An extreme *creA* mutation in *Aspergillus nidulans* has severe effects on D-glucose utilization

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***Aspergillus nidulans* wild-type and the extreme carbon catabolite derepressed mutant *creA^{d-30}* were characterized with respect to enzyme activities, metabolite concentrations and polyol pools all related to glycolysis, after growth on D-glucose. In the *creA^{d-30}* strain the enzymes hexokinase and fructose-6-phosphate reductase showed a two- and threefold increase in activity, respectively, whereas phosphofructokinase and pyruvate kinase activity decreased two- and threefold, respectively, in comparison with the wild-type strain. The most notable changes in metabolite concentrations were that fructose 2,6-bisphosphate and fructose 1,6-bisphosphate showed a 2.5-fold increase, whereas both pyruvate and citrate decreased in the *creA^{d-30}*. Striking differences were found for the polyol concentrations measured for the two strains tested. Intracellular glycerol and arabitol concentrations were 10-fold higher and erythritol fivefold higher in *creA^{d-30}*, whereas intracellular trehalose and mannitol were both decreased. The total internal polyol concentration appears to be constant at ~700 µmol (g dry wt)⁻¹. All polyols were also detected in high amounts in the culture filtrate of the *creA^{d-30}* mutant strain but no extracellular trehalose was found. The overall production of polyols in this strain was therefore much higher than in the wild-type. The high level of polyols produced and the changes in metabolite concentrations in the *creA^{d-30}* strain suggest that the differences in enzyme activities result in an altered flow through glycolysis leading to a more rapid formation of polyols which are subsequently secreted.**

Keywords: *Aspergillus nidulans*, carbon catabolite repression, glycolytic enzyme/metabolite levels, metabolic flux, energy charge

INTRODUCTION

Carbon catabolite repression provides microbial cells with a means to adapt their metabolism so that favoured carbon sources such as D-glucose, D-fructose or D-xylose are utilized preferentially in the presence of other less preferred carbon sources. Mutations in the hyphal fungus *Aspergillus nidulans* resulting in altered responses to carbon catabolite repression have been known for approximately

two decades (Arst & Cove, 1973; Bailey & Arst, 1975). The strategies devised by these authors to obtain carbon catabolite derepressed mutations were based on the selection of pseudorevertants using parental strains with a specific genetic background. Mutants able to grow on a mixture of glucose and L-proline were, for instance, selected from an ammonium-requiring *areA*-defective parental strain. Similarly, carbon catabolite derepressed mutants were obtained from a glucose non-utilizing *pdhA* mutant which itself was unable to utilize ethanol in the presence of glucose due to carbon catabolite repression of alcohol- and aldehyde dehydrogenases.

The most extreme *creA* mutant isolated is the *creA^{d-30}*. This mutant was selected as a spontaneous mutation showing resistance to the toxicity of D-mannitol in a *frA-1* background (Arst *et al.*, 1990). Of the *creA* gene thus

Abbreviations: DHA, dihydroxyacetone; DHAP, dihydroxyacetone-phosphate; F6P, fructose 6-phosphate; F1,6bP, fructose 1,6-bisphosphate; F2,6bP, fructose 2,6-bisphosphate; G1P, glucose 1-phosphate; G3P, glycerol 3-phosphate; G6P, glucose 6-phosphate; M1P, mannitol 1-phosphate; PEP, phosphoenolpyruvate; 1,3bPG, 1,3-bisphosphoglycerate; 2PG, 2-phosphoglycerate; 3PG, 3-phosphoglycerate; 6PG, 6-phospho-gluconolactone; T6P, trehalose 6-phosphate; UDPG, UDP-glucose.

identified, a number of mutant alleles have been selected which are recessive to wild-type and which typically display non-hierarchical heterogeneity of mutant phenotypes (Arst & Bailey, 1977; van der Veen *et al.*, 1994). The CREA protein was postulated to be a negatively acting regulatory protein directly involved in regulating gene expression (Arst & Bailey, 1977; Arst & MacDonald, 1975). This was confirmed by Dowzer & Kelly (1989, 1991) who cloned *creA* and found it to encode a 'zinc finger' DNA-binding protein of the Cys₂His₂ type. A fusion protein containing the 'zinc finger' domain of *A. nidulans* CREA showed sequence-specific binding to promoters of genes which are under carbon catabolite repression control, such as those of the ethanol regulon (Kulmburg *et al.*, 1993) or the proline gene cluster (Cubero & Scazzocchio, 1994). Although the direct role of the CREA protein as a repressor of gene expression has now been confirmed (Cubero & Scazzocchio, 1994; Mathieu & Felenbok, 1994), the physiological consequences of this extreme *creA*^{d-30} mutation have not yet been investigated. We therefore decided to investigate the physiological changes occurring upon growth on D-glucose in a mutant *creA* strain compared to wild-type *A. nidulans*. In this paper we present data on enzyme activities and metabolite levels related to glycolysis and energy metabolism. In addition, intracellular and extracellular polyol concentrations were studied.

METHODS

Materials. All chemicals used were of analytical quality and obtained from Merck. All enzymes and substrates were obtained from Boehringer Mannheim except for fructose 2,6-bisphosphate (F2,6bP) and pyrophosphate-dependent fructose-6-phosphate kinase which were obtained from Sigma.

Strains and growth conditions. *A. nidulans* strain WG096 (*yA2 pabaA1*) was used as the wild-type strain. The *A. nidulans* carbon catabolite derepressed mutant strain used was *creA*^{d-30} (*biA1*) (Arst *et al.*, 1990). The strains were grown on minimal medium as previously described by Pontecorvo *et al.* (1953). *p*-Aminobenzoic acid was added to a final concentration of 2 mg l⁻¹ and biotin to a final concentration of 4 µg l⁻¹ to the cultures. Both strains were grown at 37 °C for 16 h in 250–300 ml minimal medium, 0.05 % (w/v) yeast extract and 1.5 % (w/v) D-glucose in 1 l Erlenmeyer flasks using a New Brunswick orbital shaker at 250 r.p.m. All cultures were inoculated to 1 × 10⁶ spores ml⁻¹.

Preparation of cell-free extracts and protein determination. The preparation of cell-free extracts from mycelium was carried out as described by Witteveen *et al.* (1989). Protein concentrations were measured using the Sigma BCA method as described by van der Veen *et al.* (1991).

Determinations of metabolites. The method to extract metabolites from mycelium as developed by G. J. G. Ruijter & J. Visser (unpublished results) for *Aspergillus niger* was used. It is a modification of the method described by de Koning & van Dam (1992). Glycolytic metabolites were assayed enzymically according to the methods of Bergmeyer (1985a, b). F2,6bP was determined by a kinetic method by measuring the rate of PP_i-dependent phosphofructokinase as described by Van Schaftingen *et al.* (1982). All analyses were performed on a Cobas Bio Autoanalyser (Roche) which was connected to an

MS-DOS computer for datalogging. Volumes in published methods were adapted to the working volume of the apparatus (0.25 ml). Analyses were performed at 30 °C.

Enzyme determinations. All enzyme determinations were carried out using an Aminco DW-2 UV/VIS double beam spectrophotometer (Silver Spring) with an Atari computer for datalogging and calculations. The enzyme activities were measured at 37 °C essentially as described by Bergmeyer (1985a, 1985b) but using 50 mM PIPES buffer, pH 7.5, containing 100 mM KCl and 5 mM MgCl₂ in all cases.

Polyol extraction and determination. The intracellular polyol determinations were carried out as described by Witteveen *et al.* (1993). The extracellular polyol determinations were done as follows. Just before harvesting of the mycelia, a sample was taken from the culture filtrate and spun down for 5 min in an Eppendorf centrifuge to remove mycelia and debris. The sample was diluted fivefold with deionized water from a Millipore filtration unit, and the polyol concentrations were determined by high performance anion-exchange chromatography (HPAEC) on a Dionex system with a CarboPac MA1 column using isocratic elution with 0.48 M NaOH as described by Witteveen *et al.* (1993).

RESULTS

Enzyme activities

An analysis of glycolytic enzyme patterns and metabolite levels in the *A. nidulans* WG096 wild-type strain and a genotypically defined extreme derepressed *creA*^{d-30} mutant was initiated to investigate the physiological consequences of this mutation. Fig. 1 shows an overview of glycolysis with some of its branches.

After 16 h of growth, samples were taken from the mycelia and the culture broth for further analysis. The D-glucose concentration was found to be high enough to exert full carbon catabolite repression (wild-type, 35 mM; *creA*^{d-30}, 40 mM). The total biomass produced in these cultures was 0.823 and 0.725 g dry weight per 250 ml culture for wild-type and *creA*^{d-30}, respectively.

Following growth of the wild-type and *creA*^{d-30} strains on D-glucose we found differences in the activity of several glycolytic enzymes (Table 1). The first notable difference was the increased hexokinase activity in *creA*^{d-30}. The glucose 6-phosphate (G6P) pool formed by hexokinase is distributed to supply the glycolytic and pentose phosphate pathways as well as to synthesize UDP-glucose (UDPG). Moreover, G6P is required for the synthesis of trehalose. There were no significant changes found between the wild-type and the *creA*^{d-30} strain for any of the enzymes involved in G6P metabolism. At the level of fructose 6-phosphate (F6P) metabolism, however, two significant changes were found (cf. Fig. 1). Phosphofructokinase, one of the key regulatory enzymes in glycolysis in many organisms, was decreased in the *creA*^{d-30} mutant strain. Another enzyme that converts F6P into mannitol 1-phosphate (M1P) is fructose-6-phosphate reductase. The M1P formed is subsequently converted into mannitol and is initially stored. Fructose-6-phosphate reductase levels were threefold higher in the *creA*^{d-30} mutant. Interestingly, this was the opposite

Fig. 1. Part of the hexose monophosphate pathway and metabolic routes involved in polyol synthesis. Enzymes: 1, hexokinase; 2, phosphoglucose isomerase; 3, phosphofructokinase; 4, fructose-1,6-bisphosphatase; 5, aldolase; 6, triose phosphate isomerase; 7, glyceraldehyde-3-phosphate dehydrogenase; 8, phosphoglycerate kinase; 9, phosphoglyceromutase; 10, enolase; 11, pyruvate kinase; 12, glycerol-3-phosphate dehydrogenase; 13, glycerol-3-phosphate phosphatase; 14, transketolase; 15, phosphoglucomutase; 16, glucose-6-phosphate dehydrogenase; 17, trehalose-6-phosphate synthase; 18, trehalose-6-phosphate phosphatase; 19, fructose-6-phosphate reductase; 20, mannitol-1-phosphate phosphatase; 21, mannitol dehydrogenase; 22, dihydroxyacetonephosphate phosphatase; 23, glycerol dehydrogenase; 24, UDP-glucose pyrophosphorylase; 25, trehalase; 26, fructose-6-phosphate-2-kinase; 27, fructose-2,6-bisphosphatase.

Table 1. Activities of various intracellular enzymes involved in glucose metabolism in extracts of *A. nidulans* wild-type and *creA^{d-30}* mutant strains

Data are the means of two independent experiments. Strains were grown for 16 h on 1.5% (w/v) D-glucose at 37 °C. Activities are expressed in U (mg protein)⁻¹ ± sd.

Enzyme	Wild-type	<i>creA^d-30</i>
Hexokinase	0.28 ± 0.02	0.46 ± 0.02
Glucose-6-P dehydrogenase	2.18 ± 0.05	1.99 ± 0.01
Phosphoglucumutase	1.22 ± 0.04	1.56 ± 0.05
Phosphoglucose isomerase	2.24 ± 0.13	1.95 ± 0.09
Fructose-6-P reductase	0.53 ± 0.02	1.86 ± 0.03
Phosphofructokinase	1.18 ± 0.02	0.46 ± 0.02
Fructose-1,6-bisphosphatase	0.06 ± 0.005	0.14 ± 0.01
Aldolase	1.86 ± 0.14	1.83 ± 0.14
Triose phosphate isomerase	18.26 ± 0.32	16.83 ± 0.51
Pyruvate kinase	1.50 ± 0.1	0.56 ± 0.1
Citrate synthase	1.08 ± 0.01	0.64 ± 0.01

effect to that observed for phosphofructokinase. Another important enzyme activity in this connection is, of course, fructose-1,6-bisphosphatase which catalyses the reverse reaction of phosphofructokinase (cf. Fig. 1). This activity was low in both strains and although somewhat higher in the *creA^d-30* mutant, it is not likely that this increase can account for the significant physiological changes in the *creA^d-30* strain. The other glycolytic enzyme activities measured, viz. triose phosphate isomerase and aldolase, were high and not altered in the *creA^d-30* strain.

As for the other two enzymes measured, pyruvate kinase activity was threefold lower and citrate synthase activity was 1.7-fold lower in the *creA^d-30* mutant compared with the wild-type.

Metabolite levels

Data on enzyme levels alone provides only limited information as the enzyme activities *in vitro* define only the capacity of the particular enzymic steps in the pathway. By measuring metabolite levels in addition, information is

Table 2. Metabolite determinations in mycelia of *A. nidulans* wild-type and *creA^d-30* strains

Data are the means of three independent experiments. Concentrations are expressed in $\mu\text{mol (g dry wt)}^{-1} \pm \text{SD}$.

Metabolite	Wild-type	<i>creA^d-30</i>
Glucose 6-phosphate	1.23 $\pm$ 0.34	1.02 $\pm$ 0.21
Fructose 6-phosphate	0.20 $\pm$ 0.05	0.22 $\pm$ 0.04
Fructose 1,6-bisphosphate	0.34 $\pm$ 0.04	0.73 $\pm$ 0.08
Fructose 2,6-bisphosphate	0.00135 $\pm$ 0.00014	0.00275 $\pm$ 0.00021
3-Phosphoglycerate	0.08 $\pm$ 0.03	0.12 $\pm$ 0.01
2-Phosphoglycerate	0.07 $\pm$ 0.03	0.12 $\pm$ 0.04
Phosphoenolpyruvate	0.18 $\pm$ 0.11	0.29 $\pm$ 0.15
Pyruvate	3.82 $\pm$ 0.22	1.49 $\pm$ 0.48
Citrate	7.04 $\pm$ 0.67	2.42 $\pm$ 0.23
ATP	1.37 $\pm$ 0.28	1.49 $\pm$ 0.44
ADP	1.88 $\pm$ 0.43	1.66 $\pm$ 0.38
AMP	0.75 $\pm$ 0.12	0.67 $\pm$ 0.18

obtained about the actual cellular concentrations of the various reactants, viz. substrates, products and effectors from which actual metabolic rates can be calculated. The intracellular metabolite levels are presented in Table 2. The levels of G6P and F6P were quite similar in wild-type and in the *creA^d-30* strain. However, in between the phosphofructokinase and pyruvate kinase reaction steps elevated levels of the glycolytic intermediates F1,6bP, 2-phosphoglycerate (2PG), 3-phosphoglycerate (3PG) and phosphoenolpyruvate (PEP) were found. In contrast, levels of pyruvate and citrate, which depend on pyruvate kinase activity, were lower in the *creA^d-30* mutant.

Another striking change in *creA^d-30* was seen at the level of F2,6bP which is a potent activator of phosphofructokinase. Similarly to F1,6bP, a two- to threefold increase was found in the mutant. To determine whether the metabolic changes observed in glycolysis in the *creA^d-30* mutant strain would also have consequences for the energy state of the cells, the levels of the total adenine nucleotide pools present were measured. The values for the energy charge calculated on the basis of these measurements fluctuate between 0.5 and 0.6 and do not suggest drastic changes.

Polyol pools

A. nidulans forms several polyols from D-glucose. Mannitol and glycerol are derived from glycolytic intermediates, whereas arabinol and erythritol arise from pentose phosphate pathway intermediates. Initially the polyols accumulate inside the fungal cell, but finally they are also excreted. Re-utilization of these polyols requires the induction of specific enzymes which are usually under carbon catabolite repression control. We therefore compared wild-type *A. nidulans* and the *creA^d-30* mutant with respect to the intracellular and extracellular polyols formed upon growth on D-glucose (Table 3). The major polyol present in the wild-type upon growth on D-glucose was mannitol. In addition, some trehalose and erythritol

Table 3. Intracellular and extracellular polyol concentrations in *A. nidulans* wild-type and *creA^d-30* mutant strains

Data are the means of two independent experiments. Strains were grown for 16 h on 1.5% (w/v) D-glucose at 37 °C. Intracellular concentrations are expressed in $\mu\text{mol (g dry wt)}^{-1}$; extracellular concentrations are expressed in mM.

Polyol	Intracellular		Extracellular	
	Wild-type	<i>creA^d-30</i>	Wild-type	<i>creA^d-30</i>
Mannitol	623.1	469.4	0.61	2.71
Trehalose	32.7	2.3	0	0
Erythritol	26.9	139.7	0.14	2.36
Glycerol	7.1	65.6	0.18	0.53
Arabinol	2.2	28.2	0	0.16
Total	692	705	0.93	5.76

accumulated whereas only small amounts of glycerol and arabinol were found. In the *creA^d-30* strain this pattern changed completely. Both mannitol and trehalose levels were much lower in *creA^d-30*, whereas the concentrations of arabinol (10-fold) and erythritol (15-fold) were greatly increased. The total intracellular concentration of these polyols including trehalose was found to be similar in both strains [$\sim 690\text{--}700 \mu\text{mol (g dry wt)}^{-1}$]. Polyols were found in the culture filtrates of both the wild-type and the *creA^d-30* strain but no trehalose could be detected in either, whereas arabinol was only present in the culture filtrate of the *creA^d-30* mutant. Extracellular polyols were present in much higher concentrations in the medium of the *creA^d-30* strain (cf. Table 3).

Table 4 shows the total amounts of the various polyols produced by the wild-type and the *creA^d-30* mutant strains. Trehalose was the only polyol produced more in the wild-type (25-fold), whereas the other polyol levels

Table 4. Total amount of intracellular and extracellular polyols produced by *A. nidulans* wild-type and *creA*^{d-30} mutant strainConcentrations are expressed in μmol per 250 ml culture.

Polyol	Wild-type	<i>creA</i> ^{d-30}
Mannitol	666	1063
Trehalose	27	2
Erythritol	57	705
Glycerol	51	186
Arabitol	2	64
Total	803	2020

were higher in the *creA*^{d-30} strain (glycerol, threefold; erythritol, 12-fold; arabitol, 28-fold; mannitol, 1.5-fold). Also, the total amount of the polyols formed in the *creA*^{d-30} mutant was almost threefold higher than in the wild-type strain.

DISCUSSION

The enzymic data obtained suggest that a number of enzymes in D-glucose metabolism are, directly or indirectly, influenced by the CREA protein, namely hexokinase, fructose-6-phosphate reductase, phosphofructokinase, pyruvate kinase and citrate synthase.

Hexokinase, the first enzyme in glycolysis (Fig. 1), was elevated in the *creA*^{d-30} strain (Table 1) whereas phosphofructokinase was lowered. However, these changes did not alter concentrations of G6P and F6P (Table 2), suggesting that overflow may occur via routes branching off from glycolysis (Fig. 1). Two possibilities are the conversion of F6P to M1P by fructose-6-phosphate reductase which leads to the formation of mannitol and the conversion of G6P to 6-phospho-gluconolactone (6PG) by glucose-6-phosphate dehydrogenase which can lead, via the pentose phosphate pathway, to arabitol and erythritol formation. Fructose-6-phosphate reductase (Table 1) was greatly increased in the *creA*^{d-30} strain. Although the internal mannitol concentration (Table 3) is lower than in the wild-type, the total amount of mannitol formed by the *creA*^{d-30} mutant is higher (Table 4). Furthermore, high internal and external concentrations of arabitol and erythritol (Table 3) were found in the *creA*^{d-30} strain, and these high internal concentrations may have some toxic effect in the *creA*^{d-30} strain resulting in, for instance, compact growth. Because of the role polyols have in maintaining the osmotic balance (Beever & Laracy, 1986; Van Laere, 1989), it is likely that the total internal concentration of these polyols remains constant for a given set of growth conditions. Polyols which are overproduced are therefore likely to be secreted, as was found with the *creA*^{d-30} mutant, which had high extracellular levels of all polyols although the total internal polyol concentration was almost the same as that in the wild-type (Table 3).

The increased internal concentration of the metabolites

F1,6dP, 2PG, 3PG and PEP in the *creA*^{d-30} mutant (Table 2) is likely to result from the decreased activity of pyruvate kinase in the *creA*^{d-30} mutant. The increased internal concentration of these metabolites might explain the formation of glycerol in the *creA*^{d-30} mutant. Reduced pyruvate kinase activity is also reflected by lower amounts of pyruvate and citrate in the mutant.

In yeast and fungi, glycolysis is controlled mainly at the level of phosphofructokinase and pyruvate kinase (for a review see, e.g. Gancedo & Serrano, 1989). In *A. niger*, phosphofructokinase is also a key point in regulation of the glycolytic flux, which is activated by NH_4^+ , AMP and F2,6bP and is inhibited by citrate and PEP (Habison *et al.*, 1983; Arts *et al.*, 1987). In the *creA*^{d-30} strain, phosphofructokinase activity was lower than in the wild-type strain. However, the concentration of F2,6bP was higher. It is possible that this increase compensates for the lower amount of phosphofructokinase, but it is not known whether in the *creA*^{d-30} strain this results *in vivo* in phosphofructokinase activity that is comparable with that in the wild-type strain.

Another control point of glycolysis is the first step, the phosphorylation of glucose by hexokinase. In yeast, a strong correlation was found between the catalytic activity of the gene *hxxk2*, which encodes the hexokinase isoenzyme P-II, and its capacity to mediate catabolite repression (Ma *et al.*, 1989; Rose *et al.*, 1991). Furthermore, *Saccharomyces cerevisiae* hexokinase activity was recently found to be strongly inhibited by trehalose 6-phosphate (T6P) (Blázquez *et al.*, 1993). In wild-type *A. nidulans*, more trehalose was detected than in the *creA*^{d-30} strain, suggesting that the flux towards trehalose formation is higher in the wild-type or, more likely, that trehalose turnover is higher in the *creA*^{d-30} strain. Recently it has been found that hexokinase in *A. nidulans* is also inhibited by T6P, suggesting similar regulation to that in *S. cerevisiae* (G. J. G. Ruijter & J. Visser, unpublished results). Interestingly, in *S. cerevisiae* there are several putative binding sites for the carbon catabolite repressor protein MIG1 in the promoter region of *hxxk2*, but the *A. nidulans* equivalent of the latter gene has not been cloned yet.

In summary, the *creA*^{d-30} mutation is responsible for radical changes in D-glucose degradation. These changes occur mainly at the level of those enzymes known to play a role in regulating glycolysis, namely hexokinase, phosphofructokinase and pyruvate kinase. The result of these changes is a higher flux through the side chains towards formation of polyols and a possible decreased flux through the tricarboxylic acid cycle due to lower activity of pyruvate kinase. It is not known, however, if the changes found in the different enzyme levels are a result of a direct or an indirect effect of the *creA* mutation. Polyol catabolism in *A. nidulans* is known to be affected by carbon catabolite repression as shown for the utilization of glycerol (Hondmann & Visser, 1992) and erythritol (Hondmann, 1994). We have insufficient knowledge of how carbon catabolite repression affects polyol biosynthesis (cf. Fig. 1). Although this study indicates the fructose-6-phosphate reductase gene to be a potential

CREA target in mannitol biosynthesis, information about CREA-responsive steps in polyol biosynthesis at the C5, C4 and C3 level is not available. The overall conclusion from our data is that polyol turnover will be higher in *creA^d-30*.

The changes found did not disturb the energy charge in the *creA^d-30* mutant which is comparable to that of the wild-type. Besides these effects found in D-glucose degradation, it is likely that more enzyme systems will be affected by the *creA* mutation, which should be taken into account in interpreting studies of gene regulation using *creA* mutants. The accumulation of glycerol in the *creA^d-30* mutant might be the prime reason, for instance, why its *alcA* and *aldA* expression is high (Mathieu & Felenbok, 1994), although there would be no further physiological consequences in the absence of a suitable substrate.

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REFERENCES

- Arst, H. N., Jr & Cove, D. J. (1973). Nitrogen metabolite repression in *Aspergillus nidulans*. *Mol & Gen Genet* **126**, 111–141.
- Arst, H. N., Jr & MacDonald, D. W. (1975). A gene cluster of *Aspergillus nidulans* with an internally located *cis*-acting regulatory region. *Nature* **254**, 26–31.
- Arst, H. N., Jr & Bailey, C. R. (1977). The regulation of carbon metabolism in *Aspergillus nidulans*. In *Genetics and Physiology of Aspergillus*, pp. 131–146. Edited by J. E. Smith & J. A. Pateman. London: Academic Press.
- Arst, H. N., Jr, Tollervey, D., Dowzer, C. E. A. & Kelly, J. M. (1990). An inversion truncating the *creA* gene of *Aspergillus nidulans* results in carbon catabolite derepression. *Mol Microbiol* **4**, 851–854.
- Arts, E., Kubicek, C. P. & Röhr, M. (1987). Regulation of phosphofructokinase from *Aspergillus niger*: effect of fructose-2,6-bisphosphate on the action of citrate, ammonium ions and AMP. *J Gen Microbiol* **133**, 1195–1199.
- Bailey, C. & Arst, H. N., Jr (1975). Carbon catabolite repression in *Aspergillus nidulans*. *Eur J Biochem* **51**, 573–577.
- Beever, R. E. & Laracy, E. P. (1986). Osmotic adjustment in the filamentous fungus *Aspergillus nidulans*. *J Bacteriol* **168**, 1358–1365.
- Bergmeyer, H. U. (1985a). *Methods of Enzymatic Analysis*, vol. 6. Deerfield Beach, FL: VCH Publishers.
- Bergmeyer, H. U. (1985b). *Methods of Enzymatic Analysis*, vol. 7. Deerfield Beach, FL: VCH Publishers.
- Blázquez, M. A., Lagunas, R., Gancedo, C. & Gancedo, J. M. (1993). Trehalose-6-phosphate, a new regulator of yeast glycolysis that inhibits hexokinases. *FEBS Lett* **329**, 51–54.
- Cubero, B. & Scazzocchio, C. (1994). Two different, adjacent and divergent zinc finger binding sites are necessary for CREA-mediated carbon catabolite repression in the proline gene cluster of *Aspergillus nidulans*. *EMBO J* **13**, 407–415.
- Dowzer, C. E. A. & Kelly, J. M. (1989). Cloning of the *creA* gene from *Aspergillus nidulans*: a gene involved in carbon catabolite repression. *Curr Genet* **15**, 457–459.
- Dowzer, C. E. A. & Kelly, J. M. (1991). Analysis of the *creA* gene, a regulator of carbon catabolite repression in *Aspergillus nidulans*. *Mol Cell Biol* **11**, 5701–5709.
- Gancedo, C. & Serrano, R. (1989). Energy-yielding metabolism. In *The Yeasts*, vol. 3, pp. 205–259. Edited by A. H. Rose & J. S. Harrison. New York: Academic Press.
- Habison, A., Kubicek, C. P. & Röhr, M. (1983). Partial purification and regulatory properties of phosphofructokinase from *Aspergillus niger*. *Biochem J* **209**, 669–676.
- Hondmann, D. H. A. (1994). *Purification, properties and physiological significance of NAD⁺-dependent erythritol dehydrogenase from Aspergillus nidulans*, pp. 105–117. PhD thesis, Wageningen Agricultural University, The Netherlands.
- Hondmann, D. H. A. & Visser, J. (1992). Selection and characterization of *Aspergillus nidulans* mutants involved in regulation of glycerol metabolism. *J Gen Microbiol* **138**, 809–815.
- de Koning, W. & van Dam, K. (1992). A method for the determination of changes in glycolytic metabolites in yeast on a subsecond time scale using extraction at neutral pH. *Anal Biochem* **204**, 118–123.
- Kulmburg, P., Mathieu, M., Dowzer, C., Kelly, J. & Felenbok, B. (1993). Specific binding sites in the *alcR* and *alcA* promoters of the ethanol regulon for the CREA repressor mediating carbon catabolite repression in *Aspergillus nidulans*. *Mol Microbiol* **7**, 847–857.
- Ma, H., Bloom, L. M., Zhu, Z. M., Walsh, C. T. & Botstein, D. (1989). Isolation and characterization of mutations in the Hxk2 gene of *Saccharomyces cerevisiae*. *Mol Cell Biol* **12**, 5630–5642.
- Mathieu, M. & Felenbok, B. (1994). The *Aspergillus nidulans* CREA protein mediates glucose repression of the ethanol regulon at various levels through competition with the ALCR-specific trans-activator. *EMBO J* **13**, 4022–4027.
- Pontecorvo, G., Roper, J. A., Hemmons, L. M., Macdonald, K. D. & Button, A. W. J. (1953). The genetics of *Aspergillus nidulans*. *Adv Genet* **5**, 141–238.
- Rose, M., Albigh, W. & Entian, K. D. (1991). Glucose repression in *Saccharomyces cerevisiae* is directly associated with hexose phosphorylation by hexokinases PI and PII. *Eur J Biochem* **3**, 511–18.
- Van Laere, A. (1989). Trehalose, reserve and/or stress metabolite? *FEMS Microbiol Rev* **63**, 201–210.
- Van Schaftingen, E., Lederer, B., Bartrons, R. & Hers, H. G. (1982). A kinetic study of pyrophosphate: fructose-6-phosphate transferase from potato tubers. *Eur J Biochem* **129**, 191–195.
- van der Veen, P., Flipphi, M. J. A., Voragen, A. G. J. & Visser, J. (1991). Induction, purification and characterisation of arabinases produced by *Aspergillus niger*. *Arch Microbiol* **157**, 23–28.
- van der Veen, P., Arst, H. N., Jr, Flipphi, M. J. A. & Visser, J. (1994). Extracellular arabinases in *Aspergillus nidulans*: The effect of different *cre* mutations on enzyme levels. *Arch Microbiol* **162**, 433–440.
- Witteveen, C. F. B. (1993). *Gluconate formation and polyol metabolism in Aspergillus niger*, pp. 107–122. PhD thesis, Wageningen Agricultural University, The Netherlands.
- Witteveen, C. F. B., Busink, R., van de Vondervoort, P., Dijkema, C., Swart, K. & Visser, J. (1989). L-Arabinose and D-xylose catabolism in *Aspergillus niger*. *J Gen Microbiol* **135**, 2163–2171.

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