

From Model Organism to Industrial Workhorse:

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

By

Jai Andrew Denton

B.Sc. (Honours)



A thesis submitted to the University of Adelaide in total fulfilment of the requirements for admission to the degree of Doctor of Philosophy.

Genetics Discipline
School of Molecular and Biomedical Sciences
Faculty of Science
The University of Adelaide

June 2010

Table of Contents

List of Figures	v
List of Tables	vi
Abstract.....	vii
Declaration.....	ix
Acknowledgements	x
Abbreviations	xi
Chapter 1- Introduction	1
1.1 Alternative fuel	1
1.1.1 Ethanol as a fuel.....	2
1.1.2 Cellulose and cellulose saccharification	3
1.1.3 Cellulosic ethanol production	3
1.2 <i>Trichoderma reesei</i> as an industrial organism	4
1.3 <i>Aspergillus nidulans</i> as a model organism.....	5
1.4 Carbon catabolite repression.....	6
1.4.1 Carbon catabolite repression in <i>A. nidulans</i>	6
1.4.4 Carbon catabolite repression and cellulase regulation in <i>T. reesei</i>	11
1.4.5 Carbon catabolite repression in other filamentous fungi	12
1.4.6 Carbon catabolite repression in <i>Saccharomyces cerevisiae</i>	13
1.4.7 Carbon catabolite repression in bacteria	15
1.5 Regulation by ubiquitination	15
1.6 <i>ROD1</i> and <i>ROG3</i>	17
1.7 <i>RSP5</i>	18
1.8 Aims of this project.....	18
Chapter 2 - Materials & Methods.....	20
2.1 Materials	20
2.1.1 DNA modifying enzymes.....	20
2.1.2 DNA Molecular weight markers.....	20
2.1.3 Fungal strains	20
2.1.4 Oligonucleotides	21
2.1.5 Vectors	21
2.1.6 Kits and miscellaneous materials.....	22

2.1.7 Solutions and buffers	22
2.1.8 Media	23
2.2 Methods.....	23
2.2.1 Nucleic acid isolation	23
2.2.2 Transformation	23
2.2.3 Meiotic crossing.....	24
2.2.4 Diploid formation in <i>A. nidulans</i>	24
2.2.5 Haploid formation in <i>A. nidulans</i>	24
2.2.6 Polymerase chain reaction	24
2.2.7 Enzyme assays.....	25
2.2.8 Southern blot analysis	25
2.2.9 Bioinformatic analysis.....	25
Chapter 3 - <i>In silico</i> Identification of Genes Involved in Carbon Catabolite Repression..	26
3.1 Introduction	26
3.2 CreA.....	27
3.3 CreB and CreC	30
3.3.1 CreB and CreC in <i>Trichoderma reesei</i>	31
3.3.2 CreB and CreC in Saccharomycetes	32
3.4 CreD and ApyA in Ascomycota	32
3.5 Discussion	36
Chapter 4 -Characterisation of <i>cre2</i> in <i>T. reesei</i> for Strain Improvement	38
Introduction	40
Materials and Methods	42
Results.....	44
Discussion	49
Acknowledgments	52
Chapter 5 - Analysis of <i>apyA</i>	61
5.1 Introduction	61
5.2 Creation of a disruption construct and transformation	61
5.3 Phenotypic analysis.....	62
5.4 Epistatic interactions	63
5.4.1 <i>creA</i>	63
5.4.2 <i>creB</i> and <i>creC</i>	64

5.4.3 Identification of a <i>creB</i> suppressor	64
5.4.4 <i>creD</i> and <i>acrB</i>	65
5.5 Discussion	67
Chapter 6 - Deletion of <i>Aspergillus nidulans hula</i>	69
6.1 Introduction	69
6.2 Deletion of <i>hula</i>	69
6.2.1 Deletion Construct	69
6.2.2 Transformation of <i>A. nidulans</i> and <i>A. nidulans nkuA::argB</i>	69
6.2.3 Development and Transformation of an <i>A. nidulans nkuA</i> Diploid.....	70
6.3 Haplodisation of Riboflavin Independent Diploids.....	71
6.4 Confirmation of <i>hula</i> Deletion.....	72
6.5 Phenotypic Testing.....	72
6.6 Discussion	74
Chapter 7 - Discussion.....	76
Appendix A	82
References	85

List of Figures

Chapter 1

Figure 1.1 - Current approaches to alternative fuel.	2
Figure 1.2 - Representation of strain development programs prior to 1992.	5
Figure 1.3 - Protein conservation between CreD, ApyA, Rod1p and Rog3p.	10
Figure 1.4 - Model of CCR in <i>Aspergillus nidulans</i> .	10
Figure 1.5 - Model of CCR in <i>S. cerevisiae</i> .	14
Figure 1.6 - Model of Ubiquitination.	17

Chapter 3

Figure 3.1 - Diagrammatic representation of the evolutionary relationship between fungi.	28
Figure 3.2 – Protein relatedness between CreA homologues within Ascomycota.	29
Figure 3.3 - Protein relatedness between CreB homologues.	30
Figure 3.4 - Protein relatedness of CreD-like sequences amongst the Aspergilli.	34
Figure 3.5 - Protein relatedness of Rod1p and Rog3p like sequences within Saccromycetes.	35

Chapter 4

Figure 1 - Protein sequence alignment of <i>A. nidulans</i> CreB and the putative <i>T. reesei</i> orthologue.	53
Figure 2 - Complementation of <i>A. nidulans creB1937</i> by <i>T. reesei cre2</i> .	54
Figure 3 - Phenotype of <i>cre2</i> disruption strain on solid media.	55
Figure 4 - Total secreted cellulase activity of three <i>T. reesei</i> strains.	56
Figure 5 - Total secreted cellulase per gram per litre dry mycelial weight and glucose concentration.	57
Supplementary Figure 1 – Southern hybridisation of genomic DNA testing for <i>cre2::amdS</i>	60

Chapter 5

Figure 5.1 - Southern hybridisation of <i>apyA</i> disruption transformants.	62
Figure 5.2 - Solid media growth analysis of <i>apyA::AfriboB</i> epistatic interactions.	66

Chapter 6

Figure 6.1 - Southern hybridisation of genomic DNA testing for <i>nkuA::argB</i> .	71
Figure 6.2 - Southern hybridisation of genomic DNA Testing for <i>hulA::riboB</i> .	72
Figure 6.3 - Solid media growth analysis for <i>hulA</i> diploid.	73

List of Tables

Chapter 2

Table 2.1 - DNA modifying enzymes.	20
Table 2.2 - <i>A. nidulans</i> strains used in this study.	20
Table 2.3 - <i>T. reesei</i> strains used in this study.	21
Table 2.4 - Oligonucleotide primers.	21
Table 2.5 - Vectors used in this study.	22
Table 2.6 - Kits and miscellaneous materials used in this study.	22

Chapter 4

Table 1 - Total Dry Biomass in Various Carbon Sources.	58
Supplementary Table 1 - Total Secreted Cellulase & Xylanase Activity.	59

Chapter 6

Table 6.1 - Scoring for riboflavin independence.	71
--	----

Appendix A

Table A - Genomic sequence IDs used in Chapter 3.	82
---	----

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 identified 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. reesei* 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.

I give consent to this copy of my thesis when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

I also give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library catalogue, the Australasian Digital Theses Program and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

The author acknowledges that copyright of published works contained within this thesis (see below) resides with the copyright holder of those works.

Jai A. Denton

22nd 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)

Acknowledgements

I would like to acknowledge the support, guidance and patience of my supervisor Dr Joan Kelly. I hope that my odd ways and tendency to wander into her office and complain about the world every time I was bored didn't try this patience too often. I have enjoyed the time in her lab greatly and I hope we always stay in touch. I would also like to thank Dr Robin Lockington for his insight into scientific matters and for conversations about the world. I suspect anyone listening would (and sometimes did) suggest that we were a tad too cynical.

I would like to thank my fellow Kelly lab PhD students, Adrian and Vivan, for their support (and the food from Vivian). I would also like to thank the other current member of the Kelly lab, Denise, and members throughout my time here, Tash v1, Damien, Preeti, Niyom, Ainslie, Jianliang, Tash v2 and Hajer.

I would like to thank all of my family and friends for putting up with me over the duration of my PhD, in particular my mum, dad and sister. I would also like to thank Pia for putting up with my "I'll be finished soon" for so long.

The entire Genetics discipline has been amazing. The friendships and support I've gained here have made the experience all the more worthwhile.

I would like to thank Sheridan, Joan, Chris, Michelle and Jamie for providing me with assorted forms of employment. I would like to thank Joan, Robin and Adrian for critically reading this thesis.

Abbreviations

General Abbreviations

ATP	adenosine triphosphate
ATCC	American Tissue Culture Collection
aa	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

A	adenine	S	guanine or cytosine
G	guanine	W	adenine or thymidine
T	thymidine	B	not adenine
C	cytosine	D	not cytosine
R	adenine or guanine	H	not guanine
Y	cytosine or thymidine	V	not thymidine
M	adenine or cytosine	N	any nucleotide
K	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 threonine 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.