Disruption of carbon catabolite repression and investigation of amylase gene duplication in the filamentous fungus *Aspergillus oryzae*

by

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Abstract

Through many centuries of domestication, *Aspergillus flavus*, a dangerous human pathogen and producer of carcinogenic aflatoxin, has given rise to *Aspergillus oryzae*, a benign fungus widely cultivated in the Orient to produce traditional fermented foods and beverages. Today *A. oryzae* is also a source of amylases and other hydrolytic enzymes used in industry, and has been proposed to be used in an environmentally friendly treatment process that would convert the organic material in winery wastewater into protein-rich fungal biomass. Such biomass could be sold as animal feed, offsetting treatment costs and reclaiming nutrients currently being lost as waste.

To enhance the efficiency of this process, carbon catabolite repression (CCR) was disrupted in *A. oryzae*. CCR is the repression of genes encoding enzymes for the utilisation of non-preferred carbon sources in the presence of a preferred carbon source such as glucose, which is abundant in winery wastewater. To disrupt CCR, the gene *creB*, encoding a deubiquitinating enzyme, was deleted in two strains of *A. oryzae*. In *A. oryzae* RIB40, *creB* deletion increased the production of secreted cellulases, xylanases, and amylases in inducing conditions, and greatly increased the production of secreted amylases in non-inducing and repressing conditions. Repression of amylases by glucose was much weaker in the *creB*-deleted strain, indicating CCR was disrupted.

In contrast, deletion of *creB* in *A. oryzae* DAR3699, a strain used in soy fermentation, had no discernible effect on CCR. *A. oryzae* DAR3699 was shown to have weak CCR and other phenotypes characteristic of *creB* mutants. It was found to have a single base pair insertion in a putative micro-open reading frame in the promoter of *creB*, predicted to greatly reduce the efficiency of translation. Thus *A. oryzae* DAR3699 already possessed a loss-of-function mutation in *creB*.

To investigate whether *creB* deletion would be useful for winery wastewater treatment, *creB*-deleted *A. oryzae* RIB40 and its parent were grown on synthetic winery wastewater in a bench-scale bioreactor. The two strains were found to perform similarly, with indistinguishable morphology and patterns of carbon source consumption. Although no advantage of the *creB*-deleted strain was observed for this application, its robust growth and increased enzyme secretion suggest it may be useful in other industrial processes.

Whereas *A. flavus* has only one copy of the gene for alpha-amylase, various *A. oryzae* strains are known to have additional copies, which have presumably arisen by gene duplication during domestication. *A. oryzae* DAR3699 was observed to have lower amylase secretion than other strains, suggesting it might lack the additional copies. Investigation revealed that it does in fact contain a second alpha-amylase gene, but that this copy has arisen from a duplication event independent of those that produced the second and third copies in *A. oryae* RIB40. The duplications in the latter strain appear to have been mediated by complex transposition events involving a 9.1 kb transposable element of the Tc1/mariner class.

Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name in any university or other tertiary institution 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. In addition, I certify that no part of this work will, in the future, be used in a submission in my name for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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Adrian Hunter

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Contextual statement

This work is based on, and in part funded by, an ARC linkage project grant titled, "Fungal Biomass Protein: A Bioproduct Derived from a Treatment Process of Winery Waste Streams" (Project ID LP0562153), with industry partner SA Water Corporation. The proposed treatment process uses a filamentous fungus, *Aspergillus oryzae*, to consume organic material from winery wastewater within a bioreactor. Depleted of organic material, the treated wastewater would be less polluting and should be suitable for re-use for irrigation. An advantage of the treatment process would be the generation of protein-rich fungal biomass, which could be sold as nutritious animal feed. This would offset operating costs and make the process economically viable, as well as reclaiming value from a waste product, thus making the process environmentally friendly. The process was originally developed for treating starch-rich liquid wastes from potato chip manufacturing, and the strain *A. oryzae* DAR3699 had been identified as especially suitable for this purpose.

The project suffered a setback when the post-doctoral fellow who was to oversee the engineering aspects of adapting the process to winery wastewater was unable to join the project. Nevertheless, I continued with my planned role in the project, which was to disrupt carbon catabolite repression in *A. oryzae* DAR3699 by deleting the *creB* gene and investigating whether this improved the performance of the strain. I observed early in my candidature that carbon catabolite repression in *A. oryzae* DAR3699 seemed weaker than in the model fungus *Aspergillus nidulans,* and later observed that it was also weaker than in *A. oryzae* strain RIB40, a widely used strain that was the source of DNA for the genome sequencing project. Deleting *creB* in *A. oryzae* DAR3699 ultimately had only a very subtle effect on phenotype, with no discernible effect on carbon catabolite repression. I determined this to be because *A. oryzae* DAR3699 already possessed a loss-of-function mutation in the promoter of the *creB* gene. I also deleted *creB* in *A. oryzae* RIB40, and the phenotype was as expected, with reduced carbon catabolite repression and increased secretion of biomass-degrading enzymes. The production and characterisation of the *creB*Δ strains are described in the publication presented in this thesis as Chapter 2, "Deletion of *creB* in *Aspergillus oryzae* increases secreted hydrolytic enzyme activity".

Although *creB* deletion had the expected effect in *A. oryzae* RIB40 in terms of carbon metabolism and enzyme secretion, it remained to be seen whether the *creB*Δ phenotype was advantageous in wastewater treatment. As *creB* deletion had so little effect in *A. oryzae* DAR3699, I focused on comparing *A. oryzae* RIB40 *creB*Δ and its parent. The two strains were compared in a bench-scale bioreactor. As real winery wastewater is highly variable, and as the treatment process had not been optimised for real winery wastewater due to the absence of the post-doctoral fellow, I used synthetic winery wastewater as the growth medium. I observed no advantage in carbon content reduction for the *creB*Δ strain. However, this strain retained the robust growth and desirable pellet morphology of its parent, suggesting that *creB* mutation might be useful in combination with other mutations, or in different industrial processes in which the altered metabolism and superior hydrolytic enzyme secretion may be advantageous. This investigation is described in the manuscript presented in this thesis as Chapter 3, "Behavior of an *Aspergillus oryzae* strain with disrupted carbon catabolite repression in a mixed carbon source fermentation".

As with many strains used in industry, transforming *A. oryzae* DAR3699 proved difficult and time consuming, with results from each attempted transformation taking approximately one week to become apparent and many transformation attempts being necessary. This afforded time to investigate this strain more broadly. My sequencing of four genes in *A. oryzae* DAR3699 had revealed it to be extremely similar at the DNA level to *A. oryzae* RIB40. Yet *A. oryzae* DAR3699 differs strikingly from *A. oryzae* RIB40, both in morphology and in carbon metabolism; in particular, it secretes markedly lower levels of amylases. *A. oryzae* is used as a source of amylases in industry, and four strains were known to have multiple highly similar copies of the gene encoding the most abundant secreted amylase, α-amylase, which had apparently arisen from a single ancestral gene since the domestication of *A. oryzae*. It was therefore speculated that *A. oryzae* DAR3699 might not possess these additional α-amylase genes, representing the ancestral state of *A. oryzae*. Experiments revealed that *A. oryzae* DAR3699 did, in fact, contain a second copy of α-amylase, but that this copy originated independently of the additional copies in *A. oryzae* RIB40. This work culminated in the publication presented in this thesis as Chapter 4, "Independent duplications of α-amylase in different strains of *Aspergillus oryzae*".

Paper or manuscript	Contributions of principle author (candidate)	Overall contribution
Chapter 2: Adrian J. Hunter, Bo Jin, Joan M. Kelly (2011) Deletion of *creB* in *Aspergillus oryzae* Increases Secreted Hydrolytic Enzyme Activity. *Applied and Environmental Microbiology* 48:438–444	Generated both *creB* deletion strains; complemented *creB* in a *creB*Δ *niaD*$^{-}$ strain; designed, performed and interpreted all experiments with *A. oryzae* DAR3699 and all experiments with results shown in Tables 3 and 4 and Figures 1, 3, 4, 5A, and 6; co-wrote the manuscript (50% of the words).	60%
Chapter 3: Adrian J. Hunter, Pia Tille, Bo Jin, Joan M. Kelly, Christopher P. Saint. Behavior of an *Aspergillus oryzae* Strain with Disrupted Carbon Catabolite Repression in a Mixed Carbon Source Fermentation. *Unpublished manuscript*	Planned all experiments, performed bioreactor experiments presented in paper, interpreted data and wrote the manuscript.	70%
Chapter 4: A. J. Hunter, T. A. Morris, B. Jin, C. P. Saint, J. M. Kelly (2013) Independent Duplications of α-Amylase in Different Strains of *Aspergillus oryzae*. *Fungal Genetics and Biology* 79(18):5480–5487	Planned and performed all experiments, interpreted data and wrote the manuscript.	90%

1 Introduction

1.1 Food and beverage industry waste streams

1.1.1 Winery waste streams

Typical wineries produce 3 to 5 kL of wastewater for every tonne of grapes crushed (MOSSE *et al.* 2011). In the past, wastewater has been discharged directly into rivers, causing considerable pollution. The organic material in the wastewater causes depletion of oxygen from the water as it decays, leading to the death of aquatic organisms. Nitrogen and other nutrients in the wastewater promote algal blooms. Algae block sunlight, retarding the growth of aquatic flora, and their metabolism exacerbates oxygen depletion. Salts in the wastewater can also be toxic to aquatic organisms, and suspended solids can both block sunlight and smother habitats (stated in text CHAPMAN *et al.* 2001; SOUTH AUSTRALIAN EPA 2004; IOANNOU *et al.* 2015).

Associated with growing public awareness of pollution problems, environmental legislation regarding wastewater disposal has become stricter, and wineries must now treat their waste streams. Many wineries use wastewater to irrigate their grapevines and/or tree plantations established specifically for wastewater disposal. However, untreated winery wastewater can have many adverse effects on soil. Prolonged low levels of oxygen resulting from microbial decomposition of organic matter can kill plant roots. Suspended solids in the wastewater can reduce soil porosity, reducing oxygen transfer and thus further reducing oxygen levels. Extremes of pH can affect the solubility and toxicity of heavy metals present in the soil, as well as being toxic in their own right. Salts can reduce water uptake by plants. The high levels of sodium relative to divalent cations present in winery wastewater disrupt the structure of clay soils, reducing transfer of air and water. In addition, organic nitrogen from wastewater that leaches into groundwater supplies can be converted to nitrate, which is toxic and renders groundwater unfit for human consumption (stated in text CHAPMAN *et al.* 2001; SOUTH AUSTRALIAN EPA 2004). In contrast, treated winery wastewater does not appear to be harmful to soils (MOSSE *et al.* 2012).

The sources of wastewater from a typical winery have been described (CHAPMAN 1995; CHAPMAN *et al.* 2001). The sources include:

Alkali washwater Grape juice and wine are rich in potassium-hydrogen bitartrate. This adheres to equipment during storage and processing, and is deliberately precipitated during wine stabilisation, forming a solid deposit that also contains pigments, tannins and proteins. This precipitate is dissolved using caustic cleaning agents, such as sodium hydroxide, potassium hydroxide, sodium metasilicate or sodium carbonate. Sometimes these cleaning agents contain added enzymes.

Rinsewater Since high pH has adverse effects on wine quality, alkali washing is followed by washing with solutions of 2% to 5% citric or tartaric acid. Clean water is then used to remove traces of the acid, as well as for hosing the outsides of equipment and cellar floors.

Earth filtration Juice and wine may be filtered using diatomaceous earth. Rotating drum filters have their diatomaceous earth repacked using a slurry of diatomaceous earth in water. This process may require over 20 kL of water, and may need to be performed twice daily during vintage.

Cooling tower bleed Evaporative cooling towers are used to keep juice and wine cool, to prevent spoilage. A fraction of the cooling tower water must be continuously bled off to prevent salt accumulation and consequent corrosion.

Ion exchange Many wineries use an ion exchange column to acidify and/or stabilise wine, by replacing potassium and other ions with hydrogen or sodium. The column is regenerated using a mineral acid, usually sulphuric acid, or sodium chloride.

Figure 1 shows the relative contributions of these sources to the volume of wastewater produced by a large non-distilling Australian winery. Although alkali washing and rinsewater are the major contributors to the volume of wastewater produced, other components can have important effects on the quality of the wastewater. Water from earth filtration can be a major source of suspended solids; cooling tower bleed can be a major source of salts. Ion exchange column regeneration produces small volumes of wastewater which is rich in potassium. Depending on the type of column used, it is also highly acidic (pH <2) and rich in sulphate, or rich in chloride.

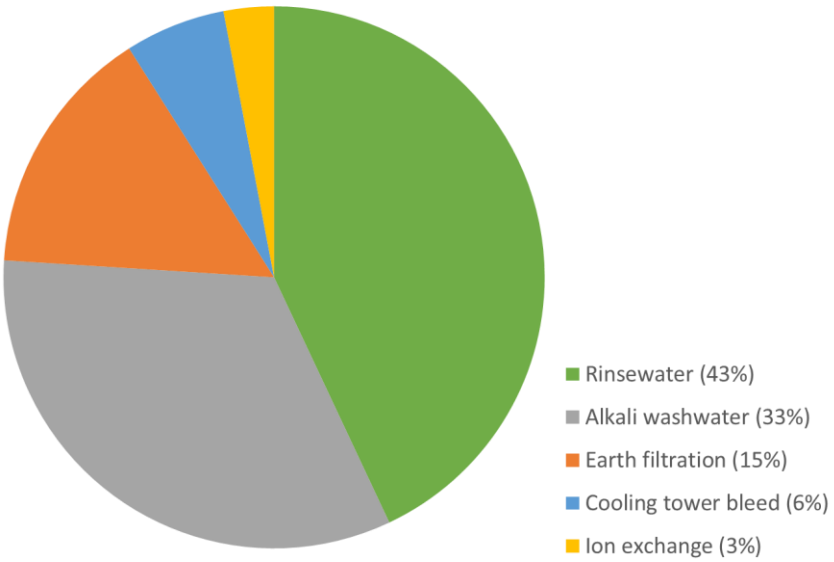

Figure 1: An example of the relative contributions of the sources of wastewater from a large non-distilling Australian winery. Adapted from Chapman *et al*. (2001)

Table 1 gives general characteristics of wastewater from Australian wineries. Vintage is a 6 to 20 week period during which winegrapes are harvested and crushed, and fermentation of grapes into wine occurs. A large winery may produce around 2 to 3 million litres of wastewater per week during vintage, declining to 1 million litres or fewer per week during non-vintage. Biological oxygen demand (BOD) is an important indicator of pollution; South Australian regulations state that water discharged into aquatic ecosystems must have a BOD of no more than 10 $mg.L^{-1}$ (SOUTH AUSTRALIAN EPA 2003). Table 1 also emphasises the variability of wastewater, which is apparent day-to-day and even hour-by-hour, necessitating facilities that can store wastewater prior to microbial treatment.

Table 1: Typical characteristics of winery wastewater. (CHAPMAN 1995)

	Vintage	Non-Vintage
Biological oxygen demand (mg/L)	1500 - 6000	500 - 3500
Total organic carbon (mg/L)	700 - 2900	250 - 1800
Total Kjeldahl nitrogen (mg/L)	34 - 60	22 - 40
Total phosphorus (mg/L)	0.1 - 0.3	0.1
Electrical conductivity (dS/m)	1.5 - 3.5	0.9 - 1.3
pH	4 - 8	6 - 10

The organic carbon in Australian winery wastewater is dominated by simple sugars, carboxylic acids and alcohols (Table 2) (CHAPMAN 1995). The major sources of organic carbon are grape juice and wine, largely from product losses. The organic carbon composition of winery wastewater resembles that of grape juice during vintage, and wine during non-vintage.

Table 2: An example of the organic composition of winery wastewater.

(CHAPMAN 1995)

	Vintage (mg/L)	Non-Vintage (mg/L)
Tartaric acid	530	350
Lactic acid	250	120
Acetic acid	100	50
Glucose	2500	230
Fructose	2500	270
Glycerol	190	120
Ethanol	2400	2900*

* samples may contain butyric acid, which co-elutes with ethanol in this HPLC analysis

These cited studies by Chapman were the most comprehensive studies of the composition of Australian winery wastewater available at the commencement of this project; therefore, they formed the basis for the design of synthetic winery wastewater used in experiments. Many subsequent studies are reviewed in Mosse (2011). The review confirms that

glucose, fructose, and ethanol continue to be the typical major contributors to organic carbon in Australian winery wastewater, though one source reported 2.22 g/L maltose in outflow from a holding tank (MALANDRA *et al.* 2003). The review cites far greater mean BOD (8858 mg/L) than Chapman (1995), possibly due to increasing use of water saving measures such as high-pressure cleaners concentrating organic materials into smaller volumes of wastewater. A later study sampled wastewaters from four wineries after on-site holding, and found that two contained around 2 g/L medium-chain fatty acids of six to ten carbons in one winery, and predominantly eight carbons in another (MOSSE *et al.* 2013). Such compounds are produced by yeasts such as *Saccharomyces cerevisiae*, the major fermenting organism of wine, and *Dekkera bruxellensis*, responsible for wine spoilage (RAZES *et al.* 1992). Given that such compounds were not reported in abundance in fresh wastewater in any studies, they are likely to have been produced during on-site holding.

In addition to wastewater, wineries also produce solid waste in the form of marc and lees. Grape marc is the stems, skins, and seeds of grapes left over after crushing; lees is the mixture of wine, yeast, bacteria, grape pulp, pectins, tannins and proteins produced after clarification of wine or grape juice by settling (CHAPMAN *et al.* 2001).

1.1.2 Potato processing industry wastewater

Like winery wastewater, potato processing wastewater from potato chip production is a nutrient-rich mixture of plant-derived organic substances produced in large quantities, and its disposal causes pollution problems (LOTZ *et al.* 1991). It contains soil particles and residues of the skin and bodies of potatoes (MUNIRAJ *et al.* 2014). Typical characteristics are summarised in Table 2.

Table 2: Typical characteristics of potato processing wastewater. (MUNIRAJ *et al.* 2014)

Total soluble starch (mg/L)	30 000 – 36 200
Total soluble COD (mg/L)	35 000 – 40 000
Total Kjeldahl nitrogen (mg/L)	400 - 620
Ammonium nitrogen (mg/L)	190
pH	4.5 - 5

1.2 *Aspergillus oryzae: A tool for wastewater treatment and biomass production*

Any treatment process of winery waste streams must reduce its potential to cause pollution. An ideal treatment process would also utilise the waste to produce something of value. The organic material and nutrients present in winery waste represent a potential energy resource, currently being lost, that could be channelled into the production of energy-rich feedstock in the form of microbial biomass. Microbes such as bacteria, algae, yeasts and filamentous fungi can grow in liquid wastewater, consuming organic materials and treating the water as they do so. The microbial biomass produced can then be used as a protein-rich food source for humans or animals.

Several properties of filamentous fungi make them particularly attractive for treating winery wastewater. Many thrive at low pH levels, which inhibit the growth of competing bacteria. Many are capable of metabolising complex mixtures of organic compounds. Microbial biomass produced from fungi is particularly nutritious, having lower amounts of nucleic acids than bacterial or yeast biomass, while protein levels are reasonably high. In terms of amino acid composition, fungal biomass protein more closely resembles animal protein than plant protein. Moreover, the cost of separating microbial biomass from treated wastewater can be a significant contributor to the costs of producing microbial biomass. Whereas bacteria or yeasts may need to be recovered by expensive centrifugation, filamentous fungi that grow as pellets or as compacted mycelial flocs may be harvested more cheaply, and with lower energy use, by filtration (JIN *et al.* 2002).

1.2.1 The filamentous fungus *Aspergillus oryzae*

Aspergillus oryzae is a filamentous fungus that has been used for the fermentation of foods and beverages in eastern Asia for over 2000 years, including *sake* (rice wine), *miso* (soybean paste) and *shoyu* (soy sauce). It arose through the ancient domestication of *Aspergillus flavus* (CHANG et al. 2015), a serious agricultural pest, human pathogen, and producer of the potent carcinogen aflatoxin. However, *A. oryzae* does not produce aflatoxin. Many strains lack multiple genes required for aflatoxin synthesis (TAO AND CHUNG 2014); those that contain all such genes have multiple missense mutations in *aflJ*, a co-activator necessary for aflatoxin production, that abolish its activity (KIYOTA *et al.* 2011). Moreover, the long history of use of *A. oryzae* in food production supports its safety, and it is classified as Generally Recognized As Safe (GRAS) by the US FDA.

The innate ability of *A. oryzae* to secrete large amounts of proteins, as well as the development of a transformation system (GOMI *et al.* 1987), have led to its widespread use in biotechnology for the expression of heterologous genes, including active human enzymes (reviewed in WARD *et al.* 2006). Sequential gene deletions can be achieved using a single genetic marker by marker rescue using the Cre-*loxP* recombination system (MIZUTANI *et al.* 2012). In common with most fungi used in industry, sexual reproduction has not been reported in *A. oryzae*, making it difficult to combine desirable traits from different strains. However, sexual reproduction was reported for *A. flavus* in 2009 (HORN *et al.* 2009), and two ideomorphs of the mating type gene *MAT1* have been found in different *A. oryzae* strains (WADA *et al.* 2012), suggesting that sexual reproduction may be possible. Strains can be combined using the parasexual cycle, and a system for promoting heterokaryon formation has been developed (WADA *et al.* 2014). Recently, the CRISPR/Cas9 gene editing system has been applied to *A. oryzae* (KATAYAMA *et al.* 2016; NODVIG *et al.* 2018), which will greatly facilitate future genetic manipulations.

The *A. oryzae* genome is 37 megabase pairs in size and contains 12,074 putative genes encoding proteins of more than 100 amino acids (MACHIDA *et al.* 2005). It is thus a third larger than the genomes of related aspergilli, the genetics model organism *Aspergillus (Emericella) nidulans* and the dangerous human pathogen *Aspergillus fumigatus*. Comparison of the three genomes revealed that the *A. oryzae* genome contains blocks that share synteny with the other two genomes, as well as blocks specific to *A. oryzae*. The blocks specific to *A. oryzae* are enriched with genes predicted to be involved with secondary metabolism that do not have direct orthologues in the other two aspergilli. Other gene categories involved in metabolism are also expanded in *A. oryzae*, including sugar uptake transporters, maltases, secreted proteases, secreted α-glucosidases, and genes involved in the uptake and metabolism of amino acids (KOBAYASHI *et al.* 2007). These findings provide theoretical backing to the use of *A. oryzae* in fermentation.

1.2.2 Use of *A. oryzae* for wastewater treatment

Industrial fermentations are performed in bioreactors, which are sealed vessels that provide tightly controlled conditions for biological fermentations. This section describes how *A. oryzae* strain DAR3699 was chosen as suitable for the treatment of starch processing wastewater, as well as optimisation of the running of a bioreactor for high treatment efficacy and fungal biomass protein yield. The demonstration that starch

processing wastewater could be feasibly treated by *A. oryzae* DAR3699 was the foundation for the proposal to treat winery wastewater using the same strain.

1.2.2.1 Use of *A. oryzae* for treatment of potato processing wastewater

A. oryzae DAR3699 was chosen from a screen of 30 microbial fungi and yeasts as being appropriate for starch processing wastewater treatment and fungal biomass protein production (JIN *et al.* 1999a). Initially one representative strain of each of 15 species of filamentous fungi and yeasts was tested for growth in 50 ml shake flask cultures of starch processing wastewater, without pre-treatment or nutrient supplementation. The cultures were grown for 24 h at 30°C under non-aseptic conditions. *A. oryzae* was represented by strain DAR3699. Of all the species tested, it was the best performer in all three criteria measured: it hydrolysed the most starch (100%), gave the greatest reduction in total organic carbon (86.7%), and had the highest growth rate (0.22 g l^{-1} h^{-1}). This and seven other strains of *A. oryzae*, as well as a total of seven other strains of *Rhizopus oligosporus*, *Rhizopus arrhizus* and *Trichoderma viride* were further tested. DAR3699 gave the third-highest yield of dry biomass (5.24 g l^{-1}), third-highest COD reduction (88.8%), and equal highest specific growth rate (0.12 h^{-1}). Among the *A. oryzae* strains, DAR3699 had the second highest protein content (40.7%) and was one of three strains to grow as compact pellets (JIN *et al.* 1999a).

Subsequent experiments focused on optimising fermentation conditions in an external airlift bioreactor. Airlift bioreactors are alternatives to the more traditional stirred-tank bioreactors. They are mixed by the action of air rising from a sparger at the bottom of the reactor, rather than by rotating impellers. Although stirred tank reactors are more widely used, airlift bioreactors have the advantages of lower power consumption (and hence lower operating costs), simpler construction, better mixing and reduced shear stresses placed on delicate fungal hyphae. An external airlift bioreactor has a separate downcomer distinct from the main riser housing the sparger (Figure 2). This permits better mixing and therefore better transfer of heat, nutrients and waste products.

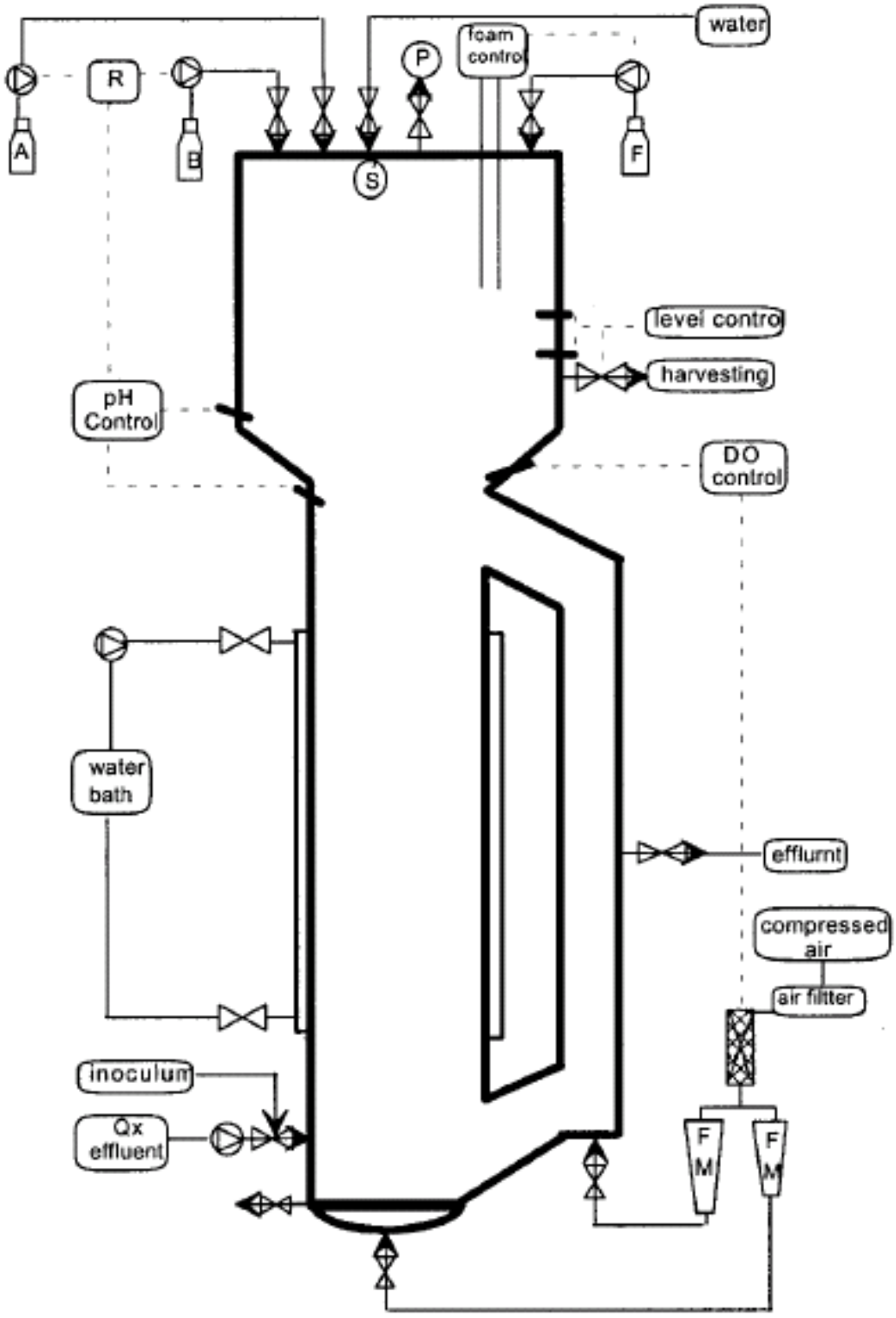

Figure 2: The external airlift bioreactor. A, acid liquid; B, alkali liquid; F, antifoam liquid; R, relay; FM, air flow meter; S, spray ball; P, pressure indicator with gas outlet; Qx, wastewater stream. From Jin *et al.* (2002)

The maintenance of a high level of dissolved oxygen is widely recognised as a critical factor determining the success of aerobic airlift bioreactor processes, particularly those involving viscous mycelial broths. The oxygen transfer in a pilot-scale (160 L) external airlift bioreactor containing starch processing wastewater and the filamentous fungus *Rhizopus oligosporus* was characterised and improved (JIN *et al.* 2001b). A key finding was that while dissolved oxygen level increased almost linearly with air flow rate in the range of 0.25 to 1.00 volumes per volume per minute (v v^{-1} m^{-1}), there was little further improvement in dissolved oxygen level as air flow rate was increased to 2.00 v v^{-1} m^{-1}. This was particularly true for highly viscous broths resulting from high biomass concentrations, such as 4 or 8 g l^{-1}. Thus it would be difficult to achieve adequate dissolved oxygen levels solely through increased airflow, especially at the high biomass concentrations required for a continuous process. Two methods were investigated for improving oxygenation. First, the use of a second small air sparger in the downcomer created a more fully aerated environment within the bioreactor. At a constant air flow rate of 1.0 v v^{-1} m^{-1}, diversion of 10% of the air flow to the second sparger led to a slight increase in mixing time, but an increase in the minimum dissolved oxygen level from 28% to 35% of saturation, and a concomitant increase in biomass productivity from 0.61 to 0.71 g l^{-1} h^{-1}. Second, operating the bioreactor at a top pressure of 1.0 bar above atmospheric pressure improved the minimum dissolved oxygen level from 21% to 63% of saturation, and the biomass productivity from 0.71 to 0.96 g l^{-1} h^{-1}. Moreover, operating under a top pressure eliminated the production of foam, eliminating the need for antifoam, which would reduce operating costs. Further increasing the top pressure to 1.5 bar above atmospheric pressure resulted in only a slight increase in dissolved oxygen and no detectable increase in biomass productivity (JIN *et al.* 2001b).

The fungus in the investigation described above grew as freely dispersed mycelia. Dispersed mycelial growth can be problematic in a bioreactor, since it tends to promote clogging around probes and blockages of sampling ports and spargers. Moreover, mycelial broth is highly viscous, particularly at high biomass concentrations, limiting oxygen and nutrient transfer. These problems are alleviated when filamentous fungi grow as discrete pellets, which are spherical agglomerates of several hyphal elements. The conditions affecting morphology of *A. oryzae* DAR3699 and two other strains growing on starch processing wastewater were investigated (JIN *et al.* 1999b). In 50 mL shake flask cultures inoculated with spore suspensions, pH values from 3.5 to 4.5 favoured compact

pellet formation for strain DAR3699. At pH 5.0, pellets were formed that had a compact nucleus but a diffuse boundary ("clumpy pellets"); pH values of 3.0, 5.5 and 6.0 produced diffuse or aggregated mycelia but not discrete pellets. The effect of inoculum quality on fungal morphology in a bench-scale (3.5 L) airlift bioreactor was then investigated. At pH 5.5, pellet inoculum led to the formation of clumpy pellets, whereas pellets were not formed when dispersed mycelia were used as the inoculum. At pH 4.0, compact pellets were formed regardless of whether the inoculum used was a spore suspension, dispersed mycelia or compact pellets. However, inoculum quality affected pellet size, with spore suspension producing the smallest pellets, and pellet inoculum producing the largest pellets. Pellet size was found to be inversely correlated to protein content, thought to be due to limited diffusion of oxygen and/or other nutrients into the centre of the larger pellets. Pellet size was also found to be affected by aeration, with higher rates of aeration resulting in the formation of smaller pellets (JIN *et al.* 1999b).

The pilot-plant scale 160 L bioreactor described above was used to determine the optimal process mode for bioreactor operation, i.e., batch, continuous or semi-continuous (JIN *et al.* 2001a). In a semi-continuous process, a portion of the broth referred to as the V_{out}/V_t ratio is removed at regular intervals. The remaining broth then serves as the new inoculum as the bioreactor is topped up with new growth media. V_{out}/V_t ratios of 0.90, 0.70 and 0.50 were tested. Lower V_{out}/V_t ratios gave slightly lower biomass productivities and required more frequent medium exchanges. All three V_{out}/V_t ratios resulted in higher biomass productivities than batch cultures, as well as avoiding the need for the preparation of numerous seed cultures, which are expensive for an industrial process. Continuous process was investigated with dilution rates ranging from 0.06 to 0.20 h^{-1}; 0.14 h^{-1} was found to be optimal for both biomass productivity and wastewater treatment, as assessed by reduction in chemical oxygen demand. Although biomass productivity was higher for the continuous process than the other process modes, the variable composition of starch processing wastewater and blocking of the air sparger created difficulty in maintaining a steady state beyond 4 days of continuous cultivation. Thus semi-continuous was deemed to be the best process mode (JIN *et al.* 2001a).

The qualities of the treated wastewater and the fungal biomass, as well as kinetic parameters, were analysed in detail using the optimised pilot plant-scale process (JIN *et al.* 2002). The 160 L bioreactor was inoculated with 8% (v/v) preculture and operated at

35°C, with the pH maintained between 4.5 and 6.5, and the aeration regulated between 0.5 and 1.2 v v^{-1} m^{-1} so as to maintain the dissolved oxygen level above 50% of saturation. There was no fungal growth observable by microscopy during the first 2 h after inoculation, but growth of hyphae and branching were visible at 3 h. The exponential growth phase lasted from 4 h to 9 h. Glucose accumulated during this time, reaching a peak of over 2.5 g l^{-1} at approximately 7 h growth, showing that the rate of starch hydrolysis exceeded the rate of glucose uptake by the cells. A semi-continuous process was run with a V_{out}/V_t ratio of 0.90. A biomass yield of 8.8 g l^{-1} was achieved, with a 0.9 g l^{-1} h^{-1} productivity. Growth was retarded beyond 48 h, corresponding to the fifth exchange of media. This may have been due to contamination and/or a reduction in dissolved oxygen resulting from clogging of the air sparger (JIN *et al.* 2002).

The fungal biomass was produced in pellets of diameter 2 to 5 mm, allowing simple separation using a rotating drum filter with a pore size of 200 μm. It had a pleasant odour, and biomass semi-dried by vacuum or pressure filtration could be stored at ambient conditions for several days without apparent deterioration in quality. The biomass was found to contain 46% protein by dry weight, as well as 16% dietary fibre, 24% total carbohydrate and only 2% fat. The levels of 8 of the 10 essential amino acids exceeded the levels of the FAO reference protein (FAO/WHO 1974), while levels of tyrosine and tryptophan were slightly lower and may need to be supplemented if the fungal biomass is used as a feed material. The treated wastewater had chemical oxygen demand, biological oxygen demand and suspended solids reduced by over 95%, while nitrogen and phosphorous were reduced by three-quarters. Residual minerals were very low, and the treated wastewater would be suitable for farm irrigation. The production of valuable fungal biomass protein, combined with efficiencies of operation such as simple biomass harvesting, asepsis during operation, low power consumption and the lack of need for wastewater pretreatments such as sterilisation, partial starch hydrolysis, nutrient supplementation or initial pH adjustment suggest the process may be economically feasible. Clogging of the air sparger after 2 to 3 days of operation remained the major obstacle to be overcome (JIN *et al.* 2002).

1.3 Carbon catabolite repression

1.3.1 CCR in winery wastewater treatment

In the presence of two or more carbon sources, micro-organisms will typically utilise energetically favourable sources preferentially to less readily metabolised sources. Carbon catabolite repression (CCR) is the process by which the presence of favourable carbon sources leads to the repression of genes necessary for the utilisation of less favoured sources. It affects the expression of hundreds of genes. It is distinct from specific induction, which is the requirement for the presence of a particular carbon source (or derivative thereof) for genes required to utilise that carbon source to be expressed (reviewed in STULKE AND HILLEN 1999; FLIPPHI AND FELENBOK 2004; KELLY 2004).

As an evolved mechanism, carbon catabolite repression must confer a selective advantage to filamentous fungi in their natural environment. However, the environment that *A. oryzae* will encounter within a bioreactor containing winery wastewater differs to that of the natural environment of *A. oryzae* in several ways. For example, due to the filtering effects of the cross-flow microscreen, the maintenance of low pH and the large monoculture inoculum, *A. oryzae* is likely to encounter reduced competition with other micro-organisms, compared with its natural environment. Therefore, there is less need to rapidly consume the most energetically favourable carbon sources before they can be consumed by competing micro-organisms. Moreover, the combinations, concentrations and relative proportions of carbon sources in winery wastewater differ from those of the natural environment of *A. oryzae*. Thus it is possible that CCR will impede *A. oryzae* growth in a bioreactor.

As discussed in the next section, mutation can create "derepressed" fungal strains with reduced CCR. Following are four potential mechanisms by which a derepressed strain might more efficiently treat winery wastewater in a bioreactor:

1. **Reduction of lag phase(s) in a batch fermentation:** When presented with several carbon sources simultaneously, a micro-organism will typically utilise them in a sequential fashion: first the most preferred source (often glucose) is consumed, then after this is depleted the second most preferred source is consumed, and so on. Concomitant with changes in carbon source utilisation are changes in growth rate, which may follow a stepwise pattern with the highest

growth rate during utilisation of the most preferred carbon source, followed by lower growth rates associated with utilisation of less preferred carbon sources. Often a lag phase follows the depletion of each carbon source, as the organism requires time to begin expressing the enzymes required for the next carbon source (reviewed in SIEGAL 2015). For example, when the yeast *S. cerevisiae* is grown on glucose media, many strains produce ethanol as a byproduct of fermentation; upon depletion of the glucose, there is typically a lag phase lasting up to several hours associated with the diauxic shift from growth on glucose to growth on ethanol (MURPHY *et al.* 2015). This suggests that, if *A. orzyae* is grown in winery wastewater in a batch culture, there may be a lag phase between the utilisation of the sugars and the ethanol. Lag phases associated with switching between carbon sources are reduced in yeasts with weaker CCR (reviewed in SIEGAL 2015), suggesting disruption of CCR may improve the speed of batch culture treatment of complex mixtures such as winery wastewater.

2. **Engineering advantages:** As well as changes in growth rate, changes in carbon source utilisation can be accompanied by changes in morphology. For example, *A. nidulans* looks markedly different when grown on glucose than on ethanol, with reduced hyphal diameter in the latter case (unpublished data). A strain with disrupted CCR would consume multiple carbon sources simultaneously and likely have a more constant micromorphology throughout the fermentation than a strain with CCR. This greater consistency may be advantageous from an engineering perspective. For example, it may be simpler to optimise the physicochemical environment within the bioreactor (temperature, pH, agitation rate, dissolved oxygen, etc.) if the fungus has only one mode of growth throughout the fermentation, rather than one for each carbon source. This may be particularly important for a filamentous fungus, where achieving the desired small pellet morphology is strongly dependent on fermentation parameters. Conversely, a more natural growth pattern in which the specific growth rate slows as carbon sources are depleted and biomass accumulates may be preferable, as it is more difficult to keep a liquid culture mixed and well oxygenated in the later stages of a fermentation when total fungal oxygen consumption is highest and the culture broth is at its most viscous.

3. **Higher feed rate in a continuous fermentation:** In continuous fermentation, growth substrate is fed to the bioreactor at a constant rate and all carbon sources are consumed simultaneously and at the same rate as they are being provided. Continuous fermentations provide advantages over batch fermentations such as eliminated time between batches, eliminated microbial lag phases, and greater potential for automation. For a fungus with ordinary CCR, the rate at which winery wastewater could be provided in a continuous process would be constrained by glucose – the steady-state glucose concentration would have to be kept low enough to minimise CCR so that other carbon sources could be consumed. In contrast, in a strain with disrupted CCR, simultaneous utilisation of carbon sources would be possible at higher glucose concentrations, allowing higher feed rates of winery wastewater and more rapid processing overall.

4. **Higher expression of genes encoding enzymes for carbon utilisation:** Even in the absence of glucose or another repressing carbon source, residual activity of the mechanism of CCR often seems to limit expression of genes involved in carbon source utilisation, and this can restrict growth. Evidence for this is that derepressed strains often grow better than their parent strains on certain non-preferred carbon sources. For example, an *A. nidulans* derepressed strain grows better than its parent on protein and on acetamide as sole carbon sources, and these differences are attributed to higher expression of proteases and acetamidase in the deprepressed strain (HYNES AND KELLY 1977). Similarly, a deprepressed strain of *Trichoderma reesei* grows better than its parent on maltose, likely due to higher expression of an α-glucosidase (DENTON AND KELLY 2011). A derepressed *A. oryzae* strain grows better than its parent on 5% xylose (but not 1% xylose), though the reason for this is unknown (ICHINOSE *et al.* 2017). If utilisation of fructose or ethanol is limited by CCR in *A. oryzae*, then disruption of CCR may lead to faster consumption of these carbon sources and thus faster treatment of winery wastewater.

1.3.2 The genetics of CCR in filamentous fungi

A fruitful method for discovering genes involved in CCR in filamentous fungi has been genetic screens for suppressors of *areA* loss-of-function mutants in the model fungus *A. nidulans*. In a system analogous to CCR, filamentous fungi do not normally express enzymes for the utilisation of nitrogen sources when a more preferred nitrogen source,

such as ammonium, is present. The gene *areA* encodes a positively acting transcription factor that permits utilisation of non-preferred nitrogen sources in the absence of ammonium. Fungal strains harbouring loss-of-function mutants of *areA* always behave as though ample ammonium were present; that is, they do not respond to nitrogen starvation by expressing enzymes for the utilisation of non-preferred nitrogen sources. Compounds such as acetamide and proline provide the cell with both a carbon source and a nitrogen source, and genes encoding proteins required for their utilisation, including acetamidase and proline permease, may be subject to regulation by both ammonium repression and carbon catabolite repression (reviewed in Caddick *et al.* 1994). Fungal strains that are wildtype except for a loss-of-function mutation in *areA* cannot grow in a medium containing repressing concentrations of glucose and acetamide as a sole nitrogen source, as they will be starved of nitrogen. However, if CCR is disturbed, the fungus will utilise acetamide as a carbon source. Since acetamide is also a nitrogen source, its utilisation relieves nitrogen starvation and permits growth. Th us mutations disabling CCR can be identified as suppressors of *areA* mutants, when mutagenised fungi are grown in appropriate media. The genes *creA*, *creB* and *creC* were first identified in this manner. Since this kind of screen has been carried out several times, it is likely that these are the only genes in *A. nidulans* that can be mutated to relieve CCR without killing the organism (reviewed in KELLY 2004).

1.3.2.1 *creA*

The *creA* gene and its orthologues are ubiquitous throughout the fungal kingdom (BENOCCI *et al.* 2017). CreA is orthologous to the well-studied Mig1 in the yeast *S. cerevisiae*, and both are negatively acting transcription factors that repress a broad range of target genes, mostly involved in utilisation of non-preferred carbon sources. However, the mechanism of CCR is quite different in yeast than in filamentous fungi. Mig1 leaves the nucleus in derepressing conditions, relieving CCR (DE VIT *et al.* 1997), whereas in *A. nidulans* CreA can still regulate CCR correctly when present at high levels in both the nucleus and the cytoplasm (ROY *et al.* 2008). CCR is mediated by glucose only in *S. cerevisiae*, but by a range of carbon sources in *A. nidulans* and other filamentous fungi (reviewed in BENOCCI *et al.* 2017). Relief of CCR requires specific induction in filamentous fungi but not in yeast (reviewed in BROWN *et al.* 2014). The latter two differences reflect the specialisation of yeast to glucose-rich environments, in contrast to filamentous fungi, which tend to be generalists that can use a variety of carbon sources.

Under repressing conditions, *creA* deletion mutants in *A. nidulans* have decreased growth rates and reduced conidiation on solid media (SHROFF *et al.* 1997), and increased branching and reduced culture viscosity in liquid media (AGGER *et al.* 2002); similar morphological changes have been observed in other species (reviewed in BENOCCI *et al.* 2017). Many enzymes required for the utilisation of non-preferred carbon sources, including alcohol dehydrogenase I and β-galactosidase, as well one or more α-amylases, are derepressed (RUIJTER AND VISSER 1997; SHROFF 1997; MOGENSEN *et al.* 2006).

As well as its effects in repressing conditions, *creA* also has effects in derepressing conditions. A microarray analysis in *A. nidulans* adds to previous findings of enhanced expression of enzymes for carbon source utilisation, finding that over 25% of the genes whose expressions increase in a *creA*Δ mutant in 1% glucose, representing repressing conditions, also increase during growth in 1% ethanol, representing derepressing conditions (SIMS *et al.* 2004).

CreA contains two highly conserved Cys_2-His_2 class zinc finger DNA-binding motifs (DOWZER AND KELLY 1991). Many CreA orthologues contain a homopolymeric stretch of Ala, Asp or His residues several residues C-terminal of the DNA binding domain; the function of this region of the protein or of the corresponding region of transcript, if any, is unknown (reviewed in KELLY 2004) . Binding of CreA to DNA has been shown to depend on the core recognition sequence 5'-SYGGRG-3' (KULMBURG *et al.* 1993; CUBERO AND SCAZZOCCHIO 1994). Frequently multiple copies of this motif are present in the promoters of genes subject to carbon catabolite repression, although some may not be functional (reviewed in KELLY 2004).

Strains of *A. nidulans* containing different mutant alleles of *creA* exhibit remarkable phenotypic heterogeneity. For example, one allele results in elevated levels of α-L-arabinofuranosidase B during growth on medium containing L-arabitol as a sole carbon source whilst having little effect on the level of endo-arabinase; another allele has the opposite effect (VAN DER VEEN *et al.* 1994). Presumably these complexities reflect the intricate binding of CreA to DNA, which is predicted to involve the link between the zinc finger domains as well as seven amino acid residues across the two domains (ESPERON *et al.* 2014).

The regulation of *creA* is complex and appears to differ among fungi. In *A. nidulans*, *creA* transcripts are more abundant in non-repressing than repressing conditions (VAUTARD *et al.* 1999), which seems counterintuitive given that CreA ought to be more active in repressing conditions. In contrast, protein levels of the CreA orthologue in the filamentous fungus *Sclerotinia sclerotiorum* appear relatively constant (CZIFERSZKY *et al.* 2002), although the *A. nidulans* and *S. sclerotiorum* studies used different derepressing media. The orthologue of CreA in *T. reesei*, Cre1, can only bind DNA after phosphorylation at amino acid 241, a serine residue (VAUTARD-MEY AND FEVRE 2000). Mutation of the corresponding serine in *S. sclerotiorum* does not affect protein localisation, which is predominantly nuclear in both repressing and derepressing conditions in *S. sclerotiorum* (VAUTARD *et al.* 1999). In *A. nidulans*, CreA localisation is predominantly nuclear in both repressing and derepressing conditions when expressed by a strong constitutive promoter (ROY *et al.* 2008). In contrast, when expressed from its native promoter, localisation is predominantly nuclear in repressing conditions but predominantly cytoplasmic in derepressing conditions (RIES *et al.* 2016). CreA negatively autoregulates its own expression, presumably by binding to the 5'-SYGGRG-3' motifs in its own promoter (reviewed in KELLY 2004), but this autoregulation is not essential for CreA function (ROY *et al.* 2008). CreA function is not wholly dependent on *de novo* translation in *T. reesei* (LICHIUS *et al.* 2014) or *A. nidulans* (RIES *et al.* 2016). Although there was one report that CreA may be ubiquitinated (RIES *et al.* 2016), this was not corroborated by mass spectroscopy analysis of the proteome (CHU *et al.* 2016) or of purified CreA (ALAM *et al.* 2017).

Of particular interest in biotechnology, RUT-C30, a cellulase hyper-producing strain of *T. reesei* that has been been widely used in industry, was created by random mutagenesis and later found to express a truncated form of Cre1 (ILMEN *et al.* 1996). Not only does this explain the reduced CCR in this strain, but the truncated form acts on target genes in a positive manner by opening the chromatin structure, facilitating transcription (MELLO-DE-SOUSA *et al.* 2014). Although *creA* homologues have now been deleted in several fungi of industrial importance (RUIJTER *et al.* 1997; ICHINOSE *et al.* 2014), there are as yet no reported attempts to engineer positively-acting CreA derivatives in other fungi.

1.3.2.2 *creB* and *creC*

Initial investigation of *creB* and *creC* was conducted in *A. nidulans areA* mutant strains. In this background, the phenotypes associated with mutations in *creB* and *creC* are indistinguishable and non-additive, and there is no phenotypic heterogeneity. Mutations in either gene cause slightly reduced colony density and conidiation in both complete and minimal media. Many genes show relief from CCR, including *amdS* encoding acetamidase, *alcA* encoding alcohol dehydrogenase, and genes involved in acetate metabolism. Uptake of proline and glutamate are greatly reduced, whereas glucose uptake is unaffected, although there is a reduction in the acidification of the growth media normally associated with glucose uptake. There is reduced utilisation of hexose and pentose sugars, amino acids and nitrate. Mutations in *creB* and *creC* also impart resistance to several toxic sugar analogues and molybdate, but hypersensitivity to acriflavine. Secretion of proteases is increased (HYNES AND KELLY 1977; LOCKINGTON AND KELLY 2001; reviewed in FLIPPHI AND FELENBOK 2004). Subsequent analyses in an *areA* wildtype background found that the phenotypes of *creC* mutant strains are slightly weaker than those of *creB* mutant strains (Robin Lockington, pers. comms.). Although *creA* mediates CCR due to many carbon sources, *creB* and *creC* are not involved in CCR due to gluconeogenic carbon sources such as acetate, which is a strongly repressing carbon source in *A. nidulans* that represses via *creA* and other genes (GEORGAKOPOULOS *et al.* 2012).

Consistent with the similar phenotypes of *creB* and *creC* mutants, CreB and CreC have been shown to physically interact in *A. nidulans in vivo* by co-immunoprecipitation (LOCKINGTON AND KELLY 2002). In carbon catabolite derepressing conditions, full-length CreB cannot be detected unless CreC is also present, although a degradation product is detectable. In addition, overexpression of CreB alone, in the absence of any CreC, is sufficient to restore glucose repression of alcohol dehydrogenase in carbon catabolite repressing conditions, although quinate utilisation under derepressing conditions is only partially restored. Overexpression of CreC in the absence of CreB does not alleviate the *creB*/*C* phenotype. Taken together, these data indicate that a major role of CreC is to stabilise CreB, although the possibility that CreC also influences the substrate specificity of CreB cannot be excluded (LOCKINGTON AND KELLY 2002).

In repressing conditions, mutation of *creC* in *A. nidulans* changed the apparent cellular distribution of a CreA::GFP fusion protein from nuclear to homogeneous and greatly reduced the abundance of full-length CreA::GFP, whereas mutation of *creB* had little effect on CreA::GFP localisation or abundance (RIES *et al.* 2016). However, it is unclear whether the observed GFP fluorescence in the nucleus corresponds to full-length CreA::GFP or one or more degradation products, given that degradation products were far more abundant than full-length CreA:GFP in that study, and that a poly-alanine rich region in the N-terminal third of CreA is important for CreA to leave the nucleus (RIES *et al.* 2016).

CreB is a functional deubiquitinating enzyme (LOCKINGTON AND KELLY 2001), but CreA is not one of its targets (ALAM *et al.* 2017). Although no targets of CreB are known, its physical interactors in *A. nidulans* tend to be involved in cellular transportation and organisation (ALAM AND KELLY 2017). In particular, Hir3 (histone transcription regulator 3) physically interacts with both CreA and CreB, suggesting Hir3 may be a component of the CCR regulatory network not yet investigated (ALAM AND KELLY 2017). As well as six *DUB* homology domains, CreB contains a region predicted to form a coiled-coil (LOCKINGTON AND KELLY 2001) and four PEST sequences (LOCKINGTON AND KELLY 2002). PEST sequences, when exposed, mark proteins for ubiquitination and rapid degradation by the proteasome (reviewed in RECHSTEINER AND ROGERS 1996). CreC contains a proline rich sequence and a series of five WD40 repeats, which are predicted to fold into a five-bladed propeller structure (TODD *et al.* 2000). Proline rich sequences and WD40 repeats are both involved in protein-protein interactions (WILLIAMSON 1994; CHEN *et al.* 2004). CreC also contains a predicted nuclear localisation sequence of the SV-40 T antigen type (LOCKINGTON AND KELLY 2002), although studies of its localisation have not been reported. CreB, when overexpressed, is predominantly cytoplasmic (ROY 2008).

1.3.2.3 *creD, acrB* and *apyA*

In contrast to *creA, creB* and *creC*, mutations in *creD* and *acrB* lead to tighter carbon catabolite repression in *A. nidulans*. Mutations in *creD* impart resistance to fluoroacetate toxicity in the presence of glucose, implying that the gene encoding acetyl coA synthetase, *facA*, may be under tighter glucose repression in *creD* mutants than in wildtype organisms (BOASE AND KELLY 2004). Mutations in *creD* and *acrB* both

suppress the derepression of *alcA* exhibited by *creB* and *creC* mutants (KELLY AND HYNES 1977), and *creD* mutants also suppress the derepression of *facA* in *creB* and *creC* mutants (KELLY AND HYNES 1977; BOASE AND KELLY 2004). Mutations in both *creD* and *acrB* confer resistance to acriflavine and hypersensitivity to molybdate, the reverse of the phenotypes of *creB* and *creC* mutants (BOASE AND KELLY 2004). In derepressing conditions, *acrB* (but not *creD*) suppresses the poor growth of *creB* and *creC* mutants on quinate. CreD interacts with the HECT ubiquitin ligase HulA *in vitro* (BOASE *et al.* 2003). Taken together, these data suggest that *creD* and *acrB* may play opposing roles to *creB* and *creC*, and hence may be involved in ubiquitination. An additional aspect of the *acrB* mutant phenotype is reduced utilisation of many carbon sources, including ethanol, acetate, starch and several simple saccharides including fructose, maltose and cellobiose. Several ω-amino acids are also poorly utilised, both as sole carbon sources and as sole nitrogen sources (BOASE *et al.* 2003).

AcrB is predicted to contain three transmembrane domains and a coiled-coil domain (BOASE *et al.* 2003). The coiled-coil domain is predicted to occur on the non-cytosolic face of the membrane. CreD contains an arrestin domain and three PY motifs (BOASE AND KELLY 2004). PY motifs can bind WW domains (CHEN AND SUDOL 1995) , PxY being a core motif than can bind with some affinity (CHEN *et al.* 1997). Arrestins play a role in signal transduction, binding phosphorylated G-proteins and altering their signalling properties (reviewed in PALCZEWSKI 1994).

ApyA was implicated in CCR by its amino acid sequence homology to the first two-thirds of CreD (BOASE AND KELLY 2004). It is named because, like CreD, it has an arrestin domain and a PY motif. Also in common with CreD, it interacts with HulA *in vitro* (BOASE AND KELLY 2004). No phenotypic effects were seen in an *apyA* mutant, although a *creD/apyA* double-mutant had strong morphological impairment, and *apyA* mutation partially suppressed the phenotypes of *creB* and *creC* mutations (DENTON 2011). Multiple copies of *apyA* did not suppress the phenotypes of a *creD* mutation (DENTON 2011). Together, these data indicate a role in CCR for *apyA* distinct from that of *creD*.

2 Statement of Authorship

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Principal Author

Name of Principal Author (Candidate)	Adrian Hunter
Contribution to the Paper	Generated both *creB* deletion strains complemented *creB* in a *creBΔ niaD*$^-$ strain; designed, performed and interpreted all experiments with *A. oryzae* DAR3699 and all experiments with results shown in Tables 3 and 4 and Figures 1, 3, 4, 5A, and 6; co-wrote the manuscript (50% of the words).
Overall percentage (%)	60%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper
Signature	Date 20 April 2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that

i. the candidate's stated contribution to the publication is accurate (as detailed above)

ii. permission is granted for the candidate to include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution

Name of Co-Author	Tom Morris
Contribution to the Paper	Designed, performed, and interpreted all qRT-PCR experiments and wrote the corresponding portions of the manuscript (10% of the words)
Signature	Date 25/6/2018

Name of Co-Author	Bo Jin
Contribution to the Paper	Evaluated and edited the manuscript
Signature	Date 30/04/2018

Name of Co-Author	Christopher Saint		
Contribution to the Paper	Evaluated and edited the manuscript.		
Signature		Date	15/6/18

Name of Co-Author	Joan Kelly		
Contribution to the Paper	Performed experiments with results shown in Figure 2; designed, performed, and interpreted microscopic examination of strain morphology; assisted with design and interpretation of all experiments; supervised laboratory work; co-wrote the manuscript (40% of the words); acted as corresponding author.		
Signature		Date	25/4/2018

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Deletion of *creB* in *Aspergillus oryzae* Increases Secreted Hydrolytic Enzyme Activity

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***Aspergillus oryzae* has been used in the food and beverage industry for centuries, and industrial strains have been produced by multiple rounds of selection. Targeted gene deletion technology is particularly useful for strain improvement in such strains, particularly when they do not have a well-characterized meiotic cycle. Phenotypes of an *Aspergillus nidulans* strain null for the CreB deubiquitinating enzyme include effects on growth and repression, including increased activity levels of various enzymes. We show that *Aspergillus oryzae* contains a functional homologue of the CreB deubiquitinating enzyme and that a null strain shows increased activity levels of industrially important secreted enzymes, including cellulases, xylanases, amylases, and proteases, as well as alleviated inhibition of spore germination on glucose medium. Reverse transcription-quantitative PCR (RT-qPCR) analysis showed that the increased levels of enzyme activity in both *Aspergillus nidulans* and *Aspergillus oryzae* are mirrored at the transcript level, indicating transcriptional regulation. We report that *Aspergillus oryzae* DAR3699, originally isolated from soy fermentation, has a similar phenotype to that of a *creB* deletion mutant of the RIB40 strain, and it contains a mutation in the *creB* gene. Collectively, the results for *Aspergillus oryzae*, *Aspergillus nidulans*, *Trichoderma reesei*, and *Penicillium decumbens* show that deletion of *creB* may be broadly useful in diverse fungi for increasing production of a variety of enzymes.**

Aspergillus oryzae is a multicellular fungus that has been used for centuries for the production of Asian foods and beverages, including sake (rice wine) and shoyu (fermented soybean). Today it is also used industrially as a source of secreted enzymes, including cellulases, amylases, proteases, β-galactosidase, and lipase, and as a host for the production of heterologous proteins (1, 2).

creB was identified in the model filamentous fungus *Aspergillus nidulans* in a screen for mutations that alleviate carbon catabolite repression (CCR) (3). CCR is a mechanism by which genes for the utilization of nonpreferred carbon sources are repressed in the presence of preferred carbon sources; organisms thus avoid wasting energy producing enzymes for the degradation of complex carbon sources when more readily metabolized carbon sources are available. *A. nidulans creB* mutants grow well and alleviate CCR of various enzymes, including acetyl-coenzyme A (acetyl-CoA) synthetase, isocitrate lyase, acetamidase, and alcohol dehydrogenase (3, 4). In addition, *A. nidulans creB* mutants show a pleiotropic range of phenotypes apparently unrelated to CCR, including slightly reduced conidiation, reduced growth with quinate, proline, or glucuronate as the sole carbon source, enhanced growth on acetamide or acrylamide, resistance to molybdate, hypersensitivity to acriflavine, and reduced acidification of liquid growth media (3–5). *creB* encodes a deubiquitinating enzyme (DUB); DUBs are cysteine proteases that specifically cleave ubiquitin from ubiquitin-conjugated protein substrates (6, 7).

Other genes identified in the same screen for mutations that reduce CCR were *creA* and *creC* (3, 4). Molecular analysis of *creA* showed that it encodes a DNA-binding regulatory protein that is the major regulatory protein involved in CCR in *A. nidulans* (8) and many other multicellular fungi subsequently analyzed. *creA* mutations lead to derepression of a wider range of enzymes than *creB* mutations, but lack-of-function alleles lead to severe morphological effects (9, 10). Molecular analysis of *creC* revealed that it encodes a WD40-containing protein (11). The pleiotropic phenotypes of mutations in *creB* and *creC* are similar and nonadditive, and overexpression of *creB* can suppress the absence of *creC*, but not vice versa, indicating that CreB is the active partner (7).

The finding that *creB* mutations in *A. nidulans* reduce CCR without causing the severe morphological effects associated with mutations in *creA* makes *creB* an attractive target for mutagenesis in industrially useful strains. To date, *creB* disruption has been reported in two industrial sources of cellulase enzymes: *Trichoderma reesei* and *Penicillium decumbens* (12, 13). The *T. reesei creB* disruptant has increased growth on maltose, increased secretion of proteases, and greatly increased total secreted cellulase and xylanase activities in the absence of glucose. The *P. decumbens creB* deletion mutant has increased cellulase and xylanase activities and increased total secreted protein levels.

We deleted the *creB* gene from *A. oryzae* and analyzed the phenotypic effects, with an emphasis on the expression and secretion of industrially relevant enzymes, and we found that a null strain showed increased activity levels. Reverse transcription-quantitative PCR (RT-qPCR) experiments showed that this increase was due to increased levels of mRNA, indicating effects at the level of transcriptional control.

MATERIALS AND METHODS

Strains and media. *A. oryzae* RIB40 (ATCC 42149) and NBRC 30105 (JCM02239) were obtained from the NITE Biological Resource Centre

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TABLE 1 Oligonucleotide primers used for RT-qPCR

Organism	Primer	Primer sequence (5′ → 3′)	Exons whose boundary was crossed	Amplicon size (bp)
A. nidulans	*alcA* F	GAGGCTCTGGACTTCTTCGCT	2 and 3	107
	alcA R	GCGATTCTGCCTTGTTCCATA	2 and 3	107
	tubC F	TAACCTGCTCAACCCTGTTCC	5 and 6	137
	tubC R	CATAGAGCACAGAGCAGTTTGGAC	5 and 6	137
A. oryzae	β-tubulin F	GGTAACCAAATAGGTGCCGC	4 and 5	80
	β-tubulin R	GAGGAGCCATTGTAAACACCG	4 and 5	80
	amyABC F	AGGCGTGTACTGTATCGGCG	6 and 7	117
	amyABC R	CGTTGAGGAGTGGATAGTAAATGG	6 and 7	117
	glaA F	AGGCAATCTTGAATAATATCGGC	1 and 2	113
	glaA R	CACGGGTCCAGGTATAGAAATAATG	1 and 2	113

(NBRC), Japan. *A. oryzae* DAR3699, isolated during soy fermentation, was obtained from CSIRO Division of Food Science & Technology, Australia (14). The *A. nidulans* "wild-type" strain had the full genotype *biA1*; *riboB2*; *niiA4*, and the *A. nidulans creB1937* strain had the full genotype *yA1 pabaA1*; *creB1937*; *riboB2* (6).

Specific growth tests were undertaken in minimal medium (containing 0.05% KCl, 0.05% $MgSO_4$, and 0.15% KH_2PO_4 plus traces of $Na_2B_4O_7$, $CuSO_4$, $FePO_4$, $MnSO_4$, $NaMoO_4$, and $ZnSO_4$, with the pH adjusted to 6.5) supplemented with the appropriate carbon and nitrogen sources, as indicated in each experiment. Unless otherwise specified, the nitrogen source was filter-sterilized 10 mM urea added after autoclaving; nitrate was not used because *creB* deletion reduces nitrate utilization. Solid medium also contained 1% agar.

Spore germination assays. Spores were scraped from plates containing 1% sucrose after growth for 8 days at 30°C, vortexed in 0.1% Tween 20 to dislodge any hyphae, and centrifuged at 1,000 × *g* for 1 min. Pelleted spores were resuspended in fresh 0.1% Tween 20, vortexed vigorously to separate clumps, and counted with a hemocytometer. Plates were inoculated with 200 μl 0.1% Tween 20 containing 500 or 5,000 spores and incubated at 30°C.

Enzyme assays. Spores were scraped from spread plates containing 1% sucrose supplemented with 0.1 M KCl to promote extensive sporulation (15). Spores were prepared as described above, except with centrifugation at 1,500 × *g* for 10 min. A suspension containing 2×10^7 spores per ml was prepared in 0.1% Tween 20, and 0.5 ml of this suspension was added to 50 ml autoclaved medium in a 250-ml Erlenmeyer flask, producing a final spore concentration of 2×10^5 spores per ml. Where necessary, xylan from oat-spelt (Fluka) was dissolved before autoclaving via extended heating on a hot plate with vigorous stirring. The starch used was analytical-grade soluble starch (Univar). Inoculated flasks were incubated at 30°C with shaking at 150 rpm. Because light has been shown to influence secreted cellulase levels in some fungi (16, 17), a glass-front incubator was used, and ordinary fluorescent indoor lighting was left on throughout incubation to maintain approximately constant ambient light. Under these conditions, all strains grew as discrete pellets. After 48 h, supernatant samples were added to a one-sixth volume of Complete protease inhibitor (Roche) and frozen at −80°C until analysis. Biomass samples were vacuum filtered through 55-mm filter paper circles (Whatman) that had been preweighed after drying at 65°C. Filters with biomass were then washed with 200 ml reverse osmosis (RO)-purified water, dried at 65°C to constant mass, and weighed. Total secreted cellulase, xylanase, and amylase activities were measured using EnzChek cellulase, xylanase, and amylase substrates (Invitrogen).

Quantitative real-time PCR. For RNA preparation, conidial suspensions were prepared from 2-day-old conidia in 0.01% Tween 20, and 4.0×10^7 spores were added to 200 ml liquid medium, producing a final spore concentration of 2×10^5 spores per ml, in 1-liter flasks. The cultures were incubated overnight at 30°C with shaking at 150 rpm. Total RNA was isolated from mycelia grown under the specified conditions, using an RNeasy Plant minikit (Qiagen) according to the manufacturer's instructions. For cDNA synthesis, total RNA was treated with DNase (Promega). cDNA first-strand synthesis was performed using a Moloney murine leukemia virus (M-MLV) reverse transcriptase kit (Promega). The design of primers and the calculation of optimum annealing temperatures for PCR were performed using NetPrimer (www.premierbiosoft.com/netprimer/). RT-qPCRs were performed according to the instructions of Applied Biosystems. All experiments were performed with SYBR green as the detector, using an ABI Prism 7000 sequence detection system with a 2-step PCR and 60°C as the annealing temperature, unless otherwise stated.

The primers used for this study are shown in Tables 1 and 2.

Statistical analysis. All claims of statistical significance are based on two-tailed two-sample Student's *t* tests assuming unequal variance.

RESULTS

The *A. oryzae* genome contains orthologues of genes involved in carbon catabolite repression in *A. nidulans*. Although many genes have been shown to be affected by CCR in *A. oryzae* (18), there has been little research into the genetics of CCR in this fungus. We used published amino acid sequences of *A. nidulans* proteins to identify putative orthologues in the *A. oryzae* genome. Clear orthologues of *creA*, *creB*, and *creC* were identified (Table 3). When sequences were aligned, the *A. oryzae* CreB protein showed 63% amino acid identity with CreB from *A. nidulans* and 42% amino acid identity with Cre2 from *T. reesei*, and there was 38% identity when all three proteins were aligned, with conservation strongest in the six DUB homology domains surrounding conserved cysteine, aspartic acid, and histidine residues required for catalytic activity. CreB in *A. nidulans* and Cre2 in *T. reesei* share 41% amino acid identity.

Creation of *A. oryzae creB* deletion and complemented strains. A deletion construct was made which lacks 2,191 bp of *creB*, beginning from 4 nucleotides past the start codon, and contains 621 bp 5′ of *creB* and 769 bp 3′ of *creB*, surrounding the *ptrA1* pyrithiamine resistance selectable marker (19). The plasmid was used to transform RIB40, and pyrithiamine-resistant colonies were obtained. To verify that a deletion of *creB* was obtained, one colony was analyzed using PCR to show that both ends of *creB* were replaced (Fig. 1). To confirm that the phenotype was due to the deletion, the strain was further transformed with a plasmid containing *creB* to produce a complemented strain for use as a control in experiments. To do this, a chlorate-resistant sector was isolated and shown to be a *niaD* mutant, as it was complemented by the *A. nidulans niaD* gene (20). This *creB*Δ;*niaD1* mutant was

TABLE 2 General oligonucleotide primers

Primer	Sequence (5′ → 3′)	Purpose
*creB*_US_F	CGTTCGCTCTCTAACTCCGTC	Amplification of upstream region of *creB* for deletion construct
*creB*_US_R	CCCCATAATTGTCACAAC	Amplification of upstream region of *creB* for deletion construct
*creB*_DS_F	GAGGGATCAGGAAGCGAG	Amplification of downstream region of *creB* for deletion construct
*creB*_DS_R	CCAGCTATGTGACCCAGG	Amplification of downstream region of *creB* for deletion construct
*ptrA*_F	GACGGGCAATTGATTACG	Amplification of *ptrA* for deletion construct
*ptrA*_R	CTATCATGGGGTGACGATG	Amplification of *ptrA* for deletion construct
*creB*_US_R_Fus	CGTATAGATCAGCGGCACCCCATAATTGTCACAAC	Assembly of *creB* deletion construct by fusion PCR
*creB*_DS_F_Fus	CTCATCGTCACCCCATGATAGGAGGGATCAGGAAGCGAG	Assembly of *creB* deletion construct by fusion PCR
*Ao_creB*_US_F2	ACCGCCAATCCACACGTC	Confirmation of replacement of *creB* at upstream end (Fig. 1)
pPTR_for_*creB*_US2	GATAGTGTTGGGGTCCATGC	Confirmation of replacement of *creB* at upstream end (Fig. 1)
*Ao*_DS_*creB*KOtest_F	TATGTAAATGGCTGTGTCCC	Confirmation of replacement of *creB* at downstream end (Fig. 1)
*Ao*_DS_*creB*KOtest_R	ACCGTTCCCAAAACCTG	Confirmation of replacement of *creB* at downstream end (Fig. 1)
*Ao*_β-tub_RT_F_bridge	TTTTGGGATGGAGAATTACG	Semiquantitative RT-PCR of *creB* mRNA levels with respect to β-*tubulin* levels in *A. oryzae* DAR3699
*Ao*_β-tub_RT_R	CTTGAAGAGCTCCTGGATGG	Semiquantitative RT-PCR of *creB* mRNA levels with respect to β-*tubulin* levels in *A. oryzae* DAR3699
*Ao_creB*_RT_F_bridge	ACCTGCTCTGCTATCTTCCG	Semiquantitative RT-PCR of *creB* mRNA levels with respect to β-*tubulin* levels in *A. oryzae* DAR3699
*Ao_creB*_RT_R	GCGAAGTTTTGATAGCGAAG	Semiquantitative RT-PCR of *creB* mRNA levels with respect to β-*tubulin* levels in *A. oryzae* DAR3699

transformed using a plasmid containing both *A. oryzae creB* and *A. nidulans niaD*, and colonies that grew on nitrate were selected and tested.

Growth of *A. oryzae creB*Δ strain. Growth phenotypes of the RIB40 parent strain, the *creB* deletion strain, and the *creB* deletion strain complemented with *creB* were tested under a range of conditions (Fig. 2). The deletion mutant strain grew well on standard minimal media such as Czapek-Dox medium, which contains 3% sucrose as a carbon source, and on richer media such as potato dextrose agar (which contains starch and 2% glucose), albeit with slightly reduced conidiation and mycelial density. The strain also grew robustly in liquid culture: after 48 h, the biomasses of *A. oryzae creB*Δ triplicate spore-inoculated cultures grown in sorbitol, xylan, or xylan plus sucrose were not significantly different from the biomass of the wild-type or complemented strain (Fig. 3A).

The deletion mutant exhibited pleiotropic phenotypes similar to those seen in *A. nidulans*, with commonalities including reduced growth on quinate, proline, or glucuronate as the sole carbon source, reduced growth on nitrate as the sole nitrogen source, enhanced growth on acetamide, resistance to molybdate, and hypersensitivity to high concentrations of acriflavine. In contrast to the case for the *creB* disruptant in *T. reesei*, improved growth was not observed on maltose, likely because secreted α-glucosidase activity would not limit growth of *A. oryzae* RIB40, which was selected for strong starch degradation (21).

Of particular note, the *A. oryzae creB*Δ strain showed greatly enhanced growth on the protein bovine serum albumin, with a slightly broader halo of degraded protein on plates containing milk. Together, these observations indicate increased protease secretion in the *creB*-deleted strain.

RIB40 and the *creB*Δ and complemented strains were germinated on glass coverslips in 1% sorbitol and 10 mM urea liquid medium and examined microscopically after 18 and 24 h. No differences in morphology, hyphal length, or the amount of branching between the strains were apparent. Similarly, when conidia of the three strains were inoculated on solid medium lack-

TABLE 3 Conservation of proteins involved in CCR in *A. nidulans* and *A. oryzae*[a]

Protein	% Identity	% Similarity
CreA	84.7	87.1
CreB	73.5	78.5
CreC	80.1	83.9
CreD	81.7	85.8
AcrB	77.2	81.6

[a] Putative *A. oryzae* orthologues of genes involved in CCR in *A. nidulans* were identified by BLAST searching of the *A. oryzae* genome, and putative amino acid sequences were determined. Orthologous amino acid sequences were aligned using Gap (GCG).

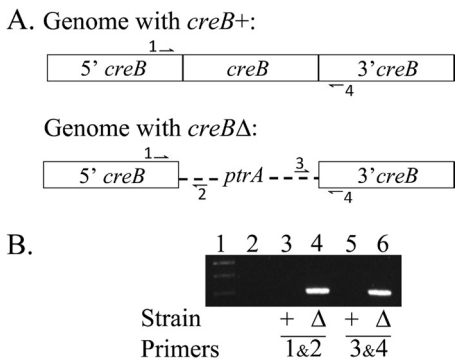

FIG 1 Verification of gene replacement. (A) Schematic showing primer binding sites. Primers (see Table 2 for details): 1, *Ao_creB*_US_F2; 2, pPTR_for_*creB*_US2p; 3, *Ao*_DS_*creB*KOtest_F; 4, *Ao*_DS_*creB*KOtest_R. (B) Agarose gel showing PCR products. Lane 1, molecular size markers; lane 2, blank; lanes 3 to 6, primers and DNA templates for strains, as indicated. +, *creB*$^+$ strain; Δ, *creB*Δ strain.

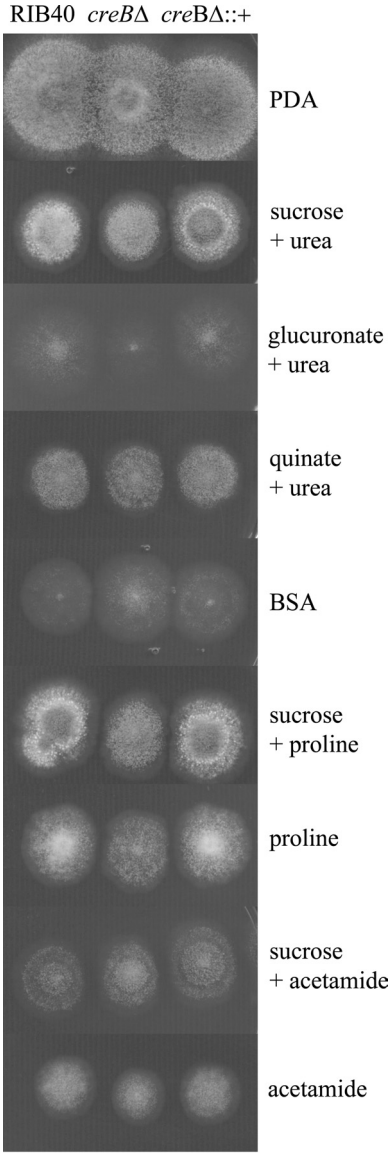

FIG 2 Phenotypic effects of deleting *creB* in *A. oryzae*. The indicated strains were grown for 4 days at 30°C on the indicated media. Sucrose was added at 1%; proline, acetamide, glucuronate, and quinate were added at 50 mM, as carbon sources; urea, proline, and acetamide were added at 10 mM, as nitrogen sources; and bovine serum albumin (BSA) was added at 0.01%. PDA, potato dextrose agar.

ing either a carbon or nitrogen source, the spidery hyphal extensions had similar diameters and were present in similar amounts. When the three strains were grown with shaking in liquid medium for RNA and enzyme analyses (see Materials and Methods), all strains grew as discrete pellets, and thus there was no apparent effect on viscosity due to deletion of *creB*.

Deletion of *creB* alleviates glucose inhibition of conidial germination. *A. oryzae* RIB40 germinates very poorly from conidial spore suspensions on 1% glucose medium and somewhat poorly on 1% sucrose medium compared to medium containing 1% fructose as the carbon source (Fig. 4; Table 4), indicating that glucose inhibits germination. This finding has not previously been reported explicitly, and the reason for it is not understood. This inhibition is not specific to the RIB40 strain, as it was also seen in *A. oryzae* NBRC 30105. Inhibition was not detected in the presence of a low concentration of glucose (0.1%) or on rich medium containing 1% glucose together with yeast extract, peptone, and amino acids (data not shown). The inhibition of conidial germination was abolished in the *creB*Δ strain (Fig. 4; Table 4).

Deletion of *creB* increases expression of cellulases and xylanases under inducing conditions. Preliminary experiments in RIB40 found that 1% sorbitol can be used as a carbon source that is neither an inducer nor a repressor of cellulases, amylases, and xylanases and that 2% sucrose causes repression of these enzymes. Subsequently, triplicate spore-inoculated 50-ml shake flask cultures of the three strains were grown under noninducing (1% sorbitol), inducing (1% xylan), and repressing (1% xylan plus 2% sucrose) conditions. After 48 h, total secreted cellulase activities were measured using EnzChek cellulase substrate and are expressed as activities per unit of dry weight biomass, normalized such that activity in the wild-type strain under inducing conditions was 100 units (Fig. 3B). Activity was barely detectable for all three strains under noninducing conditions, and the three strains' activities did not differ significantly. Under repressing conditions, activities were low but detectable and did not differ significantly between the three strains. Activities were over 2 orders of magnitude higher under inducing conditions. The wild-type and complemented strains' activities were not significantly different, whereas the *creB*Δ strain had significantly greater activity ($P < 0.05$), with the mean for the *creB*Δ strain cultures being 50% higher than that for the wild-type strain.

The same cultures were also analyzed for total secreted xylanase activity, using EnzChek xylanase substrate, and the results are expressed and normalized in the same manner as that described above (Fig. 3C). The results mirrored those for the cellulase assays, consistent with reports that cellulases and xylanases are regulated similarly in *A. oryzae* (22). Activities were barely detectable in all three strains under noninducing conditions, and the three strains' activities did not differ significantly. Under repressing conditions, activities were low but detectable and did not differ significantly between the three strains. Activities were over 3 orders of magnitude higher under inducing conditions. The wild-type and complemented strains' activities were not significantly different, whereas the *creB*Δ strain had significantly greater activity ($P < 0.05$), with the mean for the *creB*Δ strain cultures being almost double that for the wild-type strain.

Thus, deletion of *creB* increases the expression of cellulases and xylanases in the absence of CCR but does not affect the response to CCR.

Deletion of *creB* increases expression of amylases under various conditions via an increase in gene transcription. Triplicate spore-inoculated 50-ml shake flask cultures of the three strains were grown under noninducing (1% sorbitol), inducing (1% sorbitol plus 1% starch), and repressing (1% sorbitol plus 1% starch plus 2% sucrose) conditions. After 48 h, total secreted amylase activities were measured using EnzChek amylase substrate and are expressed as activities per unit of dry weight biomass, normalized such that activity in the wild-type strain under inducing conditions was 100 units (Fig. 5A).

Under all three growth conditions, the activity of the *creB*Δ strain was significantly higher than that of the wild-type or complemented strain ($P < 0.001$ for noninducing conditions, $P < 0.01$ for inducing conditions, and $P < 0.001$ for repressing conditions).

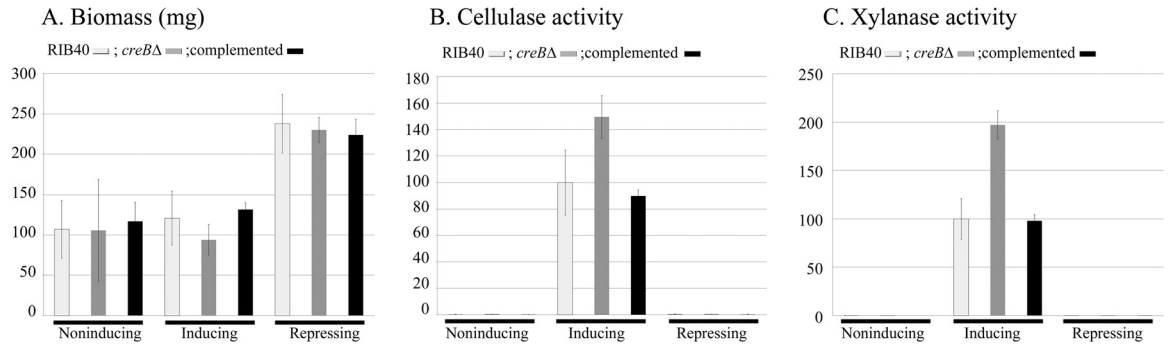

FIG 3 Deletion of *creB* increases secretion of cellulases and xylanases under inducing conditions. Triplicate spore-inoculated 50-ml shake flask cultures of the three strains were grown under noninducing (1% sorbitol), inducing (1% xylan), and repressing (1% xylan plus 2% sucrose) conditions. After 48 h, biomass and supernatants were harvested, and total secreted enzyme activities were measured using EnzChek cellulase substrate or EnzChek xylanase substrate and are expressed as activities per unit of dry weight biomass, normalized such that activity in the wild-type strain under inducing conditions was 100 units. (A) Growth in liquid medium. (B) Total secreted cellulase activity. (C) Total secreted xylanase activity.

The activities of the wild-type and complemented strains were not significantly different from one another under any growth condition. The *creB*Δ strain produced readily detectable activity under noninducing conditions, with more than double the wild-type activity under inducing conditions and 40-fold more than the wild-type activity under repressing conditions.

The addition of 2% sucrose reduced amylase activities in the wild-type and complemented strains ($P < 0.01$) about 35-fold. It also reduced amylase activities in the mutant strain, but only by one-third. This indicates a high level of carbon catabolite derepression of one or more genes encoding starch-degrading enzymes in the mutant strain.

To investigate the molecular basis of these observations, glucoamylase (*glaA*) and α-amylase (*amyA*, *amyB*, and *amyC*) transcript levels in the wild-type and *creB*Δ strains were measured under all three growth conditions, using quantitative real-time PCR. Representative results from three independent experiments are shown in Fig. 5. There are three genes for secreted α-amylase in *A. oryzae* RIB40, with almost identical nucleotide sequences, which are all expressed (23, 24); our measurements indicate the total transcript levels of these three genes combined. The transcript levels of both glucoamylase (Fig. 5B) and α-amylase (Fig. 5C) were significantly higher ($P < 0.01$) in the *creB*Δ strain than in the wild-type strain under all three growth conditions, reflecting the higher secreted total amylase activities observed. Thus, *creB* deletion increases total secreted amylase activity by increasing transcript levels of multiple amylase-encoding genes.

The finding that deletion of *creB* in *A. oryzae* elevates the levels of glucoamylase and α-amylase transcripts and almost abolishes repression led us to look at an *A. nidulans* example, as the effects of *creB* on transcription have not been published. We chose *alcA*, encoding alcohol dehydrogenase I, as it has been well characterized at the plate test and enzyme activity levels and shows derepressed expression in medium containing both an inducer and a repressor (3). To investigate the molecular basis of this derepressed expression, transcript levels of the wild-type and *creB1937* null strains grown under uninduced, ethyl methyl ketone (EMK)-induced, and EMK-plus-glucose-repressed conditions were measured using quantitative real-time PCR. Representative results from three independent experiments are shown in Fig. 5D. The data were assessed for the effects of *creB1937* on the elevation of transcript levels, the inducibility of the system, and derepression. The transcript levels in the uninduced cultures showed that there was very low basal transcription, with no evidence of elevation of *alcA* transcription due to *creB1937*. The transcript levels increased significantly ($P < 0.001$) in the induced samples compared to the uninduced levels, with the increase being greater in the *creB1937* strain than in the wild-type strain. While the strains induced with EMK showed very large elevations in transcription of *alcA*, there

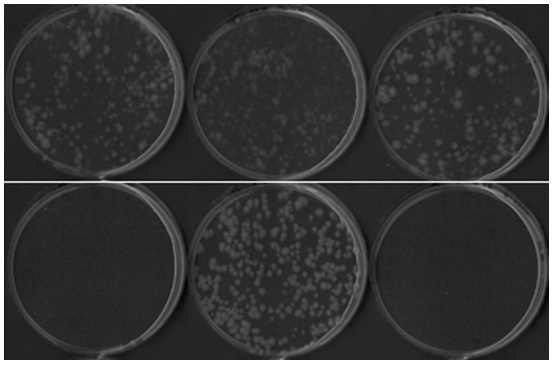

FIG 4 Inhibition of germination of *A. oryzae* by glucose. Strain RIB40, the *creB*Δ strain, and the *creB*Δ::*creB*$^+$ strain (left to right) were grown from 500 spores for 2 days at 30°C on 1% fructose (top) or 1% glucose (bottom).

TABLE 4 Deletion of *creB* alleviates inhibition of spore germination in the presence of repressing carbon sources[a]

Carbon source	Spore germination[b]		
	Wild-type strain	*creB*Δ strain	Complemented strain
1% fructose	+++	+++	+++
1% sucrose	++	+++	++
1% sucrose + 1% glucose	+	+++	+
1% glucose	—*	+++	—*
0.1% glucose	+++	+++	+++

[a] Five hundred spores were spread on agar plates containing the indicated carbon sources as described in Materials and Methods.
[b] Symbols indicate the numbers of colonies visible after 2 days of incubation. —, 0 colonies; +, 2 colonies; ++, 75 to 80 colonies; +++, >200 colonies. Similar trends were observed on plates inoculated with 5,000 spores (data not shown). * indicates colonies present at higher-density plating.

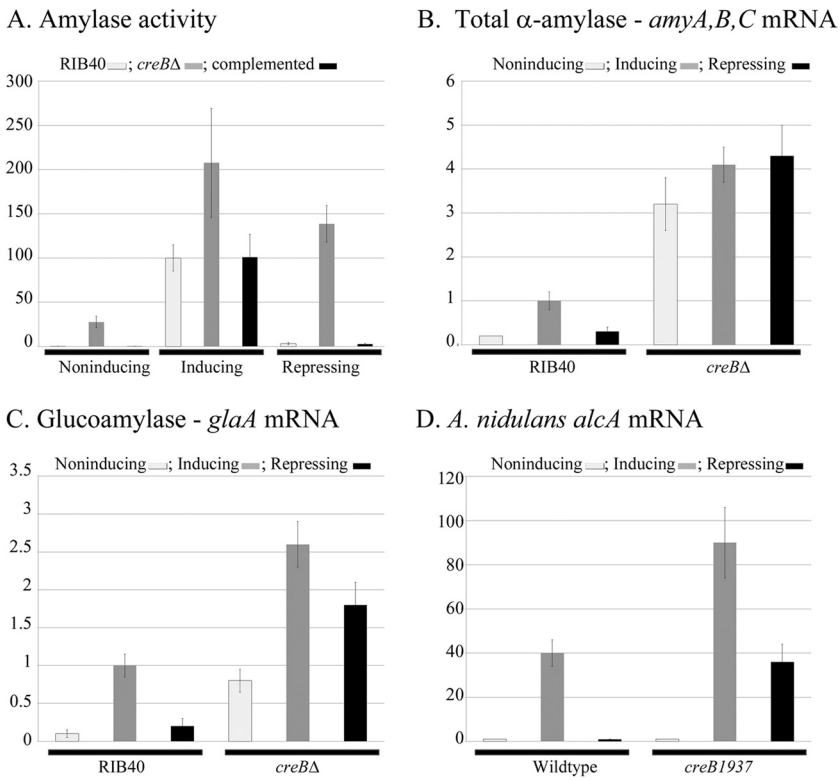

FIG 5 Deletion of *creB* increases secretion of amylases under various conditions via an increase in gene transcription. (A) Triplicate spore-inoculated 50-ml shake flask cultures of the three strains were grown under noninducing (1% sorbitol), inducing (1% sorbitol plus 1% starch), and repressing (1% sorbitol plus 1% starch plus 2% sucrose) conditions. After 48 h, biomass and supernatants were harvested, and total secreted amylase activities were measured using EnzChek amylase substrate and are expressed as activities per unit of dry weight biomass, normalized such that activity in the wild-type strain under inducing conditions was 100 units. (B) qRT-PCR analysis of total α-amylase transcript levels in *A. oryzae* RIB40 and the *creB*Δ strain. (C) qRT-PCR analysis of glucoamylase A transcript levels in *A. oryzae* RIB40 and the *creB*Δ strain. Strains were induced using starch (1%) or repressed using sucrose (2%) and were grown at 30°C for 24 h. Transcript levels were standardized against β-tubulin levels. (D) qRT-PCR analysis of *alcA* transcript levels in *A. nidulans* wild-type and *creB1936* strains. Strains were induced using EMK (50 mM) or repressed using glucose (1%) and were grown at 37°C for 16 to 18 h. Transcript levels were standardized against β-tubulin levels. Results shown are fold changes compared to the wild type induced with ethanol.

was a significant decrease ($P < 0.001$) when a repressor was added. In the wild type, this decrease was to the uninduced level; however, in the *creB1937* strain, there remained, on average, a 20-fold increase in *alcA* transcript levels compared to that in the uninduced culture. These experiments were replicated using ethanol rather than EMK as the inducer of *alcA*, and the absolute levels of induction were reduced as expected, but the same results regarding elevation and repression were apparent. Thus, in *A. nidulans*, *creB1937* leads to both elevation and partial derepression of *alcA* transcription.

***A. oryzae* DAR3699 has a phenotype similar to that of RIB40 *creB*Δ.** DAR3699, an *A. oryzae* strain from the CSIRO collection, was originally isolated during soy fermentation. Previous analyses found it to secrete high levels of amylase, to have a good growth rate, and to grow as compact pellets suitable for biomass production (14). As these properties make it suitable for bioreactor use, we included it in our phenotypic tests. DAR3699 showed phenotypes that were similar to those of the RIB40 *creB*Δ strain, including molybdate resistance, strong growth on acetamide, weak growth on proline, quinate, and arabinose, and high protease secretion. As indicated above, glucose inhibited the germination of RIB40 spores, but *creB* deletion abolished this inhibition; this inhibition was also absent in DAR3699. Furthermore, when we deleted *creB* in the DAR3699 strain, the phenotype was unchanged from that of DAR3699. These phenotypes indicated that DAR3699 might be a *creB* mutant strain and that this might contribute to its useful properties. We sequenced 8 kb of genomic DNA covering at least 200 bp upstream and 100 bp downstream of the *creA*, *creB*, and *creC* loci and found no differences between RIB40 and DAR3699 for *creA* and *creC*. The DAR3699 *creB* locus contains a single base pair insertion in a putative upstream open reading frame, lengthening it to 46 codons (Fig. 6). To test whether this change led to reduced transcription, RNAs were extracted from RIB40 and DAR3699 grown as surface colonies on liquid medium containing 1% glucose, and primers that amplify *creB* or β-*tubulin* were used in semiquantitative RT-PCRs. No reduction was detected in the amount of *creB* mRNA in DAR3699 compared with RIB40; thus, if the insertion in DAR3699 affects CreB levels, the effects are likely to be at the translational level. This suggestion is supported by findings in *Saccharomyces cerevisiae* which have shown that translation efficiency decreases markedly as upstream open reading frame length is increased, with 36 codons reducing translation by about 95% (25).

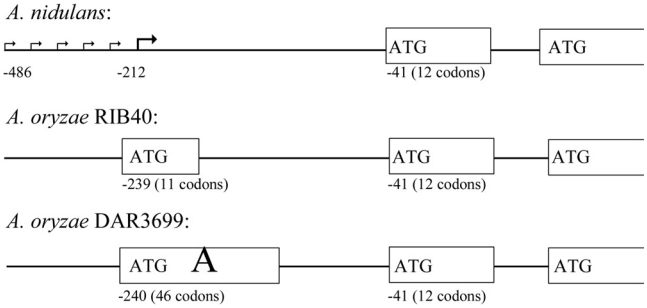

FIG 6 Analysis of DAR3699 *creB* locus. The schematic shows a representation of the region at the 5′ end of the *creB* gene in *A. nidulans*, *A. oryzae* RIB40, and *A. oryzae* DAR3699 (not to scale). The top line represents the 5′ region of *A. nidulans creB*, showing the mapped start points of transcription (6) and an upstream open reading frame of 12 codons that is conserved in *A. oryzae*. The middle line represents the 5′ region of *A. oryzae* RIB40 *creB*, showing a putative 11-codon upstream open reading frame spanning the site that is the major start point of transcription in *A. nidulans*. The bottom line represents the 5′ region of *A. oryzae* DAR3699 *creB*, showing the effect of the insertion of one base pair into the upstream open reading frame sequence, lengthening it to 46 codons.

DISCUSSION

A. oryzae is used widely in the sake and soy brewing industries, as it produces and secretes a variety of amylases, cellulases, and proteases to break down carbohydrates and proteins in rice, wheat, and soybean to produce nitrogen and other nutrients, and these enzymes are also used to accelerate hydrolysis of substrates in fish sauce fermentation (1). Production strains have been selected over centuries for enhanced properties, but further improvements are possible.

Many of the genes encoding industrially important enzymes are not expressed when a good carbon source is available, due to the transcriptional control mechanism of CCR. For example, the gene expression profiles of *A. oryzae* cells were analyzed for mycelia grown in glucose-rich and glucose-poor media. A key finding was that cultures grown with wheat bran mimicked the glucose-depleted cultures and showed a diverse gene expression profile for hydrolytic enzymes, most probably due to a relaxation of CCR (18). Thus, an important approach to strain development is to perturb CCR in production strains, using information from model fungi and techniques that allow precise gene manipulations, to increase production of useful enzymes. Gene targeting has advantages over random mutagenesis, particularly in introducing multiple desired changes in organisms where genetic crosses cannot be undertaken due to the lack of a sexual cycle. Even though functional mating type genes were recently identified in *A. oryzae* (26), because production strains have a range of lesions in their genomes, molecular rather than meiotic approaches will still be preferable to maintain the overall phenotypes.

In *A. nidulans*, deletion of *creA*, encoding the major CCR repressor, has severe effects on morphology, so to avoid these effects, we chose to disrupt the *A. oryzae creB* gene. In *A. nidulans*, the regulatory deubiquitinating enzyme CreB is proposed to be involved in carbon metabolism by two possibly distinct mechanisms (3, 4). First, under carbon catabolite-repressing conditions, null alleles lead to derepressed expression of enzymes, including alcohol dehydrogenase I and acetamidase. Second, on a range of carbon sources generally not considered to be repressing, null alleles show altered growth, such as increased growth on maltose and decreased growth on D-quinate. Complete details of the mechanism of action of CreB are not fully understood, but the reduced growth on compounds such as quinate and proline is due to a direct targeting of permeases by CreB that is required to prevent premature turnover (27; N. Kamglangdee and J. M. Kelly, unpublished data). We demonstrate here that when *A. oryzae* lacks the CreB deubiquitinase, it produces higher levels of amylase, cellulase, xylanase, and protease activities. For cellulase and xylanase, these increased levels were apparent when no repressing carbon source had been added to the medium but not when a repressing carbon source was exogenously added, whereas amylase expression was increased under both conditions. Thus, we propose that *A. oryzae* CreB is involved not only in derepression of some enzymes but also in the expression of some enzymes even in the absence of CCR, consistent with observations in *A. nidulans* (3–6). The molecular basis of the *creB* deletion derepression phenotypes is not understood for any organism, so to determine whether there are effects on transcription, glucoamylase and α-amylase transcript levels were compared between the wild-type and *creB*Δ strains, and enzyme activity changes were found to be mirrored at the transcript level. This finding is significant, and because there are no published data about the transcriptional effects of *creB* mutations in *A. nidulans*, we examined *alcA* expression. *alcA* encodes alcohol dehydrogenase I, has been well characterized at the plate test and enzyme activity levels, and shows derepressed activity in medium containing both an inducer and a repressor (3). Comparisons between the wild-type and *creB*Δ strains showed that enzyme activity changes were mirrored at the transcript level in this organism as well. Importantly, this evidence indicates that the increased amount of expression, at least for the systems tested, has a transcriptional component and thus is likely due to a direct involvement of CreB in CCR rather than a consequence of the effects of *creB* on permeases (27). Although effects on permeases may alter intracellular inducer levels for some systems, e.g., at least in the case of ethanol induction of *alcA* in *A. nidulans*, no permease is required.

During our phenotypic analysis of the mutant strain, we found that glucose and, to a lesser degree, sucrose inhibit germination of *A. oryzae* RIB40 conidia. This inhibition of conidial germination was abolished in the *creB*Δ strain. Although we do not understand the molecular basis of this observation, this may provide a novel way of selecting new mutant strains: if spores are subjected to mutagenesis and then germinated in glucose-containing medium, the population of spores that regenerate is likely to be enriched for mutants with phenotypes overlapping those of the *creB*Δ strain. Furthermore, the germination of the *creB*Δ strain in the presence of glucose may be useful in industrial processes in which the preculture or primary fermentation is inoculated with conidia.

A. oryzae DAR3699 was already known to have many valuable properties for biotechnological use. Among 15 fungal strains tested, it had the highest protein content and shared the highest specific growth rate in starch processing wastewater, as well as having strong secreted amylase activity and a compact pellet morphology ideal for use in a bioreactor (14). In a pilot plant-scale air lift bioreactor, the strain efficiently converted carbon and other nutrients in starch processing wastewater into protein-rich fungal biomass (28). Yet little is known of the genetics of this strain. We have shown that *A. oryzae* DAR3699 has a mutation in the promoter of its *creB* gene; that it has many phenotypes consistent with loss of *creB* function, including strong growth on acet-

amide, weak growth on proline, quinate, or arabinose, resistance to molybdate, high protease secretion, and little or no inhibition of spore germination by glucose; and that deleting *creB* in this strain does not change the phenotype. *A. oryzae* arose through the ancient domestication of *Aspergillus flavus* and has undergone genetic changes during its centuries of use in Asian food and beverage production (29). Our data suggest that for *A. oryzae* DAR3699, one of these genetic changes is likely to have been partial or complete loss of *creB* function. This change may have been selected for the consequent increase in protease secretion, as this strain is used in soy fermentation, which is initially limited by low levels of free nitrogen.

In addition to being mutated in the model fungus *A. nidulans* (3), *creB* has now been mutated in three industrially useful fungi, diverse among the Ascomycetes: *A. oryzae*, *P. decumbens* (13), and *T. reesei* (12). In every case, loss of *creB* function has resulted in increased activities of multiple secreted hydrolases of industrial importance. Furthermore, we have shown that *A. oryzae* DAR3699, a strain useful in both solid-state food production and industrial wastewater treatment, is likely to have lost *creB* function. Taken together, these findings indicate that deletion of *creB* homologues may be broadly useful in a variety of fungi for producing a range of enzymes.

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Contribution to the Paper	Planned all experiments, performed bioreactor experiments presented in paper interpreted data, wrote the manuscript.
Overall percentage (%)	70%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

iv. the candidate's stated contribution to the publication is accurate (as detailed above);

v. permission is granted for the candidate to include the publication in the thesis; and

vi. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Name of Co-Author	Christopher Saint
Contribution to the Paper	Evaluated and edited the manuscript, acted as corresponding author
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Behaviour of an *Aspergillus oryzae* strain with disrupted carbon catabolite repression in a mixed carbon source fermentation

Abbreviated running headline: Mixed carbon source fermentation

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SIGNIFICANCE AND IMPACT OF THE STUDY

Carbon catabolite repression (CCR) is often undesirable in industrial bioprocesses as it can limit the production of useful enzymes. Deletion of *creB* is known to alleviate CCR and increase enzyme secretion in filamentous fungi, potentially stimulating bioconversion. However, *creB* deletion has not been investigated in the context of industrial fermentation. This is the first report of a *creB*-deleted strain grown in a bioreactor. In synthetic winery wastewater, *creB*-deleted *Aspergillus oryzae* retained the robust growth and bioconversion capabilities of its parent, as well as the desirable pellet morphology that enables optimized harvesting of this protein-rich biomass.

ABSTRACT

The filamentous fungus *Aspergillus oryzae* is used in food and beverage production as a source of hydrolytic enzymes. Deletion of *creB,* encoding a deubiquitinating enzyme, has previously been shown to increase enzyme production and reduce carbon catabolite repression. We grew wildtype and *creB*-deleted strains in a bench-scale stirred tank bioreactor. In these fermentation trials, the strains were grown in media based on wastewater produced by Australian wineries containing glucose, fructose, and ethanol. Supplementation with 5 mmol l^{-1} urea enabled rapid consumption of the sugars. Both strains showed a strong preference for consuming the glucose first, with appreciable consumption of fructose beginning only when glucose concentrations were reduced to around 0.7 g l^{-1}. Consumption of ethanol was detectable only after the fructose concentration was similarly reduced. The overall patterns of carbon source consumption, growth and morphology were similar in the two strains.

KEYWORDS

Aspergillus oryzae, bioreactor, carbon catabolite repression, *creB*, glucose, mixed carbon source

Introduction

In the presence of glucose or other readily metabolized carbon sources, most micro-organisms reduce the expression of genes encoding enzymes for the utilization of complex or non-preferred carbon sources. This reduced expression is called carbon catabolite repression (CCR), and its targets include genes encoding many enzymes important in biotechnology such as amylases, cellulases and xylanases. Overcoming or circumventing CCR is therefore a key objective in optimizing many industrial fermentation processes.

The major regulator of CCR in filamentous fungi is *creA*, which encodes a DNA-binding transcriptional repressor (Dowzer and Kelly 1991). The *creA* gene has been studied in many species, and its disruption leads to increased expression of many genes affected by CCR, but also to impaired growth and aberrant morphology (Hynes and Kelly 1977). Less well studied is *creB*. In the model fungus *Aspergillus nidulans*, mutations in *creB* have a milder effect on growth than *creA* mutations and cause pleiotropic effects on carbon metabolism including alleviation of CCR in a subset of those genes affected by *creA*, as well as increased production of some enzymes (Hynes and Kelly 1977). The

creB gene encodes a deubiquitinating enzyme that works in a complex with CreC, a WD40 repeat-containing protein (Lockington and Kelly 2001; Lockington and Kelly 2002). The only protein known to be targeted by CreB is QutD, a cell membrane quinate permease (Kamlangdee 2008). CreA is not a direct target of CreB (Alam *et al.* 2017). Proteins that co-purify with CreB tend to be involved in cellular materials transport and organization (Alam and Kelly 2017). Among fungi used in industry, *creB* has been disrupted in *Trichoderma reesei* and *Penicillium decumbens*, and this increased cellulase production in both species (Denton *et al*. 2011; Zhou *et al*. 2012).

Aspergillus oryzae is a domesticated filamentous fungus that has been used in East Asia for centuries for fermenting soy sauce, *sake*, and other foods and beverages. The generation and properties of *A. oryzae creBΔ* have been described (Hunter *et al*. 2013). Growth is slightly reduced on solid media but no difference in growth was detected in shake flasks. The *creBΔ* strain secretes higher levels of cellulases and xylanases than its parent in inducing conditions, and higher levels of amylases in non-inducing, inducing, and repressing conditions. It also secretes higher levels of proteases and, unlike wildtype *A. oryzae*, its spores germinate efficiently in the presence of high concentrations of glucose.

Winery wastewater is produced in large quantities and is rich in glucose, fructose, and ethanol (reviewed in Mosse *et al*. 2011). The high carbon content makes treatment of winery wastewater by conventional means costly. *A. oryzae* shows potential for treating winery wastewater in an environmentally friendly manner, by consuming its organic

materials and converting them into protein-rich fungal biomass (Zhang *et al*. 2008). This biomass could be used as animal feed, helping to off-set the operating costs of the treatment process as well as extracting value from a nutrient-rich resource that is usually lost as waste. Here we report growing *A. oryzae* wildtype and *creBΔ* strains in synthetic winery wastewater in shake flasks and in a laboratory scale stirred tank bioreactor. Our aims are to compare the *creBΔ* strain to its parent and to investigate the behavior of both strains in a mixed carbon source fermentation.

Results and discussion

Before progressing to bioreactor experiments, we performed preliminary investigations of the growth of *A. oryzae* in synthetic winery wastewater in shake flasks (results not presented). As real winery wastewater is highly variable, both throughout the year and even hour by hour (reviewed in Mosse *et al*. 2011), we designed synthetic winery wastewater to ensure consistency between experiments (Table 1). Doubling the concentration of salt and trace element solution in synthetic winery wastewater supplemented with 3 g l^{-1} $(NH_4)_2SO_4$ did not improve growth of *A. oryzae* after 72 hours, indicating the concentrations of salts and trace elements are not limiting in these conditions. Growth of *A. oryzae* after 48 h was the same in synthetic winery wastewater as in modified synthetic winery wastewater lacking ethanol, indicating *A. oryzae* is not inhibited by ethanol at a concentration typical for South Australian winery wastewater. *A. oryzae* grew well in synthetic winery wastewater supplemented with 3 g l^{-1} $(NH_4)_2SO_4$ at 30, 34, and 37 °C, without large differences in carbon content reduction or biomass

accumulation between temperatures. Both wildtype and *creBΔ* strains grew well at 30 °C in synthetic winery wastewater supplemented with 3 g l^{-1} $(NH_4)_2SO_4$ and 10 mmol l^{-1} citric acid as buffer and set to pH 3.0, 4.0, 5.0, or 6.0, again without large differences in carbon content reduction or biomass generation. Thus *A. oryzae* grows robustly in synthetic winery wastewater in a broad range of temperature and pH conditions. 30 °C and pH 6.0 were chosen as conditions for further experiments.

Growth of both *A. oryzae creBΔ* and its parent was poor in a stirred-tank bioreactor containing synthetic winery wastewater without nitrogen supplementation, which was expected as real winery wastewater is deficient in nitrogen (Chapman 2001). Therefore wildtype *A. oryzae* was grown in duplicate shake flasks containing synthetic winery wastewater supplemented with 0–20 mmol l^{-1} urea. Based on sugar consumption during 48 h incubation, 5 mmol l^{-1} urea supplementation was found to be optimal (Figure 1); additional urea did not markedly improve sugar consumption. Therefore 5 mmol l^{-1} urea supplementation was used in subsequent bioreactor experiments.

Results for two 48-h fermentations with each strain are shown in Figure 2. Glucose was the preferred carbon source for both strains, and there was little consumption of fructose before glucose concentrations decreased to approximately 0.7 g l^{-1}. Fructose concentrations were reduced to undetectable levels by each strain in at least one bioreactor run, whereas glucose concentrations tended to stabilize at 0.1–0.2 g l^{-1}. Early in the fermentations, ethanol was lost at a gradual and constant rate due to evaporation rather than consumption. After 36–40 hours, sugar concentrations were low, and ethanol

concentrations began decreasing more rapidly due to consumption in addition to evaporation. The performance of the two strains appeared similar. Both strains consumed over 90% of the sugars and much of the ethanol after 48 h, and grew as white fluffy pellets (Figure 3). This is a desirable morphology as the fluffiness presents a high surface area of mycelium in contact with the substrate. Importantly, the pellet form of fungal biomass can lead to low viscosity of the fermentation broth, which is beneficial for enhancing energy and mass transfer, and consequently the fungal growth and production rates. Furthermore, pellets can be easily and cheaply separated from the substrate by filtration (Jin *et al*. 2002).

To more closely analyze CCR in the fermentations shown in Figure 2, concentrations of non-preferred carbon sources (fructose and ethanol) were plotted as functions of concentrations of preferred carbon sources (either glucose or total sugars, meaning glucose plus fructose) in Figure 4. These graphs eliminate time as a variable, reducing the influence of experimental variation between repeat fermentations. If there were hypothetical perfect CCR, these graphs would run directly left from the starting point in the top-right corner of the plot, indicating complete consumption of the preferred carbon source before any consumption of the non-preferred carbon source. In the complete absence of CCR, given equal starting concentrations of carbon sources, the graphs would be linear from the starting point to the origin, indicating simultaneous utilization of carbon sources. Figure 4 (a) shows that glucose is preferred over fructose to the same degree in wildtype and in *creBΔ* strains. Thus there is a mechanism of glucose repression of fructose consumption that is independent of *creB*. The mechanism is likely to involve

157 *creA*, as deletion of the homologue of *creA* in *Saccharomyces cerevisiae*, *mig1*, causes
158 glucose to be consumed simultaneously with otherwise non-preferred sugars maltose
159 (Klein *et al*. 1997) and sucrose (Klein *et al*. 1999). Figures 4 (b) and (c) show that CCR
160 of ethanol consumption appeared to be modestly reduced in the *creBΔ* strain, indicated by
161 graphs slightly closer to the center of the plots than those of the wildtype strain. Greater
162 consumption of ethanol in the presence of sugars in *A. oryzae creBΔ* is consistent with
163 our data about mRNA levels in *A. nidulans* (Hunter *et al*. 2013). In *A. nidulans*, the
164 major enzyme for ethanol utilization is alcohol dehydrogenase I, encoded by *alcA* which
165 is subject to CCR (Lockington *et al.* 1985). Mutation of *creB* in *A. nidulans* strongly
166 derepresses *alcA* expression, permitting levels of *alcA* mRNA in the presence of glucose
167 20-fold higher than in a $creB^{+}$ strain, similar to the levels seen in the $creB^{+}$ strain in the
168 absence of glucose (Hunter *et al*. 2013). *A. oryzae* is also known to have a catabolic
169 alcohol dehydrogenase gene, *adhB*, which is subject to glucose repression (Maeda *et al.*
170 2004). Thus *creB* is involved in CCR of ethanol catabolism in both *A. nidulans* and *A.*
171 *oryzae*.
172
173 Some fungi, including the ascomycete *Candida boidinii* (GenBank: AFI55138.1),
174 produce glucose isomerase (xylose isomerase, EC 5.3.1.5), which can interconvert
175 glucose and fructose. To investigate whether *A. oryzae* might have produced glucose
176 isomerase during the experiments described above, wildtype and *creBΔ* strains were
177 grown in separate shake flasks containing synthetic winery wastewater supplemented
178 with 5 mmol l^{-1} urea, but lacking either glucose or fructose. After 24, 52, 72, or 96 h
179 incubation, no converted sugar could be detected (results not shown). Thus neither *A.*

oryzae strain produced detectable glucose isomerase activity when grown in modified synthetic winery wastewater.

To our knowledge, this is the first report of a *creB*-deleted fungal strain grown in a bioreactor. The strain performed well, with similar morphology and carbon source consumption to its parent. This contrasts with *creA*-deleted strains, which have aberrant morphology and reduced growth rate in *A. nidulans*, not only on solid media but also in batch bioreactor cultures (Agger *et al*. 2001; David *et al.* 2005). Deletion of *creB* slightly reduces growth on solid media in *A. nidulans* (Hynes and Kelly 1977), *T. reesei* (Denton *et al*. 2011), and *A. oryzae* (Hunter *et al*. 2013), but was not found to reduce growth in *A. oryzae* grown in shake flasks (Hunter *et al*. 2013). In this study we observed no inhibition of growth in the *creB*-deleted strain, either in shake flasks or in the bioreactor. Thus *creB*-deleted strains may be particularly useful in liquid-phase industrial fermentations in which fungal growth is beneficial, particularly in processes in which increased enzyme secretion is desirable. Deletion of *creB* may further enhance strains already possessing beneficial mutations. For example, an *A. oryzae creAΔ /creBΔ* double mutant was found to have superior secreted α-amylase activity to strains possessing either mutation alone (Ichinose *et al*. 2014).

Materials and Methods

Strains

A. oryzae RIB40 (also called ATCC42149 or NBRC 100959), obtained from the NITE Biological Resource Center, was used as the wildtype strain in this study. The generation of *A. oryzae* RIB40 *creBΔ* was described in Hunter *et al.* (2013).

Media

Synthetic winery wastewater was designed to represent wastewater from a typical South Australian winery during vintage. The composition is given in Table 1.

Spores were harvested from spread plates containing *Aspergillus* nitrogen-free medium (Todd *et al.* 2007) with 2.2% agar, 10 mmol l^{-1} urea, and 0.1 mol l^{-1} KCl to promote extensive sporulation (Song *et al.* 2001).

Shake flask experiments

All shake flask experiments were performed with 50 ml cultures in 250 ml Erlenmeyer flasks, with the rubber seal removed from the caps and the caps sitting loosely on the flasks to facilitate aeration. Inoculated flasks were incubated at 30°C unless otherwise indicated, with shaking at 150 rpm. As *A. oryzae* can respond to light (Hatakeyama *et al.* 2007), a glass-fronted incubator was used, and fluorescent indoor lighting was left on throughout incubation to maintain approximately constant ambient light.

Bioreactor experiments

The bioreactor used was a BioFlo III 3.3-l stirred tank reactor with a 2.5 l working volume (New Brunswick Scientific, USA). To produce the inoculum, 0.01% Tween 20

was used to harvest spores from a spread plate inoculated 5 days prior, and the suspension was pelleted by centrifugation, resuspended in fresh 0.01% Tween 20 in a 20 ml McCartney bottle, and vortexed vigorously to break up spore clumps. Spore concentration was determined using a haemocytometer, and 2×10^8 spores in 10 ml 0.01% Tween 20 were added to 50 ml synthetic winery wastewater supplemented with 5 mmol l^{-1} urea in a 250 ml Erlenmeyer flask. This spore suspension was incubated for 6 h as described for shake flask experiments, producing a pre-culture with a high concentration of very fine pellets.

After autoclaving the bioreactor, glucose, fructose and ethanol were added from a single filter-sterilized stock solution. Reactor conditions were: temperature, 30 °C; stirring rate, 200 rpm; aeration rate, 0.5 v v^{-1} min^{-1}; pH, 6.0 controlled with 1 mol l^{-1} H_2SO_4 and 2 mol l^{-1} NaOH. A baffle was not used as preliminary experiments found too much fungal growth stuck to the baffle; the sensors and sampling ports appeared to introduce sufficient turbulence. The condenser was connected to a recirculating ice water bath to minimize loss of ethanol due to evaporation. Fluorescent indoor lighting was left on throughout inoculum incubation and bioreactor experiments as for the shake flask experiments.

Analytical methods

To measure concentrations of sugars or ethanol, culture samples were centrifuged at 10,000 g for 5 min and the supernatants diluted 5 or 10 times and filtered using 0.45 µm filters. Concentrations were measured with a Varian Pro Star HPLC machine, with a

Rezex ROA organic acid column (300 × 7.8 mm, Phenomenex, Australia), refractive index detector (Model 350, Varian, Australia), 4 mmol l^{-1} H_2SO_4 mobile phase, 65 °C column temperature and 0.6 ml min^{-1} flow rate.

To measure biomass, contents of shake flasks were vacuum-filtered through 55-mm filter paper circles (Whatman) that had been pre-weighed after drying at 65 °C. Filters with biomass were then washed with 200 ml reverse osmosis-purified water, dried at 65 °C to constant mass, and weighed.

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Conflict of interest

No conflict of interest declared.

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360 **Table 1 Composition of synthetic winery wastewater used in this study**. The

361 composition is based on wastewater from South Australian wineries during vintage.

362 Total ammonia nitrogen is from Zhang *et al*. (2008). Other characteristics are from

363 Chapman *et al*. (2001).

Typical composition of winery wastewater	**Composition of synthetic winery wastewater**
Glucose 2500 mg l^{-1}	Glucose 2500 mg l^{-1}
Fructose 2500 mg l^{-1}	Fructose 2500 mg l^{-1}
Ethanol 2400 mg l^{-1}	Ethanol 2500 mg l^{-1}
Total Kjeldahl nitrogen 34–60 mg l^{-1}	Proteose peptone 250 mg l^{-1} (= total Kjeldahl nitrogen 40 mg l^{-1})
Total ammonia nitrogen 6.0–21.1 mg l^{-1}	Ammonium sulphate 0.25 mmol l^{-1} (= 9 mg l^{-1} ammonium)
	1× Salt and trace element solution (Todd *et al.* 2007)
pH 4–8	pH to 6.0 using concentrated NaOH

364

365

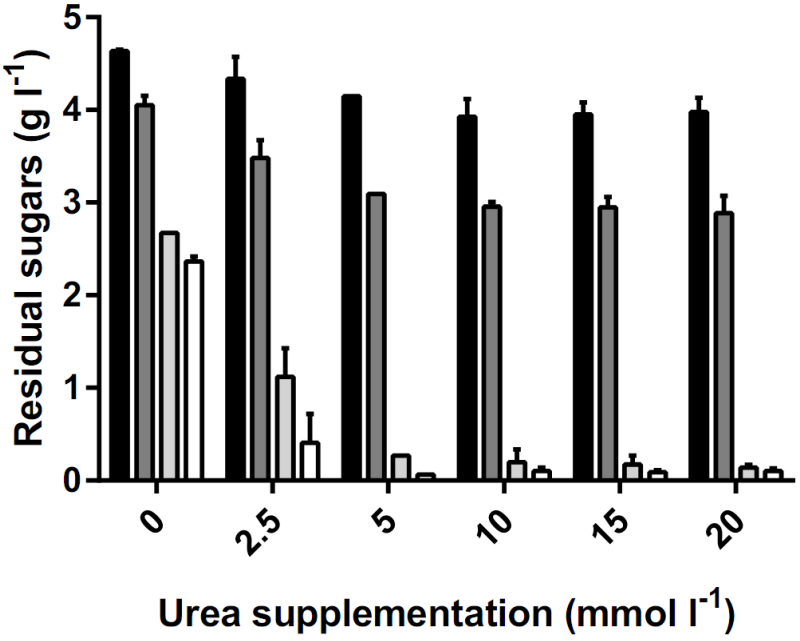

Fig. 1 Optimization of concentration of urea supplementation of synthetic winery wastewater. *A. oryzae* was incubated in synthetic winery wastewater in shake flasks as described in the methods. Means and standard deviations of duplicate flasks are shown. 5 mmol l^{-1} urea was found to be optimal for fungal consumption of sugars. (■) 20 h, (▩) 26 h, (▩) 42 h, (□) 48 h

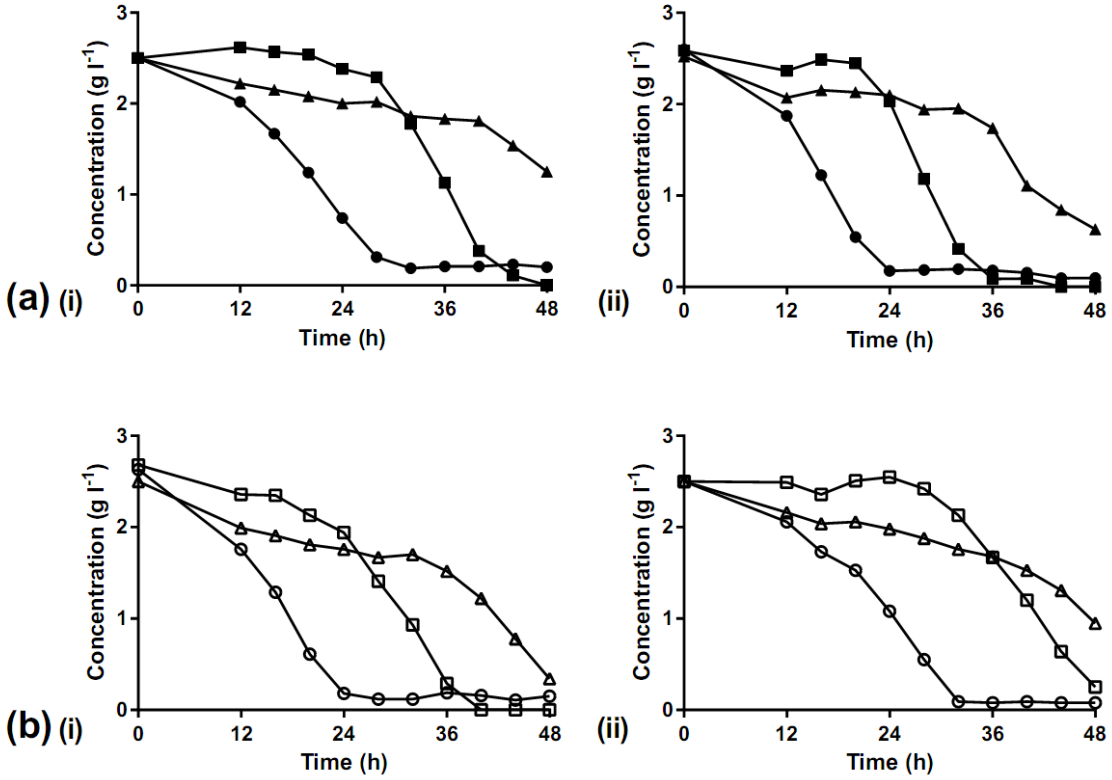

Fig. 2 Consumption of carbon sources in synthetic winery wastewater by *A. oryzae* strains. *A. oryzae* was grown in synthetic winery wastewater in a stirred tank bioreactor as described in the methods. (a) (i) and (ii) show duplicate fermentations with wildtype *A. oryzae*. (b) (i) and (ii) show duplicate fermentations with *A. oryzae creBΔ*. (●) and (○), glucose; (■) and (□), fructose; (▲) and (△), ethanol.

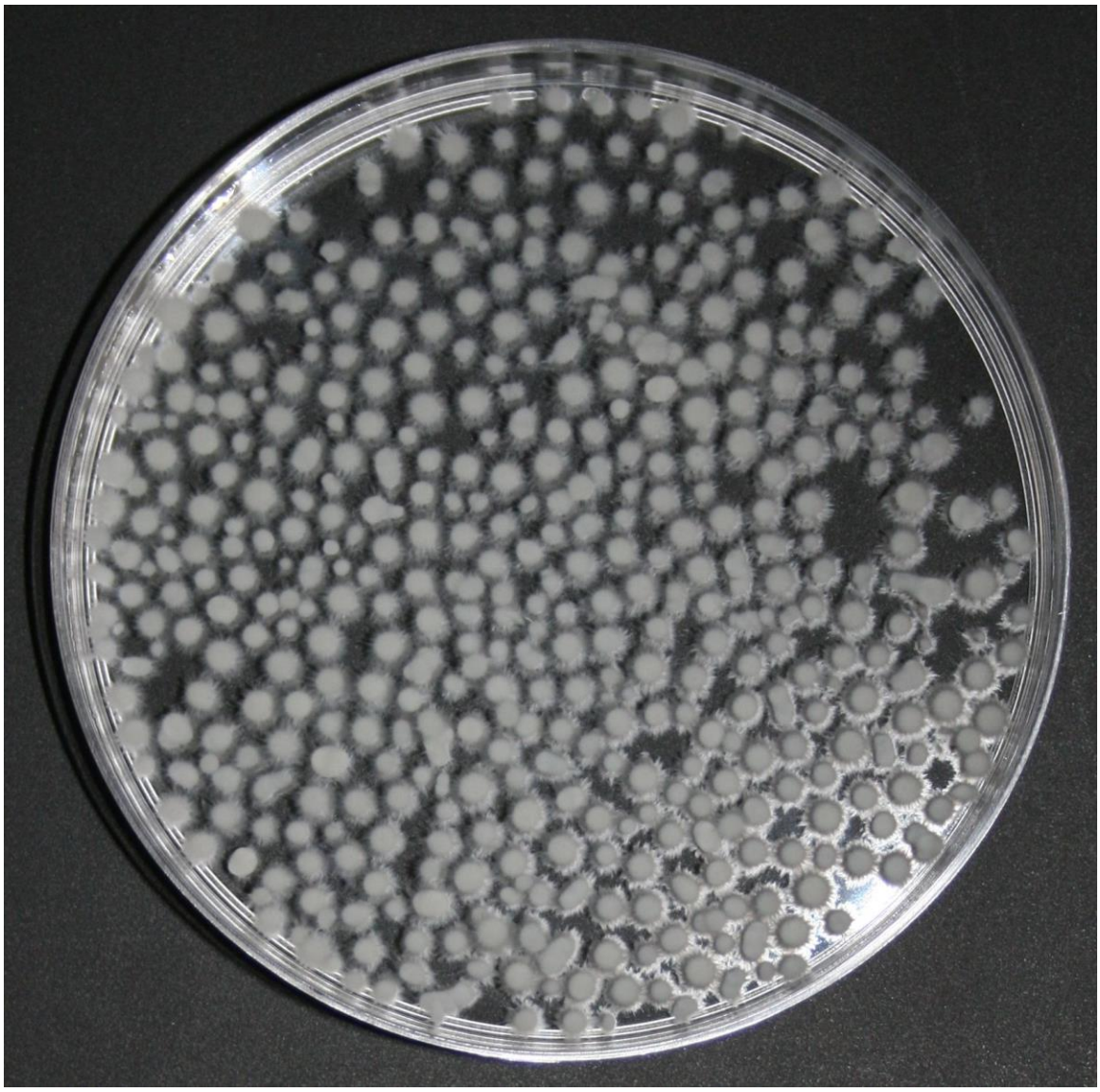

Fig. 3 Morphology of *A. oryzae creBΔ*. The fungus was grown 48 h in a stirred-tank bioreactor containing synthetic winery wastewater supplemented with urea as described in Materials and Methods. It grew as fluffy white pellets, shown here in a standard 80 mm Petri dish.

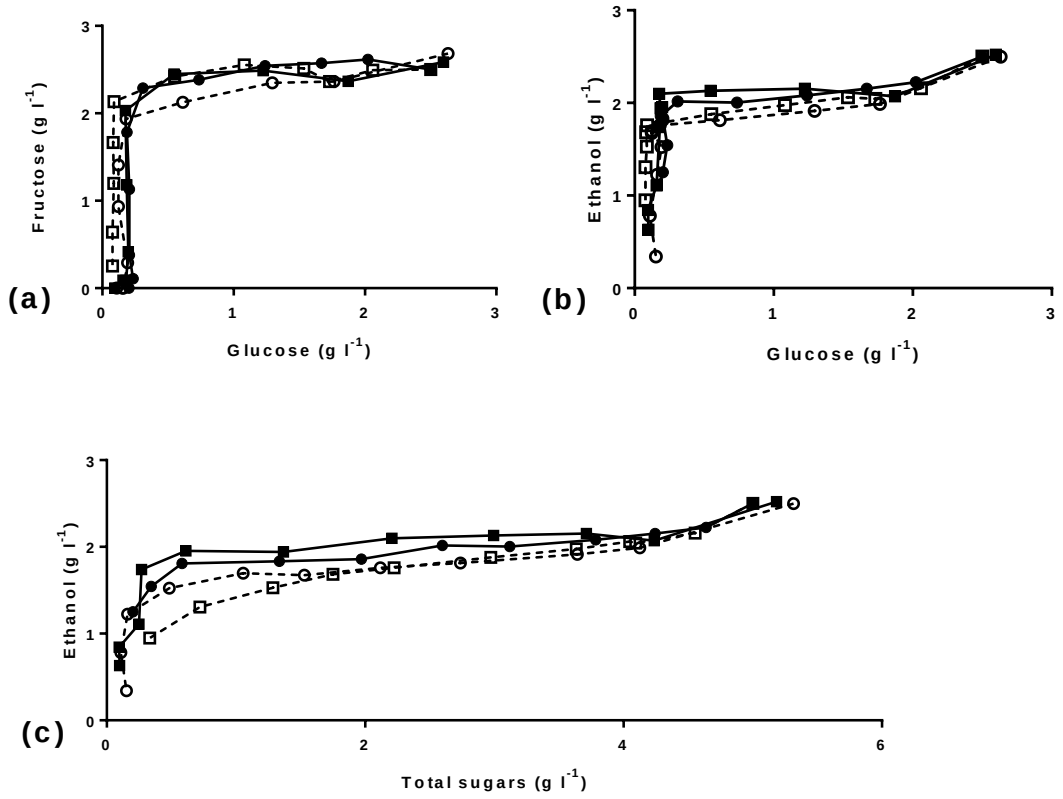

Fig. 4 Consumption of non-preferred carbon sources as a function of concentrations of preferred carbon sources for the fermentations shown in Figure 2. In each plot, the starting points of each fermentation are to the upper right, and fermentations progress toward the origin. (●) and (■) with solid lines indicate duplicate fermentations with wildtype *A. oryzae*. (○) and (□) with dashed lines indicate duplicate fermentations with *A. oryzae creBΔ*. (a) Consumption of fructose as a function of glucose concentration. (b) Consumption of ethanol as a function of glucose concentration. (c) Consumption of ethanol as a function of total sugar concentration. Note that ethanol concentrations reduced due to evaporation as well as consumption.

4 Statement of Authorship

Title of Paper	Independent duplications of α-amylase in different strains of *Aspergillus oryzae*
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Principal Author

Name of Principal Author (Candidate)	Adrian Hunter
Contribution to the Paper	Planned and performed all experiments, interpreted data and wrote manuscript.
Overall percentage (%)	90%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.
Signature	Date 20 April 2018

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

vii. the candidate's stated contribution to the publication is accurate (as detailed above);

viii. permission is granted for the candidate in include the publication in the thesis; and

ix. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Bo Jin
Contribution to the Paper	Evaluated and edited the manuscript.
Signature	Date 30/04/2018

Name of Co-Author	Joan Kelly
Contribution to the Paper	Supervised laboratory work, assisted with data interpretation and experiment planning, evaluated and edited the manuscript.
Signature	Date 25/04/2018

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Independent duplications of α-amylase in different strains of *Aspergillus oryzae*

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ABSTRACT

Aspergillus oryzae is a filamentous fungus that has arisen through the ancient domestication of *Aspergillus flavus* for making traditional oriental foods and beverages. In the many centuries *A. oryzae* has been used for fermenting the starch in rice to simple sugars, it has undergone selection for increased secretion of starch-degrading enzymes. In particular, all *A. oryzae* strains investigated thus far have two or more copies of a gene encoding α-amylase, whereas *A. flavus* has only one. Here we investigate the duplications leading to these copies in three *A. oryzae* strains. We find evidence of at least three separate duplications of α-amylase, an example of parallel evolution in a micro-organism under artificial selection. At least two of these duplications appear to be associated with activity of transposable elements of the *Tc1/mariner* class. Both involve a 9.1 kb element that terminates in inverted repeats, encodes a putative transposase and another putative protein of unknown function, and contains an unusual arrangement of four short internal imperfect repeats. Although "unusual *Mariners*" of this size have previously been identified in *A. oryzae*, *Aspergillus fumigatus* and *Aspergillus nidulans*, this is the first evidence we know of that at least some of them are active in modern times and that their activity can contribute to beneficial genetic changes.

1. Introduction

Aspergillus oryzae is a filamentous fungus that has been used for centuries in the production of traditional oriental foods and beverages. In particular, it is used in the fermentation of rice for *sake* production, during which it secretes large amounts of amylases for the saccharification of starch into glucose for subsequent fermentation by yeasts. Great quantities of *A. oryzae* amylases are also prepared and used in food production industries (Berka et al., 1992).

Alpha-amylase (EC 3.2.1.1) catalyses the hydrolysis of internal α-1,4-glycosidic bonds in starch and related molecules and is the major secreted amylase in *A. oryzae* solid-state culture (Oda et al., 2006). The *A. oryzae* α-amylase is known as Taka-amylase and has been studied extensively at the levels of transcription initiation (Tanaka et al., 2000; Tani et al., 2000), protein folding (Kawata et al., 1998), crystal structure (Swift et al., 1991), glycosylation (Eriksen et al., 1998), secretion kinetics (Santerre Henriksen et al., 1999), and reaction kinetics (Batlle et al., 2000); there have also been many investigations of the optimal conditions for α-amylase production and activity.

Given the importance of α-amylase in rice fermentation and the length of time *A. oryzae* has been used to ferment rice, it is unsurprising that *A. oryzae* has experienced considerable selection for increased α-amylase secretion (Hara et al., 1992). In 1989 four groups independently reported that *A. oryzae* has multiple copies of α-amylase. Using Southern blotting, two copies were detected in *A. oryzae* NBRC 30105 (Tsukagoshi et al., 1989) and NRC401013 (Gines et al., 1989), and three copies in *A. oryzae* RIB40 (Tada et al., 1989) and DSM63303 (Wirsel et al., 1989). To our knowledge, no-one has reported an *A. oryzae* strain with fewer than two α-amylase copies. The genome of *A. oryzae* RIB40 was subsequently sequenced, confirming it had exactly three copies of α-amylase on different chromosomes (Machida et al., 2005). These copies have almost identical nucleotide sequences, differing at only three sites across a region spanning 3.2 kb. *A. oryzae* has arisen through the ancient domestication of *Aspergillus flavus* (Geiser et al., 1998), which has only one α-amylase gene (Fakhoury and Woloshuk, 1999). Thus it is likely that the additional copies of α-amylase have arisen through gene duplication in *A. oryzae* during its domestication.

Gene duplication can result from a variety of genetic changes associated with transposable elements (reviewed in Gray, 2000). Although transposable elements have long been considered genetic parasites, the last two decades have seen the description of many examples of beneficial genetic changes mediated by transposable elements (reviewed in Sinzelle et al., 2009). Transposable elements may be especially beneficial as a source of genetic variation in filamentous fungi such as *A. oryzae* that lack a known sexual cycle; indeed, transposition of transposable elements of the *Tc1/mariner*

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superfamily has been shown to be enhanced by stress conditions in *A. oryzae* (Ogasawara et al., 2009). Members of the *Tc1*/*mariner* superfamily are the predominant transposable elements in *A. oryzae*, comprising approximately half of all transposable elements and repetitive sequences (Galagan et al., 2005 Supplementary data §5). They characteristically integrate at TA dinucleotide sites, and their excision leaves duplicates of the TA integration site separated by two or three base pairs (reviewed in Plasterk et al., 1999).

Here we argue for the involvement of *Tc1*/*mariner*-type transposable elements in the duplications of α-amylase in *A. oryzae* strains NBRC 30105 and RIB40. In addition, we show that *A. oryzae* DAR3699, a strain used in soy fermentation (FRR Culture Collection catalogue, http://www.foodscience.afisc.csiro.au, accessed 2/16/2008) and shown to be an effective degrader of starch (Jin et al., 1999), also has two copies of α-amylase but that its second copy has arisen independently of that of the other strains previously characterised. Thus in an example of parallel evolution, duplication of an α-amylase gene has occurred at least three times in different *A. oryzae* strains.

2. Materials and methods

2.1. Strains

A. oryzae strains used were DAR3699 from the FRR culture collection (http://www.foodscience.afisc.csiro.au), NBRC 30105 (also called JCM02239) from the NITE Biological Resource Center (http://www.nbrc.nite.go.jp/NBRC2/NBRCDispSearchServlet), and RIB40 (also called ATCC42149 or NBRC 100959), also from the NITE Biological Resource Center.

2.2. Southern hybridization

Southern hybridization was performed using the DIG High Prime DNA Labelling and Detection Kit (Roche) according to the manufacturer's instructions. The probe to detect α-amylase bound a region of the α-amylase coding sequence and was constructed using the primers Ao_amy_F (AGGGAATGGGCTTCACAG) and Ao_amy_R (GGCGTTGAGGAGTGGATAG) and Taq polymerase (NEB). The probe to detect the 9.1 kb element bound a region indicated in Fig. 1 and was constructed using the primers 9kb_detect_F (ATGCCTCCACCCTCAACG) and 9kb_detect_R (CCCTCACGGACACTCTTGCT) and Taq polymerase (Roche).

2.3. PCR

Primers used for showing *A. oryzae* DAR3699 lacks a copy of α-amylase on chromosome 5 in the location where one is present in *A. oryzae* RIB40 were Ao_amy_C5_F (CTCATGGGCAGGAAACTTGG) and Ao_amy_C5_R (AGACCGAAGGACTTGAAACACC). This was done using Taq polymerase (NEB) according to the manufacturer's instructions. All other PCR was performed using Taq polymerase (Roche) according to the manufacturer's instructions. Primers used in generating Fig. 3A were Ao_amy_copy1_fwd (CCAGGCTCGCACTATGTATG), Ao_amy_copy2_fwd (GGACGGGATTGGATGAGG), Ao_amy_copy3_fwd (CAATAGTCATCTAACGCCTCG), and Ao_amy_copyX_rev (CGGCTGCTCGGTCTACTAC) with an annealing temperature of 55 °C, an extension time of 45 s, and 30 cycles. Primers used to investigate chromosome 3 of *A. oryzae* NBRC 30105 were Ao_amy_copy2_fwd given above and Ao_amy_C3_R (CGATAATACCACTCCCCAAAGC), used with annealing temperatures from 50 to 70 °C, an extension time of 200 s, and 40 cycles.

2.4. Sequence alignment

Fig. 2 was created by aligning 300 nt of each sequence using MUSCLE alignment with a terminal gap open score of zero, but otherwise using the default parameters of Geneious (Drummond et al., 2010). Other alignments used in creating Figs. 2 and 5 but not shown were generated using CLUSTAL.

2.5. Amylase assays

Fresh spores were point-inoculated onto 1% Aspergillus nitrogen-free media (Cove, 1966) containing 10 mM urea and 1% soluble starch (BDH). After two days' growth at 30 °C, the plates were

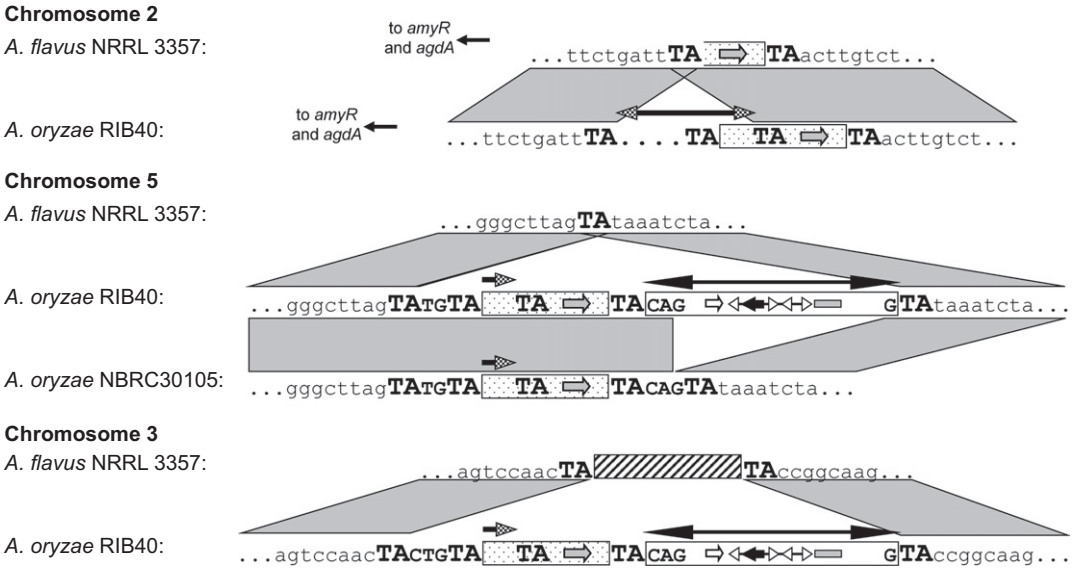

Fig. 1. Schematic of chromosomal regions about α-amylase genes in *A. oryzae* RIB40 and corresponding regions of other strains. Not to scale. Grey shaded areas indicate regions of homology on the corresponding chromosome between strains. TA dinucleotides are indicated in large capital letters. Other nucleotides mentioned in the text are indicated in smaller capital letters. Nucleotides not mentioned in the text are written in lower case. Corresponding pairs of inverted repeats are indicated by chequered, black, or white triangles. The repeats in the Aot1 complex indicated by white triangles all terminate in TA dinucleotides, which are not depicted due to space constraints. Gray arrow, α-amylase gene; white arrow, gene for a putative transposase *tpnA*; black arrow, putative gene of unknown function; grey box, region to which probe for detecting 9.1 kb element binds; speckled box, 3.2 kb conserved region in *A. oryzae* or part thereof in *A. flavus*; white box, 9.1 kb conserved *Mariner*-like element; striped box, 2.8 kb region unique to *A. flavus*.

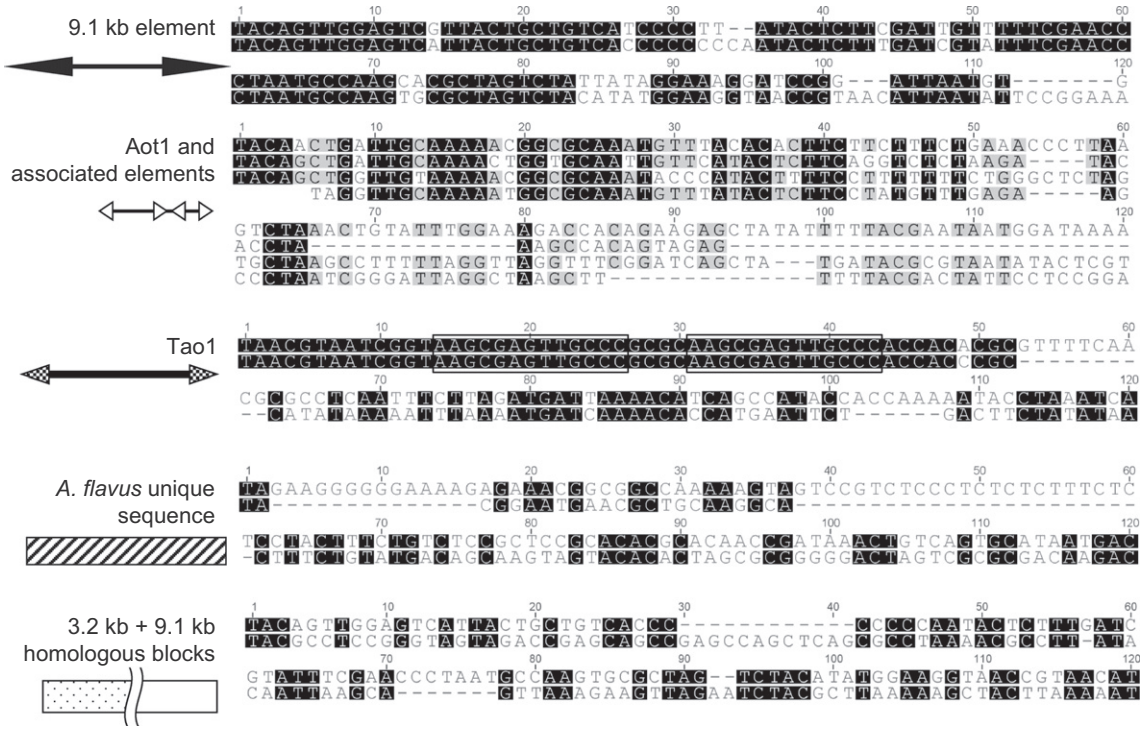

Fig. 2. Alignments of various sequences depicted in Fig. 1. In each alignment, the top sequence corresponds to the left end of the element as depicted in Fig. 1, and the bottom sequence corresponds to the reverse complement of the right end of the element. In the Aot1 alignment the four sequences from top to bottom correspond to the indicated repeats from left to right, respectively. The reverse complements of the second and fourth repeats are shown. 13 bp perfect direct repeats within the Tao1 inverted repeats are indicated by boxes. The 9.1 kb conserved element, the Aot1-associated cluster of repeats, and Tao1 all end in imperfect inverted repeats terminating in TA dinucleotides, typical of *Mariner*-class elements. The sequence unique to *A. flavus* chromosome 3 that is not present in *A. oryzae* RIB40 does not end in inverted repeats and does not appear to be a transposable element. The beginning of the 3.2 kb conserved block and the end of the 9.1 kb conserved block show little or no sequence similarity.

flushed with 0.033% iodine solution, which darkly stained remaining starch in the growth media. Total secreted amylase activity was estimated by the diameter of unstained haloes around the colonies.

3. Results

3.1. Arrangement of α-amylase genes in A. oryzae RIB40

The following is based on examination of the *A. oryzae* RIB40 genomic sequence (Machida et al., 2005). Three copies of α-amylase are present, on chromosomes 2, 5, and 3 (Fig. 1). The copy on chromosome 2 is part of a cluster of genes associated with starch utilisation, and occurs downstream of genes encoding an α-glucosidase and the maltose-induced transcriptional activator AmyR. Similar gene clusters occur in *Aspergillus nidulans* (NCBI accession number AF208225) and in *A. flavus* (http://www.aspergillusflavus.org/genomics/). The α-amylase copies on chromosomes 5 and 3 are not part of a similar cluster; therefore, the copy on chromosome 2 is the original α-amylase gene, ancestral to the other two copies. Each of the three α-amylase copies lies within a separate 3.2 kb block of highly conserved sequence. The 3.2 kb blocks containing the α-amylase copies on chromosomes 5 and 3 are identical in sequence, whereas the 3.2 kb block on chromosome 2 differs from the other two blocks by two mismatches upstream of α-amylase and one mismatch within the α-amylase coding region, coding for glutamine instead of arginine. In addition, immediately downstream of the 3.2 kb blocks on chromosomes 5 and 3 are 9.1 kb blocks of highly conserved sequence that differ by only one mismatch. The 3.2 kb and 9.1 kb blocks on chromosomes 5 and 3 thus form a pair of 12.3 kb blocks of highly conserved sequence differing by a single mismatch. Both 9.1 kb blocks and all three 3.2 kb blocks are immediately flanked by TA dinucleotides, in common with transposable elements of the *Tc1/mariner* superfamily. The 9.1 kb blocks terminate in imperfect inverted repeats of approximately 83 bp (Fig. 2). Each encodes a putative transposase previously designated *tpnA* and a putative transposon-like element previously designated Aot1 (NCBI accession number AB072434). Aot1 was identified based on 41 bp imperfect inverted repeats terminating in TA dinucleotides. Examination of the full sequence of the 9.1 kb element reveals that these are two in a cluster of four repeats of similar length, each terminating in a TA dinucleotide, in the arrangement depicted in Fig. 1. Only 53 bp separate the TA dinucleotides of the second and third repeat. The first and second repeats are separated by 1.9 kb, and the third and fourth by 1.6 kb. A putative gene between the first two repeats encodes a hypothetical protein of 194 amino acids (NCBI accession number AP007157). A third highly conserved copy of the entire 9.1 kb element occurs on chromosome 3 77 kb downstream of the copy depicted in Fig. 1. BLASTN searching of the nr database at the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed that *Aspergillus niger* also contains a copy of this element with greater than 99.9% sequence conservation. BLASTN revealed no other sequences with similarity to this element across its full length, although sequences of 7–9 kb described as "unusual *Mariners*" have previously been identified in the genomes of *A. flavus*, *A. oryzae* and *A. nidulans* (Galagan et al., 2005 Supplementary data §5).

3.2. Comparison to A. flavus

The genome of the sequenced strain of *A. flavus*, NRRL 3357, is highly similar to that of *A. oryzae* RIB40, with the 16 largest genomic scaffolds of *A. flavus* essentially corresponding to the 16 arms of the eight chromosomes of *A. oryzae* (Payne et al., 2006). Because *A. flavus* is the ancestor of *A. oryzae*, examination of the regions of the *A. flavus* genome corresponding to the loci into which the

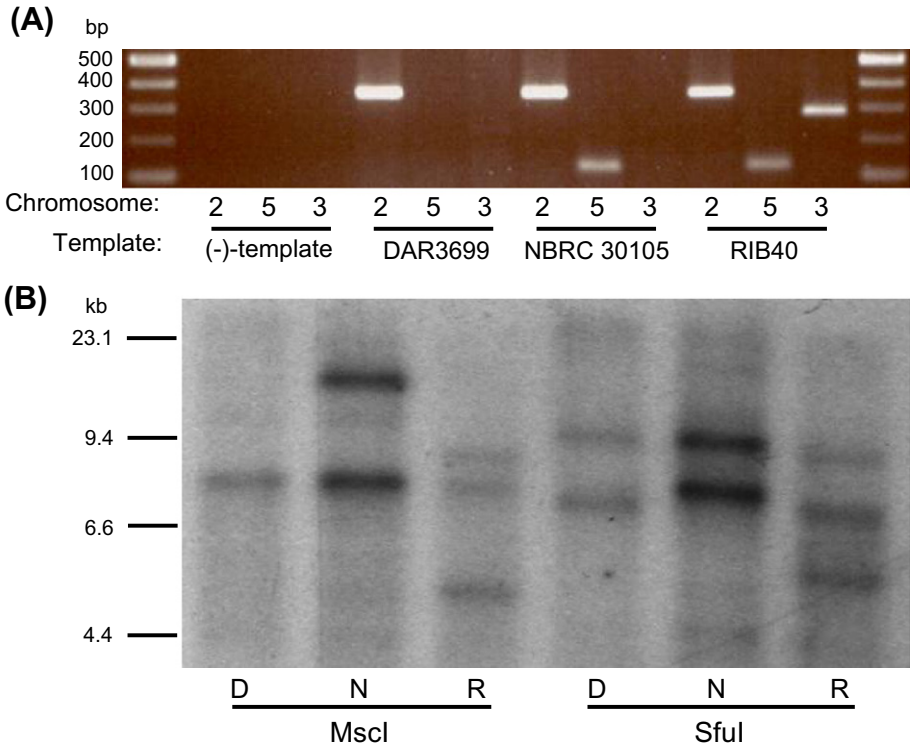

Fig. 3. Copies of α-amylase in different strains of *A. oryzae*. A, PCRs to detect the 3.2 kb block containing an α-amylase gene in genomic locations corresponding to those in *A. oryzae* RIB40. The reactions to detect the block on each chromosome use a common reverse primer binding near the 5′-end of the 3.2 kb block, and different forward primers binding to sequences shortly upstream of the 3.2 kb block that are unique to chromosomes 2, 5, and 3. B, Hybridization of a probe binding the α-amylase coding sequence to genomic DNA of *A. oryzae* strains DAR3699 "D", NBRC 30105 "N" and RIB40 "R" digested with MscI or SfuI. The restriction fragment lengths expected to bind the probe for chromosomes 2, 5, and 3 of RIB40 are 7.0, 4.7, and 7.9 kb respectively for MscI digestion and 8.8, 5.4, and 6.9 kb respectively for SfuI digestion. The restriction fragment lengths expected if the 9.1 kb *Mariner*-like element excised from chromosome 5 are 10.9 kb for MscI digestion and 7.2 kb for SfuI digestion.

amylase genes duplicated in *A. oryzae* RIB40 provides insight into the nature of these duplications.

The sole copy of α-amylase in *A. flavus* NRRL 3357 occurs on chromosome 2 and, similar to α-amylase in *A. nidulans* and the α-amylase on chromosome 2 in *A. oryzae*, lies downstream of genes encoding an α-glucosidase and AmyR. In all three of the mismatches that distinguish the 3.2 kb block on *A. oryzae* RIB40 chromosome 2 from the other two 3.2 kb blocks, the corresponding base pair in *A. flavus* matches that on *A. oryzae* RIB40 chromosome 2, further confirming that this is the ancestral copy. There is 1.9 kb of sequence between α-amylase and α-glucosidase in *A. oryzae* RIB40 that is not homologous to any sequence in *A. flavus* NRRL 3357. This sequence includes imperfect 52 bp inverted terminal repeats ending in TA dinucleotides and has been previously designated as the transposon-like element Tao1 (NCBI accession number AB021710). The sequences in *A. flavus* homologous to the sequences flanking Tao1 are contiguous and share a common TA dinucleotide, corresponding to the site of insertion of Tao1. Thus the insertion of Tao1 into the *A. oryzae* genome was typical for a transposable element of the *Tc1*/*mariner* superfamily.

Chromosome 5 of *A. flavus* lacks the 3.2 kb and 9.1 kb blocks described earlier that are present on chromosome 5 of *A. oryzae*. In *A. flavus*, sequences homologous to sequences upstream and downstream of these blocks in *A. oryzae* are contiguous, and share a common TA dinucleotide that was presumably the site of insertion of these blocks in *A. oryzae*. *A. oryzae* also contains an additional four base pairs, TATG, immediately upstream of the 3.2 kb block that is not present in *A. flavus*.

A. flavus chromosome 3 contains sequence homologous to that immediately upstream of the 3.2 kb sequence block on *A. oryzae* chromosome 3, and sequence homologous to that immediately downstream of the 9.1 kb sequence block on *A. oryzae* chromosome 3, flanking and an additional 2.8 kb of sequence not present in *A. oryzae* RIB40. This sequence is immediately flanked by TA dinucleotides. It contains several short putative open reading frames, although BLASTN searches of all Aspergillus genomes available at the Broad Institute (http://www.broadinstitute.org/annotation/genome/aspergillus_group/Blast.html), and all sequences in the nr database at the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi) using default parameters did not identify any other sequence with similarity across more than 10% of its length. In *A. flavus*, sequences homologous to the sequences flanking the additional copy of the 9.1 kb element on chromosome 3 of *A. oryzae* RIB40 are contiguous and share a common TA dinucleotide, which presumably corresponds to the site of the insertion of the element in the *A. oryzae* RIB40 lineage.

3.3. Comparison to *A. oryzae* NBRC 30105 and *A. oryzae* DAR3699

A. oryzae NBRC 30105 was shown to have two copies of α-amylase by Southern hybridization (Tsukagoshi et al., 1989). We have used PCR and Southern hybridization to compare this strain to *A. oryzae* RIB40 (Fig. 3) and examined sequence data from Tsukagoshi et al. (1989) in light of information now available from the *A. oryzae* RIB40 genome. PCR was performed with three pairs of primers designed to detect the three copies of the 3.2 kb conserved block in *A. oryzae* RIB40. The three primer pairs all included one common reverse primer binding within the 3.2 kb conserved block, and each included a separate forward primer binding to a region shortly upstream of one of the 3.2 kb blocks. Separate reactions using each of these primer pairs and *A. oryzae* RIB40 genomic DNA all generated a product. When genomic DNA from *A. oryzae* NBRC 30105 was used instead, only the reactions detecting the 3.2 kb blocks on chromosomes 2 and 5 produced a product

(Fig. 3A), indicating the two copies of α-amylase in this strain corresponded to two of the α-amylase copies in *A. oryzae* RIB40. Sequencing of the PCR product that detected the 3.2 kb block on chromosome 5 confirmed the genomic sequence at the upstream boundary of the 3.2 kb block in *A. oryzae* NBRC 30105 matches that of *A. oryzae* RIB40 (data not shown). However, Southern hybridization to *A. oryzae* NBRC 30105 genomic DNA of a probe binding the α-amylase coding region did not produce a band for chromosome 5 the same size as that seen in *A. oryzae* RIB40 (Fig 3B). Examination of sequence data presented by Tsukagoshi et al. (1989) reveals why: *A. oryzae* NBRC 30105 lacks the 9.1 kb *Mariner*-like element immediately downstream of the 3.2 kb block on chromosome 5 in *A. oryzae* RIB40. Instead, the 3.2 kb block is followed by a pair of TA dinucleotides flanking the sequence CAG. The molecular weights of the bands observed for *A. oryzae* NBRC 30105 match what would be expected for *A. oryzae* RIB40 lacking the 9.1 kb element. Thus it appears that the 9.1 kb element has excised from this location in the *A. oryzae* NBRC 30105 lineage, leaving a canonical footprint for a member of the *Tc1/mariner* superfamily.

We have shown *A. oryzae* DAR3699 also contains at least two copies of α-amylase by Southern hybridization to the α-amylase coding region following digestion with the restriction endonucleases SfuI (Fig. 3B) and PciI (data not shown). However, Southern hybridization following digestion with MscI produced only a single band at 7.0 kb, consistent with a large duplication spanning the 7.0 kb MscI fragment. Of the three PCRs described above, only the one designed to detect the 3.2 kb conserved block in chromosome 2 produced a product when using *A. oryzae* DAR3699 genomic DNA. Taken together, these data indicate the duplication leading to the second α-amylase copy in *A. oryzae* DAR3699 was likely independent of those in the other two strains, and that this duplication included sequence upstream of the 3.2 kb block. We have also sequenced across the region of *A. oryzae* DAR3699 chromosome 5 where *A. oryzae* RIB40 contains 12.3 kb of conserved sequence including α-amylase but *A. flavus* does not, and found the sequence of *A. oryzae* DAR3699 to be identical to that of *A. flavus* NRRL 3357, proving *A. oryzae* DAR3699 does not possess a copy of α-amylase at this location (data not shown). The PCR and Southern hybridization results are consistent with *A. oryzae* DAR3699 retaining the ancestral α-amylase on chromosome 2 and having one additional α-amylase copy in an undetermined location.

We investigated whether the 9.1 kb element that had excised in the *A. oryzae* NBRC 30105 lineage was present elsewhere in that genome or in the *A. oryzae* DAR3699 genome using Southern hybridization (Fig. S1). For *A. oryzae* RIB40, the expected pattern of three restriction fragments was detected. For *A. oryzae* DAR3699, two bands of hybridization were seen for each of the three restriction endonucleases used. For *A. oryzae* NBRC 30105, two bands were seen for XhoI and BglII, but one dark band and two fainter bands were seen for MfeI; this is most likely due to an MfeI site within the region to which the probe binds in one of the 9.1 kb elements in this strain. Thus the results are consistent with *A. oryzae* strains DAR3699 and NBRC 30105 both having two copies of the 9.1 kb element.

4. Discussion

Through centuries of growth on starch-rich substrates under domestication, *A. oryzae* has undergone selection for increased amylase secretion. How this selection has been manifest is of interest with regard to the development of industrial *A. oryzae* strains with even higher amylase production. Here we have shown that selection has resulted in duplication of an α-amylase gene on at least three occasions: once in the RIB40 and NBRC 30105 lineage to chromosome 5, once again in the RIB40 lineage to chromosome 3, and once again in the DAR3699 lineage to an undetermined location. Although we have not analysed the contribution of each α-amylase copy to the total secreted amylase activity, we found that *A. oryzae* RIB40, the strain with the highest α-amylase gene copy number, also has the highest total secreted amylase activity as estimated by a starch halo test (data not shown). These observations suggest not only that gene duplication is a common natural mechanism for increasing amylase secretion, but also that creating further duplications may be a viable route for producing amylase hyper-secreting strains. We have also implicated transposition events involving members of the *Tc1/mariner* superfamily in at least two of these duplications, based on the 12.3 kb duplicated regions in *A. oryzae* RIB40 both being flanked by TA dinucleotides, and each either inserting into a specific TA dinucleotide in the case of chromosome 5, or replacing a region flanked by TA dinucleotides in the case of chromosome 3. This supports the notion that transposable elements are important in evolution, perhaps especially so in fungi such as *A. oryzae* that lack a known sexual cycle.

In Fig. 4 we present one possible sequence of events that could have led to the arrangement of α-amylases observed in *A. oryzae* strains NBRC 30105 and RIB40. An ancestral *A. flavus* strain resembled *A. flavus* NRRL 3357. Insertion of the DNA transposon Tao1 into a TA dinucleotide upstream of α-amylase led to Predicted Intermediate 1, and might have occurred before or after the domestication of *A. flavus*. Use of Predicted Intermediate 1 for rice fermentation then selected for a duplication of a 3.2 kb block of sequence containing α-amylase and part of Tao1, and was associated with the arrival of the 9.1 kb *Mariner*-like element. Whether Tao1 played any functional role in this duplication is unknown. *A. oryzae* NBRC 30105 is a descendant of Predicted Intermediate 2 in which the 9.1 kb *Mariner*-like element has excised. Chromosome 3 of *A. oryzae* NBRC 30105 is represented by a question mark, because a pair of PCR primers that amplified a portion of *A. oryzae* RIB40 genomic DNA spanning this region failed to produce a product from *A. oryzae* NBRC 30105 genomic DNA (data not shown), suggesting further uncharacterised chromosomal re-arrangements have occurred. In a separate lineage leading to *A. oryzae* RIB40, further growth on rice selected for a duplication of the 3.2 kb block and the 9.1 kb block together to chromosome 3, displacing a block of unneeded sequence flanked by TA dinucleotides. The mechanism underlying this duplication is unclear, as there is little or no sequence similarity between the upstream end of the 3.2 kb block and the downstream end of the 9.1 kb block (Fig. 2). It may have involved insertion and excision of some other element to leave what appears to be a *Tc1/mariner*-class element footprint immediately upstream of the 3.2 kb block on chromosome 3.

An alternative sequence of events has *A. oryzae* NBRC 30105 as a descendant of the RIB40 lineage in which α-amylase has been lost from chromosome 3. This seems less likely, as an environment that selected for the emergence of three α-amylase copies would presumably continue to exert selective pressure to maintain them.

Gene duplication as a mechanism for increasing α-amylase production in response to environmental selection has occurred not only in fungi, but also in animals. Per haploid genome, healthy humans can have from one copy of the salivary amylase gene *AMY1* to a cluster of up to seven copies arranged as tandem repeats (Groot et al., 1989); higher copy numbers correlate with higher levels of salivary amylase and are more common in populations with high-starch diets (Perry et al., 2007). Amplification of amylases has also been characterised in the mouse and rat (Sugino, 2007), chicken (Benkel et al., 2005), and in several species of *Drosophila* (Schaeffer et al., 2003; Zhang et al., 2003 and references therein).

In the Supplementary data to the *A. nidulans* genome paper it was noted in passing that the genomes of *A. nidulans*, *A. fumigatus*, and *A. oryzae* all contain "unusual *Mariners*" of 7–9 kb that encode both a transposase and a conserved protein of unknown function

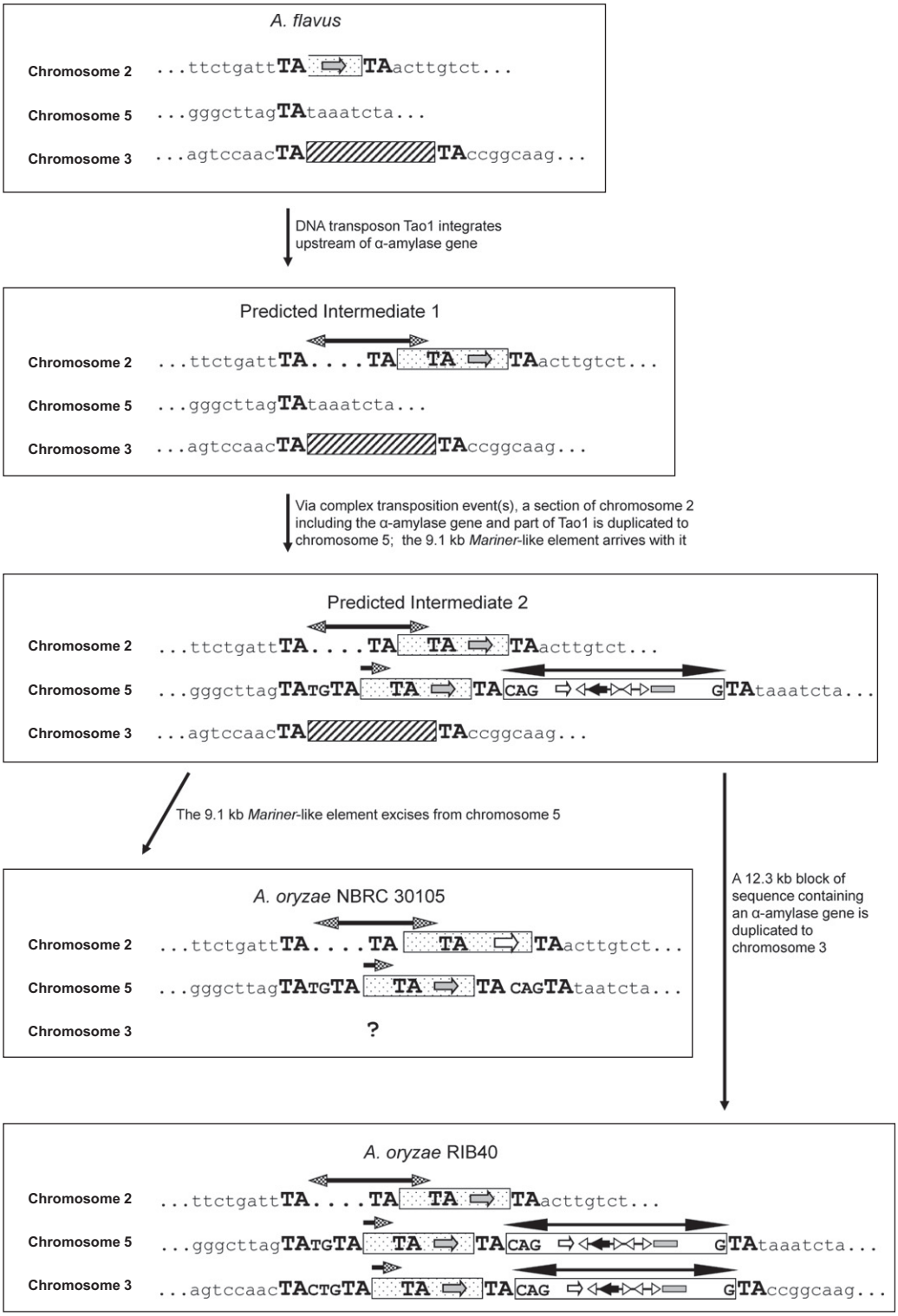

Fig. 4. Schematic of one possible sequence of events leading to the arrangement of α-amylases in *A. oryzae* strains NBRC 30105 and RIB40. Symbols are as in Fig. 1.

(Galagan et al., 2005 Supplementary data §5). The 9.1 kb blocks of sequence downstream of the α-amylase genes on chromosomes 5 and 3 appear to be examples of such *Mariners*. At the position on chromosome 5 in which *A. oryzae* RIB40 has a 9.1 kb *Mariner*-like element, *A. oryzae* NBRC 30105 chromosome 5 instead contains the nucleotides CAG, flanked by a pair of TA dinucleotides. This matches the canonical footprint of an element of the *Tc1/mariner* superfamily, indicating the 9.1 kb element has most likely excised in the *A. oryzae* NBRC 30105 lineage. The CAG nucleotides might be derived from the first two base pairs and the last base pair of the 9.1 kb element, which would be consistent with the source of the three base pairs left between TA dinucleotides by the excision of *crawler*, another *Tc1/mariner* class transposable element in *A. oryzae* (Ogasawara et al., 2009). Alternatively, they may simply be derived from the first three nucleotides of the 9.1 kb element. The apparent excision of the 9.1 kb element in the *A. oryzae*

NBRC 30105 lineage is the first evidence we know of that at least some of the 7–9 kb elements present in the three Aspergilli may be active. The arrangement of the four inverted repeats in this element is unusual, though a set of four repeats in a similar arrangement has been observed about the element *Tan1* in *Aspergillus niger* (Nyyssonen et al., 1996). *Tan1* is also a member of the *Tc1/mariner* superfamily. The 9.1 kb element has a GC content of 44%, lower than the average GC content of the genomes of either *A. oryzae* (48%, Galagan et al., 2005) or *A. niger* (50%, Pel et al., 2007). This and the extreme conservation between the copies of the 9.1 kb element in *A. oryzae* and *A. niger* suggest both might have received the element through horizontal transfer from an unidentified third species.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2011.01.006.

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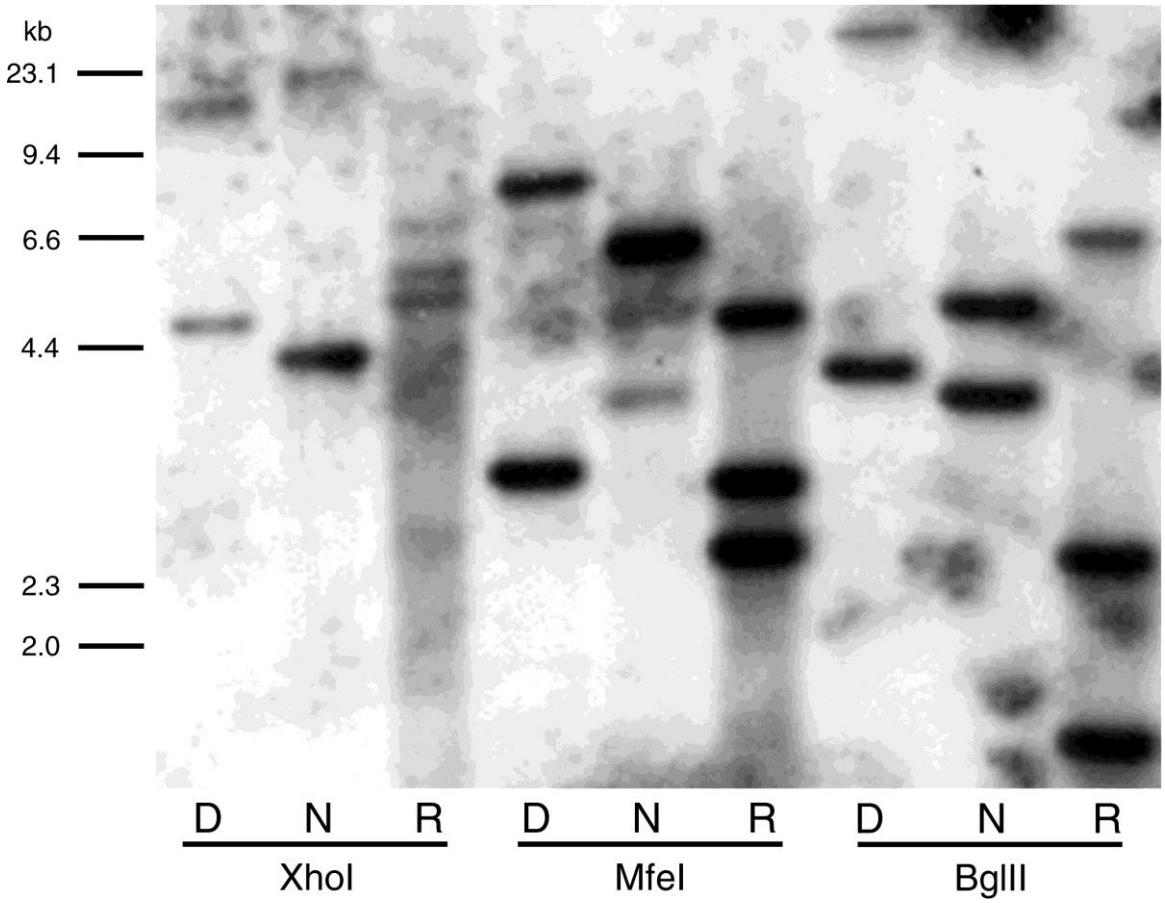
Appendix A. Supplementary material



Supplementary Fig. S1. Hybridization of a probe binding the 9.1 kb element to genomic DNA. DNA samples from *A. oryzae* strains DAR3699 “D”, NBRC 30105 “N”, and RIB40 “R” were digested with XhoI, MfeI, or BglII. The region to which the probe binds is depicted in Fig. 1. For *A. oryzae* RIB40, the restriction fragment lengths expected for the 9.1 kb elements on chromosome 5, on chromosome 3 near α-amylase, and on chromosome 3 distant from α-amylase, are 4.8 kb, 5.2 kb and 6.3 kb respectively for XhoI, 2.3 kb, 2.7 kb and 4.2 kb respectively for MfeI, and 5.2 kb, 2.3 kb and 1.6 kb respectively for BglII.

5 Discussion

5.1 Amylase gene copy number

The domestication of plants, animals, and micro-organisms has been a tremendous driver of human prosperity since the beginning of agriculture. Although the often dramatic phenotypic changes accompanying domestication have long been recognised, only recently have we become able to understand the genetic basis of these changes. In the centuries or millenia *A. oryzae* has been grown on starch-rich rice, there has been strong selection for higher secretion of starch-degrading amylase enzymes. Independent groups discovered multiple copies of the gene encoding α-amylase in several strains of *A. oryzae* (Gines et al. 1989; Tada et al. 1989; Tsukagoshi et al. 1989; Wirsel et al. 1989); more recently, it was shown that the wild ancestor of *A. oryzae*, *A. flavus*, has only a single copy (Fakhoury and Woloshuk 1999). We have shown that the increase in copy number of the α-amylase gene in *A. oryzae* has happened not only once, but independently in different strains. Thus increase in α -amylase gene copy number was not an extremely improbable once-off event, but something that occurred multiple times in an example of parallel evolution.

Remarkably, a comparable post-domestication increase in α-amylase copy number occurred in the domestic dog, *Canis lupus familiaris* (Axelsson et al. 2013). Whereas the wild ancestor of the dog, the grey wolf (*Canis lupus lupus*), has a single copy of the pancreatic α-amylase gene *AMY2B*, domestic dogs have 4–30 copies per diploid genome, and higher copy numbers correlate with higher α-amylase expression levels and activities (Axelsson et al. 2013). This reflects the change from the mainly carnivorous diet of wolves to the starch-rich diet of dogs, which are believed to have fed on starch-rich human refuse and scraps early in their domestication. Humans similarly vary widely in the copy number of the gene encoding salivary amylase, *AMY1*. In human populations with long histories of consuming starch-rich diets due to agriculture, the average copy number of salivary amylase has increased (Perry et al. 2007), and higher copy numbers correlate with slightly higher α-amylase expression levels and activities (Carpenter et al. 2017). That our own genomes have undergone such similar changes to those of dogs and *A. oryzae* – all within the last few tens of thousands of years and all in response to changes in human culture – reflects the deep kinship humanity has with these two domesticated species.

5.2 Carbon catabolite repression in A. oryzae

Subsequent to Hunter *et al.* (2013), there have been three published reports describing manipulations of CCR in *A. oryzae*, all involving mutation of *creB*.

Ichinose *et al.* (2014) deleted *creA* and *creB* both singly and in combination, and assayed α-amylase activity in several conditions. Surprisingly, no effect of *creB* deletion was observed on α-amylase activity under non-inducing, repressing conditions (Figure 4a of their paper). Although we did not test such conditions, we did test both non-inducing, non-repressing and inducing, repressing conditions. In both cases the *creBΔ* strain produced vastly more α-amylase activity, and in both cases the difference was very highly significant ($P < 0.001$); mRNA levels measured by qPCR reflected enzyme activity levels (HUNTER *et al.* 2013). Ichinose *et al.* (2014) also tested inducing, repressing conditions, and found approximately 3-fold higher α-amylase activity in the *creBΔ* strain compared to its parent – much less than the approximately 40-fold increase we observed (HUNTER *et al.* 2013). Although both studies used strains derived from *A. oryzae* RIB40, they used quite different growth media, which might explain the apparent discrepancies. Ichinose *et al.* (2014) used rich media containing yeast extract and peptone, with 1% glucose as a source of repression, whereas Hunter *et al.* (2013) used simple media containing urea as a nitrogen source and 2% sucrose as a source of repression. Possibly the glucose or some other component of the rich media exerted stronger CCR that was less mitigated by *creB* deletion, or the rich media may have contained low concentrations of gluconeogenic repressing carbon sources that exert CCR through mechanisms independent of *creB*.

Ichinose *et al.* (2017) describes further characterisation of the strains described in Ichinose *et al.* (2014), focusing on the production of cellulolytic and xylanolytic enzymes. Consistent with Hunter *et al.* (2013), *creB* deletion modestly increased activity levels of both types of enzymes in inducing, non-repressing liquid culture. Across both papers by Ichinose *et al.*, there was a loose trend whereby the quantities of secreted enzymes by the four strains tended to order wildtype < *creBΔ* < *creAΔ* < *creAΔ*/*creBΔ*, although the exact order varied according to the enzyme and growth conditions.

A. oryzae is the first fungus in which manipulation of *creD* in a species used in biotechnology has been reported (TANAKA *et al.* 2017). Deletion of *creD* increased CCR, as in *A. nidulans*, and reduced α-amylase production. Two phosphorylated serine residues in CreD were identified, although neither phosphorylation nor dephosphorylation

mimics of these residues influenced interaction with HulA, and both mimics only slightly reduced α-amylase production. However, the dephosphorylation mimic enhanced the *creB*Δ phenotype by further increasing α-amylase activity, whereas *creD* deletion and especially the phosphorylation mimic suppressed the *creB*Δ phenotype. That manipulation of *creD* had a phenotypic effect even in a *creB*-null strain implies CreD must not operate solely by influencing CreB.

5.3 Reproducibility between biological replicates

One factor that made comparing strains difficult in liquid culture tests was the substantial variability between replicate shake flasks. Flasks inoculated at the same time and grown side by side varied in the number and size of fungal pellets, in the proportion of the mycelium that grew on the side of the flask rather than as suspended pellets, and in the overall amount of growth. This variability persisted despite using a single inoculum to inoculate replicate flasks, preparing a single batch of medium to aliquot to replicate flasks, rejecting any flasks with visible scratches or other damage, using uniform loose-fitting hard plastic lids rather than potentially variable hand-made stoppers or lids with rubber inserts that may grip the tops of flasks to varying degrees, and rinsing all flasks with distilled water after cleaning to remove all traces of detergent (small quantities of detergent can profoundly alter fungal morphology; see, for example, Lee *et al.* 2017). Growing flasks for 48 hours rather than 24 reduced variability but did not eliminate it. For enzyme assays, best results were obtained by determining dry biomass in each flask and expressing secreted enzyme activity per unit dry weight.

5.4 Future perspectives

5.4.1 Improving reproducibility

One technique for reducing variability between shake flasks that was not attempted is silanisation of the flasks, which should prevent mycelia sticking to and growing on the sides of the flasks. Such growth is probably stronger than pellet growth, as solid phase is a more natural growth mode for filamentous fungi, and the shaking of the flasks frequently exposes the sides to air, providing more oxygen. As growth on the sides of flasks varied markedly between replicate flasks, silanisation may be a powerful technique for improving reproducibility.

The flasks used to inoculate replicate bioreactor experiments contained tiny pellets in suspension, with no visible growth on the sides of the flasks. Nevertheless, it is

speculated that some of the variation between replicate bioreactor experiments may have resulted from variation between inocula. If this is the case, one way to create more consistent inocula may be to produce each one across three flasks that are then combined immediately before inoculation.

5.4.2 Winery wastewater and *creB* mutant strains

Although this thesis only describes fungal growth in synthetic winery wastewater, the feasibility of growing *A. oryzae* in sterilised real winery wastewater has also been demonstrated (ZHANG *et al.* 2008). *A. oryzae* was grown in shake flasks on wastewater from a South Australian winery produced during vintage. With nitrogen supplementation, *A. oryzae* reduced the wastewater COD by 91.6% after 54 h, and at 48 h, biomass protein content was 35.3%, corresponding to a productivity of 0.382 g l^{-1} h^{-1}.

Water conservation measures are driving more wineries toward smaller volumes of more highly concentrated effluent. This should favour the economics of bioreactor-based treatment processes, as smaller bioreactors have lower capital and operating costs. Higher concentrations of organic material in more concentrated effluents should enhance fungal growth, though whether this will be offset by higher concentrations of toxins or cleaning product residues would need to be determined empirically. Higher concentrations of organic material would impose stronger CCR, strengthening the case for testing strains with defective CCR.

We observed no advantage of *creB* deletion in treating synthetic winery wastewater in this study, though any small advantage would have been obscured by variability between experimental replicates. Future studies should investigate growth on non-synthetic winery wastewater, as it remains to be seen whether *creB* deletion influences susceptibility to any of the toxic compounds present in low concentrations in winery wastewater, such as tannins and other polyphenolics (MOSSE *et al.* 2013).

5.4.3 Solid winery wastes and *creB* mutant strains

As well as treating winery wastewater, fungi also have potential for treating solid winery wastes such as grape marc. Fungi can convert grape marc into a fermented product with increased digestibility and protein content, which is more valuable and nutritious as animal feed than untreated grape marc (ZEPF AND JIN 2013). Given that disruption of

creB increases cellulase secretion in all fungi tested thus far, *creB* deletion seems more likely to be beneficial in processing solid cellulosic biomass such as grape marc than in processing liquid waste. Increased secretion of cellulytic and hemicellulytic enzymes could improve performance in two ways. One is by liberating more sugars from plant cell walls for the fungus to use as an energy source. Jin *et al.* (2016) found that sugar utilisation was strongly correlated with the ability of different fungal strains to generate protein through grape marc fermentation. The other way is by helping the fungus access more digestible nutrients behind cell walls within plant cell cytoplasms, such as starch, protein, and amino acids. In particular, protein and amino acids are nitrogen sources, and grape marc has scarce nitrogen accessible to micro-organisms outside the tough plant cell walls. Jin *et al.* (2016) also found that adding wine lees or yeast extract to grape marc markedly improved the ability of the fungi to generate protein and increase digestibility, probably because these are nitrogen-rich supplements. Fungi with *creB* deleted may be advantageous not only because their improved secretion of hydrolytic enzymes improves their ability to access nitrogen-rich cell cytoplasms, but also because *creB* deletion increases protease secretion (HYNES AND KELLY 1977; HUNTER *et al.* 2013), which would help convert protein inside or outside cell walls into assimilable nitrogen. This would be analogous to the selection of the *creB* mutation in *A. oryzae* DAR3699, which may have occurred because the increased protease secretion improved access to nitrogen during fermentation of soy, which initially has little accessible nitrogen (HUNTER *et al.* 2013).

Jin *et al.* (2016) compared the performance of 13 fungal strains from *Aspergillus*, *Rhizopus*, and *Trichoderma* genera in their ability to rapidly produce protein from grape marc and improve digestibility. *A. oryzae* RIB40, *A. oryzae* DAR3699, and *T. reesei* RUTC30 were identified as the best performers. Remarkably, two of these three strains are known to have defective CCR. *T. reesei* RUTC30 has a mutated *cre1*, the orthologue of *creA* in *Trichoderma* sp., that encodes a dominant negatively-acting truncated protein that greatly reduces CCR, as well as activating target genes (MELLO-DE-SOUSA *et al.* 2014); this strain performed far better than its parent strain, *T. reesei* QM6a, or three other *Trichoderma* strains tested. We have shown that *creB* in *A. oryzae* DAR3699 has a loss-of-function mutation that likely explains the weak CCR in this strain (HUNTER *et al.* 2013). These findings further support the notion that *creB* mutation would be beneficial for treating solid wastes. The third of the best-performing strains identified by Jin *et al.* (2016), *A. oryzae* RIB40, is the strain in which I deleted *creB*, creating a mutant strain

with increased production of cellulases, xylanases and proteases (HUNTER *et al.* 2013). It would therefore be of great interest to investigate the performance of *A. oryzae* RIB40 *creB*Δ in grape marc bioconversion. Ideally, the performance of the *creB* mutants of *A. oryzae*, *A. nidulans*, *T. reesei* and *P. decumbens* would all be compared to their parent strains, allowing a broad analysis of how deleting *creB* in distantly related ascomycete fungi influences solid waste treatment.

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