  220

            661  CAAATCAGCGATTTCCATGTTGCCACTCGCTTTAATGATGATTTCAGCCGCGCTGTACTG  720
            623  CAAATCAGCGATTTCCATGTTGCCACTCGCTTTAATGATGATTTCAGCCGCGCTGTACTG  682
            221  Q  I  S  D  F  H  V  A  T  R  F  N  D  D  F  S  R  A  V  L  240

            721  GAGGCTGAAGTTCAGATGTGCGGCGAGTTGCGTGACTACCTACGGGTAACAGTTTCTTTA  780
            683  GAGGCTGAAGTTCAGATGTGCGGCGAGTTGCGTGACTACCTACGGGTAACAGTTTCTTTA  742
            241  E  A  E  V  Q  M  C  G  E  L  R  D  Y  L  R  V  T  V  S  L  260

            781  TGGCAGGGTGAAACGCAGGTCGCCAGCGGCACCGCGCCTTTCGGCGGTGAAATTATCGAT  840
            743  TGGCAGGGTGAAACGCAGGTCGCCAGCGGCACCGCGCCTTTCGGCGGTGAAATTATCGAT  802
            261  W  Q  G  E  T  Q  V  A  S  G  T  A  P  F  G  G  E  I  I  D  280
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841  GAGCGTGGTGGTTATGCCGATCGCGTCACACTACGTCTGAACGTCGAAAACCCGAAACTG  900
803  GAGCGTGGTGGTTATGCCGATCGCGTCACACTACGTCTGAACGTCGAAAACCCGAAACTG  862
281  E  R  G  G  Y  A  D  R  V  T  L  R  L  N  V  E  N  P  K  L    300

                                                        lacZ3
901  TGGAGCGCCGAAATCCCGAATCTCTATCGTGCGGTGGTTGAACTGCACACCGCCGACGGC  960
863  TGGAGCGCCGAAATCCCGAATCTCTATCGTGCGGTGGTTGAACTGCACACCGCCGACGGC  922
301  W  S  A  E  I  P  N  L  Y  R  A  V  V  E  L  H  T  A  D  G    320

          >>
961  ACGCTGATTGAAGCAGAAGCCTGCGATGTCGGTTTCCGCGAGGTGCGGATTGAAAATGGT  1040
923  ACGCTGATTGAAGCAGAAGCCTGCGATGTCGGTTTCCGCGAGGTGCGGATTGAAAATGGT  982
321  T  L  I  E  A  E  A  C  D  V  G  F  R  E  V  R  I  E  N  G    340

1021 CTGCTGCTGCTGAACGGCAAGCCGTTGCTGATTCGAGGCGTTAACCGTCACGAGCATCAT  1100
938  CTGCTGCTGCTGAACGGCAAGCCGTTGCTGATTCGAGGCGTTAACCGTCACGAGCATCAT  1042
341  L  L  L  L  N  G  K  P  L  L  I  R  G  V  N  R  H  E  H  H    360

1081 CCTCTGCATGGTCAGGTCATGGATGAGCAGACGATGGTGCAGGATATCCTGCTGATGAAG  1160
1043 CCTCTGCATGGTCAGGTCATGGATGAGCAGACGATGGTGCAGGATATCCTGCTGATGAAG  1102
361  P  L  H  G  Q  V  M  D  E  Q  T  M  V  Q  D  I  L  L  M  K    380

1141 CAGAACAACTTTAACGCCGTGCGCTGTTCGCATTATCCGAACCATCCGCTGTGGTACACG  1200
1103 CAGAACAACTTTAACGCCGTGCGCTGTTCGCATTATCCGAACCATCCGCTGTGGTACACG  1162
381  Q  N  N  F  N  A  V  R  C  S  H  Y  P  N  H  P  L  W  Y  T    400

1201 CTGTGCGACCGCTACGGCCTGTATGTGGTGGATGAAGCCAATATTGAAACCCACGGCATG  1260
1163 CTGTGCGACCGCTACGGCCTGTATGTGGTGGATGAAGCCAATATTGAAACCCACGGCATG  1222
401  L  C  D  R  Y  G  L  Y  V  V  D  E  A  N  I  E  T  H  G  M    420

1261 GTGCCAATGAATCGTCTGACCGATGATCCGCGCTGGCTACCGGCGATGAGCGAACGCGTA  1320
1223 GTGCCAATGAATCGTCTGACCGATGATCCGCGCTGGCTACCGGCGATGAGCGAACGCGTA  1282
421  V  P  M  N  R  L  T  D  D  P  R  W  L  P  A  M  S  E  R  V    440

1321 ACGCGAATGGTGCAGCGCGATCGTAATCACCCGAGTGTGATCATCTGGTCGCTGGGGAAT  1380
1283 ACGCGAATGGTGCAGCGCGATCGTAATCACCCGAGTGTGATCATCTGGTCGCTGGGGAAT  1342
441  T  R  M  V  Q  R  D  R  N  H  P  S  V  I  I  W  S  L  G  N    460

1381 GAATCAGGCCACGGCGCTAATCACGACGCGCTGTATCGCTGGATCAAATCTGTCGATCCT  1440
1343 GAATCAGGCCACGGCGCTAATCACGACGCGCTGTATCGCTGGATCAAATCTGTCGATCCT  1402
461  E  S  G  H  G  A  N  H  D  A  L  Y  R  W  I  K  S  V  D  P    480

1441 TCCCGCCCGGTGCAGTATGAAGGCGGCGGAGCCGACACCACGGCCACCGATATTATTTGC  1500
1403 TCCCGCCCGGTGCAGTATGAAGGCGGCGGAGCCGACACCACGGCCACCGATATTATTTGC  1462
481  S  R  P  V  Q  Y  E  G  G  G  A  D  T  T  A  T  D  I  I  C    500

1501 CCGATGTACGCGCGCGTGGATGAAGACCAGCCCTTCCCGGCTGTGCCGAAATGGTCCATC  1560
1463 CCGATGTACGCGCGCGTGGATGAAGACCAGCCCTTCCCGGCTGTGCCGAAATGGTCCATC  1522
501  P  M  Y  A  R  V  D  E  D  Q  P  F  P  A  V  P  K  W  S  I    520

1561 AAAAAATGGCTTTCGCTACCTGGAGAGACGCGCCCGCTGATCCTTTGCGAATACGCCCAC  1620
1523 AAAAAATGGCTTTCGCTACCTGGAGAGACGCGCCCGCTGATCCTTTGCGAATACGCCCAC  1582
521  K  K  W  L  S  L  P  G  E  T  R  P  L  I  L  C  E  Y  A  H    540

1621 GCGATGGGTAACAGTCTTGGCGGTTTCGCTAAATACTGGCAGGCGTTTCGTCAGTATCCC  1680
1583 GCGATGGGTAACAGTCTTGGCGGTTTCGCTAAATACTGGCAGGCGTTTCGTCAGTATCCC  1642
541  A  M  G  N  S  L  G  G  F  A  K  Y  W  Q  A  F  R  Q  Y  P    560

1681 CGTTTACAGGGCGGCTTCGTCTGGGACTGGGTGGATCAGTCGCTGATTAAATATGATGAA  1740
1643 CGTTTACAGGGCGGCTTCGTCTGGGACTGGGTGGATCAGTCGCTGATTAAATATGATGAA  1702
561  R  L  Q  G  G  F  V  W  D  W  V  D  Q  S  L  I  K  Y  D  E    580

1741 AACGGCAACCCGTGGTCGGCTTACGGCGGTGATTTTGGCGATACGCCGAACGATCGCCAG  1800
1703 AACGGCAACCCGTGGTCGGCTTACGGCGGTGATTTTGGCGATACGCCGAACGATCGCCAG  1762
581  N  G  N  P  W  S  A  Y  G  G  D  F  G  D  T  P  N  D  R  Q    600

                                                        lacZ5>>
1801 TTCTGTATGAACGGTCTGGTCTTTGCCGACCGCACGCCGCATCCAGCGCTGACGGAAGCA  1860
1763 TTCTGTATGAACGGTCTGGTCTTTGCCGACCGCACGCCGCATCCAGCGCTGACGGAAGCA  1822
601  F  C  M  N  G  L  V  F  A  D  R  T  P  H  P  A  L  T  E  A    620

1861 AAACACCAGCAGCAGTTTTTCCAGTTCCGTTTATCCGGGCAAACCATCGAAGTGACCAGC  1920
1823 AAACACCAGCAGCAGTTTTTCCAGTTCCGTTTATCCGGGCAAACCATCGAAGTGACCAGC  1882
621  K  H  Q  Q  Q  F  F  Q  F  R  L  S  G  Q  T  I  E  V  T  S    640
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1921  GAATACCTGTTCCGTCATAGCGATAACGAGCTCCTGCACTGGATGGTGGCGCTGGATGGT  1980
1883  GAATACCTGTTCCGTCATAGCGATAACGAGCTCCTGCACTGGATGGTGGCGCTGGATGGT  1942
 641  E  Y  L  F  R  H  S  D  N  E  L  L  H  W  M  V  A  L  D  G    660

1981  AAGCCGCTGGCAAGCGGTGAAGTGCCTCTGGATGTCGCTCCACAAGGTAAACAGTTGATT  2040
1943  AAGCCGCTGGCAAGCGGTGAAGTGCCTCTGGATGTCGCTCCACAAGGTAAACAGTTGATT  2002
 661  K  P  L  A  S  G  E  V  P  L  D  V  A  P  Q  G  K  Q  L  I    680

2041  GAACTGCCTGAACTACCGCAGCCGGAGAGCGCCGGGCAACTCTGGCTCACAGTACGCGTA  2100
2003  GAACTGCCTGAACTACCGCAGCCGGAGAGCGCCGGGCAACTCTGGCTCACAGTACGCGTA  2062
 681  E  L  P  E  L  P  Q  P  E  S  A  G  Q  L  W  L  T  V  R  V    700

2101  GTGCAACCGAACGCGACCGCATGGTCAGAAGCCGGGCACATCAGCGCCTGGCAGCAGTGG  2160
2063  GTGCAACCGAACGCGACCGCATGGTCAGAAGCCGGGCACATCAGCGCCTGGCAGCAGTGG  2122
 701  V  Q  P  N  A  T  A  W  S  E  A  G  H  I  S  A  W  Q  Q  W    720

2161  CGTCTGGCGGAAAACCTCAGTGTGACGCTCCCCGCCGCGTCCCACGCCATCCCGCATCTG  2200
2123  CGTCTGGCGGAAAACCTCAGTGTGACGCTCCCCGCCGCGTCCCACGCCATCCCGCATCTG  2182
 721  R  L  A  E  N  L  S  V  T  L  P  A  A  S  H  A  I  P  H  L    740

2221  ACCACCAGCGAAATGGATTTTTGCATCGAGCTGGGTAATAAGCGTTGGCAATTTAACCGC  2260
2183  ACCACCAGCGAAATGGATTTTTGCATCGAGCTGGGTAATAAGCGTTGGCAATTTAACCGC  2242
 741  T  T  S  E  M  D  F  C  I  E  L  G  N  K  R  W  Q  F  N  R    760

                    lacZ6>>
2281  CAGTCAGGCTTTCTTTCACAGATGTGGATTGGCGATAAAAAACAACTGCTGACGCCGCTG  2320
2243  CAGTCAGGCTTTCTTTCACAGATGTGGATTGGCGATAAAAAACAACTGCTGACGCCGCTG  2302
 761  Q  S  G  F  L  S  Q  M  W  I  G  D  K  K  Q  L  L  T  P  L    780

2341  CGCGATCAGTTCACCCGTGCACCGCTGGATAACGACATTGGCGTAAGTGAAGCGACCCGC  2380
2303  CGCGATCAGTTCACCCGTGCACCGCTGGATAACGACATTGGCGTAAGTGAAGCGACCCGC  2362
 781  R  D  Q  F  T  R  A  P  L  D  N  D  I  G  V  S  E  A  T  R    800

2401  ATTGACCCTAACGCCTGGGTCGAACGCTGGAAGGCGGCGGGCCATTACCAGGCCGAAGCA  2440
2363  ATTGACCCTAACGCCTGGGTCGAACGCTGGAAGGCGGCGGGCCATTACCAGGCCGAAGCA  2402
 801  I  D  P  N  A  W  V  E  R  W  K  A  A  G  H  Y  Q  A  E  A    820

2461  GCGTTGTTGCAGTGCACGGCAGATACACTTGCTGATGCGGTGCTGATTACGACCGCTCAC  2500
2423  GCGTTGTTGCAGTGCACGGCAGATACACTTGCTGATGCGGTGCTGATTACGACCGCTCAC  2462
 821  A  L  L  Q  C  T  A  D  T  L  A  D  A  V  L  I  T  T  A  H    840

2521  GCGTGGCAGCATCAGGGGAAAACCTTATTTATCAGCCGGAAAACCTACCGGATTGATGGT  2560
2483  GCGTGGCAGCATCAGGGGAAAACCTTATTTATCAGCCGGAAAACCTACCGGATTGATGGT  2522
 841  A  W  Q  H  Q  G  K  T  L  F  I  S  R  K  T  Y  R  I  D  G    860

2581  AGTGGTCAAATGGCGATTACCGTTGATGTTGAAGTGGCGAGCGATACACCGCATCCGGCG  2620
2543  AGTGGTCAAATGGCGATTACCGTTGATGTTGAAGTGGCGAGCGATACACCGCATCCGGCG  2582
 861  S  G  Q  M  A  I  T  V  D  V  E  V  A  S  D  T  P  H  P  A    880

2641  CGGATTGGCCTGAACTGCCAGCTGGCGCAGGTAGCAGAGCGGGTAAACTGGCTCGGATTA  2680
2603  CGGATTGGCCTGAACTGCCAGCTGGCGCAGGTAGCAGAGCGGGTAAACTGGCTCGGATTA  2642
 881  R  I  G  L  N  C  Q  L  A  Q  V  A  E  R  V  N  W  L  G  L    900

2701  GGGCCGCAAGAAAACTATCCCGACCGCCTTACTGCCGCCTGTTTTGACCGCTGGGATCTG  2740
2663  GGGCCGCAAGAAAACTATCCCGACCGCCTTACTGCCGCCTGTTTTGACCGCTGGGATCTG  2702
 901  G  P  Q  E  N  Y  P  D  R  L  T  A  A  C  F  D  R  W  D  L    920

2761  CCATTGTCAGACATGTATACCCCGTACGTCTTCCCGAGCGAAAACGGTCTGCGCTGCGGG  2800
2723  CCATTGTCAGACATGTATACCCCGTACGTCTTCCCGAGCGAAAACGGTCTGCGCTGCGGG  2762
 921  P  L  S  D  M  Y  T  P  Y  V  F  P  S  E  N  G  L  R  C  G    940

2821  ACGCGCGAATTGAATTATGGCCCACACCAGTGGCGCGGCGACTTCCAGTTCAACATCAGC  2860
2783  ACGCGCGAATTGAATTATGGCCCACACCAGTGGCGCGGCGACTTCCAGTTCAACATCAGC  2822
 941  T  R  E  L  N  Y  G  P  H  Q  W  R  G  D  F  Q  F  N  I  S    960

2881  CGCTACAGTCAACAGCAACTGATGGAAACCAGCCATCGCCATCTGCTGCACGCGGAAGAA  2920
2843  CGCTACAGTCAACAGCAACTGATGGAAACCAGCCATCGCCATCTGCTGCACGCGGAAGAA  2882
 961  R  Y  S  Q  Q  Q  L  M  E  T  S  H  R  H  L  L  H  A  E  E    980

2941  GGCACATGGCTGAATATCGACGGTTTCCATATGGGGATTGGTGGCGACGACTCCTGGAGC  2980
2903  GGCACATGGCTGAATATCGACGGTTTCCATATGGGGATTGGTGGCGACGACTCCTGGAGC  2942
 981  G  T  W  L  N  I  D  G  F  H  M  G  I  G  G  D  D  S  W  S   1000

3001  CCGTCAGTATCGGCGGAATTCCAGCTGAGCGCCGGTCGCTACCATTACCAGTTGGTCTGG  3040
2963  CCGTCAGTATCGGCGGAATTCCAGCTGAGCGCCGGTCGCTACCATTACCAGTTGGTCTGG  3002
1001  P  S  V  S  A  E  F  Q  L  S  A  G  R  Y  H  Y  Q  L  V  W   1020
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3061  TGTCAAAAA                                  3069
3023  TGTCAAAAA                                  3031
1021  C  Q  K                                    1029
```

Appendix 4.1 Sequencing Over The *cyc*A-*CYC1* Fusion

The *Xho*I site is indicated (in red), between the *cyc*A promoter (black) and *CYC1* (blue) promoter sequences. The position of the proposed transcriptional start site of the *cyc*A gene is indicated by thick underlining. The translational start site of the *lac*Z sequence (green) is indicated by double underlining. The annealing site of the CPD5 primer used to check the *cyc*A-*lac*Z translational fusions is shown.

CPD5>>

GTTGCAAGAGATGTTAACACACCCTGCCAACTCACATATTAGTCACATAGCTCCCAACCCAGAAAGCAGT
TTGCGGGTAAATGAGTACGCACAAAAGCAATCCAGACATGAATCCACCGACTCGTCAAAAACCGAAACAT
GACCGTCCCCTCGGGCGGGAACATATTCGGGTACTTCTTTTTTGCCCGCTCCGCCTCTTCTTTCTCAGAG
AACTTGGGACCGGGGTAGTTAACGACTTTACCATTGCGTGTTGGAACGACGCGGCGCGCAGGATGTGAGG
GCGGGCGAAATTTATCTGGTTGCGCGAGGACGCGCGGATTTTGGCTGTTGTCACTTGAGGAATTTGTTGA
TGCGCAGCGGTGCGTCGGTGGGAGCCTGCCGATGCGGGAGGACCGGGACAGGAATAGGATTGCTCGTGCT
GAGGGAACACCTCGGGGAAGGAGGAATGGGGGAATGGCTGATGGGGGCATTCTGTACGAATTGCTGGTTC
TTGGCTGCGATTTCTCTATATGCTAGCTTCTGGTCGCCGGCATAACATTTTGGTGTTGATATAATCATGT
GACTTCTGCCGCCGGGAATAAGGCATCAAGGCATCAAGGCACAAACACATTTTTTCTAATGGCCGCTAAG
GCATCAGGCCACTTCGGATTAGGGCCGGGGAGAGCGGGAAAAACTCGCCATGACTAGGCGAATGAAAGGA
TGCAGATTTGTTATTACGGGGAGGGCTACTCCGGCCTCCGTAGCCCACCGTTGCCCATTCCCCGAGACAG
ACAGTGCAGAGCTCCAAGTAACCAGCGTCTCTATGCGCTGGAATGAGGTCATGCCATGACGGCATGCAGA
ATCACCAATCATCTTTTACAGTATAGTAGTTAGGCTCTATGATAGATAGATGTCATAGAAGGTGCATTGT
TGCTATCTAGAGCTGCATAACTGAGCCCTTAGACGTAGTATATAGGATTACAATAGTCTCTAAATAAAGC
TTCATCCAGCCAGGCGTTATTTGCAGTGACCGATTCCTGACCTCAGACCCGGCAGCGCCCGTTACTCTGA
GCACAGTGCTGAATCATCCTACCTCTGATTGGTCAATTCCCAGCTCGAGCAGATCCGCCAGGCGTGTATA
TAGCGTGGATGGCCAGGCAACTTTAGTGCTGACACATACAGGCATATATATATGTGTGCGACGACACATG
ATCATATGGCATGCATGTGCTCTGTATGTATATAAAACTCTTGTTTTCTTCTTTTCTCTAAATATTCTTT
CCTTATACATTAGGTCCTTTGTAGCATAAATTACTATACTTCTATAGACACGCAAACACAAATACACACA
CTAAATTAATAATGACCGGATCCGGAGCTTGGCTGTTGCCCGTCTCACTGGTGAAAAGAAAAACCACCCT

Appendix 4.2 Raw Data For The *S. Cerevisiae* β-Galactosidase Assays

The β-galactosidase assays were carried out on cultures grown in 1% glucose (**A**) and glycerol (**B**). BWG1-7A and LPY22 are wildtype and *hap1* strains of *S. cerevisiae*, respectively. The cytochrome *c* gene promoter fragments used in the constructs are from the *S. cerevisiae CYC1* gene and the *A. nidulans cycA* gene.

A

Strain	Promoter Fragment (s)	β-galactosidase Activity (units/min/ml)				Standard Error
		Sample 1	Sample 2	Sample 3	Average	
BWG1-7A only	n/a	0	0	0	0	0
BWG1-7A + R141	0.18 kb *CYC1*	5.986	7.363	2.174	5.174	1.552
BWG1-7A + R142	1.1 kb *CYC1*	13.086	10.055	6.567	9.903	1.883
BWG1-7A + R182	0.18 kb *CYC1* + 1 kb *cycA*	9.225	5.691	6.275	7.064	1.094
LPY22 only	n/a	0	0	0	0	0
LPY22 + R142	0.18 kb *CYC1*	8.108	5.529	5.591	6.409	0.850
LPY22 + R141	1.1 kb *CYC1*	16.959	6.452	8.759	10.723	3.188
LPY22 + R182	0.18 kb *CYC1* + 1 kb *cycA*	15.072	3.646	8.188	8.969	3.322

B

Strain	Promoter Fragment (s)	β-galactosidase Activity (units/min/ml)				Standard Error
		Sample 1	Sample 2	Sample 3	Average	
BWG1-7A only	n/a	0	0	0	0	0
BWG1-7A + R142	0.18 kb *CYC1*	8.313	6.948	4.909	6.723	0.989
BWG1-7A + R141	1.1 kb *CYC1*	14.043	16.6	12.324	14.322	1.242
BWG1-7A + R182	0.18 kb *CYC1* + 1 kb *cycA*	8.112	7.581	4.762	6.818	1.040
LPY22 only	n/a	0	0	0	0	0
LPY22 + R142	0.18 kb *CYC1*	3.690	21.447	10	11.713	5.197
LPY22 + R141	1.1 kb *CYC1*	17.349	53.205	11.633	27.396	13.010
LPY22 + R182	0.18 kb *CYC1* + 1 kb *cycA*	13.511	17.766	12.863	14.713	1.538

Appendix 4.3 ANOVA Analysis of β-Galactosidase Activites

ANOVA analysis (three factor) was carried out as detailed in Campbell (1974). The null hypothesis (Ho) was that the samples are equal (ie drawn from populations with the same mean). Ho was rejected if the calculated F value was larger than the F critical value (one tailed test, probability level 5%).

	Sum of Squares	Degrees of freedom	Mean Sum of Squares	F value	Critical F value
Plasmids	142.99	2	71.49		
Strains	74.59	1	74.59		
Strains & plasmids	748.34	5	149.67	0.498	6.61
Carbon source	93.20	1	93.20		
Carbon source & plasmid interaction	677.45	5	135.49	0.688	6.61
Strain & carbon source interaction	40.32	3	13.44		
Strain & carbon source & plasmid interation	-1371.40	11	-124.67	-0.108	3.59
Total	405.49	28	14.48		

Appendix 4.4 Results Of T-Tests (Paired Two Sample For Means)

T-test analysis (Paired two sample for means) was carried out using the Microsoft Excel program. The null hypothesis (Ho) was that the two samples have the same mean (ie $\mu = \mu o$). The alternative hypothesis (Ha) for the one-tailed test was that ie $\mu > \mu o$, or $\mu < \mu o$. Ho was rejected if the calculated T value was outside the T critical value (2.92).

Transformant Strain, Host Strain	Carbon Source(s)	T value
R142 (BWG1-7A), R141 (BWG1-7A)	1% Glucose	-3.68* [1]
R142 (BWG1-7A), R182 (BWG1-7A)	1% Glucose	-1.05 [7]
R141 (BWG1-7A), R182 (BWG1-7A)	1% Glucose	2.22
R142 (LPY22), R141 (LPY22)	1% Glucose	-1.83
R142 (LPY22), R182 (LPY22)	1% Glucose	-1.00 [7]
R141 (LPY22), R182 (LPY22)	1% Glucose	2.71
R141 (LPY22), R141 (BWG1-7A)	1% Glucose	-0.36 [4]
R142 (LPY22), R142 (BWG1-7A)	1% Glucose	-0.78
R182 (LPY22), R182 (BWG1-7A)	1% Glucose	-0.84 [5]
R142 (BWG1-7A), R141 (BWG1-7A)	1% Glycerol	-6.69* [2]
R142 (BWG1-7A), R182 (BWG1-7A)	1% Glycerol	-0.35 [7]
R141 (BWG1-7A), R182 (BWG1-7A)	1% Glycerol	8.41* [8]
R142 (LPY22), R141 (LPY22)	1% Glycerol	-1.79
R142 (LPY22), R182 (LPY22)	1% Glycerol	-0.77 [7]
R141 (LPY22), R182 (LPY22)	1% Glycerol	1.11
R141 (LPY22), R141 (BWG1-7A)	1% Glycerol	-1.11 [4]
R142 (LPY22), R142 (BWG1-7A)	1% Glycerol	-0.90
R182 (LPY22), R182 (BWG1-7A)	1% Glycerol	-5.70* [6]
R141 (BWG1-7A), R141 (BWG1-7A)	1% Glucose, 1% Glycerol	-2.53 [3]
R142 (BWG1-7A), R142 (BWG1-7A)	1% Glucose, 1% Glycerol	-1.57
R182 (BWG1-7A), R182 (BWG1-7A)	1% Glucose, 1% Glycerol	0.23 [5]
R141 (LPY22), R141 (LPY22)	1% Glucose, 1% Glycerol	-1.11 [3]
R142 (LPY22), R142 (LPY22)	1% Glucose, 1% Glycerol	-0.90
R182 (LPY22), R182 (LPY22)	1% Glucose, 1% Glycerol	-1.26

Note: The promoter fragments contained within the reporter constructs are as follows: R141, 1.1 kb *S. cerevisiae CYC1*; R142, 0.18 kb *S. cerevisiae CYC1*; R182, 0.18 kb *S. cerevisiae CYC1* and 1 kb *A. nidulans cycA*. Strains BWG1-7A and LPY22 are wildtype and *hap1*, respectively.
* indicates a rejection of Ho

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