# From Model Organism to Industrial Workhorse:

Analysis of genes in *Aspergillus nidulans* and disruption of *cre2* for *Trichoderma reesei* strain improvement.

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#### Abstract

Carbon catabolite repression is a regulatory system whereby an organism can sequentially utilise carbon sources based on their available energy. This system results in the repression of genes encoding enzymes responsible for the utilisation of poorer carbon sources when preferable ones are available. Carbon catabolite repression has been extensively studied in the filamentous fungus *Aspergillus nidulans*. Repression is mediated via CreA, a zinc finger DNA binding protein, which is in turn, either directly or indirectly, regulated by an ubiquitination / deubiquitination system involving CreB, CreC and CreD.

Previous work demonstrated that the A. nidulans genome contains a CreD homologue, ApyA, and that both of these proteins interact with an ubiquitin ligase, HulA. This relationship was proposed to be similar to Rod1p and Rog3p and their interaction with the ubiquitin ligase Rsp5p in Saccharomyces cerevisiae. Both apyA and hulA were targeted for disruption to facilitate phenotypic analysis and the study of epistatic interactions. Deletion of hulA was shown to be lethal in an A. nidulans haploid, but viable as a heterozygote in an A. nidulans diploid. The only detectable phenotypes of this deletion in a heterozygous diploid were increased sensitivity to molybdate and acriflavine. A strain containing a disruption of *apyA* did not demonstrate any detectable phenotypes, however, the apyA disruption allele showed epistatic interactions with mutations in creB, creC and creD. The disruption of apyA partially suppressed the phenotype of sensitivity to allyl alcohol in the presence of glucose displayed by strains containing mutations in creB and creC. However, the level of suppression exhibited by the disruption of *apyA* was not as strong as that shown by the *creD34* mutation. A strain containing mutations in both creD and apyA demonstrated severe morphological deficiencies on minimal media as well as stronger resistance to acriflavine than creD34 alone, and resistance to molybdate.

Bioinformatic analysis of CreD and CreD-like proteins, including ApyA, from sequenced members of the Aspergilli and Rod1p, Rog3p and related proteins from members of Saccharomycetes suggested that the arrestin-like proteins, a group to which these belong, are subject to frequent gene duplication events. The number and range of sequenced fungal genomes also allowed a bioinformatic examination of the conservation of proteins involved in the carbon repression mechanisms across the fungal kingdom. A homologue of CreA was identified only within the members of Ascomycota that were examined, but putative homologues of CreB and CreC were identified across the fungal kingdom. The Saccharomycetes were an exception to this as a CreC homologue was not indentified and the CreB homologue was highly divergent or absent.

The filamentous fungus, *Trichoderma reesei* is an important source of cellulases for use in the textile and alternative fuel industries. Previous studies have suggested a benefit for the manipulation of carbon catabolite repression for strain improvement, as the industrially significant strain RUTC-30 contains a mutation in *cre1*, the *T. reesei creA* homologue. The *T. reesei* orthologue of the *A. nidulans creB* gene, designated *cre2*, was shown to be functional in carbon repression through complementation of a *creB* mutation in *A. nidulans*. This gene was targeted for disruption in *T. ressei* as disruption in *A. nidulans* leads to carbon derepression of some systems without the severe morphological effects of strains containing *creA* mutations. A *T. reesei* strain containing a *cre2* disruption exhibited phenotypes similar to the *A. nidulans creB* mutant strain on solid media and had elevated cellulase levels.

# Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution to Jai Denton and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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Jai A. Denton 22<sup>nd</sup> of June, 2010

"Disruption of *Trichoderma reesei cre2*, encoding an ubiquitin C-terminal hydrolase, results in increased cellulase activity" by JA Denton and JM Kelly. (In Submission)

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# Abbreviations

#### **General Abbreviations**

ATP	adenosine triphosphate	
ATCC	American Tissue Culture Collection	
аа	amino acid	
bp, kb, mb	basepair, kilobase pairs, megabase pairs	
CCR	carbon catabolite repression	
°C	degrees Celsius	
DIG	Digoxigenin	
DNA	deoxyribonucleic acid	
DUB	deubiquitinating enzyme	
FGSC	Fungal Genetics Stock Center	
GPCRs	G protein-coupled receptors	
gDNA	genomic deoxyribonucleic acid	
g, mg, μg, ng	gram, milligram, microgram, nanogram	
GFP	green fluorescent protein	
JGI	Joint Genome Initiative	
l, ml, μg	litre, millilitre, microlitre	
M, mM	molar, millimolar	
NCBI	National Center for Biotechnology Information	
nt	nucleotide	
PCR	polymerase chain reaction	
RNA	ribonucleic acid	

#### Nucleotide abbreviations

А	adenine	S	guanine or cytosine
G	guanine	W	adenine or thymidine
Т	thymidine	В	not adenine
С	cytosine	D	not cytosine
R	adenine or guanine	Н	not guanine
Y	cytosine or thymidine	V	not thymidine
Μ	adenine or cytosine	Ν	any nucleotide
Κ	guanine or thymidine		

#### Protein Domains

Arrestin: Domains mirrored at either end of a protein with Ig-like beta sandwich fold that are involved in protein-protein interactions.

C2: A calcium dependent membrane targeting module.

DUB: An ubiquitin hydrolase domain.

HECTc: A C-terminal ubiquitin-transferase domain.

PEST: Proline, glutamic acid, serine and theronine rich regions involved in protein turnover.

PPXY & PXY: One or two proline residues next to any residue followed by a tyrosine.

WW: Two highly conserved tryptophan residues involved in protein-protein interactions.

Zinc finger: Involved in DNA or RNA binding. Use zinc ions to stabilise their folding.

# **Chapter 1- Introduction**

The use of microorganisms in the production of fuel is being extensively investigated with the aim of supplementing or replacing traditional fossil fuels, reducing greenhouse gas production and producing useful by-products from waste sources.

Trichoderma reesei has been widely employed for industrial use due to its high enzyme production, specifically cellulolytic enzymes, and ease of culture. However *T.* reesei lacks the extensive body of genetic and molecular biology resources of the model organism *Aspergillus nidulans*. The aim of this study is the further dissection of carbon catabolite repression (CCR) in *A. nidulans* and the application of knowledge generated in the model organism for the targeted genetic manipulation of *T. reesei* to improve enzyme production. This study also aims to strengthen support for the use of *A. nidulans*, not just as a general model organism, but also as a specific model for industrially significant filamentous fungi that lack an extensive history of genetic analysis.

#### **1.1 Alternative fuel**

The culmination of research in numerous fields, including climate change, economics, politics and transportation, is that the reliance on petroleum based fuel needs to be reduced. The surge in globalisation has been greatly facilitated by the availability of petroleum as a cheap and high density fuel. However, given that oil reserves are finite, research into alternatives needs to be accelerated.

Several alternative fuels are being investigated, including bio-diesel, butanol, methanol and ethanol, produced from feed stock or waste material (Figure 1.1). One of the most mature of these alternatives is ethanol production from feedstock, but it is often met with contention due to the fuel versus food debate; that is farming land is required for fuel production rather than food production. An alternative strategy for ethanol production is cellulosic ethanol, the production of ethanol from cellulose rich waste, a by-product from many industries.



Figure 1.1 - Current approaches to alternative fuel. Taken from Sheehan, 2009 (119)

#### 1.1.1 Ethanol as a fuel

Ethanol has only 67% of the energy content compared with the same volume of gasoline, although it has a higher octane rating, 98, compared to gasoline, 80 (octane rating is a measure of a fuels ability to resist auto-ignition) (Reviewed in (38)). The reduction in energy content of ethanol can be offset by the higher octane rating, which allows for a combustion motor to have higher valve compression, also giving rise to improved efficiency. Standard petroleum can be supplemented with ethanol as a way of improving octane rating, giving rise to improved engine performance (151). There are two common forms of supplemented petroleum, E10, 10% ethanol, and E25, 25% ethanol. These blended ethanol fuels also have a higher biodegradability than petrol, with even the E10 blend showing a significant increase in biodegradability (124).

In the analysis of several studies relating to ethanol production from corn, Farrell *et al.* (32) have suggested that current corn based ethanol production in the United States has negligible benefits in reducing green house gases. However, they suggest that even in its infant state, in 2006, the production of cellulosic ethanol produces much less green house gas per unit of energy (32). The United States Department of Agriculture released a report, based on a survey of ethanol producers, that highlighted the variability in feedstock price as a major factor inhibiting the production of ethanol (118). These aspects further strengthen the case for ethanol production from a waste product.

#### 1.1.2 Cellulose and cellulose saccharification

Cellulose, a structural polymer and the most abundant biopolymer on the planet, consists of stacked long chains of glucose molecules arranged in  $\beta$ -1,4 linkages, strengthened via hydrogen bonds both within each chain, and between interlocking chains. This crystalline structure allows cellulose to perform its role as a structural molecule and as such does not facilitate saccharification. The enzymatic saccharification of cellulose to the glucose dimer cellobiose occurs by the hydrolysis of the  $\beta$ -1,4 linkages in cellulose via two classes of cellulases, cellobiohyrolases (exocellulases) and endoglucanases (endocellulases). Endoglucanases act by preferentially cleaving the internal bonds of the crystalline structure breaking each molecule into smaller ones, while cellobiohyrolases preferentially cleave cellobiose molecules from the end of the cellulose chains. Finally  $\beta$ -glucosidases are required to convert the cleaved cellobiose to glucose. Classification of cellulases into a specific class is dependent on sequence homology and optimal activity. However, the activity of a specific cellulase is often not limited to a single class, and a single cellulase may cleave cellulose both internally and externally at reduced efficiencies for their non-specific activity (Reviewed in (20, 113)). The cellobiohyrolases class can be divided into two further classes based on their activity. In *T. reesei* there are two major cellobiohyrolases, Cbh1 and Cbh2. Cbh1 preferentially cleaves cellobiose from the reducing end of the cellulose molecule, while Cbh2 preferentially cleaves from the non reducing end (10). Optimal cellulose degradation was achieved via cellulase mixtures containing both types of cellobiohyrolases and an endoglucanase, demonstrating synergy between the cellulase classes (57, 149, 157). Strategies to improve saccharification of cellulose rich waste need to consider this synergy by seeking to improve the production of a range of cellulases.

#### 1.1.3 Cellulosic ethanol production

Ethanol production from cellulose often involves four steps: a pre-treatment step, to disrupt the cellulose structure; a hydrolysis step, to generate simple sugars for fermentation; a fermentation step, to produce ethanol; and a distillation step, to refine the ethanol. However, these steps are often varied or combined in the optimisation of the process, such as continuous fermentation methods of cellulosic ethanol production (13). This is a system whereby a substrate, in this case biomass, is constantly fed into the reactor and a product, in this case ethanol, is extracted. This reaction is conducted for long enough to achieve a steady state within the reactor. This thesis addresses improvements to the hydrolysis step, specifically enzymatic hydrolysis via improvements to cellulase production through strain manipulation.

# 1.2 Trichoderma reesei as an industrial organism

The isolate of *T. reesei*, QM 6a, was initially identified as *Trichoderma viride* when isolated from canvas and cotton during World War II (104). QM 6a was subjected to numerous rounds of mutagenesis selecting strains that have improved cellulase activity, including those produced at Natick utilising a linear accelerator (82) and those selected at Rutgers utilising UV and nitrosoguandine (Figure 1.2). The strain Rut C-30 was produced as part of the latter (Figure 1.2) and continues to be industrially significant. *T. reesei* strain, QM 6a, has been shown through molecular analysis to be a clonal derivative of *Hypocrea jecorina* (68) but will be referred to as *T. reesei* throughout this thesis. Since its isolation, *T. reesei* has become industrially invaluable due in part to its ability to produce large amounts of cellulytic enzymes but also due its non-pathogenic properties, and the efficiency with which it produces and secretes heterologous proteins. The study of *T. reesei* has been aided by the release of the complete genome sequence of QM 6a (83), and the initiation of RUT C-30<sup>1</sup> genome sequencing.

A sexual cycle has been characterised in *T. reesei* through the identification of the mating type locus (117). QM 6a was found to be of mating type *MAT1-2*, while a natural isolate of *H. jecorina* was found that is *MAT1-1*. The complementary mating types allow not only the crossing of QM 6a with the *H. jecorina* isolate, but also the crossing of RUT C-30 and QM 9414, two strains that have undergone extensive mutagenesis, to the *H. jecorina* isloate. This development is a monumental step forward in the field of strain improvement. Given the finite number of selectable markers available, a sexual cycle allows the development of strains containing multiple gene disruptions without having to utilise numerous selectable markers. This sexual cycle may also prove useful in the characterisation of mutagenised strains, making it easier to identify specific mutations that are improving cellulase production. One drawback identified within the study was that QM 6a contains a mutation that prevents the formation of fruiting bodies, referred to as female sterility (117), that was subsequently shown to be caused by a mutation in *ste3 (112)*. However this could potentially be overcome by a series of outcrosses and further characterisation of the *ste3* mutation causing this infertility.

<sup>&</sup>lt;sup>1</sup> http://genome.jgi-psf.org/

NOTE: This figure is included on page 5 of the print copy of the thesis held in the University of Adelaide Library.

Figure 1.2 - Representation of strain development programs prior to 1992. (Adapted from (31))

#### 1.3 Aspergillus nidulans as a model organism

Over the past 60 years *A. nidulans* has been used extensively as a model organism due to its rapid well defined growth, amenity to genetic analysis and ease of genetic manipulation. *A. nidulans* is a filamentous fungus that has been invaluable for the study of numerous cellular mechanisms from cell division to gene regulation. Of particular interest to this study is gene regulation in response to available sources of carbon and nitrogen. *A. nidulans* will grow on defined simple solid media as compact colonies, producing mature conidiating colonies in two days when grown at 37°C, and it can also grow as a vegetative mycelial form in submerged liquid culture. When grown on solid media *A. nidulans* demonstrates colony inhibition, allowing numerous colonies to be directly compared on a single Petri dish. *A. nidulans* is haploid, allowing the direct examination of recessive phenotypes, but can be forced into heterokaryon and diploid states as all laboratory strains are homothallic and derived from a single isolate. There is also a robust system for transformation (135) with numerous selectable markers characterised. The development of strains containing a deletion of ku70, named *nkuA* in *A. nidulans*, has improved homologous transformation frequencies from about two percent to great than 95%, allowing rapid targeted gene disruption (88). In addition the genome sequence of *A. nidulans*, as well as that of several other *Aspergilli*, has been released allowing bioinformatic analysis (34).

#### **1.4 Carbon catabolite repression**

CCR is a regulatory system that responds to environmental carbon sources, whereby particular carbon sources can be utilised preferentially. In the presence of easily metabolised carbon sources like glucose, genes encoding enzymes or permeases involved in the utilization of complex carbon sources, such as starch or cellulose, are repressed. The mechanisms of CCR has been characterised in a number of bacteria and fungi.

#### 1.4.1 Carbon catabolite repression in A. nidulans

The identification of CCR mutants in *A. nidulans* was facilitated by the exploitation of the *areA* mutant phenotype. AreA is a DNA binding activator protein required for nitrogen derepression. In the presence of a strongly repressing carbon source an *A. nidulans areA* mutant is unable to utilise nitrogen sources that also act as carbon sources (6). Mutation screens to identify mutations affecting CCR were conducted that exploited the inability of *areA* mutants to utilise acetamide or proline as a nitrogen source in the presence of glucose. In this screen mutations that caused derepression of gene responsible for acetamide or proline utilisation would allow growth. These mutations fell into three classes, *creA*, *creB* and *creC* (6, 9, 54).

#### 1.4.1.1 CreA

The *creA* gene in *A. nidulans* encodes a  $C_2H_2$  zinc finger DNA binding protein (26, 27) that binds to the recognition site 5'-SYGGRG-3' (69), and these sites have been identified in the promoter regions of genes normally subject to CCR (69, 93).

A comprehensive phenotypic and molecular analysis of numerous *creA* mutant strains has been conducted (122). Several strains, including those containing *creA1*, *creA204* and *creA331*, have smaller colonies than wildtype with normal conidiation on complete medium. Other strains, including those containing *creA220*, *creA221* and *creA322*, show less severe affects on size but poorer conidiation, and finally there are a class of morphological mutants that demonstrate both poor conidiation and severe reduction in growth, and these include strains containing *creA303*, *creA304* and *creA225*. As well as varying morphology, these mutants demonstrate varying level of derepression of genes normally subject repression by glucose. When wildtype *A. nidulans* is grown on starch and glucose the genes encoding amylases, the enzymes responsible for starch

saccharification, are tightly repressed. The *creA* mutant strains all demonstrate various degrees of derepression of amylases in the presence of glucose. Mutations in *creA* also lead to various levels of deregulation of alcohol dehydrogenase. These mutations have been grouped into two broad classes, missense mutation within the DNA binding zinc finger region, including *creA1*, *creA204*, *creA331*, *creA306* and *creA225*, and those that are predicted to result in the truncation of the CreA peptide, including *creA303*, *creA304*, *creA220* and *creA221*.

The cellular localisation of CreA has been investigated utilising CreA:GFP, CreA fused with green fluorescent protein. CreA is present in the nucleus in both repressing and derepressing conditions, suggesting that CCR is not mediated via exclusion of CreA from the nucleus, and mutations in *creB*, *creC* or *acrB* do not affect cellular localisation of CreA (107). A possibility is that CreA requires posttranslational modification or protein-protein interaction to function as a repressor.

#### 1.4.1.2 CreB and CreC

There were two other loci identified as suppressors of the inability of a strain containing areA217, a non-reverable loss of function mutation, to use proline or acetamide as a sole nitogen source, creB and creC (54). Genetic analysis mapped these to chromosome II but showed that they segregate independently from one another (54). The phenotypes of the creB and creC mutations are very similar, although the creB mutant phenotype is generally more severe. Unlike creA, allelic variants of creC and creB display the same range of phenotypes, only differing in severity. In addition mutations in creB and creC, when present together in a double mutant strain, do not have an additive phenotype (54). This suggests that CreB and CreC are involved in the same pathway or act together as part of a complex. Mutations in *creB* or *creC* result in resistance to molybdate and sensitivity to acriflavine when grown on solid complete media. As well as the deregulation of amdS, that the mutations were selected for, the mutants display deregulation of genes encoding enzymes responsible for the metabolism of several other carbon sources such as starch and ethanol. In addition, creB and creC mutants have poor utilisation of quinate as a sole carbon source and proline as either a sole nitrogen or carbon source. This poor utilisation suggests a function for CreB and CreC in addition to the mediation of CCR. The creB gene is allelic to molB, a previously identified mutation that confers resistance to molybdate (5, 7, 8).

The *creB* gene encodes a 767aa functional C-terminal ubiquitin protease (76) or deubiquitinating (DUB) enzyme. CreB contains six DUB domains, responsible for ubiquitin protease activity, four PEST regions, implicated in other proteins as signals for proteolysis, and a coiled-coil region, potentially involved in substrate recognition (76). The *creC* gene encodes a 630aa protein containing five WD40 repeats, involved in protein-protein interactions, and a proline rich region (137). Orthologues of CreB and CreC have been identified in members of plantae, animalia and fungi suggesting an early origin of these proteins.

Under both repressing and derepressing conditions, CreB and CreC are present together in a high molecular weight complex (77). This complex has been proposed to stabilise or modify target proteins by the removal of ubiquitin. Over-expression of CreB can compensate for the lack of CreC, but not vice versa, suggesting that CreB is the active component (77). CreC may play a role in stabilisation, by masking the PEST region, of CreB (77).

There is preliminary evidence indicating that CreA is an ubiquitinated protein, and thus an *A. nidulans* diploid strain containing epitope tagged versions of CreA and CreB, CreAHAGFP, CreA epitope tagged with HA and GFP, and CreBFLAG, CreB epitope tagged with FLAG, were developed to ascertain if CreA is a target of CreB. When CreAHAGFP was precipitated using the HA tag, CreBFLAG was detected in the precipitate (62). However, in the reverse experiment CreA was not detected when CreBFLAG was precipitated. Thus further analysis needs to be conducted to determine whether CreB and CreA are part of a protein complex.

Due to the poor utilisation of proline and quinate by strains containing *creB* mutations, CreB was proposed to play a role in the regulation of permeases, specifically proline permease, PrnB, and quinate permease, QutD. To examine the relationship between QutD and CreB, *A. nidulans* strains were created that contained QutDHA, QutD epitope tagged with HA, and CreBFLAG, CreB epitope tagged with FLAG. In co-immunoprecipitation experiments CreB could be detected in the precipitate when a anti-HA antibody was used and QutD could be detected in the precipitate when a anti-FLAG antibody was used (62). A strain containing the *creB1937* mutation and QutDHA had lower levels of QutD but higher levels of the *qutD* mRNA when compared to a strain with a functional *creB (62)*, demonstrating the post translational effects of CreB on QutD

stability. Moreover, QutD was found to be an ubiquitinated protein (62), supporting the hypothesis that CreB acts to stabilise QutD through ubiquitin modulation.

#### 1.4.1.3 CreD, ApyA and HulA

The *creD34* mutation was identified as a suppressor of the *creC27* mutant phenotype of sensitivity to fluoroacetamide in the presence of glucose and was found to suppress the same phenotype in a strain containing *creB15* (64). The *creD34* mutation also suppresses the *creB15* and *creC27* phenotypes of sensitivity to allyl alcohol or fluoroacetate in the presence of glucose (64). This suppression suggested that CreD has an antagonistic relationship to the CreB/CreC complex and hence *creD34* leads to stronger repression of genes that are subjected to CCR, highlighted by resistance of *creD34* to fluoroacetete in the presence of glucose. The *creD34* mutation also confers resistance to acriflavine in rich media and shows poor utilisation of pyrrolidinone as a sole nitrogen source, the opposite phenotypes to mutations in *creB* or *creC* (64).

The *creD* gene encodes a 597aa protein containing one arrestin\_N and one arrestin\_C domain, as well as a single PPxY motif and two PxY motifs. PY motifs in other proteins have been shown to interact with the WW domains (18). The *creD34* mutation is a G-to-T substitution at nucleotide 642 resulting in a premature stop codon, truncating after prior to the two PXY motifs after 427aa (11).

Examination of the NCBI sequence database revealed two proteins similar to CreD in *S. cerevisiae*, Rog3p and Rod1p (11). The presence of two homologues within the *S. cerevisiae* genome led to the identification of a CreD paralogue within the *A. nidulans* genome, ApyA (11), that shared 44.2% identity with CreD, 29.9% identity with Rod1p and 31.3% identity with Rog3p (Figure 1.3). ApyA contains the arrestin\_N and arrestin\_C domains and the PPXY motif but lacks the two C-terminal PXY motifs (11). A homologue of Rsp5p, a *S. cerevisiae* HECT type ubiquitin ligase known to interact with Rod1p and Rog3p, was also indentified within the *A. nidulans* genome and was designated HulA.

As the *creD34* mutant does not have severe phenotypes, it was proposed that ApyA is able to partially compensate for the loss of CreD. However, a strain containing *creD34* and multiple copies of *apyA*, expressed from the endogenous promoter, was found to be indistinguishable from the parent strain containing *creD34* (11).

Using the bacterial-2-hybrid system to detect protein-protein interactions, neither CreD nor ApyA directly interact with CreA but both CreD and ApyA interact with HulA (11). The interaction of CreD with an ubiquitin ligase supports the model of an antagonistic relationship between CreD and CreB/CreC, whereby a CreD/HulA containing complex adds ubiquitin to a target protein while CreB/CreC containing complex removes ubiquitin.



**Figure 1.3 - Protein conservation between CreD, ApyA, Rod1p and Rog3p.** Amino acid identity and similarity percents based on pair-wise BestFit alignments. Amino acid identities are in bold type and similarities are in normal type (11).

#### 1.4.3.3 Model of carbon catabolite repression in A. nidulans

The proposed model of carbon repression involves the antagonistic modification, either directly or indirectly, of CreA via two complexes, one containing CreB and CreC and one containing CreD and HulA (Figure 1.4).



**Figure 1.4** - **Model of CCR in** *A. nidulans.* A model of carbon repression proposing the antagonistic relationship between complexes containing CreB/CreC and CreD/HulA, adding and removing ubiquitin, wherein the addition of ubiquitin occurs in derepressing conditions, inactivating CreA, and the removal and absence of ubiquitin in repressing conditions results in the binding of CreA to target promoters.

#### 1.4.4 Carbon catabolite repression and cellulase regulation in *T. reesei*

In T. reesei the functional homogloue of A. nidulans creA, is cre1 (55, 126). Cre1 recognises a consensus sequence, 5'-GCGGAG-3', which matches the A. nidulans CreA consensus binding site, 5'-SYGGRG-3' (126). These binding sites are present in the promoter regions of cre1 (55), xyn1 (80), encoding a xylanase enzyme, and cbh1 (130), encoding a cellulase enzyme. The industrial strain RUT C-30 contains a mutation in cre1, labelled cre1-1, which truncates the protein within the second zinc finger after 96 amino acids. This mutation is similar to the severe creA mutations in A. nidulans, creA303 and creA304, that also result in protein truncations prior to the end of the zinc finger binding region (123). The transformation of full length cre1 into RUT C-30 restores cbh1 glucose repression, demonstrating the involvement of *cre1* in the repression of the major cellulase (55). The cre1-1 mutation and a cre1 deletion in a QM 6a background mimic A. nidulans in that they have reduced colony diameter and conidiation (87). These mutants show a higher expression of the major cellulases (and a xylanse), cbh1, cbh2, eql1 and xyn1, as well as higher cellulase activity in both repressing, glucose, and non-repressing, sorbitol, growth conditions (87). In *T. reesei*, phosphorylation of Cre1 at Ser<sub>241</sub> is required for correct functioning of Cre1 (23). The authors examined the binding of mutated Cre1 proteins, with either the serine changed to alanine or a truncation prior to Ser<sub>241</sub>, and found that they were still capable of binding to the *chb1* promoter. However the serine to alanine mutant strain was not capable of derepression, but the truncation was able to derepress (23). This is not fully consistent with unpublished work in A. nidulans where a creA gene under the gpd promoter encoding a form of CreA where a large section was removed, including the corresponding serine residue, complements a creA deletion, allowing both repression and derepression (R. Lockington, personal communication).

In addition to Cre1 mediated repression, cellulase and xylanase encoding genes are regulated via Xyr1 and Ace2, transcriptional activators, Ace1, a transcriptional repressor, and the HAP2/3/5 complex. Ace1 has been identified as a repressor of cellulase expression through yeast two hybrid analysis and found to contain three Cys<sub>2</sub>-His<sub>2</sub>-type zinc fingers, binding the sequence 5'-AGGCA-3' (110). A strain containing a deletion of *ace1* has higher expression of all primary cellulases in the presence of sophorose and cellulose, relative to the parental strain (3). Deletion of *ace1* makes no significant difference to growth on glucose but results in improved growth on cellulose and inhibited growth on sorbitol (3), and the negative effect on sorbitol growth suggests other roles for Ace1, potentially in the transcriptional regulation of related regulatory pathways.

In *T. reesei*, the Ace2 protein is required for the induction of the *T. reesei* cellulases, *cbh1*, *cbh2*, *egl1* and *egl2*, in cellulose, but not sophorose media (4). Ace2 is a Cys<sub>6</sub> zinc finger DNA binding protein that binds to the 5'-GGCTAATAA-3' region of the *cbh1* promoter (4).

Homologues of the *A. nidulans* genes *hapB*, *hapC* and *hapE*, that form the HAP complex, were identified in *T. reesei* (156). The *T. reesei* protein complex, Hap2/3/5, was shown to bind the CCAAT box in the promoter region of the major cellulase encoding gene *cbh2* (156). However, the Hap complex may not be specifically involved in cellulase expression as the promoter for *cbh1* lacks a CCAAT box (155). Also the transcripts of these genes, *hap2*, *hap3* and *hap5*, were also shown to be present at similar levels in both cellulase inducing and repressing growth conditions (156). It is likely that the HAP complex plays and important role in general response environmental response asthe HapB/C/E complex in *A. nidulans* has been shown to regulation of gene expression in response to oxidative stress (134).

The *T. reesei* homologue of the *Aspergillus niger* xylanase activator XlnR, Xyr1, was identified as an activator for cellulase encoding genes (103). Xyr1 was shown to compete with Ace1 for 5'-GGCTAA-3' inverted elements within the *xyn1* promoter (103). A recent study determined that Xyr1 binds not only 5'-GGCTAA-3', but also 5'-GGC(A/T)<sub>3</sub>-3' motifs (33). The deletion of *xyr1* prevents the induction of cellulase encoding genes by cellulase inducers cellulose and sophorose (127), while constitutive expression can improve cellulase production (81).

Cre1 represses the transcription of *xyr1* in glucose rich growth conditions (81). This regulation may suggest a mechanism whereby cellulase expression in the *cre1*- strain exceeds the wild type when grown in the absence of glucose. It also provides a clear link between the negative and positive regulation of the cellulase encoding genes. Ace1 repression is proposed to be mediated via a competitive binding relationship with Xyr1 (103) and both have been shown to bind to the *cbh1* promoter region (74).

#### 1.4.5 Carbon catabolite repression in other filamentous fungi

Homologues of *creA* have been identified in a diverse range of other fungi, including *Botrytis cinerea* (140), *Fusarium oxysporum* (60), *Gibberella fujikuroi* (140), *Sclerotinia sclerotiorum* (145), *Metarhizium anisopliae* (114) and *Aspergillus niger* (29).

CreA, in each of these organisms, has been implicated as the transcriptional regulator of CCR. While *creA* homologues have been cloned from a number of fungi, further analysis is often lacking. In *A. niger* a mutation screen was conducted exploiting *areA*, in a similar fashion to the *A. nidulans* mutation screens, to isolate *creA* mutants (109). The *A. niger* findings supported the *A. nidulans* work in that the mutants demonstrated a range of morphologies and varying degrees of derepression (109). In addition CreA in *A. niger* regulates the expression of arabinase encoding genes, meaning these genes provide an alternative for studying carbon derepression (109). In *S. sclerotiorum*, the mutation of Ser<sub>266</sub> in Cre1 to alanine abolishes repression, mimicking *cre1* loss of function, but does not change nuclear localisation (146). However other genes implicated in CCR remain largely unstudied outside of *A. nidulans*.

#### 1.4.6 Carbon catabolite repression in *Saccharomyces cerevisiae*

CCR in *S. cerevisiae* has been extensively studied with numerous components of the regulatory network identified and characterised. Amongst the best studied of these is the protein kinase Snf1p and the transcriptional repressor Mig1p.

Strains of *S. cerevisiae* containing a mutated form of *MIG1* showed reduced glucose repression (52, 89), similar to the *creA* mutations in *A. nidulans (26)*. Like CreA, Mig1p is a zinc-finger DNA binding transcriptional repressor that binds at the consensus sequence (5'-WWWWNSYGGGG-3') (79). The localisation of Mig1p is regulated by phosphorylation by the kinase Snf1p (94). The migration of Mig1p was viewed in live cells by the insertion of Mig1p-GFP fusion into a strain containing a *MIG1* deletion. This strain exhibits normal glucose repression (25). In the presence of glucose or fructose as sole carbon sources Mig1p is localised to the nucleus, while in the presence of glycerol or sucrose, non-repressing carbon sources, Mig1p is transported to the cytoplasm (25). The movement of Mig1p was shown to be rapid and independent of translation, with movement into the nucleus after the addition of glucose after 30 seconds and movement into the cytoplasm after 2-3 minutes after the removal of glucose (25). It has been shown that repression by Mig1p is dependent on Ssn6p-Tup1p (141) and that in a yeast-2-hybrid assay Mig1p physically interacts with Ssn6p (139).

A screen for mutations that affected CCR identified two mutants, *SSN6* and *TUP1*, that were shown to have similar phenotypes (105). Moreover, co-immunoprecipitation experiments have shown that the proteins are present in a complex (147). Subsequent

analysis of this complex demonstrated that one Ssn6p is bound with four Tup1p subunits (144).

*SNF1*, <u>s</u>ucrose <u>n</u>on-<u>f</u>ermenting, mutants have been identified in several screens for poor utilisation of various carbon sources, including glycerol or ethanol (19), raffinose (158), maltose (30) and sucrose (90). *SNF1* encodes a 72kDa protein kinase that is central to CCR in *S. cerevisiae* (15). In response to low glucose Snf1p has been shown to be activated by phosphorylation (148). The exact process of this activation is not fully understood, but it involves upstream activity of at least three protein kinases Pak1p, Tos3p and Elm1p (50). Snf1p has been shown to regulate Mig1p by phosphorylation of two serine residues, as when these are mutated Snf1p mediated phosphorylation of Mig1p cannot occur (94) and Mig1p is not shuttled out of the nucleus (25).

The central component to CCR in yeast is proposed to be the phosphorylation of Mig1p by Snf1p, which causes Mig1p to dissociate from the Ssn6p-Tup1p complex (97) (Figure 1.5).



**Figure 1.5** - **Model of CCR in** *S. cerevisiae*. In low concentrations glucose Snf1p is phosphorylated and transported to the nucleus, where it phosphorylates Mig1p and Sip4p. The phosporylation of Mig1p determines its localisation, causing it to be exported from the nucleus, disrupting Mig1p associated with the Ssn6p/Tup1p complex subsequently preventing repression.

#### 1.4.7 Carbon catabolite repression in bacteria

CCR has been identified and studied in bacteria, with the greater part of research being conducted in *Escherichia coli* or *Bacillus subtilise*. Competition within the environment for preferable carbon sources between various microorganisms gives rise to this finely tuned control of carbon source use. With growth on glucose as a baseline *E. coli* demonstrates an increase in a number of genes regulated as the quality of carbon source decreases (75). This ability to preferentially select the carbon source that allows for the highest growth rate is a key aspect in the evolution of CCR in microorganisms.

Global regulation in *E. coil* has been proposed to be via the cAMP-CRP, system whereas in *B. subtilis* the proposed system is via CcpA/HPrK (Reviewed in (39)).

#### 1.4.7.1 The cAMP-CRP system

In *E. coli* CCR is regulated via the cAMP-CRP system and depends on the phosphorylation state of the glucose transporter EIIA<sup>Gic</sup>. The phosphorylation status of EIIA<sup>Gic</sup> is mediated via the phosphoenolpyruvate-carbohydrate phosphotransferase system (PTS) and the ratio between phosphate donor phosphoenolpyruvate (PEP) and pyruvate. The phosphate from EIIA<sup>Gic</sup> is transferred to available glucose, resulting in dephosphorylated EIIA<sup>Gic</sup>. In its phosphorylated form the glucose transporter EIIA<sup>Gic</sup> binds and activates adenylate cylase, leading to cyclic AMP (cAMP) synthesis. The abundance of cAMP leads to the formation cAMP-CRP complex which activates catabolic genes by binding to their promoter. The non-phosphorylated EIIA<sup>Gic</sup> is unable to activate adenylate cylase, but binds and inactivates metabolic enzymes and transporters such as LacY, responsible for lactose uptake, a regulatory system referred to as inducer exclusion (Reviewed in (39)).

#### 1.4.7.2 The CcpA/HPrK System

In *B. subtilis* CCR is primarily regulated by the histidine protein (HPr), which can be phosphorylated at either Ser46 or His15. When cellular concentrations of fructose-1,6bisphosphate and ATP are high, as they are in the presence of a preferred carbon source, HPr is phosphorylated at Ser46 by HPr kinase. The phosphorylated HPr(Ser-P) binds to CcpA, and in turn this complex binds to palindromic operator sequences, called *cre* sites, on the DNA repressing transcription of catabolic genes Reviewed in (39)).

#### **1.5 Regulation by ubiquitination**

Optimal cellular function requires a protein to be present at an appropriate concentration, time and cellular location. To facilitate this organisms have evolved

multiple levels of regulation to facilitate the transcription of genes, translation of transcripts and translocation of proteins at appropriate levels and amounts. Included in this regulatory repertoire are post translational modification of proteins, including ubiquitination, the addition of ubiquitin (Ub) to a substrate, and deubiquitination, the removal of Ub from a substrate. Ub is a highly conserved 76 amino acid protein that is present in all eukaryotic cells examined. The process of ubiquitination involves three enzyme complexes, E1, Ub activating, E2, Ub conjugating, and E3, Ub ligase.

Ub activation via the E1 complex is an ATP dependant process whereby a thioester bond forms between the C-terminal of Ub and a cytosine within the E1. Typically organisms have a single E1 enzyme, such as Uba1p in *S. cerevisiae* and Uba1 in *Drosophila melanogaster* (73, 85). The Ub is transferred to a cytosine within the active domain of the E2 complex. Finally the Ub is transferred to a substrate either directly or via an E3 intermediate. The E3 Ub ligases are divided into two broad categories, HECT type, which interact directly with the Ub, such as HulA in *A. nidulans* (11), and RING type, which facilitate the transfer of Ub from the E2 to the substrate.

Poly-Ub chains, of four or more Ub, have been shown to cause a protein to be targeted for degradation by the 26S proteasome, whereas less than four Ub moieties modify the target protein, affecting protein function (16, 49, 100) (Figure 1.6). Ub can be hydrolysed from specific substrates by DUB enzymes, either stabilising the substrate or returning them to non-ubiquitinated function (47) (Figure 1.6).

NOTE: This figure is included on page 17 of the print copy of the thesis held in the University of Adelaide Library.

**Figure 1.6** - **Model of Ubiquitination.** Diagram showing the ubiquitination (black arrow) and deubiquitination (red arrow) systems. Ubiquitin (Ub) is ligated to a target substrate via a three protein complex process. The E1 complex activates the Ub, whereas the E2 and E3 complexes facilitate the ligation of the Ub to a target substrate. Poly-ubiquitinated proteins are targeted to the 26S proteasome for degradation, while the addition of less than four ubiquitin molecules can cause conformational changes to the substrate (in gray square). The DUB enzyme prevents the Ub-substrate complex from degradation or returns it to the original function by removing Ub. (Taken from (62))

#### 1.6 ROD1 and ROG3

*ROD1* was cloned by transforming *S. cerevisiae* using a high copy number plasmid library, culturing the transformants on *o*-dinitrobenzene and selecting resistant colonies (150). The over-expression of *ROD1* was shown to confer resistance to zinc and calcium, while the deletion was shown to confer sensitivity to these compounds (150). Rod1p contains two PY motifs that when modified prevent binding to Rsp5p (1). This modification also relieved the resistance to *o*-dinitrobenzene (1). Rod1p was also found to be phosphorylated, at serine 447, by the Snf1-kinase and that Snf1p was required for *o*-dinitrobenzene resistance (121).

*S. cerevisiae ROG3* has been largely unstudied with only a single paper related to its function identified in literature searches. *ROG3* was isolated as a suppressor of the *mck1 mds1* double mutant temperature sensitive phenotype (1). It shares 43% sequence identity with Rog3p and, like Rod1p, over-expression confers *o*-dinitrobenzene resistance

suggesting that they are functional paralogues (1). Rog3p was also shown to interact with Rsp5p through its PY motifs (1).

# 1.7 RSP5

S. cerevisiae Rsp5p, a 95-kDa protein (53), has become one of the most extensively studied HECT type ubiquitin ligases. Rsp5p, like all other members of the Nedd4 family of ubiuitin ligases, contains a C2 domain, several WW domains and a catalytic HECT domain (Reviewed in (56)). Rsp5p, like many Nedd4 family members, has been linked to a diverse range of cellular functions. It has been linked to the degradation of permeases involved in uracil and nitrogen uptake (43), which could directly impact on CCR. Rsp5p has also been implicated in the stress response pathway. In response to environmental stress factors Rsp5p has been shown to regulate stress response elements at the post translational level (41). Moreover, Rsp5p has been implicated in intracellular protein trafficking, numerous nuclear functions, fatty acid synthesis and the mevalonate pathway (Reviewed in (61)). An example of this intracellular trafficking is the regulation via ubiquitination of Gap1p, a general amino acid transporter, in response to nitrogen. In high nitrogen environments, Gap1p is poly-ubiquitinated and shuttled to the vacuole for degradation, whereas in low nitrogen environments, Gap1p is mono-ubiquitinated and trafficked to the plasma membrane for amino acid uptake (44). The targeting of Gap1p via Rsp5p is facilitated by two arrestin-like proteins Bul1p and Bul2p (44). Interestingly Bul1p, Bul2p and Rsp5p have been implicated in the degradation of Rod1p (2).

#### 1.8 Aims of this project

Prior to the commencement of this work a paralogue of *creD*, *apyA*, was identified within the *A. nidulans* genome (11). Given the predicted sequenced similarity of ApyA to CreD and the limited phenotypic effects identified due to *creD* mutants, it was proposed that ApyA and CreD may have overlapping function, and as such compensate to some degree for each other. To examine this, one aim of this study was to generate an *A. nidulans* strain containing a disruption of *apyA* and once obtained to assess epistatic interactions between the *apyA* disruption and other genes involved in CCR.

A HECT type ubiquitin ligase, HulA, was shown to physically interact with both CreD and ApyA in a bacterial-2-hybrid system (11). This interaction suggests a role for HulA in the addition of ubiquitin to substrates involved in CCR and as such *hulA* warrants further investigation. To facilitate this investigation the second aim of this study was to ascertain the effects of the deletion of *hulA*.

The understanding of CCR in *A. nidulans* allows the identification of gene targets in other filamentous fungi for strain improvement strategies. In the industrial fungus *T. reesei*, mutations in *cre1*, the *creA* homologue, have lead to higher cellulase expression. Thus the third aim of this study was to determine if *T. reesei* contains a homologue of *A. nidulans creB*, and if so to determine the level of conservation between the *A. nidulans* and *T. reesei* homologues, and to investigate the phenotype of disrupting *creB* in *T. reesei*, particularly concentrating on phenotypes relevant to improving cellulase production and hence cellulosic ethanol production.

# **Chapter 2 - Materials & Methods**

# 2.1 Materials

General laboratory chemicals and growth media were of analytical research grade and were purchased from a range of commercial manufacturers.

#### 2.1.1 DNA modifying enzymes

#### Table 2.1 - DNA modifying enzymes

Enzyme	Manufacturer
Klenow fragment	Geneworks, Adelaide, SA, Australia
T4 DNA ligase	New England Biolabs, Beverly, MA, USA
Lysing enzymes	Sigma Chemical Co., St. Louis, MO, USA
Taq DNA polymerase	New England Biolabs, Beverly, MA, USA
Restriction endonucleases	New England Biolabs, Beverly, MA, USA

All enzymes were used according to the manufacturer's instructions, in the

appropriate reaction buffers.

#### 2.1.2 DNA Molecular weight markers

DNA Molecular weight markers used were 100bp DNA ladder and 1kb DNA ladder purchased from New England Biolabs, Beverly, MA, USA.

#### 2.1.3 Fungal strains

 Table 2.2 - A. nidulans strains used in this study. All strains are veA1 mutants.

Strain	Genotype	Origin
wild-type	biA1;riboB2	Derived from Pateman et al. (1967) (98)
wild-type bi	biA1; niiA4	Derived from Pateman et al. (1967) (98)
wild-type ribo	yA1 pabaA1; riboB2	Derived from Pateman et al. (1967) (98)
creA204	yA1 creA204; riboB2	Derived from Hynes and Kelly (1977) (54)
creB1937a	creB1937;riboB2	Derived from Lockington and Kelly (2001) (76)
creB1937b	yA1 pabaA1;creB1937;riboB2	Derived from Lockington and Kelly (2001) (76)
creC27	biA1;creC27	Derived from Hynes and Kelly (1977)(54)
creD34	creD34; riboB2	Derived from Kelly and Hynes (1977)(64)
acrB2	biA1;acrB2;riboB2	Derived from FGSC869
C26-1-1-10	yA2 pabaA1; argB2	Upshall et al.(1986) (142)
Bi#9	biA1; pyroA2 nkuA::argB; riboB2 niiA4	This work
Bi#11	biA1; pyroA2 nkuA::argB; riboB2 niiA4	This work
Bi#15	biA1; pyroA2; riboB2 niiA4	This work
Bi#17	biA1; pyroA2; riboB2 niiA4	This work
Arg#1	pabaA1; riboB2	This work
Arg#2	pabaA1; argB?; pyroA2 nkuA::argB; riboB2	This work

Arg#5	pabaA1; argB?; pyroA2 nkuA::argB; riboB2	This work
Arg#13	pabaA1; argB?; pyroA2 nkuA::argB; riboB2	This work
nkuAΔ pyroA2 nkuA::argB; riboB2		Nayak <i>et al.</i> (2006) (88)
Bi9Arg5 (diploid)	biA1; pyroA2 nkuA::argB; riboB2 niiA4 / pabaA1; argB?; pyroA2 nkuA::argB; riboB2	This work
Bi11Arg5 (diploid)	biA1; niiA1; pyroA1; riboB2 nkuA::argB / pabaA1; argB?; pyroA2 nkuA::argB; riboB2	This Work

Table 2.3 - T. reesei strains used in this study.	All QM 6a derived strains are mating type <i>Mat1-2</i> .
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Strain	Genotype	Origin
QM 6a	Wild type	Supplied by M. Penttilä.
RUT C30	Mutagenesis strain	ATCC 56765
VTT-D 02877	cre1::amds	Nakari-Setala <i>et al.</i> (2009) (87)
JMKTR2-6	cre2::amdS	This study
CBS999.97	Mat1-1	Supplied by M. Schmoll

# 2.1.4 Oligonucleotides

Custom oligonucleotides were purchased from Geneworks (Adelaide, Australia).

Primer name	Primer sequence (5' to 3')	Use
apyAUp8-11	GTT GAA GAA GGC CGT GAT	Amplification of <i>apyA</i>
apyAdownstream2	TGC CTA AAC TGC CTC AAG	Amplification of <i>apyA</i>
hulAF	TCG CCC TGT GGA GTA TTG AT	Amplification of hulA
hulAR	CGC GGA GAA GAT CAA ACA AG	Amplification of hulA
RiboBgIIIL	TTC AAA GAT CTA GTC AAT TTA TGG CCA TTT GC	Amplification of A. nidulans riboB
RiboBgIIIR	TTC AAA GAT CTG TGG GCT GAT ATG CCA ATT C	Amplification of A. nidulans riboB
CBUP1	CCC ATT GCT GTC TCG CTA TT	Amplification of T. reesei cre2
CBDOWN2	AAG GCA AGA TGT GTC GGA AC	Amplification of T. reesei cre2

#### Table 2.4 - Oligonucleotide primers

#### 2.1.5 Vectors

Vectors used are presented in Table 2.4. All vectors were propagated in *E. coli* strain DH5 $\alpha$ .

#### Table 2.5 - Vectors used in this study.

Plasmid	Description	Source
pBAfRiboB	Aspergillus fumigatus riboB locus cloned into pBluescript	Supplied by M. Hynes
рВаруА	apyA amplicon cloned into pBluescript	This Work
pBapyA::AfRiboB	<i>apyA</i> amplicon cloned into pBluescript disrupted with the <i>Aspergillus fumigatus riboB</i> locus	This Work
pBhulA	hulA amplicon cloned into pBluescript	This Work
pBhulA::riboB	hulA amplicon cloned into pBluescript disrupted with       This Work         the Aspergillus fumigatus riboB locus       This Work	
pBluescript II SK+	General purpose cloning vector	Stratagene
pPL3	riboB locus cloned into pUC19	Oakley <i>et al.</i> (1987) (92)
pTRcre2	T. reesei cre2 cloned into pBluescript	This Work
pTRcre2∆amds	<i>T. reesei cre2</i> cloned into pBluescript disrupted with <i>A. nidulans amdS</i>	This Work

#### 2.1.6 Kits and miscellaneous materials

Table 2.6 - Kits and miscellaneous materials used in this study.

DIG-High Prime DNA Labelling and Detection Starter Kit	Roche Diagnostics, Germany
DNeasy <sup>®</sup> Plant Mini Kit	Qiagen, Valencia, USA
EnzChek <sup>®</sup> cellulase substrate *blue fluorescent, 339/452	Invitrogen, Carlsbad, USA
EnzChek <sup>®</sup> Amylase Fluorometric (Green) Assay Kit	Invitrogen, Carlsbad, USA
Hybond-N+ nylon membrane	Amersham, UK
QIAquick PCR Purification Kit	Qiagen, Valencia, USA
QIAquick Gel Purification Kit	Qiagen, Valencia, USA
RNeasy <sup>®</sup> Plant Mini Kit	Qiagen, Valencia, USA
Wizard <sup>®</sup> Plus SV Minipreps DNA purification systems Kit	Promega, USA
Fuji RX Medical X-ray Film	Fuji Photo Film Co., Ltd., Japan

#### 2.1.7 Solutions and buffers

All solutions and buffers were prepared using Millipore water and where appropriate, were autoclaved. Solutions not able to be autoclaved were sterilised by filtrations through a 0.2  $\mu$ m filter where needed. Solutions and all other buffers routinely used in this study were as follows:

10 x Loading dye	0.25% bromophenol blue, 0.25% xylene cyanol FF, 25% Ficoll 400
1 x SSC	0.15 M NaCl, 0.015 M Na <sub>3</sub> C <sub>6</sub> H <sub>5</sub> O <sub>7</sub> .2H <sub>2</sub> O, pH 7.2
1% SDS	sodium dodecyl sulfate
1 x TAE	40 mM Tris base, 20 mM NaAc, 2 mM EDTA, pH 7.8 with glacial acetic
	acid
1 X TE	10 mM Tris-HCl (pH 8.0), 1 mM EDTA

#### 2.1.8 Media

*A. nidulans* was grown on complete and minimal media as described by Cove (1966) (21). *T. reesei* was grown on the above media, on potato dextrose agar (Oxoid LTD., England) and also on media described by Seiboth *et al.* (1997) (115). Carbon sources were added at a final concentration of 1% (w/v) unless otherwise stated. Nitrogen sources were added to a final concentration of 10 mM unless otherwise stated. All *T. reesei* growth tests were initially conducted on detergent free solid media but for photography, TritonX-100 was added to *T. reesei* media at 0.01% (vol/vol) to reduce colony diameter.

Standard bacterial medium was used as described in Sambrook & Russell (2001) (111). Where required, ampicillin was added to a final concentration of  $50\mu$ g/ml, unless otherwise stated.

#### 2.2 Methods

Standard molecular techniques were performed as outlined in Sambrook & Russell (2001) (111).

#### 2.2.1 Nucleic acid isolation

*A. nidulans* gDNA was isolated from conidia using the QIAGEN DNeasy<sup>®</sup> Plant Mini Kit as per the manufacturer's instructions or from wet mycelium by the method of Lee and Taylor (1990). *T. reesei* gDNA was isolated from conidia using the QIAGEN DNeasy<sup>®</sup> Plant Mini Kit as per the manufacturer's instructions. Total RNA was isolated from *A. nidulans* and *T. reesei* mycelia, using the QIAGEN RNeasy<sup>®</sup> Plant Mini Kit as per the manufacturer's instructions. Plasmids were isolated from *E. coli* using the Promega Wizard<sup>®</sup> Plus SV Minipreps DNA purification systems Kit as per the manufacturer's instructions.

#### 2.2.2 Transformation

*E. coli* was transformed as described in Sambrook *et al.* (2001) (111). *A. nidulans* was transformed based on the method described by Tilburn *et al.* (1983) (135), and transformants were selected for riboflavin independence conferred by the *riboB+* gene in pPL3 (92), pBapyA::AfRiboB or pBhulA::riboB. *T. reesei* was transformed using linearised plasmid based on the method described by Pentillä *et al.* (1987) (99). Transformants were selected on medium containing 10 mM acetamide as the sole nitrogen source using the *amdI9* variant of the *amdS* gene from *A. nidulans*.

#### 2.2.3 Meiotic crossing

The *A. nidulans* strains to be crossed were inoculated on 1% complete medium, 5mm apart and grown for two days at 37°C. Pieces of agar from the mycelia interface between these colonies were taken and allowed to form heterokaryons for by growth on selective medium with nitrate as the sole nitrogen source for two days at 37°C. These plates were sealed with parafilm for seven to 14 days to allow cleistothecia formation. These cleistothecia were subsequently picked and tested for the products sexual crossing.

The *T. reesei* sexual cross was conducted as per Seidl *et al.* (117) between JKTR2-6 (*cre2::amdS*) and CBS999.97 (MAT1-1) on PDA at 25°C with 16 hours of light in a 24 hour period. Fruiting bodies formed between the strains at approximately day 16 post inoculation and spores were collected from the lid of the petridish at day 22.

#### 2.2.4 Diploid formation in A. nidulans

Two A. nidulans haploid strains were inoculated on 1% complete medium, 5mm apart and grown for two days at 37°C. Pieces of agar from the mycelia interface between these colonies were taken and allowed to form heterokaryons for by growth on selective medium for three days at 37°C. Strong sections of growth were transferred to new selective medium.

#### 2.2.5 Haploid formation in *A. nidulans*

Diploid strains were broken down into haploid via growth on Benlate (DuPont, Delaware, USA Active Constituent 500 g/kg Benomyl). Haploid sectors were taken from diploid strains inoculated on 1% complete media containing between 0.001% - 0.0001% benlate.

#### 2.2.6 Polymerase chain reaction

Optimal conditions for amplification of DNA fragments via Polymerase Chain Reaction (PCR) were determined for each set of template and primers. PCR primers were designed using Primer3<sup>2</sup> (108). Finnzymes Phusion Polymerase was typically used for all reactions as per the manufacturer's instructions. The reactions were performed on PTC-200 (MJ Research) and Mastercycler gradient (Eppendorf) machines.

PCR products were purified using the QIAGEN QIAquick<sup>®</sup> PCR Purification or Gel Extraction Kits.

<sup>&</sup>lt;sup>2</sup> http://frodo.wi.mit.edu/primer3/

#### 2.2.7 Enzyme assays

Enzyme assays methods are detailed in Chapter 4 Materials and Methods. The assays were conducted using the Invitrogen EnzChek<sup>®</sup> cellulase, xylanse and amylase systems as per the manufacturer's instructions.

#### 2.2.8 Southern blot analysis

DNA for Southern blot analysis was separated by gel electrophoresis through 1% agarose in 1x TAE buffer, denatured by treatment with 0.25M HCl for 10 minutes and transferred to Hybond N+ membrane (Amersham) by alkaline transfer in 0.4M NaOH for four hours, as recommended by the manufacturer.

For Digoxigenin (DIG) labelled probes 300-1000ng of DNA was labelled using the Roche DIG High Prime DNA Labeling and Detection Starter Kit II as per the manufactures instructions.

DNA filters were prehybridised for 2 hours in the buffer supplied with DIG High Prime DNA Labeling and Detection Starter Kit II 50°C, respectively.

Filters were washed with 2 x SSC, 0.1% SDS for at least 15 minutes at room temperature, and then twice with 0.5 x SSC, 0.1% SDS at 65°C. Hybridisation was detected using expose to X-ray film after treatment with a DIG antibody, as per the manufacturer's instructions.

#### 2.2.9 Bioinformatic analysis

Phylogenetic analysis was conducted using Geneious V4.8 (28) and described in greater detail in Chapter 3. Genomic sequences were taken from the Broad Institute, the Joint Genome Initiative or NCBI, as detailed in Figure 3.1 and listed in Appendix A.
# Chapter 3 - *In silico* Identification of Genes Involved in Carbon Catabolite Repression

## **3.1 Introduction**

The efficient use of available carbon sources is evolutionarily advantageous and as such proteins involved in this system might be conserved across the entire fungal kingdom if they arose early in fungal speciation. It is also possible that other cellular processes could adapt this mechanism involving regulation and fine-tuning if selection was relaxed, for example due to a change in environment. CreA-like proteins have been identified as functional regulators of CCR, in several members of Ascomycota (63). However, orthologues of other proteins involved in CCR in *A. nidulans*, CreB, CreC and CreD, remain unstudied. The abundance of sequenced fungal genomes allows the identification of these orthologues in numerous fungi, including those outside Ascomycota. The aim of this study is to investigate the level of conservation of CCR regulatory proteins CreA, CreB, CreC and CreD across the fungal kingdom.

The identification of these homologues in fungal species with sequenced genomes was conducted using one-way tBLASTn analysis with the *A. nidulans* protein sequences as queries. The comparison scoring matrices used in these analyses were blosum62, for Ascomycota, and blosum45, for Basidiomycota and Zygomycota. The alignment type was gapped with low sequence complexity filter off. This analysis normally resulted in numerous sequence hits (returned multiple sequences with sufficient similarity) but with a clear homologue. For instance using this approach with A. nidulans CreA on the M. grisea genome results in 36 hits, the highest having a score of 401, with the next closest having a score of 77. Only sequence hits with a score above 100 were pursued further. The evolutionary relationship between the fungi used within this study, as based on previous work (58), and the presence of CreA, CreB, CreC or CreD in their genome is depicted in Figure 3.1. A possible homologue of CreB was identified in all members of the fungal kingdom analysed, but CreA lacked this level of conservation. In Basidiomycota, many species contained sequences similar to the CreA zinc finger region, but almost double the size of CreA, and as such it is difficult to see if these sequences share a common direct lineage with A. nidulans CreA. However, A. nidulans CreA can function with large internal deletions (R. Lockington, personal communication) and therefore size might not be a critical component to CreA conservation. CreC was preserved in most

members of the fungal kingdom but absent in the class Saccharomycetes. Sequence alignments and phylogentic trees were constructed with the software package Geneious (28). Sequences were aligned using Geneious alignment with blosum62 scoring matrix for CreA and blosum45 for CreB and CreC due to the greater range of species used. Both scoring matrices were used for CreD, with blosum62 being used for comparison within the same fungal class and blosum45 being used when all sequences were compared. The alignment method was global with free end gaps and a gap opening penalty of 12, an extension cost of three and 25 refinement iterations. Phylogenetic trees were constructed based on these alignments using the Geneious tree builder with the Jukes-Cantor distance model and a Neighbor-Joining method. A total of 1,000 replicates were conducted using the Jackknife resampling method.

## 3.2 CreA

Previous studies have identified a CreA homologue in Sordariomycetes (126), Leotiomycetes (140), Schizosaccharomycetes (133) and Saccharomycetes (89). In addition analysis described in this chapter identified homologues in Pezizomycetes (Tuber melanosporum) and Dothideomycetes (Cochliobolus heterostrophus and Mycosphaerella fijiensis) (see Figure 3.2). The grouping of Scr1, the Schizosaccharomyces pombe homologue of CreA, with CreA and away from Mig1p, the S. cerevisiae homologue of CreA, is significant because the subphylum Taphrinomycotina has been shown to be basal to the rest of Ascomycota (58). The CreA homologues in basidiomycota are difficult to determine. Within Coprinus cinerea and Laccaria bicolor the predicted size of the most similar sequence is 430aa and 460aa respectively, similar to A. nidulans 416aa, but the most similar sequences in other members of Basidiomycota are much larger. The Postia placenta sequence is 738aa, Cryptococcus neoformans, 735aa, Malassezia globosa, 760aa, and Ustilago maydis, 784aa. A homologue was not identified in the division Zygomycota or in *Batrachochytrium dendrobatidis*, a member of the division Chytridiomycota. This suggests that if these sequences share a common ancestor with A. nidulans CreA they have diverged greatly and hence an in silico approach to CreA homologue identification is not useful outside of Ascomycota.







Figure 3.2 - Protein relatedness between CreA homologues within Ascomycota. A phylogenetic tree using the neighbour-joining method containing homologues of *A. nidulans* CreA across multiple members of Ascomycota. The tree is rooted using the *Coprinus cinerea* sequence most similar to CreA. Branch support values represent 10,000 Jackknife resamples and bar indicates substitutions per site. Appendix A contains accession number or genome positions.



**Figure 3.3 - Protein relatedness between CreB homologues.** A phylogenetic tree using the neighbour-joining method containing homologues of *A. nidulans* CreB throughout the fungal kingdom. The tree is rooted using the *Homo sapian* sequence most similar to CreB, UBH1. Branch support values represent 10,000 Jackknife resamples and indicated distance represents substitutions per site. Appendix A contains accession number or genome positions.

## 3.3 CreB and CreC

Using the *A. nidulans* CreB sequence as a template, a tBLASTn search was conducted on the genome sequences of fungal species listed in Figure 3.1. Homologues were identified based on sequence similarity, position of the conserved ubiquitin hydrolase domain within the protein and the overall length of the protein sequence. Of particular relevance to this thesis is the presence of a homologous sequence within *T. reesei*, identified as protein 122405 within the genome sequence database. In addition all members of the class Sordariomycetes that were analysed contain a homologue of CreB with a pairwise identity, relative to *A. nidulans*, of greater than 45%. Given CCR mutants have great potential for industrial exploitation and that CreA lacks easily identifiable homologues in distantly related fungi, CreB provides an excellent target for gene disruption for strain development, although there is the possibility that CreB function will not be conserved. Within each of the fungi a sequence similar to CreB was identified, and a sequence similar to CreC was also found with the exception of the class Saccharomycetes (Section 3.3.2).

## 3.3.1 CreB and CreC in Trichoderma reesei

Identification of T. reesei homologues of creB and creC is described in detail in chapter four, but bioinformatic analysis of their control regions is described here. 1000bp immediately upstream from each proposed start of translation were analysed using 'find motif' within Geneious. The CreA consensus binding sequence 5'-SYGGRG-3' is expected to be present every 256bp, assuming equal distribution and frequency of nucleotides, as it is bidirectional. Thus four binding sites are expected to be present in a 1000bp region by chance. The promoter region of *T. reesei cre2* (creB homologue) contains eight Cre1 (CreA) consensus-binding sequences and that of T. reesei cre3 (creC homologue) contains six consensus-binding sequences. Previous research highlighted the importance of inverted overlapping Cre1 binding sequences in T. reesei (80) and an inverted Cre1 binding site is present in the 1000bp upstream of both cre2 and cre3. Identical analysis was also conducted in A. nidulans, and no inverted overlapping consensus sequences were identified in the 1000bp upstream of either creB or creC although each contained eight consensus binding sequences, above the expected four. Two genes known to be regulated by CreA, alcA (96) and amyA (86) contain 12 and eight, respectively, consensus binding sequences within their promoter region. Also in T. reesei the transcription of xyr1, an activator of cellulase encoding genes, has been shown to be regulated via Cre1

mediated CCR (81). Cre1/A might be involved in the regulation of *cre2/B* and *cre3/C*, but experimental analysis is needed to determine if these sites occur by chance or are a further mechanism of fine tuning this regulatory system.

## 3.3.2 CreB and CreC in Saccharomycetes

The phylogenetic analysis of CreB sequences suggests that the nearest Saccharomycetes CreB homologue, UBP9p, is highly divergent, forming a basal clade away from other fungi (Figure 3.3). However, tBLASTn analysis against the A. nidulans genome sequence using S. cerevisiae UBP9p as the query sequence has CreB as the highest hit, scoring 239, with the next sequence hit scoring only 90. This suggests that UBP9p has undergone rapid divergence from the CreB ancestor, or that CreB has been lost in Saccharomycetes. Ubp9 remains largely unstudied, but has been implicated in DNA repair in vitro (101). Analysis of Saccharomycetes genomes using tBLASTn for homologues of CreC found no clear homologues. The nearest sequence in Pichia stipitis, Candida tropicalis and Candida albicans is a subunit of a protein complex that coats membrane bound vesicles, called coatomer, corresponding to ANID 03026.1 in A. nidulans. In S. cerevisiae the nearest sequence is a protein involved in ribosome biogenesis (SCRG 05461.1). This tBLASTn analysis also revealed a hit to Tup1p (SCRG 05472.1), a glucose repression regulatory protein, but RcoA in A. nidulans has higher similarity to Tup1p and is the likely homologue (48). The absence of a clear CreC homologue further suggests the highly divergent nature of this regulatory network within Saccharomycetes.

## 3.4 CreD and ApyA in Ascomycota

The previous study that identified ApyA as a CreD-like sequence similar to Rod1p and Rog3p suggested that the genes encoding these sequences were the result a gene duplication (11). At that time very few genomes of the genus Aspergillus or the class Saccaromycetes were publicly available. In light of recent sequencing programs the paralogous relationship between Rod1p and Rog3p and CreD and ApyA can be investigated by examining the genomes of closely related species for similar sequences.

Using CreD as a query sequence in tBLASTn analysis, as described in 3.1, reveals the presence of CreD like proteins within all the Aspergillus genomes on the Broad Aspergillus comparative genome database<sup>3</sup>. CreD like sequences were present in *A. nidulans*, which contains two (CreD and ApyA), *A. oryzae*, which contains three, *A. niger*, which contains

<sup>&</sup>lt;sup>3</sup> http://www.broadinstitute.org/annotation/genome/aspergillus\_group

two, *Aspergillus flavus*, which contains two, *Aspergillus fumigatus*, which contains one, *Aspergillus terreus*, which contains one, *Aspergillus clavatus*, which contains two, and *Neosartorya fischeri*, which contains one. Currently none of those sequences have been studied except CreD and ApyA, and without knowing the function of these sequences, speculation as to the significance of single or multiple CreD-like sequences is difficult. Phylogenetic grouping of ApyA with CreD (Figure 3.4), coupled with the absence of *apyA* within the genomes of other Aspergilli, suggests that ApyA is a duplication of CreD that occurred after the divergence of *A. nidulans* from other Aspergilli. However *A. nidulans* CreD and ApyA only share 44.2% identity (11), suggesting that the duplication is not recent or that there has been rapid divergence.

To examine the origin of Rod1p and Rog3p, tBLASTn analysis was used as above on the genomic sequences of *S. cerevisiae, Saccharomyces castellii, Saccharomyces mikatae, Saccharomyces paradoxus, Saccharomyces bayanus, Saccharomyces kluyveri, Candida glabrata, Candida albicans, Candida tropicalis, Pichia stipitis* and *Kluveromyces waltii* from the NCBI genome taxonomy database<sup>4</sup> with Rod1p as the query sequence. Phylogenetic grouping of these sequences (Figure 3.5) has Rod1p and Rog3p forming distinct clades with species closely related to *S. cerevisiae.* There is also a basal grouping of *S. catellii* and *C. glabrata* with these clades. These groups also form a larger clade, such that both of the sequences from *C. tropicalis* and *C. albicans,* the only sequence from *P. stipitis* and one each of the sequences from *S. kluyveri* and *K. waltii* are represented in clades separate from the Rod1p/Rog3p clade.

<sup>&</sup>lt;sup>4</sup> http://www.ncbi.nlm.nih.gov/Taxonomy



Figure 3.4 - Protein relatedness of CreD-like sequences amongst the Aspergilli. Branch support values represent 10,000 Jackknife resamples and indicated distance represents substitutions per site. Appendix A contains accession number or genome positions.



Figure 3.5 - Protein relatedness of Rod1p and Rog3p like Sequences within Sacchromycetes. A phylogenetic tree using the neighbour-joining method containing Rod1p like sequences from members of Sacchromycetes. Sequences are numbered according to order within the tree, except *S. cerevisiae* that has protein names included in the label. Appendix A contains accession number or genome positions. The genome of each species, except *Pichia stipitis*, contains two sequences similar to Rod1p. Branch support values represent 10,000 Jackknife resamples and indicated distance represents substitutions per site. Appendix 1 contains accession number or genome positions.

## **3.5 Discussion**

Previous studies have shown that CreA is conserved, both in sequence and function, across multiple members of Ascomycota. The expanded sequence analysis presented here shows that clear CreA homologues are present only in the Ascomycota, but homologues of CreB, CreC and CreD were identified across the fungal kingdom. There is increased interest in manipulating components of CCR, specifically *creA* mutants, in industrially significant members of Ascomycota, such as *T. reesei* (67), but due to the difficulty of identifying CreA homologues outside of Ascomycota and the severe morphology of *creA* mutants, the CreB and CreC homologues provide identifiable targets for directed strain improvement. It is possible that these homologues are conserved at the sequence level but not the functional level and that aims to develop industrially useful strains based on CreB and CreC will not affect carbon regulation. However deletion of these homologues and determining their function would still be of scientific interest as it will provide insights into the evolution of components, originally part of the same gene regulation system, for different cellular processes in different fungi.

CCR is different in the domesticated fungus S. cerevisiae, when compared to A. nidulans. Bioinformatic analysis suggests that the selection maintaining the CCR system, involving CreA, CreB and CreC, has been relaxed within class Saccharomycetes. There was an absence of a CreC like sequence within the genomes of the four members of Saccharomycetes. As CreC interacts with CreB in A. nidulans (77) the absence of a CreC homologue implies an altered function of CreB homologues within Saccharomycetes. The grouping of the Saccharomycetes CreA homologue as the basal group within Ascomycota (Figure 3.2) or the grouping of the Saccharomycetes CreB homologue as the basal group to the rest of the fungal kingdom (Figure 3.3) suggests that these sequences have undergone recent rapid change. Such observation within S. cerevisiae would not be surprising as the domestication process involving repeated growth in sugar rich substrates would be expected to relax selection on some sequences, particularly those responsible for CCR. However, these findings were also observed in P. stipitis, C. albicans and C. tropicalis suggesting that the event that caused this rapid change occurred within the early stages of Saccharomycetes speciation. Also the involvement of the S. cerevisiae, Ubp9p, in DNA repair is consistent with the hypothesis that the finely controlled CCR system studied in A. nidulans could be utilised for other important cellular processes in the absence of strong selection.

In silico analysis of the Aspergilli is unable to reveal the origin of ApyA, except to suggest that it is either an ancestral event that has been lost in other Aspergilli or that it is a recent duplication that has undergone rapid change. As arrestin containing proteins, like CreD, ApyA, Rod1p, and Rog3p, are known to interact with HECT-type ubiquitin ligases (1), and this interaction can determine ligation targets, it is possible that duplication of arrestin containing proteins allowed the evolution of unique regulatory systems. This is consistent with the presence of multiple gene duplication events as phylogenetic analysis in both the Aspergilli and the Saccharomycetes suggests. The adaptation to unique conditions by the evolution of a new mechanism of gene regulation could be facilitated by this duplication of arrestin containing proteins. The genomic sequencing of fungi closely related to *A. nidulans* would potentially allow the identification of the gene duplication event.

# Chapter 4 -Characterisation of *cre2* in *T. reesei* for Strain Improvement

Statement of authorship and contribution for the manuscript entitled "Disruption of *Trichoderma reesei cre2*, encoding an ubiquitin C-terminal hydrolase, results in increased cellulase activity" by JA Denton and JM Kelly. (Unpublished)

Jai Denton (Candidate)

Contributions by this author are: All work described within the manuscript and writing of the manuscript.

I, Jai Denton, certify these contributions.

22<sup>nd</sup> of June, 2010

Joan Kelly (Co-author)

Contributions by this author are: Supervision, initial project conception and editing of the manuscript.

I, Joan Kelly, certify these contributions.

22<sup>nd</sup> of June, 2010

Disruption of *Trichoderma reesei cre2*, encoding an ubiquitin C-terminal hydrolase, results in increased cellulase activity.

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Working Title: Disruption of *T. reesei cre2* increases cellulase production.

ABSTRACT:

The filamentous fungus, *Trichoderma reesei* (*Hypocrea jecorina*), is an important source of cellulases for use in the textile and alternative fuel industries. To improve the cellulase production ability of *T. reesei*, a gene known to be involved in carbon regulation in *Aspergillus nidulans* was targeted for disruption. The *T. reesei* orthologue of the *A. nidulans creB* gene, designated *cre2*, was shown to be functional in carbon repression through complementation of a *creB* mutation in *A. nidulans*. A *T. reesei* strain containing a *cre2* disruption exhibited phenotypes similar to the *A. nidulans creB* mutant strain on solid media and had elevated cellulase levels. That the phenotype was due to the *cre2* disruption was confirmed as it segregated with the disruption marker in a *T. reesei* sexual cross.

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## Introduction

Environmental concerns associated with climate change coupled with the uncertainty surrounding oil supply have caused an increased interest in alternative fuel production.

Cellulosic ethanol, produced from cellulose rich waste, has emerged as an environmentally sound alternative to petroleum based fuels. Bioethanol production generally involves two steps: the saccharification of cellulose rich waste using cellulase enzymes secreted by filamentous fungi to release glucose; and the fermentation of glucose to ethanol using yeast. The cost of ethanol production could be reduced if improvements to cellulase production by filamentous fungi were made.

The filamentous fungus, *Trichoderma reesei* (*Hypocrea jecorina*), has been used extensively for cellulase production and the release of the complete genome sequence presents new opportunities for targeted genetic manipulation to increase cellulolytic enzyme production. In the presence of glucose, genes encoding cellulase enzymes are not expressed due to carbon repression (Reviewed (67, 128)). An industrially important strain of *T. reesei*, Rut-C30, was generated by selection for improved cellulase production after multiple rounds of mutagenesis, resulting in its genome containing numerous mutations. Currently three independent mutations have been characterised within Rut-C30: a mutation in the gene encoding a carbon mediated transcriptional repressor, *cre1* (55); an 85 kb deletion containing 29 genes (116); and a frame shift within the gene encoding the glucosidase II alpha subunit (37). Targeted genetic manipulation of the *T. reesei* wild type strain could result in even higher cellulase production, due to targeted changes in the absence of potentially deleterious background changes that are present in mutagenised strains.

Genetic analysis in the model filamentous fungus, *Aspergillus nidulans*, has provided a framework to study the mechanism of carbon repression in *T. reesei*. In *A. nidulans* there are three genes in which mutations result in deregulation of carbon repression: *creA*, the *A. nidulans* orthologue of *cre1*, encoding a zinc finger DNA binding transcriptional repressor (26, 27); *creB*, encoding a ubiquitin C-terminal hydrolase (76); and *creC*, encoding a WD40 repeat protein shown to be present in a complex with CreB (77). Orthologues of *creA* have been identified and studied in numerous fungi including *Humicola grisea (131)*, *Cochliobolus carbonum* (138), *Gibberella fujikuroi* (140) and *Botrytis cinerea* (140), however *creB* or *creC* remain largely unstudied in other fungi.

Mutations in the *creB* and *creC* genes lead to deregulation of carbon repression of some genes, but not the severe morphological impairment caused by *creA* mutations (54). In *A. nidulans* there is evidence that CreA is a direct target of CreB suggesting that CreB functions in carbon repression via CreA (62). Disruption of carbon source mediated repression without severe morphological impairment could potentially lead to the development of industrially useful fungal strains, and as such *creB* represents an ideal candidate for targeted disruption.

The deletion of *cre1* within the progenitor strain of *T. reesei*, QM 6a, has recently been characterised (87). As with *A. nidulans* strains containing *creA* mutations, deletion of *cre1* resulted in morphological impairment although to a lesser degree. These deletions of *cre1* demonstrated higher total secreted protein and enzymatic activity of endoglucanases and xylanases, but not proteases. Cellulase regulation in *T. reesei* has also been shown to involve transcriptional regulators: Ace1 (3, 110), a transcriptional repressor; and Ace2 (4) and Xyr1(103, 127), transcriptional activators. It has recently been shown that *Cre1 represses xyr1 transcription*, and it was proposed that Ace1 might also play a role in *xyr1* repression (81). Deletion of *xyr1* in *T. reesei* resulted in loss of transcription of the major cellulase encoding genes, *cbh1*, *cbh2* and *egl1 (127)*, and furthermore there was no secreted cellulase or xylanase activity detected in the *xyr1* deletion strain after 72 hours growth in inducing conditions (127). Xyr1 has also been implicated in the expression of the cellulase, Egl3 (120) and the induction of the lactose metabolism pathway by lactose (129).

There is no sequence similar to *ace2* in the *A. nidulans* genome, highlighting the differences between cellulase regulation in the two filamentous fungi. However, sequences similar to *ace1*, *stzA* (17) and *xyr1*, *xlnR* (132) are present in the genome. In this study we have shown bioinformatically that the *T. reesei* genome contains orthologues of the *A. nidulans creB* and *creC* genes. There have been no reports of mutations in *creB* and *creC* orthologues in *T. reesei*, thus it was possible that mutations in the *T. reesei* orthologues of *creB* or *creC* would not result in the equivalent phenotypes seen in *A. nidulans*, as regulation of cellulase encoding genes in *T. reesei* could have been independent of the CreB/CreC complex. In order to investigate these postulations the *cre2* gene was cloned and shown to be a functional orthologue of the *A. nidulans creB* gene. A *T. reesei* strain containing a disruption within the *cre2* encoding region was

generated and was found to have increased extracellular cellulase activity in both the presence and absence of glucose.

## **Materials and Methods**

## Strains

The A. nidulans strains used in this study were creB1937 (yA1 pabaA1; creB1937; riboB2) and wild-type (yA1 pabaA1; riboB2). The T. reesei strains used in this study were QM 6a (wild type), CBS999.97 (containing MAT1-1, (117)), VTT-D 02877 (containing cre1::amdS, (87)) and JKTR2-6 (containing cre2::amdS, this study). Escherichia coli strain DH5 $\alpha$  (supE44  $\Delta$ lacU169 ( $\Phi$ 80 lacZ  $\Delta$ M15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1) was used to propagate plasmids.

## Media

Solid and 5ml liquid fungal media were based on that described by Cove (21) or potato dextrose agar (PDA) where stated, while 50ml liquid medium was as described by Seiboth *et al.* (115). *A. nidulans* and *T. reesei* were grown at 37°C and 30°C respectively. Nitrogen sources were added to a final concentration of 10mM and carbon sources at 1% (wt/vol), unless otherwise stated. Riboflavin and para amino benzoic acid were added to media at final concentrations of 2.5µg per ml and 0.5µg per ml, respectively, when required. All *T. reesei* growth tests were initially conducted on detergent free solid media but for photography, TritonX-100 was added to *T. reesei* media at 0.01% (vol/vol) to reduce colony diameter.

## T. reesei Sexual Cross

The *T. reesei* sexual cross was conducted as per Seidl *et al.* (117) between JKTR2-6 (*cre2::amdS*) and CBS999.97 (MAT1-1) on PDA at 25°C with 16 hours of light in a 24 hour period. Fruiting bodies formed between the strains at approximately day 16 post inoculation and spores were collected from the lid of the petridish at day 22.

## Transformation

*A. nidulans* was transformed based on the method described by Tilburn *et al.* (135), and transformants were selected for riboflavin independence conferred by the *riboB+* gene in pPL3 (92). *T. reesei* was transformed using linearised plasmid based on the method described by Pentillä *et. al* (99). Transformants were selected on medium containing 10 mM acetamide as the sole nitrogen source using the *amdI9* variant of the *amdS* gene from *A. nidulans*. Bacteria were transformed as described in Sambrook *et al.* (111).

#### Molecular Methods

Molecular methods were as described by Sambrook *et al.* (111). Bacterial plasmids were purified using the Wizard Plus SV Minipreps DNA Purification System (Promega, USA). Fungal DNA was extracted using the DNeasy Plant Mini Kit DNA purification system (Qiagen, USA). All PCRs were performed using the high fidelity polymerase, Phusion (Genesearch, Australia). Southern analysis was performed using DIG Highprime Labelling and Detection Kit (Roche, Australia).

#### Generation of *cre2* Constructs

A 4505 nucleotide region encompassing the Cre2 coding region, including approximately 500 bp upstream of the putative start codon and approximately 200 bp downstream of the putative stop codon, was amplified using primers CBUP1 (5'CCCATTGCTGTCTCGCTATT<sup>3'</sup>) and CBDOWN2 (5'AAGGCAAGATGTGTCGGAAC<sup>3'</sup>). The amplicon was cloned into pBluescript generating pTRcre2 for use in complementation analysis. The disruption construct, pTRcre2 $\Delta$ amdS, was generated through ligation of the *A. nidulans amdS* encoding region, containing the *amdI9* promoter mutation, into a MscI restriction site. This restriction site occurs prior to the codon for His<sup>426</sup>, which is within the 300 amino acid ubiquitin hydrolase domain and prior to His<sup>428</sup> and Asp<sup>437</sup> shown to be active sites (51) (Figure 1).

#### **Analytical Methods**

Mycelia from fungal strains QM 6a, VTT-D 02877, the *cre1* disruption, and JKTR2-6, the *cre2* disruption, were cultured either in small 5ml cultures in *A. nidulans* medium described by Cove (21), on in larger 50ml cultures in *T. reesei* medium described by Seiboth *et al.* (115).

For the 5 ml cultures, mycelia for inoculation were harvested after growth for 28 hours in 1% (w/v) glucose medium. The mycelia were washed with liquid carbon free medium, blotted with paper towel and 50mg of mycelium was added to 5ml culture medium in 10ml culture bottles. For cellulase assays, three conditions were used: 1% glucose and 10mM ammonium tartrate; 1% glucose, 1mM sophorose and 10mM ammonium tartrate. Cultures were grown at 30°C, shaken at 200 RPM, and were harvested 6 hours, 12 hours and 18 hours post inoculation.

For the 50ml cultures, mycelia for inoculation were harvested after growth for 24 hours in 1% (w/v) glucose medium. The mycelia were washed with liquid carbon free medium, blotted on paper towel and 200mg was added to 50ml culture medium in 250ml Erlenmeyer flasks. Cultures were grown at 30°C, shaken at 200 RPM, and were harvested 12 hours, 24 hours and 36 hours post inoculation.

Total cellulase or xylanase enzyme activity was assayed either using the ENZ-CHEK cellulase assay substrate or the ENZ-CHEK xylanase assay kit (Invitrogen, USA) as per the manufacturer's instructions. These systems allow for the determination of relative cellulase or xylanase activity between strains within a single experiment by the measurement of fluorescence at 360/460nm produced by cellulase or xylanase activity upon the substrates. The manufacturer's standards indicate that a fluorescence of 40000 corresponds to approximately 6mU/mL of cellulase activity for the cellulase substrate while a fluorescence of 1000 corresponds to approximately 450mU/mL of xylanase activity using the xylanase kit. Data presented for the 5ml cultures is based on biological duplicates that were each assayed in duplicate. The data presented for the 50ml cultures is based on biological triplicates that were assayed in duplicate.

The biomass from each of the 50ml cultures was harvested by filtration and dried at 65°C and weighed.

A Rezex ROA-Organic analysis column (300 x 7.8mm, Phenomenez, Australia) and a refractive index detector (Model 350, Varian, Australia) were used to analyse glucose concentrations. The mobile phase was ultra pure water at a flow rate of 0.6 ml/min and the column was maintained at 35°C.

Statistical analysis was conducted using Microsoft Excel 2007. Standard deviations were calculated using standard Excel functions on biological and experimental replicates as stated.

#### Results

#### The *T. reesei* Genome Contains Homolgoues of *creB* and *creC*

The *T. reesei* genome was examined to identify potential orthologues of the *A. nidulans creB* and *creC* genes. Using protein sequence for CreB (AAL04454) and CreC (AAF63188) from the National Centre for Biotechnology Information website (www.ncbi.nlm.nih.gov), TBLASTN analysis was performed against the *T. reesei* translated nucleotide database (genome.jgi-psf.org/Trire2/Trire2.home.html). Both the identified genomic region and subsequent protein of the *T. reesei* CreB and CreC orthologues,

designated Cre2 (Protein ID 122405) and Cre3 (Protein ID 64608) were aligned using the Geneious software package (Biomatters Ltd, USA) to the *A. nidulans* orthologues. The Cre2 amino acid sequence showed 43% identity to *A. nidulans* CreB (Fig. 1), while the Cre3 sequence showed 48% identity to *A. nidulans* CreC.

#### Cre2 is a Functional Orthologue of CreB

An *A. nidulans* strain that contained the *riboB2* and *creB1937* mutations was cotransformed with pTRcre2, containing *T. reesei cre2*, and pPL3 containing *riboB+*. The *creB1937* mutation leads to sensitivity to allyl alcohol in the presence of glucose, poor use of proline and increased sensitivity to acriflavin. Six riboflavin independent transformants were tested for complementation of these *creB1937* phenotypes (Fig. 2). Transformants T3 and T6 were morphologically similar to wildtype (Fig. 2a), and also showed complementation of the phenotypes caused by *creB1937* of sensitivity to allyl alcohol in the presence of glucose (Fig. 2b), poor proline use, and acriflavine hypersensitivity (data not shown). T1, T2, T4 and T5 showed varying degrees of partial complementation, but no expression analysis was conducted to determine the basis for this. Thus the *T. reesei cre2* gene is a functional orthologue of *creB*, and *cre2* can be expressed in *A. nidulans* from the *T. reesei cre2* promoter.

#### Generation of a cre2 Disruption Strain

A plasmid, pTRcre2∆amdS, was constructed to disrupt *cre2* in *T. reesei*, in which the *A. nidulans amdS* gene containing the *amdSI9* promoter mutation was inserted into the *cre2* ORF disrupting the gene within the ubiquitin C-terminal hydrolase domain. A strain containing a disruption of the endogenous *cre2* gene, JKTR2-6, was generated by transformation of QM 6a with this plasmid. After purification, homologous recombination at the *cre2* locus, required for disruption of the ORF, was verified using Southern analysis (Supplementary Figure 1).

#### Phenotypic Effects of cre2 Disruption

The *cre2* disruption strain demonstrated similar mycelial growth to QM 6a on solid minimal media with glucose as the carbon source, but showed reduced conidiation (Fig. 3a). The *cre2* disruption grew much better than QM 6a on solid medium containing maltose (Fig. 3b). Improved growth on maltose, linked to increased  $\alpha$ -glucosidase activity has also been seen in *A. nidulans* strains containing *creB* mutations (54). Similar growth of both QM 6a and the *cre2* disruption strain on cellobiose as a sole carbon source suggested limited effects on  $\beta$ -glucosidase activity (Fig. 3c). As is seen with *creB* mutations

in *A. nidulans* strains, the *cre2* disruption also showed poorer growth on medium containing proline as the sole nitrogen source (Fig. 3d). However, unlike *creB* mutations in *A. nidulans*, which also grow poorly on lactose, mutations in *cre2* in *T. reesei* had no observable effect on lactose use in solid medium (data not shown) but effects were apparent in liquid conditions (see below). The *cre2* disruption strain also had higher protease activity than QM 6a on medium containing 5% (vol/vol) liquid soy milk, as assessed by the size of the cleared zone around the colonies, but not on medium also containing 1% glucose. Growth tests were also conducted on solid medium containing 1% fructose, 1% glycerol, 1% sorbitol, 1% cellulose, 1% xylose or 1% xylan as a sole carbon source with 10mM ammonium tartrate as the nitrogen source and 1% glucose as the sole carbon noticeable difference was observed between the parent strain, QM 6a, and the cre2 disruption.

Growth tests were also performed in 50ml liquid culture. Dry biomass weights for a range of carbon sources for each of the three strains are shown in Table 1. The *cre2* disruption strain had significantly (P<0.05) less biomass than QM 6a on glucose at 24 and 36 hours, but significantly (P<0.001) greater growth than the *cre1* disruption. Solid media growth tests demonstrated a greatly improved growth on maltose for the *cre2* disruption and extremely poor growth for the *cre1* disruption that was corroborated in the liquid culture experiments. The *cre2* disruption had three times more biomass than QM 6a at 24 hours post mycelial inoculation while the *cre1* disruption failed to grow. Liquid culture analysis showed that disrupting *cre2* reduced growth on lactose relative to QM 6a, a result that reflects the phenotype of *creB* mutations in *A. nidulans* but was not detectable for the *T. reesei* mutation on solid medium. The disruption of *cre1* or *cre2* had no noticeable effect in medium with 2% glycerol as the sole carbon source, as both strains had similar amounts of biomass compared to QM 6a, while the *cre1* disruption grew very poorly.

Total secreted cellulase activity was assayed in the culture media from strains grown in various conditions (Fig. 4, 5 & Supplementary Table 1). In the 5ml cultures containing glucose without an inducer, all of the strains showed very low cellulase activity (results not shown). In medium containing glucose and sophorose, the *cre1* and *cre2* disruption strains showed similarly high levels of secreted cellulase activity after 12 hours (Fig. 4a). QM 6a had limited cellulase activity after 6 and 12 hours, but the activity had increased to levels similar to the disruption strains by 18 hours. Glucose levels were determined using HPLC, and found to be 0.03% at 12 hours and 0 at 18 hours in the QM 6a cultures (not shown), and thus by 18 hours the cultures contained no source of repression. In medium containing sorbitol and sophorose the *cre2* disruption strain showed higher cellulase activity than either QM 6a or the *cre1* disruption strains (Fig. 4b) at all time points.

Assays were also undertaken in 50ml cultures, using lactose as an inducer, to allow accurate measurement of mycelial dry weights, and thus ascertain activity per gram dry weight. The cellulase activity per gram dry weight in 2% lactose was higher in the *cre2* disruption compared to QM 6a (Fig. 5c) across all three time points, particularly at 24 hours. The total cellulase activity for the *cre2* disruption was also much higher than QM 6a at both 12 and 24 hours but only slightly higher at 36 hours (Supplementary Table 1). The three strains were also grown in 50ml of medium with 2% powdered cellulose as a sole carbon source, however reliable growth weights were not obtained due to limited growth and problems separating mycelium from the powered cellulose. Total activity was also much higher in the *cre2* disruption strain on 2% powdered cellulose than either QM 6a or the *cre1* disruption (Supplementary Table 1).

Cellulase activity for QM 6a was not detected until the glucose concentration had reached or was extremely close to zero, as the cellulase encoding genes are subject to glucose repression. In the *cre1* disruption strain cellulase activity was detected in all conditions tested, including in the presence of 2% glucose and the absence of the inducer indicating that at least one major exocellulase was not subject to carbon repression in the *cre1* disruption strain (Fig. 5b). The *cre2* disruption did not lead to cellulase derepression in the lactose induced cultures containing 2% glucose, as there was no detectable cellulase activity until the 36 hour time point in 2% lactose and glucose. After 36 hours glucose concentrations in the cultures had dropped to 0.6% for the *cre2* disruption and 0.3% for QM 6a, but no activity could be detected in the QM 6a cultures. In the 5ml cultures containing 1% glucose and sophorose as the inducer, the *cre2* disruption had detectable levels of cellulase activity across all three time points. These findings suggest that the disruption of *cre2* increases the concentration of glucose necessary to repress cellulase encoding genes in lactose containing cultures.

Total secreted xylanase activity was also measured in 2% glucose, 2% glucose/2% lactose and 2% lactose cultures. As with the cellulase assays the *cre2* disruption was

elevated in both total activity and total activity by weight when compared to QM 6a on 2% lactose (not shown).

## Meiotic Segregation of the Disruption Phenotype with the *amdI9* Phenotype

As with *creB* mutations in *A. nidulans,* the *cre2* disruption strain in *T. reesei* grew considerably better on medium containing maltose as the sole carbon source, relative to QM 6a, the disruption parental strain, and CBS999.97, a strain shown to undergo sexual reproduction with QM 6a (117). In order to show that the phenotypes seen were linked to the *cre2* disruption and not a fortuitous change elsewhere in the genome, a meiotic cross was undertaken between JKTR2-6, the *cre2* disruption, and CBS999.97 to demonstrate that the phenotype always segregated with the *amdI9* phenotype in the progeny of a sexual cross. Of the 38 progeny screened 23 demonstrated use of acetamide as a nitrogen source due to the *amdI9* marker and improved growth on maltose whereas the other 15 showed weak growth on acetamide and maltose media. The segregation of the *cre2* disruption phenotype with the *amdI9* marker, used to disrupt *cre2*, proves that the phenotype seen in the disrupted strain is linked to the *cre2* disruption, and not due to fortuitous changes elsewhere in the genome.

## Discussion

*T. reesei* Cre2 is conserved with *A. nidulans* CreB throughout the protein, particularly within the region encoding the ubiquitin C-terminal hydrolase domain. However there was the possibility that the identified *cre2* gene may not encode a functional orthologue of the *A. nidulans creB* gene but another separate ubiquitin C-terminal hydrolase, or that the functions of Cre2 are not conserved between these distantly related filamentous fungi. Therefore, complementation of the *A. nidulans creB1937* mutation by the *T. reesei cre2* gene was tested in order to investigate whether *cre2* is the functional orthologue of *creB*. CreB mediated repression of alcohol dehydrogenase and regulation of proline permeases are proposed to operate via separate mechanisms, and thus the ability of the *T. reesei cre2* to complement both the sensitivity to allyl alcohol in glucose medium, and the poor use of proline as a nitrogen source, indicates that both functions are conserved between the two orthologues.

While it is not uncommon for filamentous fungal genes expressed from their endogenous promoters to function in other fungal species, such as the *amdS* gene encoding acetamidase (65), CreB forms part of a large complex with CreC (77), and is likely to require multiple protein-protein interactions for its functions. Therefore the complementation of the *creB1937* mutation by *cre2* is of importance as it demonstrates that the CreB/CreC complex previously identified in *A. nidulans*, and also the targets of CreB deubiquination, are conserved between distant filamentous fungi despite relatively low conservation outside of the ubiquitin C-terminal hydrolase domain.

Growth testing on both solid and liquid media revealed similar phenotypes between *T. reesei* and *A. nidulans* strains containing *cre2/creB* mutations. These growth tests supported the initial hypothesis that a strain with disrupted *cre2* will lack the extreme morphological impairment of *cre1* mutants. However the disruption of *cre2* does lead to somewhat impaired growth on glucose and lactose, evident in liquid culture.

The *cre2* disruption demonstrated elevated cellulase activity when sophorose, lactose or cellulose was used as an inducer. In this study, cellulase activity was detected for the *cre2* disruption grown in glucose medium with sophorose (Fig. 4a) but not in glucose medium lacking sophorose. There was considerable difference between the *cre2* disruption and QM 6a for total secreted cellulase activity in sorbitol plus sophorose medium (Fig. 4b). The *cre2* disruption produced double the cellulase activity corrected for weight compared to QM 6a and the *cre1* disruption when grown on 2% lactose (Fig. 5).

Total secreted cellulase activity was also higher for the *cre2* disruption in both 2% lactose and in 2% cellulose across the three time points (Sup. Table 1). The elevated cellulase activity in medium containing sophorose (Fig. 4), lactose (Fig. 5), or cellulose (Sup. Table 1), demonstrates that the elevated cellulase activity in induced conditions is a robust phenotype of the *cre2* disruption. The *T. reesei* carbon repression defective strain, RUT C-30, has previously been shown to have elevated levels of  $\beta$ -diglucoside permease activity (66). The elevated levels in the presence of glucose in the *cre1* and *cre2* disruption strains are potentially related to the reduced repression of genes encoding both permeases and cellulases.

The disruption of *cre2* resulted in elevation of cellulase activity in the absence of glucose in all carbon sources tested when an inducer for cellulase production was present. Relative to the *cre1* disruption strain the *cre2* disruption showed reduced cellulase production in the presence of glucose, but this reduction was less than that of the parental strain, QM 6a. This suggests that *cre2* disruption exhibits an intermediate level of glucose repression. This partial deregulation allows for supplementation of industrial media, such as those that are whey or solka flok based, with potentially repressing sugars to improve biomass.

Southern analysis and previous studies in *A. nidulans* strongly indicate that the *cre2* phenotypes were the result of a double cross over event at the *cre2* locus inserting *A. nidulans amdS* into the coding region. The discovery of a sexual cycle in *T. reesei* opens the way for alternatives for strain improvement but also allows for the first time traditional genetic analysis of industrial strains, allowing an alternative to molecular approaches. A meiotic cross was used to demonstrate that the selectable marker phenotype, strong growth on acetamide, and the *cre2* disruption phenotype, strong growth on maltose, always co-segregate, thus confirming that the disruption phenotype was due to the *cre2* disruption and not fortuitous alteration to the genome.

Since the disruption of *cre2* increases the amount of cellulase activity in both the presence and absence of a repressing carbon source such as glucose without the morphological deficiencies seen due to the *cre1* disruption, targeting *creB* orthologues for disruption in other industrially useful filamentous fungi, such as *Aspergillus oryzae*, *Trichoderma harzianum* or *Aspergillus niger* may also prove beneficial. While the disruption of *cre2* has increased secreted cellulase activity, further improvements could potentially be made use the *cre2* disruption strain as a foundation for further targeted

genetic manipulation. Potential manipulations include the over-expression of the *T. reesei* orthologue of *creD* (11), shown in *A. nidulans* to increase derepression in a *creB1937* background (R. Lockington, personal communication). Protease encoding genes have previously been targeted for disruption to improve heterologous protein expression in both *A. oryzae* (59, 154) and *A. niger (143). T. reesei* proteases could be targeted using this strategy to potentially improve extracellular cellulase activity.

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Figure 1 - Protein sequence alignment of *A. nidulans* CreB and the putative *T. reesei* orthologue. A denotes identical amino acids. A denotes similar amino acids. Solid underline denotes the ubiquitin hydrolase domain,  $Asp^{54}$  to  $Tyr^{474}$ . The position of the *A. nidulans amdS* encoding region is indicated, between  $Tyr^{425}$  and  $His^{426}$ . Arrows denote the active sites,  $His^{428}$  and  $Asp^{437}$  of the ubiquitin hydrolase domain (51).



**Figure 2 - Complementation of** *A. nidulans creB1937* by *T. reesei cre2. A. nidulans creB1937* mutant strain transformed with *Trichoderma reesei cre2.* Strains were incubated at 37°C on a) 1% glucose, 10mM ammonium tartrate; b) 1 % glucose, 10mM ammonium tartrate, 10mM allyl alcohol; c) key to strains. Plates were supplemented with riboflavin and para amino benzoic acid.



**Figure 3 - Phenotype of** *cre2* **disruption strain on solid media.** Strains were grown at 30°C on a) 1% glucose, 10mM ammonium tartrate & 0.01% triton-X; b) 1% maltose, 10mM ammonium tartrate & 0.01% triton-X; c) 1% cellobiose, 10mM ammonium tartrate & 0.01% triton-X; d) 1% glucose, 10mM proline & 0.01% triton-X; e) Key to strains.







**Figure 5 - Total secreted cellulase per gram per litre dry mycelial weight and glucose concentration.** Cellulase secretion in *T. reesei* strains QM 6a, *cre2* disruption and *cre1* disruption by biomass produced 12, 24 and 36 hours post transfer. Total secreted cellulase activity was measured using the EnzChek Cellulase Substrate expressed as fluorescence. Dry weights are shown in Table 1 and levels of total cellulase activity are shown in Supplemental Table 1. Glucose concentrations were determined by HPLC analysis. Error margins indicated standard deviations that are the result biological and experimental triplicates. The carbon sources for each of the cultures are a) 2% glucose, b) 2% glucose and 2% lactose, c) 2% lactose, d) 2% sorbitol and e) 2% glycerol. The strain key is f).

Table 1 - Total Dry Biomass in Various Carbon Sources. Biomass was harvested from strains grown on carbon sources as shown for 12, 24 &36 hours at 30°C. The strains are QM 6a and the disruptions of *cre1* or *cre2*. Dry weight is shown in mg per ml. Error margins indicatedstandard deviations that are the result biological triplicates.

	12 hours			24 hours			36 hours		
Carbon Source	QM6a	cre2-	cre1-	QM6a	cre2-	cre1-	QM6a	cre2-	cre1-
2.0% Glucose	3.37 ± 0.41	2.72 ± 0.1	1.21 ± 0.10	5.19 ± 0.29	4.40 ± 0.25	$2.25 \pm 0.06$	$6.00 \pm 0.45$	4.97 ± 0.06	3.57 ± 0.12
2.0% Maltose	0.52 ± 0.07	0.85 ± 0.02	$0.32 \pm 0.05$	1.20 ± 0.26	$3.64 \pm 0.07$	0.26 ± 0.02	3.89 ± 0.83	5.32 ± 0.41	0.26 ± 0.10
2.0% Lactose	0.56 ± 0.03	$0.53 \pm 0.04$	0.57 ± 0.05	1.05 ± 0.08	0.62 ± 0.12	0.81 ± 0.05	2.29 ± 0.11	1.27 ± 0.08	1.55 ± 0.04
2.0% Glycerol	0.84 ± 0.11	0.85 ± 0.08	0.73 ± 0.10	2.09 ± 0.32	$2.06 \pm 0.08$	1.96 ± 0.05	3.71 ± 0.28	3.21 ± 0.08	3.44 ± 0.17
2.0% Sorbitol	$0.55 \pm 0.06$	$0.54 \pm 0.06$	$0.32 \pm 0.07$	$0.89 \pm 0.08$	$0.94 \pm 0.02$	0.31 ± 0.06	1.48 ± 0.05	1.50 ± 0.12	$0.51 \pm 0.06$
2.0% Glucose / 2.0% Lactose	1.02 ± 0.11	0.78 ± 0.03	$0.48 \pm 0.02$	4.12 ± 0.23	2.73 ± 0.13	$1.28 \pm 0.03$	5.51 ± 0.31	$5.04 \pm 0.23$	2.81 ± 0.36

**Supplementary Table 1 - Total Secreted Cellulase & Xylanase Activity.** Cellulase or Xylanase secretion in *T. reesei* strains QM 6a, *cre2* disruption and *cre1* disruption measured using the EnzChek Cellulase Substrate or the EnzChek Xylanase Assay Kit uncorrected for dry weight. Time indicated as hours post transfer, grown at 30°C. Error margins indicated standard deviations that are the result of assay duplicates and biological triplicates.

Cellulase Activity	12 hours			24 hours			36 hours		
Carbon Source	QM6a	cre2-	cre1-	QM6a	cre2-	cre1-	QM6a	cre2-	cre1-
2.0% Glucose	0	0	80 ± 10	0	0	6050 ± 1400	0	0	29200 ± 2100
2.0% Lactose	540 ± 180	900 ± 220	1480 ± 500	14300 ± 1130	21100 ± 1300	9180 ± 630	23900 ± 1130	$25600 \pm 600$	24000 ± 800
2.0% Glucose / 2.0% Lactose	0	0	5900 ± 500	0	0	32300 ± 1800	0	470 ± 200	62500 ± 2500
2.0% Sorbitol	0	50 ± 10	130 ± 40	0	440 ± 130	190 ± 40	0	700 ± 80	780 ± 50
2.0% Glycerol	110 ± 340	1570 ± 370	5090 ± 840	240 ± 80	750 ± 130	2170 ± 1140	0	100 ± 50	0
2.0% Cellulose Powder	0	68 ± 29	148 ± 40	208 ± 80	480 ± 500	250 ± 70	3000 ± 2200	11800 ± 760	630 ± 80
Xylanase Activity							•		
2.0% Glucose	0	0	34 ± 10	0	0	1160 ± 290	0	0	5840 ± 440
2.0% Lactose	540 ± 180	900 ± 210	1490 ± 500	14280 ± 1130	21080 ± 1300	9180 ± 630	23880 ± 1130	25630 ± 600	23960 ± 790
2.0% Glucose / 2.0% Lactose	0	0	1130 ± 120	0	0	9810 ± 980	0	50 ± 90	17610 ± 1980



Supplementary Figure 1 - Southern hybridisation of genomic DNA testing for *cre2::amdS*. Southern hybridisation of genomic DNA from strains 1) QM 6a, 2) JKTR2-4, 3) JKTR2-6, 4) VTT-D 02877 restricted with EcoRV 5) QM 6a, 6) JKTR2-4, 7) JKTR2-6, 8) VTT-D 02877 restricted with BgIII and probed with *cre2*. The expected banding pattern for: EcoRV is endogenous 8kb and disruption 6.7kb and 3.2kb; BgIII endogenous 5.7kb and 4.8kb and disruption 6.1kb, 4.8kb and 2.4kb.

# Chapter 5 - Analysis of apyA

# **5.1 Introduction**

ApyA was identified bioinformatically due to sequence similarity with CreD, and subsequent analysis using the bacterial-2-hybrid system to detect protein-protein interactions demonstrated an interaction of ApyA and CreD with the ubiquitin ligase HulA (11). The homology with CreD and interaction with HulA suggest that ApyA could be involved in CCR. Mutations in *creD*, a paralogue of *apyA*, have been shown to suppress the phenotype of carbon derepression caused by mutations in *creB* and *creC*. However *creD* mutations have few affects on phenotypes other than this epistatic interaction, possibly due to the ability of ApyA to compensate for the deficiency of CreD. In order to investigate the possibility of ApyA being involved in CCR, *apyA* was targeted for disruption, and epistatic interactions between *apyA* and other CCR genes were investigated by crossing the disrupted *apyA* strain to strains containing mutations in *creA*, *creB*, *creC*, *creD* and *acrB*.

## 5.2 Creation of a disruption construct and transformation

The *apyA* genomic region was amplified and cloned into pBluescript using the primers apyAUp8-11 and apyAdownstream2, creating the plasmid pBapyA. The *A. fumigatus riboB* gene was used to disrupt the *apyA* open reading frame, as it is able to complement the riboflavin dependent growth in *A. nidulans* but does not undergo homologous recombination at the *A. nidulans riboB* locus. An internal 254bp EcoRV BglII fragment, including half of the Arrestin\_N domain, was removed from pBapyA and the *A. fumigatus riboB* locus was ligated in, to produce a plasmid called pBapyA::AfriboB. This plasmid was used to transform the *A. nidulans* NkuAΔ strain and riboflavin independent transformants were selected.

Genomic DNA from the parent strain, NkuA $\Delta$ , and three of the riboflavin independent transformants, ApyA7, ApyA11 and ApyA14, was restricted with Sall and SacII. Southern hybridisation analysis, using an *apyA* probe, was conducted. The expected hybridised band sizes for Sall are 3692bp for wild type and two bands, 2543bp and 1731bp, in the case of a gene replacement. Expected sizes for SacII bands are 5454bp for wild type and two bands of 4263bp and 2562bp for a gene replacement. Both ApyA7 and ApyA11 gave the expected pattern for a strain that had undergone a gene replacement at the *apyA* locus (Figure 5.1). The banding pattern in ApyA14 suggests that this strain is the
product of a single homologous recombination event as opposed to double homologous recombination required for gene replacement. Further analysis was conducted using the ApyA11 strain.



**Figure 5.1** - **Southern hybridisation of** *apyA* **disruption transformants.** Genomic DNA from wild type and candidate *apyA* disruption strains digested with Sall, lanes 3-6, and SacII, lanes 8-11. Probe generated from the entire *apyA* PCR product. Expected sizes for SalI are 3692bp for wild type and two bands 2543bp and 1731bp for a disruption resulting from double homologous recombination. Expected sizes for SacII are 5454bp for wild type and two bands 4263bp and 2562 bp for a disruption. Lane 1) 1kb Ladder, 2) no sample loaded, 3) gDNA from NkuAΔ, 4) gDNA from ApyA7, 5) gDNA from ApyA11, 6) gDNA from ApyA14, 7) no sample loaded, 8) gDNA from NkuAΔ, 9) gDNA from ApyA7, 10) gDNA from ApyA11, 11) gDNA from ApyA14

## **5.3 Phenotypic analysis**

The growth of ApyA11 was compared to the growth of  $nkuA\Delta$  and wild-type Ribo on a variety of growth conditions. On minimal medium, 1% glucose supplemented with 10mM ammonium tartrate, ApyA11 did not differ in morphology from  $nkuA\Delta$  (Figure 5.2 iA). Phenotype testing was conducted to test whether disruption of apyA mimics creD34phenotypes, specifically poor use of pyrrolidinone, resistance to acriflavine and sensitivity to molybdate. When grown on complete medium supplemented with 7µg per ml of acriflavine both  $nkuA\Delta$  and ApyA11 grew slightly better than the wild-type (Figure 5.2 iiE). This image shows ApyA11 growing better than nkuA $\Delta$ , but the difference between these strains was not robust and, normally their growth was similar, and increasing the acriflavine slightly, to 8µg per ml, abolished the growth of either strain (Figure 5.2 iiiD). It is most likely that any differences in resistance to acriflavine are an aspect of the genetic background of  $nkuA\Delta$ . (Subsequent analysis of  $nku\Delta$  progeny suggests that this resistance is unlinked to *scbA*, Section 5.4.3.) Neither ApyA11 nor  $nkuA\Delta$  displayed any difference to wild-type on minimal medium with pyrrolidinone (Figure 5.2 iiiC) as a nitrogen source or minimal medium supplemented with molybdate (Figure 5.2 iiC) despite mutations in the related gene *creD* leading to clear phenotypes of these media.

ApyA11 was no more sensitive to allyl alcohol in the presence of glucose (Figure 5.2 iiF) nor did it cause poor use of quinate (Figure 5.2 iiD) or proline (Figure 5.2 iiB). Phenotypic testing of ApyA11 also failed to reveal any altered use of a series of carbon sources, including maltose, fructose, arabinose, ethanol, starch, lactose and sodium acetate. It appears, in respect to these tests, that ApyA11 has the phenotype of the parent.

### 5.4 Epistatic interactions

The *creD34* mutation was identified as a suppressor of the sensitivity to allyl alcohol due to the *creC27* mutation and later shown to similarly suppress the *creB15* phenotype. This demonstrated an epistatic interaction between CreD and CreB and CreC. To investigate the involvement of ApyA in CCR, episatic interactions between the *apyA* disruption and other CCR mutants were investigated by exploiting meiotic crossing in *A. nidulans*. The strains containing mutations in *creA*, *creB*, *creC*, *creD* and *acrB* were riboflavin dependent to allow the following of *apyA::AfriboB*, as only strains containing the *apyA* disruption will be riboflavin independent. Strains were obtained that contained *apyA::AfriboB* and *creA204*, *creB1937*, *creC956*, *creD34* or *acrB2* mutations.

Each of the double mutant strains were tested on medium containing either 1% (w/v) glucose, fructose, maltose, starch, arabinose, lactose or sodium acetate with 10mM ammonium tartrate and medium containing either 10mM ammonium tartrate, glutamine, sodium nitrate, proline, pyrrolidinone or urea with 1% (w/v) glucose. Phenotypic testing was also conducted on media containing the toxic compounds acriflavine, allyl alcohol, molybdate and fluroacetate.

#### 5.4.1 creA

The strain containing *apyA::AfriboB*, ApyA11, was crossed to a strain containing the *creA204* mutation. To assess the absence of CCR in a double mutant, containing both *creA204* and *apyA::riboB*, the strains were grown on media containing 1% glucose and 10mM ammonium tartrate supplemented with either 10mM allyl alcohol or 1% starch (Figure 5.2 i). The plates containing starch were flooded with iodine, a stain to visualise the use of starch. These tests for derepression show no difference between the strain containing *creA204* and the double mutant strain. Even when the concentration of allyl alcohol was lowered to 5mM no difference could be detected (not shown).

## 5.4.2 creB and creC

It was proposed that due to the similarity between CreD and ApyA, mutations in *apyA* may also suppress the phenotypes of *creB* and *creC* mutations. To examine this, strains containing either *creB1937* or *creC956* and *apyA::AfriboB* were produced through meiotic crossing. The *creB1937 apyA::AfriboB* and *creC956 apya::AfriboB* double mutant strains grew more strongly on glucose minimal media containing allyl alcohol, indicating suppression of the *creB1937* and *creC956* pehnotype.

Analysis of these colonies with partial suppression of allyl alcohol sensitivity demonstrated growth on quinate as a sole carbon source or proline as a sole nitrogen source identical to parental *creB1937* or *creC956*. This demonstrates that *apyA::AfriboB* does not show an epistatic interaction with regard to these *creB1937* and *creC956* phenotypes. Moreover, the double mutant strains had no phenotypic differences, relative to the *creB1937* and *creC956* parental strains, on the carbon or nitrogen sources listed in Section 5.4.

## 5.4.3 Identification of a creB suppressor

However, in these crosses there were four classes of progeny on 1% glucose and 10mM allyl alcohol medium. There were the two parental classes, but also there were two levels of suppression of the sensitivity of *creB1937* and *creC956* to allyl alcohol, a strong suppression unlinked to *apyA::AfriboB* (not shown) and a weak repression linked to *apyA::AfriboB* (Figure 5.2 iiF).

Phenotypic testing of the progeny from the *creB1937xApyA*11 meiotic cross revealed a class of progeny that displayed *creB1937* phenotypes on quinate as a sole carbon source and proline as a sole nitrogen source, were riboflavin dependant, demonstrating an absence of the *apyA::AfRibo* disruption, but were able to grow on minimal media containing allyl alcohol and glucose. This ability to grow in the presence of allyl alcohol suggests supression of the *creB1937* phenotype and hence an unidentified mutation segregating within the cross. To determine the origin of this unidentified mutation, a meiotic cross was conducted between the *creB1937* and *nkuA* $\Delta$  strains. Suppression of the *creB1937* allyl alcohol phenotype was also present among the progeny suggesting that the original *nkuA* $\Delta$  strain harbours an unidentified mutation. This mutation has been designated *scbA1*, <u>s</u>uppressor of <u>creB</u>.

## 5.4.4 creD and acrB

Phenotypic testing on minimal medium, 1% glucose with 10mM ammonium tartrate, revealed considerably weaker growth of the *creD34; apyA::AfriboB* double mutant strain compared to either of the parental strains (Figure 5.2 iiiA). The poor growth of the double mutant strain, relative to either parent, was also observed across the entire range of carbon and nitrogen sources tested (not shown). In all these phenotypic tests, with varying carbon and nitrogen sources, the strain containing *acrB2* and *apyA::AfriboB* was indistinguishable from the *acrB2* parent (Figure 5.2 iiiA, not shown).

The strain containing both the *apyA::AfriboB* and *creD34* mutations was more resistant to acriflavine than a strain containing *creD34* (Figure 5.2 iiiD). Strains containing mutations in *creD* or *acrB* have previously been shown to be resistant to acriflavine, with *acrB* mutations demonstrating a higher level of resistance (11, 12). A range of acriflavine concentrations were tested and it was found that the *creD34; apyA::AfriboB* double mutant strain had a higher level of acriflavine resistance than strains with the *creD34* or *acrB2* mutations (Figure 5.2 iiiD-F). In contrast to this, the strain containing *apyA::AfriboB* and *acrB2* routinely grew slightly worse on acriflavine that the *acrB2* parental strain (Figure 5.2 iiiD-F).

The presence of *acrB2* and *creD34* both confer sensitivity to molybdate, with *acrB2* resulting in higher sensitivity. However, the strain containing both *creD34* and *apyA::AfriboB* had similar growth on minimal medium supplemented with 6.6mM molybdate and non-supplemented minimal medium (Figure 5.2 iiiB). This resistance was not seen in the strain containing both *acrB2* and *apyA::AfriboB*. This resistance was preserved at higher concentrations (not shown).

In the phenotypic tests conducted a strain containing *acrB2* and *apyA::AfriboB* was indistinguishable from the *acrB2* parent (Figure 5.2 iii) on all media tested except acriflavine. When tested on the range of acriflavine the *acrB2 / apyA::AfRiboB* strains routinely demonstrated slightly less resistance to acriflavine (Figure 5.2 iiiD-F).





### **5.5 Discussion**

Disruption of *apyA* has no effect on the phenotype of the strain, as detectable in the range of conditions tested here. The strain containing *apyA::AfriboB*, ApyA11, grows as well as the parent strain on glucose minimal medium, and does not display any of the phenotypes caused by the *creD34* mutation.

A. nidulans strains containing mutations in creB or creC lack the level of carbon mediated repression of alcA, encoding alcohol dehydrogenase, that is seen in the wildtype. This can be demonstrated by their inability to grow on minimal medium containing varying concentrations of allyl alcohol, as allyl alcohol is toxic when metabolised by alcohol dehydrogenase. The presence of a mutation in creD suppresses the inability of creB and creC mutants to repress alcA, and thus the double mutant strains grow on glucose medium supplemented with allyl alcohol. While these strains are capable of growth, it is not to wild type levels, suggesting that the double mutants have less alcA repression than wild-type. On medium containing ethanol as a sole carbon source, these double mutant strains grow similar to wild-type (64), indicating that the growth is not due to lower levels of alcA per se. Whether the presence of an apyA mutation would also suppress the inability of strains containing creB and creC mutations to suppress alcA was tested. This was found to be the case, but the level of *alcA* suppression was less than that conferred by the presence of the creD34 mutation. Given the sequence similarity between CreD and ApyA and that they both interact with HulA, it was proposed that the presence of *apyA* may compensate for mutations in *creD*, although multiple copies of apyA were unable to suppress the phenotype of creD mutations (11). The suppression of creB and creC mutations by the disruption of apyA suggests that creD and apyA are involved in overlapping functions.

A shared function between CreD and ApyA is further supported by the reduced growth of a strain containing both *apyA::AfriboB* and *creD34*. This poor growth of the double mutant on minimal media, relative to either parent, suggests that critical functions of ApyA can be compensated for by the function of CreD and vice versa. It is not uncommon for paralogues to share similar functions but also retain unique functions. The gene duplication of *creD*, proposed in Chapter 3, could be further explored by the deletion of *creD* in a member of the Aspergilli that contains a single *creD* like sequence (Chapter 3.3), such as *A. terreus* or *A. fumigatus*. It would be proposed that the deletion of the *creD* homologues from these species would result in a phenotype akin to that seen

in the *A. nidulans* double mutant, *creD34; apyA::AfriboB*, supporting the hypothesis that ApyA and CreD retain shared function. Analysis of strains containing mutations in *apyA*, *creD* and either *creB* or *creC* was not conducted as part of this research.

# Chapter 6 - Deletion of Aspergillus nidulans hulA

# 6.1 Introduction

Boase and Kelly (2004) identified a HECT type ubiquitin ligase, HulA, which was shown to interact with CreD and ApyA in bacterial-2-hybrid analyses (11). As CreD is known to affect CCR, it was proposed that this involvement is via a CreD/HulA complex. To investigate the involvement of HulA in CCR, a deletion of *hulA* was to be developed and crossed to other strains containing mutations in genes implicated in CCR to investigate epistatic interactions. However Rsp5p, the *S. cerevisiae* homologue of HulA, is required for viability (43) and therefore it was possible that the deletion of *hulA* in a haploid strain would not be viable, and thus a deletion would need to be constructed in a diploid strain.

# 6.2 Deletion of hulA

## **6.2.1 Deletion Construct**

A 3901bp amplicon including *hulA* was generated through PCR by using custom primers, hulAF and hulAR (Tabel 2.4). This amplicon was cloned into pBluescript at the EcoRV restriction site, generating a 6859bp plasmid designated pB*hulA*. This plasmid was restricted with the BglII enzyme, which excised a 2125bp fragment from the *hulA* ORF that encodes the three WW domains and the start of the HECTc domain. *A. nidulans riboB* was amplified using the custom primers RiboBglIIL and RiboBglIIL (Table 2.4), generating a 2101bp amplicon with incorporated BglII restriction sites at either end. This amplicon containing *riboB* was restricted with BglII and inserted into the pBhulA plasmid in place of the excised BglII fragment, resulting in the plasmid *pBhulA::riboB*.

## 6.2.2 Transformation of A. nidulans and A. nidulans nkuA::argB

To delete *hulA*, the wild-type ribo strain was transformed with *pBhulA::riboB* and eleven riboflavin independent colonies were analysed, but were not due to gene replacement events.

During this work information about the  $nkuA\Delta$  strain was published (88) which allows >95% integration via homology, which is needed for gene replacement. This  $nkuA\Delta$  strain was also transformed with pBhulA::riboB, and seven riboflavin independent colonies were examined. As the gene used to delete *hulA* was the *A. nidulans* endogenous *riboB* gene, rather than the *A. fumigatus riboB* gene that was used for the *apyA* construct (Section 5.2), a homologous integration event could occur at either the *hulA* or *riboB* loci. Seven riboflavin independent transformants were analysed using Southern analysis and shown not to be *hulA* deletions. There were also a number of colonies that grew poorly on the transformation plates and did no streak out from conidia on selective medium (medium lacking riboflavin). This is indicative of an integration event that is lethal (88).

### 6.2.3 Development and Transformation of an A. nidulans nkuA Diploid

A method to determine if a specific deletion is lethal involves transformation of an *A. nidulans* diploid strain, looking for a replacement of a single copy of the gene in question. This method relies on two homologous recombination events between the deletion construct and the target gene. A diploid strain of *A. nidulans* that was homozygous for the deleted copy of *nkuA* was developed to help facilitate this recombination event.

The *nkuAD* strain was crossed to two strains, C26-1-1-10 (yA2 pabaA1; argB2) and wild-type bi (biA1; niiA4), in order to obtain nkuAD strains with various requirements. Phenotype testing was used to determine the genotype of twenty progeny from each cross with respect to pabaA, riboB, niiA, biA and pyroA. Arginine dependent strains were avoided as they did not contain *nkuA::arqB*, however genotype at the *arqB* locus cannot be determined in strains containing *nkuA::argB* using phenotypic testing alone. Four progeny from each cross, Bi#9, Bi#11, Bi#15, Bi#17, Arg#1, Arg#2, Arg#5 and Arg#13, genotyped with respect to nkuA using Southern analysis with argB as a probe. As nkuA was deleted with *arqB*, two hybridising bands would indicate that the strain contained an extra copy of argB and hence nkuA::argB. Subsequently, PCR was used to differentiate between *nkuA::arqB* and *nkuA*. Genomic DNA from the selected progeny was digested with EcoRI, a restriction enzyme that does not cut within *argB*. The southern analysis indicates that Bi#9, Bi#11, Arg#2, Arg#5 and Arg#13 contain the second copy of argB and hence contain *nkuA::argB* (Figure 6.1). Three strains were selected that contained the deleted nkuA, riboB2 and at least one other auxotrophic marker that allowed the generation of the diploid. These strains were Bi#11, Bi#9 and Arg#5. Two stable diploids were generated that were riboflavin dependent, Bi11Arg5 and Bi9Arg5.

The strain Bi9Arg5 was used in a subsequent experiment, where it was transformed with *pBhulA::riboB*. A total of ten riboflavin independent colonies were selected.



**Figure 6.1** - **Southern hybridisation of genomic DNA testing for** *nkuA::argB*. Southern hybridisation of genomic DNA from strains 1) Bi#9, 2) Bi#11, 3) Bi#15, 4) Bi#17, 5) Arg#1, 6) Arg#2, 7) Arg#5, 8) Arg#13 and 9) *nkuA* $\Delta$  that were restricted with EcoRI and probed with *argB*. The purple arrow indicates endogenous *argB*, while the blue arrow indicates hybridisation to the *nkuA::argB* sequence.

# 6.3 Haplodisation of Riboflavin Independent Diploids

Four of the riboflavin independent diploid transformants, HulDip1, 3, 4 and 5, were grown on complete medium supplemented with Benlate which causes them to produce haploid sectors (136). The only possibility that a haploid sector is riboflavin independent is if it contains the *hulA::riboB* construct. Due to the deletion of *nkuA*, the *hulA::riboB* construct can only integrate though homology, but as *A. nidulans riboB* was used as the marker it is possible that the *hulA::riboB* will integrate via homology at the *riboB* locus. If deletion of *hulA* is lethal, and a single copy sufficient in a diploid, then haploid sectors will be either 50:50 *ribo+:ribo-*, indicating integration at *riboB*, or all *ribo-*, indicating integration at *hulA*. If deletion of hulA is not lethal, then all the transformants will generate sectors that are 50:50 *ribo+:ribo-*. Four riboflavin independent diploids from the transformation of Bi9Arg5 with *phulA::riboB* were subjected to benlate induced haplodisation. Sectors from each of the strains were tested for riboflavin independence (Table 6.1).

Strain	Number of Strains	Ratio <i>riboB-</i> : <i>riboB+</i>
HulDip1	15	7:8
HulDip3	7	7:0
HulDip4	8	8:0
HulDip5	18	18:0

 Table 6.1 - Scoring for riboflavin independence.
 Haploid sectors from four diploids scored for riboflavin independence.

The ratio of riboflavin independence to dependence in haploid sectors of HulDip1 suggests integration at the *riboB* locus or that the deletion of *hulA* is not lethal. However,

the absence of riboflavin independent haploid sectors from the other three diploids demonstrates that the deletion of *hulA* is lethal and that the integration in HulDip1 must be at the *riboB* locus.

## 6.4 Confirmation of hulA Deletion

Southern hybridisation analysis was used to determine if the HulDip5 strain contained a deletion of *hulA*. The digestion of the genomic DNA from the diploids with Sall will result in the native *hulA* band of 9771bp and two *hulA* hybridising deleted bands of 6499bp and 3238bp if the transformed strain is the result of a homologues integration event at a single *hulA* locus. When their genomic DNA was digested with Sall, transferred to a nylon membrane and probed with a *hulA* probe, HulDip5 showed the pattern expected of a strain deleted for one *hulA* gene (Figure 6.2). This demonstrates that HulDip5 contains both a deleted copy of *hulA* and the endogenous sequence.



**Figure 6.2 - Southern hybridisation of genomic DNA testing for** *hulA::riboB.* Southern hybridisation of genomic DNA from strains 1) Bi9Arg5, 2) HulDip5 restricted with Sall and hybridised with a *hulA* probe. The purple arrow indicates native *hulA*, while the blue arrow indicates hybridisation to the *hulA::riboB* sequence in HulDip5.

## 6.5 Phenotypic Testing

The diploid strain containing the deletion of *hulA*, HulDip5, was compared to the parent diploid strain, Bi9Arg5, on a range of solid growth media. The phenotypes of *creD* or *acrB* mutants, such as resistance to acriflavine and poor use of pyrrolidinone, and the phenotypes of *creB* or *creC* mutants, such as poor use of proline and quinate, resistance to molybdate and loss of CCR, are recessive, that is the phenotypes are not present when

diploids contain a single faulty copy of these genes. As the deletion of *hulA* is lethal only haploinsufficiency phenotypes can be assessed. Relative to Bi9Arg5, HulDip5 demonstrates reproducible sensitivity to 10mM molybdate (Figure 6.3B) and to 7µg per ml acriflavine (Figure 6.3F). Strains containing *creD34* are resistant to acriflavine and are normally tested on 1% complete containing 10µg per ml of acriflavine. Neither Bi9Arg5 nor HulDip5 grew at this concentration, but when a range of concentrations were tested (6µg to 10µg per ml) it was found that at a concentration of acriflavine of 6µg per ml both strains grew. At a concentration of 7µg per ml growth of HulDip5 was much less than BiArg5 (Figure 6.3F) and at 8µg per ml HulDip5 did not grow.



**Figure 6.3** - **Solid media growth analysis for** *hulA* **diploid.** Colonies were grown on solid media for three days, at 37°C, unless otherwise indicated, on either A) 1% glucose plus 10 mM ammonium tartrate, B) 1% glucose plus 10 mM ammonium L-tartrate and 11mM molybdate, C) 50mM quinate plus 10 mM ammonium L-tartrate, D) 1% glucose plus 10mM 2-pyrrolidinone, E) 1% glucose plus 10mM proline, F) 1% complete plus 7µg per ml acriflavine or G) 1% glucose plus 10 mM ammonium L-tartrate and 10mM allylalcohol. Key to strains is in H).

#### 6.6 Discussion

The lethal effect of deleting *hulA*, the *A. nidulans* homologue of *S. cerevisiae RSP5*, is consistent with both the work in *S. cerevisiae* (43) and recent work in *A. nidulans* (40). Due to the lethality of *hulA* deletion, the aim of this work, to investigate any epistatic interactions between *hulA* mutations and mutations in other genes involved in CCR could not be achieved.

An unusual aspect of the HulDip5 phenotype is that this strain was sensitive to both acriflavine and molybdate, unlike CCR mutants. Haploid strains containing mutations in creB or creC are resistant to molydbate while being sensitive to acriflavine, while strains containing mutations in creD are sensitive to molydbate and resistant to acriflavine, but none of these phenotypes are dominant. The causes of these phenotypes have remained unstudied in A. nidulans, with the exception of early work conducted on molybdate resistance (7, 8). One of the identified resistance mutations, molB, was later shown to be an allele of *creB*. These authors propose two methods for resistance to molybdate, one affecting uptake of molybdate related to galactose use or phosphatase activity (7), and one related to detoxifying cells, where a nitrate reductase potentially sequesters molybdate (8). A recent study of acriflavine resistance in Trichophyton rubrum has identified 132 clones that were differentially expressed in response to acriflavine (95). Included in these clones are number of genes, with clear A. nidulans homologues, involved in metabolism, including genes encoding a short chain dehydrogenase (ANID 03234.1) and an alpha-L-rhamnosidase (ANID 11954.1) and a number of genes involved in DNA repair including a DEAD helicase (ANID 07014.1) and DNA polymerase gamma (ANID\_02094.1). It is possible that these metabolic genes are under the regulation of CCR, hence CreD mutation would result in resistance and CreB/CreC mutation result in sensitivity, but this fails to explain HulDip5 sensitivity to acriflavine.

Rsp5p has been shown to regulate permeases, including both uracil permease (35) and a general amino acid permease, Gap1p (125), act on a maltose transporter (78) and be involved in post-transcriptional activation in response to glucose (24) as well as numerous other cellular processes. Given the diverse action of Rsp5p, HulA would be expected to have a range of different targets and be important in a number of cellular processes. This could explain the phenotypes observed.

Due to the lethality of a *hulA* deletion, this work provides little new evidence for the involvement of HulA in CCR. The strain developed by Gournas *et al.* (40) containing a

copy of *hulA* that produces a protein with a deletion of aa 4 to 93, removing the C2 domain, called HulA $\Delta$ C2, would provide an opportunity for further study of the involvement of HulA in CCR. The HulA $\Delta$ C2 strain exhibited poor growth and conidiation unless the media was supplemented with sodium phosphate as well as poor growth rate and low fertility (40). Thus, to study whether HulA functions in an antagonistic fashion to the CreB/CreC complex, HulA $\Delta$ C2 could be crossed with strains containing mutations in genes involved in CCR. Another option would be to extend on experiments described by Kamlangdee, 2007 (62), where the ubiquination of CreA was examined. Investigating the ubiquination of CreA in HulA $\Delta$ C2 could provide insight into HulA activity on the primary carbon repressor.

An alternative strategy for the study of HulA involvement in CCR would be the development of a FLAG or GFP epitope-tagged HulA fusion protein. The development of a strain expressing the fusion could facilitate microscopic examination of the localisation of HulA in response to carbon growth conditions, or proteomic analysis to identify other proteins within a complex with HulA. A strain containing a tagged HulA could be crossed with strains containing tagged version of proteins that are candidates for interaction with HulA, and co-immunopreciptation experiments and western analysis could be used to confirm the presence in the same complex.

# **Chapter 7 - Discussion**

Historically microbial fungi were used as model organisms for fundamental research due to their rapid growth, sexual cycle, simple growth requirements and the ease of mutagenesis. Included in this pivotal fungal research was the initial discovery and characterisation of cell cycle regulators in S. cerevisiae (42) and initial characterisation of y-tubulin in A. nidulans (91). Since the establishment of A. nidulans and S. cerevisiae as model organisms a large range of tools have become available including extensive genetic maps, annotated genomic sequence databases and a series of robust molecular tools, which has strengthened their use as eukaryote models. However, one of the most interesting uses of fungal genetics is in the enhancement of industrial microbes. The industrial exploitation of filamentous fungi has been optimised via two complementary approaches: the optimisation of growth conditions of a particular strain, pioneered in fields like citric acid production in A. niger (22), and direct strain manipulation, such as mutagenesis approaches in T. reesei (104) or A. niger (36). However, these optimisation approaches share similar shortcomings, in that without a clear understanding of the underpinning biology they rely on numerous rounds of artificial selection and thus problems like the local optima phenomenon occur. Developments in fungal genetics can assist these optimisation approaches, either by a greater understanding of the biology of the fungi in relation to growth conditions or by direct targeted genetic manipulation rather than random mutagenesis.

The study of model fungi, such as *S. cerevisiae* and *A. nidulans*, has allowed the dissection of numerous conserved cellular processes. Included in this, with obvious industrial applications, is the understanding of sequential optimal use of available carbon sources or CCR. In both organisms CCR is directly regulated by a zinc finger DNA binding transcriptional repressor, CreA and Mig1p in *A. nidulans* and *S. cerevisiae* respectively. However, other proteins implicated in the regulation of CCR appear to differ greatly. In *S. cerevisiae* the phosphorylation state of the protein kinase Snf1p has been shown to directly regulate Mig1p and hence CCR (Figure 1.5) but mutation of the *A. nidulans SNF1*-like gene has no effects on CCR (M. Hynes, Personal Communication), and in *A. nidulans* it has been proposed that the ubiquitination state of CreA, or a protein in a complex with CreA, is a primary regulator of CCR (Figure 1.4). The model of CCR in *A. nidulans* involves CreA (63), CreB (76, 77), CreC (77, 137), CreD (11) and HulA (11), whereby the ability of

CreA to act as a repressor is regulated by an antagonistic ubiquitination of either CreA or a protein in complex with CreA (62), which does not affect the stability or localisation of CreA (107). The isolation of the *creD34* mutation and the subsequent characterisation of *creD* lead to the identification of a similar arrestin protein encoding gene, *apyA* (11). Both CreD and ApyA were shown to interact with the HECT type ubiquitin ligase HulA (11). These findings implicated both HulA and ApyA in CCR and made them ideal targets to study for the further dissection of CCR. The process for analyses used for the study of both *apyA* and *hulA* were gene deletions.

The Nedd4 family of HECT type ubiquitin ligases is named for the mammalian Nedd4, identified as being differentially expressed in the central nervous system in mouse (70, 71). Like Rsp5p, Nedd4 has been implicated in numerous cellular processes including cell proliferation, endocytosis and protein trafficking (Reviewed in (106)). Moreover, Nedd4 and other mammalian members of the Nedd4 family have been implicated in various pathologies, including cancer, altered auto-immune response and Liddle syndrome (106). However, mice homozygous for the *Nedd4* deletion are viable but neonatal lethal, possibly due to incorrect localisation of insulin growth factor receptors (IGF1-R) (14). This neonatal lethality was rescued by the knockdown of Grb10, which restores IGF-1R membrane localisation (14).

Deletion of *hulA*, encoding a NEDD4 family HECT type ubiquitin ligase, was found to be lethal in a haploid strain but a diploid strain heterozygous for a *hulA* deletion was viable (Section 6.3). This finding was consistent with observations of *RSP5* in *S. cerevisiae* (43) and Nedd4 in mouse (14). Given that Rsp5p and Nedd4 have been implicated in so many cellular functions, and that the deletion of *creA* in *A. nidulans* is not lethal, the lethality due to *hulA* deletion is most likely not related to any affect on CCR. An approach to studying the cause of the *hulA* deletion lethality in *A. nidulans* would be to identify suppressors of this phenotype. This could be achieved via a mutation screen, whereby HulDip5 would be exposed to a mutagen and haploidised on media supplemented with benlate but lacking riboflavin. Haploidisation only produces riboflavin dependant sectors due to the lethality of the *hulA* disruption and hence any riboflavin independent sectors generated would either contain a suppressor of the lethality of the *hulA* deletion or restore *riboB* function. To pursue the involvement of HulA in CCR alternative approaches need to be used including epitope fusion and the use of the HulAΔC2 strain (Section 6.6, (40)). An advantage of dissecting this system in *A. nidulans* rather than *S. cerevisiae* is that *A. nidulans* is a multi-cellular organism, and thus it potentially provides a better model for the study of NEDD4 type ubiquitin ligases because both cell autonomous and cell-cell interaction effects can be uncovered.

Arrestin proteins have been extensively studied in metazoans as they have been shown to interact with G protein-coupled receptors (GPCRs). GPCRs number at least 800 in the *H. sapian* genome, and have near ubiquitous prevalence in biological systems (72). They have also been suggested as drug targets in a range of diseases (72). The interaction between arrestin-like proteins and GPCRs has generated interest in the study and targeting of arrestins for potential therapeutic applications (Reviewed in (102)).

In A. nidulans an arrestin-like protein PalF associates with the GPCR, PalH, to mediate cellular response to environmental pH, with the PalF arrestin N domain interacting with two regions of the PalH cytoplasmic tail (45). The ubiquitination of PalF has been shown to be a critical step in the signalling of ambient alkaline pH to downstream elements of the pathway (46). In S. cerevisiae, Bul1p and Bul2p are arrestinlike proteins that have been shown to interact with Rsp5p (152, 153). BUL1 mutations have a temperature sensitive phenotype that is complemented by both BUL1 and BUL2 (152). These proteins share 51% identity and strains lacking both have a wide range of phenotypes, such as salt sensitivity and inability to use glycerol, that are not present in either the BUL1 or BUL2 single mutant strains (152). Interestingly BUL2 mutants are not temperature sensitive (152). These finding suggest significant redundancy between Bul1p and Bul2p but not a total overlap of function. Bul1p and Bul2p, like Rod1p and Rog3p and CreD and ApyA are arrestin-like proteins, the divergence of which has been discussed in Section 3.3. The presence of BUL1 and BUL2 within the S. cerevisiae genome is consistent with the hypothesis that the duplication of arrestin-like proteins, which interact with an Ub ligase, allows for rapid evolution of unique targeting systems and hence the development of new regulatory systems use existing cellular machinery. Improving the resolution of available genomes, that is having numerous sequenced genomes of members from numerous closely related geneses, would allow a detailed assessment of the evolution of arrestin-like proteins. The divergent nature of these protein sequences could potentially be due to positive selection for the evolution of beneficial function subsequent to a gene duplication event. Analysis of the genomic sequences of closely related arrestin-like proteins from these genomes would allow the comparison of missense versus silent mutations, which can detect the presence of positive selection on a

sequence. This genome resolution and the nature of arrestin-like proteins could potentially be used as a model for gene duplication and, when coupled with analysis of function, evolution of novel gene function.

The *in silico* based analysis was further supported by phenotypic analysis of *apyA* disruption (Chapter 5). As with *BUL1* and *BUL2*, the mutation of either *apyA* or *creD* did not show overlapping phenotypes but the double mutant strain, containing *apyA::AfriboB* and *creD34*, exhibited morphological effects and phenotypes of resistance to acriflavine and molybdate (Section 5.4.4). As proposed in Section 5.5, to investigate the functions of CreD and ApyA further, the homologue of *creD* could be deleted in Aspergilli that lack additional CreD-like sequences such as *A. terreus* or *A. fumigatus*. This would provide insight into the function of the highly conserved, amongst fungi, arrestin-like protein CreD and potentially explore conserved function between recently diverged proteins.

The exploitation of microorganisms provides great potential to meet the requirements caused by the rapid depletion of petroleum based fuels, either directly, via the production of product that can be utilised as a fuel, or indirectly, via the production of heterologous proteins to catalyse specific reactions. The manipulation of CCR in members of Ascomycota has been demonstrated to have industrial potential (67). As the understanding of CCR regulation in both *A. nidulans* and *S. cerevisiae* grow, and it becomes apparent that other than the utilisation of a zinc finger transcription repressor these systems have little in common, the questions arise: Is there a typical system of CCR regulation? Have different systems arisen in all fungal lineages? If not is there a fungus with a typical system that can be used as a model of CCR regulation for the entire fungal kingdom? This last question is of particular industrial microorganisms, they are lacking extensive molecular tools and knowledge. Thus realising the potential of many industrial microorganisms will be arduous without the ability to exploit the body of knowledge from a model organism like *A. nidulans* or *S. cerevisiae*.

Members of the *Trichoderma* genus, such as *T. harzianum* and *T. reesei*, have been exploited for their abundant cellulase production and extensive steps have been taken to improve enzyme production within these species. The release and annotation of the *T. reesei* genome allowed the identification of clear homologues of the *A. nidulans* CCR regulators, CreB (Cre2) and CreC (Cre3) (Chapter 4 and Section 3.1.2.1). The *T. reesei cre2* sequence was capable of complementing the *creB1937* mutation in *A. nidulans* (Chapter 4

Figure 2), demonstrating the conservation of this system at a sequence level. While sequence conservation does not demonstrate functional conservation, this does suggest that the CCR system in *A. nidulans* of a zinc finger protein being mediated via an Ub system might be conserved across Ascomycota. The conservation of CCR and validity of *A. nidulans* as a model for the fungal kingdom could be investigated via the complementation of *creB1937* with the introduction of *creB* homologues from multiple members of the fungal kingdom, either under their endogenous promoter or the *A. nidulans creB* promoter.

While the study of a complex regulatory system across an entire kingdom is of evolutionary significance, the main aim of this study is manipulation of CCR for industrial exploitation. The deregulation of CCR, via cre1 mutation, in T. reesei has been shown to improve cellulase production (87). However, as with A. nidulans there were morphological effects of *cre1* mutations, including slower growth in liquid culture (87). This study demonstrated that a disruption of *cre2* grew quicker than the *cre1* mutant but slower than QM 6a (Chapter 4 Table 1). In addition, the cre2 disruption strain generally performed better than QM 6a and the cre1 disruption in both raw cellulase activity (Chapter 4 Supplementary Table 1) and cellulase activity adjusted for biomass (Chapter 4 Figure 4 & 5). As well as providing support for the early divergence of the A. nidulans CCR regulatory system within Ascomycota, these observations support the idea of creB mutation for strain development. A benefit of targeting *creB* or more generally CCR for manipulation programs is that it provides a way to deregulate the whole pathway without specific knowledge of the inner workings of the pathway. Postia placenta, a brown rot fungus belonging to Basidiomycota, has been proposed to degrade cellulose via depolymerising of the cellulose structure. When grown on cellulose P. placenta up regulates iron reductases, quinone reductase and a range of oxidases, as well as numerous hemicellulases and a single endogluconase (84). As the degradation of cellulose is complex involving multiple protein families and not just the traditional two classes of cellulases, the over-expression of just the hemi-cellulases and the endoglucanase would be ineffective at cellulose degradation. This need to deregulate multiple related pathways is why the disruption of CCR would provide an opportunity for large improvements. A homologue of the global CCR regulator, CreB, was identified in P. placenta (Section 3.1.2 and Appendix A) and hence would provide a starting point for strain improvement in this potential industrial organism.

What has been suggested above is that the *A. nidulans* CCR regulatory method is conserved amongst Ascomycota. By studying *creB* homologues in other and diverse members of the fungal kingdom, such as *P. placenta*, it would be possible to determine if the *A. nidulans* CCR system is as wide as suggested by bioinformatic work in Chapter 3. *S. cerevisiae* is often considered atypical, however analysis of proteins implicated in *A. nidulans* CCR suggest that the entire Saccharomyetces family is atypical not only to Ascomycota but to the entire fungal kingdom (Figure 3.2 & 3.3), a finding not predicted by more general sequence analysis (58). It is possible that CCR regulation, similar to what is seen in *A. nidulans*, evolved early in fungal speciation and has remained largely unchanged except in Saccharomycetes. It is possible an event after speciation branching caused a relaxation of selection pressure on these sequences, which in turn caused their loss or significant change.

# **Appendix A**

Table A - Genomic Sequence IDs used in Chapter 3.

#### Figure 3.2

Organism	Genome or Protein ID
Acremonium chrysogenum	CAB77624.1
Aspergillus fumigatus	Afu2g11780
Aspergillus nidulans	ANID_06195.1
Aspergillus oryzae	AO090026000464
Botrytis cinerea	CAA76329.1
Candida albicans	orf19.5326
Candida tropicalis	CTRG_01933.3
Chaetomium globosum	CHGG_03907.1
Cochliobolus heterostrophus	112709
Cochliobolus heterostrophus	112709
Coprinus cinerea	CC1G_12253.2
Gibberella fujikuroi	CAA76330.1
Magnaporthe grisea	MGG_11201.6
Microsporum gypseum	MGYG_07563.1
Mycosphaerella fijiensis	57885
Neurospora crassa	NCU08807.4
Saccharomyces cerevisiae	YGL035C
Schizosaccharomyces japonicus	SJAG_03473.2
Schizosaccharomyces octosporus	SOCG_03212.3
Schizosaccharomyces pombe	SPBC1D7.02c
Sclerotinia sclerotiorum	SS1G_09934.1
Sporotrichum thermophile	109919
Thermoascus aurantiacus	AAT34979.1
Trichoderma reesei	AAB01677.1
Trichophyton tonsurans	TESG_03982.1
Tuber melanosporum	GSTUMT00002587001

### Figure 3.3

Coccidioides posadasii

Coprinus cinereus

Cochliobolus heterostrophus

#### Organism

Organism	Genome or Protein ID or Genome Position
Aspergillus fumigatus	Afu4g12910
Aspergillus nidulans	ANID_03587.1
Aspergillus niger	fge1_pm_C_10269
Aspergillus oryzae	Supercontig 1: 848292-850274
Batrachochytrium dendrobatidis	BDEG_07348.1
Botrytis cinerea	BC1G_07236.1
Candida albicans	orf19.2026
Candida tropicalis	CTRG_01163.3
Chaetomium globosum	CHGG_01824.1
Coccidioides immitis	CIHG_07314.1

CPSG\_01616.1

CC1G\_00541.2

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Cryptococcus neoformans	CNAG_02395
Homo sapian	NCBI AAC23551.1
Laccaria bicolor	301336
Magnaporthe grisea	MGG_09907.6
Malassezia globosa	NCBI EDP43336
Melampsora laricis-populina	25890
Microsporum gypseum	MGYG_01656.1
Neurospora crassa	NCU08378.4
Phycomyces blakesleeanus	60032
Pichia stipitis	10538
Postia placenta	63810
Rhizopus oryzae	RO3G_12395.3
Saccharomyces cerevisiae	YER098W
Schizosaccharomyces japonicus	SJAG_00546.2
Schizosaccharomyces octosporus	SOCG_00293.3
Schizosaccharomyces pombe	SPBC1703.12
Sporobolomyces roseus	10343
Sporotrichum thermophile	43420
Trichoderma reesei	122405
Trichoderma virens	35377
Trichophyton tonsurans	TESG_07573.1
Ustilago maydis	UM00376.1

## Figure 3.4

# Organism

# **Genome or Protein ID**

-
Aspergillud terreus 1
Aspergillus clavatus 1
Aspergillus clavatus 2
Aspergillus flavus 1
Aspergillus flavus 2
Aspergillus fumigatus 1
Aspergillus nidulans ApyA
Aspergillus nidulans CreD
Aspergillus niger 1
Aspergillus niger 2
Aspergillus oryzae 1
Aspergillus oryzae 2
Aspergillus oryzae 3
Neosartorya fischeri 1
Saccharomyces cerevisiae

ATEG\_05869.1 ACLA\_082820 ACLA\_044550 AFL2G\_02816.2 AFL2G\_02962.2 Afu6g07900 ANID\_03265.1 ANID\_04170.1 fge1\_pg\_C\_40217 fge1\_pg\_C\_11000239 AO090003000144 AO090023000029 AO090012000020 NFIA\_053600 YOR018W

### Figure 3.5

Organism Aspergillus nidulans CreD Candida albicans 1 Candida albicans 2 Candida glabrata 1 Candida glabrata 2 Candida tropicalis 1 Candida tropicalis 2 Kluyveromyces waltii 1 Kluyveromyces waltii 2 Pichia stipitis 1

Saccharomyces bayanus 1 Saccharomyces bayanus 2 Saccharomyces castellii 1 Saccharomyces castellii 2 Saccharomyces cerevisiae Saccharomyces cerevisiae Saccharomyces kluyveri 1 Saccharomyces kluyveri 2 Saccharomyces mikatae 1 Saccharomyces mikatae 2 Saccharomyces paradoxus 1 Saccharomyces paradoxus 2 **Genome or Protein ID or Genome Position** ANID 04170.1 orf19.3041 orf19.1509 XP 444854 XP\_448682 CTRG 04804.3 CTRG 01434.3 AADM01000362.1 Interval: 2,496 -> 4,811 AADM01000111.1 Interval: 65,318 -> 63,339 89396 AACG02000052.1 Contig02.606 Interval: 22140 -> 27294 AACG02000107.1 Contig02.577 Interval: 5652 -> 10785 AACF01000100.1 Interval: 18,153 -> 20,360 AACF01000039.1 Interval: 63,898 -> 61,631 YOR018W YFR022W AACE03000004.1 Interval: 792980 -> 798899 AACE03000006.2 Interval: 374936 -> 379877 AACH01000426 Interval: 5,105 -> 2,592 AACH01000177 Interval: 5,204 -> 7,402 AABY01000041.1 Interval: 57500 -> 61419 AABY01000021.1 Interval: 58379 -> 62328

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