

Isolation of *Aspergillus niger creA* mutants and effects of the mutations on expression of arabinases and L-arabinose catabolic enzymes

George J. G. Ruijter, Sipo A. Vanhanen,† Marco M. C. Gielkens,
Peter J. I. van de Vondervoort and Jaap Visser

Author for correspondence: Jaap Visser. Tel: +31 317 484439. Fax: +31 317 484011.
e-mail: office@algemeen.mgim.wau.nl

Section Molecular Genetics
of Industrial
Microorganisms,
Wageningen Agricultural
University, Dreijenlaan 2,
6703 HA Wageningen,
The Netherlands

***Aspergillus niger* mutants relieved of carbon repression were isolated from an *areA* parental strain by selection of colonies that exhibited improved growth on a combination of 4-aminobutanoic acid (GABA) and D-glucose. In addition to derepression of the utilization of GABA as a nitrogen source in the presence of D-glucose, three of the four mutants also showed derepression of L-alanine and L-proline utilization. Transformation of the mutants with the *A. niger creA* gene, encoding the repressor protein CREA, re-established the *areA* phenotype on GABA/D-glucose, identifying the mutations as *creA*^d. The *creA* gene mapped on chromosome IV by linkage analysis and contour-clamped homogeneous electric field hybridization. The *creA* mutants obtained were used to study the involvement of CREA in repression by D-glucose of arabinases and L-arabinose catabolism in *A. niger*. In wild-type *A. niger*, α -L-arabinofuranosidase A, α -L-arabinofuranosidase B, endo-arabinase, L-arabinose reductase and L-arabitol dehydrogenase were induced on L-arabinose, but addition of D-glucose prevented this induction. Repression was relieved to varying degrees in the *creA* mutants, showing that biosynthesis of arabinases and L-arabinose catabolic enzymes is under control of CREA.**

Keywords: carbon repression, *creA*, L-arabinose, arabinase, *Aspergillus niger*

INTRODUCTION

Carbon repression is a global regulatory mechanism in which the presence of D-glucose or other rapidly metabolizable carbon sources represses expression of genes involved in the utilization of less-favoured carbon sources (for reviews on carbon repression in fungi see Ruijter & Visser, 1997; Scazzocchio *et al.*, 1995; Kelly, 1994). It allows micro-organisms to cope smoothly with changes in the carbon sources present in their environment. Genetic analysis has shown that carbon repression in *Aspergillus nidulans* is mediated by a major regulatory gene, *creA*, which acts in a negative manner (Arst & MacDonald, 1975; Arst & Bailey, 1977). Mutations in *A. nidulans creA* result in derepres-

sion of a variety of activities which are normally repressed by D-glucose (Arst & Cove, 1973; Bailey & Arst, 1975; Hynes & Kelly, 1977). *creA* mutations were isolated by several strategies including selection of pseudorevertants of *areA*-defective strains. The *creA* gene has been cloned and sequenced from both *A. nidulans* (Dowzer & Kelly, 1989, 1991) and *Aspergillus niger* (Drysdale *et al.*, 1993) and has been shown to encode a DNA-binding protein containing two zinc fingers of the Cys₂His₂ type, which are very similar to the zinc fingers of MIG1, the repressor involved in glucose repression of the *GAL* and *SUC2* genes in *Saccharomyces cerevisiae* (Nehlin & Ronne, 1990; Nehlin *et al.*, 1991).

Plant cell walls consist of complex polysaccharides of which L-arabinan is one of the constituents. Mycelial fungi are able to degrade these polysaccharides into monomeric sugars, which are subsequently metabolized further. The L-arabinan-degrading system of *A. niger* consists of two distinct α -L-arabinofuranosidases (ABFs) (ABFA and ABFB) and an endo-1,5- α -arabinanase (ABNA) (Van der Veen *et al.*, 1991). The expression of

† Present address: Department of Biological Sciences, University of Durham, South Road, Durham, DH1 3LE, UK.

Abbreviations: ABFA, α -L-arabinofuranosidase A (EC 3.2.1.55); ABFB, α -L-arabinofuranosidase B (EC 3.2.1.55); ABNA, endo-1,5- α -L-arabinanase (EC 3.2.1.99); CHEF, contour-clamped homogeneous electric field; GABA, 4-aminobutanoic acid; PNP-A, *p*-nitrophenyl α -L-arabinofuranoside.

these three enzymes is under the control of pathway-specific induction and carbon repression. Three arabinase-encoding genes, *abfA*, *abfB* and *abnA*, have been cloned and characterized (Flipphi *et al.*, 1993a, b, c, 1994). In the promoters of these three genes several putative CREA-binding sites, i.e. sequence elements identical to the *A. nidulans* CREA motif 5'-G/CPyG GPuG-3' (Kulmburg *et al.*, 1993; Cubero & Sczozochio, 1994) as well as some elements common to all arabinase genes can be found (Flipphi *et al.*, 1994).

Although the *A. niger creA* gene has been cloned, the selection of *A. niger creA* mutants has thus far never been described. By conventional mutagenesis we have now isolated four *A. niger creA* mutants which are useful to establish whether various metabolic systems are under CREA control. As an example, we have studied L-arabinan degradation and L-arabinose catabolism.

METHODS

A. niger strains, isolation of mutants and linkage analysis. All strains used were descendants from *A. niger* N400 (CBS 120.49). N402 (*cspA1*) was used for contour-clamped homogeneous electric field (CHEF) analysis. NW141 (*areA1 bioA1 cspA1 pyrA13*) was isolated from NW140 (*areA1 bioA1 cspA1*; L. Fraissinet-Tachet & J. Visser, unpublished). N616 (Bos *et al.*, 1988) was used as a test strain for genetic analysis.

UV-mutagenesis and isolation of *pyrA* mutants were performed as described by Goosen *et al.* (1987). The survival of the spores for the selection of the *pyrA13* marker was more than 50%. Selection of the *creA* mutants was done after UV-mutagenesis, resulting in 38% survival. Irradiated conidia (4.5×10^7) of strain NW141 were plated on 10 minimal medium (MM) plates (see below) containing 1% (w/v) 4-aminobutanoic acid (GABA) and 1% (w/v) D-glucose. After 4 d incubation at 30 °C, 80 colonies were picked and tested on various combinations of carbon and nitrogen sources.

Linkage analysis was performed as described by Bos *et al.* (1988). Test strain N616 contains genetic markers on six linkage groups: *fwnA1* (I), *hisD4* (II), *lysA7* (III), *leuA1* (IV), *nicA1* (V) and *pabA1* (VI).

Medium and culture conditions. Mycelium was cultured on minimal medium (MM; Pontecorvo *et al.*, 1953) containing 0.02% (v/v) of a trace metal solution (Vishniac & Santer, 1957) and appropriate carbon and nitrogen sources. Where necessary, media were supplemented with 4 µg biotin l⁻¹, 1 mg nicotinamide l⁻¹, 1.4 mg *p*-aminobenzoic acid l⁻¹, 200 mg histidine l⁻¹, 200 mg leucine l⁻¹, 365 mg lysine l⁻¹ and 1.22 g uridine l⁻¹. For plate tests, MM was solidified with 1.5% (w/v) agar unless stated otherwise. For expression studies, strains were grown for 26 h on MM containing 10 mM ammonium tartrate, 0.05% (w/v) yeast extract and 2% (w/v) sucrose in an orbital shaker at 250 r.p.m. at 30 °C. Mycelium was then harvested, washed with MM, transferred to MM containing 10 mM ammonium tartrate and 1% (w/v) carbon source as indicated and incubated for another 4 h.

Transformation of *A. niger*, Northern analysis and CHEF analysis. Transformation of *A. niger* was essentially performed as described by Kusters-van Someren *et al.* (1991) using the *A. niger pyrA* gene (Goosen *et al.*, 1987) as a selective marker. Plasmid pCRA006 (A. P. MacCabe & J. Visser, unpublished results) contains the *A. niger creA* and *pyrA* genes (the *A. niger creA* gene was isolated by heterologous

hybridization with a probe constructed from *A. nidulans creA*; the identity of the gene was established by comparing the restriction map of the cloned gene with the sequence of *A. niger creA* published by Drysdale *et al.*, 1993).

For Northern analysis, total RNA was isolated using TRIzol (Life Technologies) according to the manufacturer's instructions. Total RNA (20 µg for each sample) was run on formaldehyde-containing gels as described by Sambrook *et al.* (1989) and transferred onto Hybond-N (Amersham) membranes by capillary blotting in 10 × SSC. Hybridization was done at 42 °C in buffer containing 50% (w/v) formamide, 0.75 M NaCl, 50 mM sodium phosphate (pH 7.4), 10 mM EDTA, 2 × Denhardt's, 0.1% (w/v) SDS and 10% (w/v) dextran sulphate. Northern blots were washed at 65 °C to a final stringency of 0.2 × SSC/0.1% (w/v) SDS. Probes were prepared using the random priming method (Sambrook *et al.*, 1989). The following DNA fragments were used as probes: a 1.5 kb *Pst*I fragment from plasmid pC2X1 (containing the C-terminal region of *abfA*; Flipphi *et al.*, 1993c); a 1.7 kb *Eco*RI-*Xho*I fragment from plasmid pB2 (containing the *abfB* full-length cDNA; Flipphi *et al.*, 1993a); a 1.1 kb *Eco*RI-*Xho*I fragment from plasmid pC2N4 (containing the *abnA* full-length cDNA; Flipphi *et al.*, 1993b); and a 0.9 kb *Eco*RI fragment from plasmid p28S (containing the *Agaricus bisporus* gene encoding 28S rRNA; Schaap *et al.*, 1996). RNA levels were quantified by laser densitometric scanning of autoradiograms (Ultrascan XL, LKB).

CHEF analysis was performed according to Verdoes *et al.* (1994) with chromosomal DNA of *A. niger* N402. Hybridization was performed using an 840 bp *Xho*I-*Eco*RI fragment of pCRA004 (containing an internal fragment of *creA*; A. P. MacCabe & J. Visser, unpublished results) as a probe.

Preparation of cell extracts and enzyme assays. Preparation of cell extracts and assay of L-arabinose reductase and L-arabitol dehydrogenase were performed as described by Witteveen *et al.* (1989). Enzyme assays were performed on a COBAS Bio autoanalyser (Roche) connected to an MS-DOS computer for data logging. Biochemicals were from Boehringer. Protein concentration in extracts was determined as described previously by Witteveen *et al.* (1989) using the bicinchoninic acid method (Sigma). ABF activity was determined by measuring hydrolysis of *p*-nitrophenyl α-L-arabinofuranoside (PNP-A; Sigma) as described previously by Van der Veen *et al.* (1991).

Western blotting. Denaturing electrophoresis in 10% (w/v) polyacrylamide gels containing 0.1% (w/v) SDS was performed as described by Laemmli (1970) in a Mini-V system (Life Technologies). Protein was blotted onto nitrocellulose filters and blots were then incubated with specific antisera, followed by staining with alkaline-phosphatase-labelled goat anti-mouse IgG and alkaline-phosphatase-labelled goat anti-rabbit IgG as described by the manufacturer (Bio-Rad). Antibodies raised against *A. niger* ABFA, ABFB and endo-arabinase A have been described previously by Van der Veen *et al.* (1991).

Polyol extraction and determination. Extraction and determination of intracellular polyols was done as described by Witteveen *et al.* (1994).

RESULTS

Isolation and characterization of *A. niger creA*^d mutants

A. niger creA mutants were isolated as pseudorevertants of an *areA* loss-of-function mutation. Following UV-

Table 1. Growth properties of *A. niger creA* mutants

Growth characteristics were determined on plates as described in Methods except that nitrate was omitted from the medium; 2.5 mM uridine was added; plates were solidified with agarose; 5 mM GABA, L-alanine or L-proline was used in the presence and absence of 1% (w/v) D-glucose. Growth score: +, very poor growth; ++, poor growth; +++, moderate growth; +++++, good growth.

Strain	Relevant genotype	Morphology*	Growth on:					
			GABA	GABA + D-glucose	L-Proline	L-Proline + D-glucose	L-Alanine	L-Alanine + D-glucose
NW141	<i>areA1</i>	Normal	+	+	+	+	++	+
NW142	<i>areA1 creA1</i>	Type A	++	++	+	+	++	+
NW143	<i>areA1 creA2</i>	Type B	++	++++	+	++	++	+++
NW145	<i>areA1 creA4</i>	Type B	++	++++	+	++	++	+++
NW146	<i>areA1 creA5</i>	Normal	+	+++	+	++++	++	++

* Morphology classes: normal, white mycelium and good sporulation; type A, yellow mycelium and moderate sporulation; type B, white mycelium and poor sporulation.

mutagenesis of the *areA1* strain NW141, strongly growing colonies were selected on MM containing 1% (w/v) D-glucose and 1% (w/v) GABA. Preliminary growth tests on solid media containing different combinations of carbon and nitrogen sources revealed four putative *creA* mutants which were characterized in more detail. These four mutants were clearly derepressed for using GABA as a nitrogen source in the presence of D-glucose (Table 1). Three of the four mutants, *creA2*, *creA4* and *creA5*, also showed derepression for L-alanine and L-proline utilization. Growth of the parental *areA* strain NW141 on GABA was very poor and comparable to growth on GABA + D-glucose. The same was observed for L-proline and L-proline + D-glucose. *A. nidulans areA* strains grow rather well on these amino acids, but growth is reduced when D-glucose is added, due to repression by D-glucose of amino acid metabolism (Scazzocchio *et al.*, 1995). For *A. niger* this is only observed with L-alanine. The explanation for this behaviour is that GABA and L-proline, and to a lesser extent L-alanine, are poor carbon substrates for *A. niger*. In the presence of D-glucose, the amino acids are only required as a nitrogen source, but as D-glucose represses amino acid metabolism, growth is still very poor.

Three classes of morphology were observed (Table 1). *creA5* exhibited a normal morphology, i.e. comparable to the parental *areA1* strain NW141. *creA2*, *creA4* and to a lesser extent *creA1* had difficulty forming conidiospores. The mycelium of *creA1* was yellow, unlike the parental strain and the other three mutants, which formed the usual white mycelium.

To test complementation of the mutations with the *A. niger creA* gene, the mutants were transformed with plasmid pCRA006, which contains the *A. niger creA* and *pyrA* genes (A. P. MacCabe & J. Visser, unpublished results). For all four mutants, uridine prototrophic strains were obtained. Growth of these transformants on GABA/D-glucose was indistinguishable from the

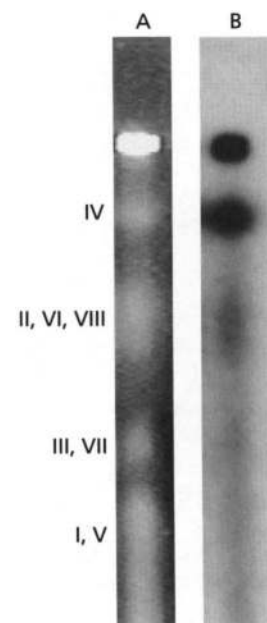


Fig. 1. Chromosome assignment of *creA* by CHEF analysis. Lane A, ethidium-bromide-stained chromosomes of wild-type *A. niger* strain N402 separated by CHEF electrophoresis. Lane B, hybridization of the CHEF blot obtained from the gel shown in lane A with the 840 bp *XhoI-EcoRI* fragment of *creA*. Linkage groups of *A. niger* N402 are indicated by Roman numerals on the left.

areA pyrA⁺ strain NW140. In addition, whereas the morphology of *creA1*, *creA2* and *creA4* was clearly different from parental strain NW141, their *pyrA*⁺ *creA*⁺ transformants had reverted to NW140 morphology.

The genetic localization of the *creA1* (strain NW142) and *creA2* (strain NW143) mutations was determined by linkage analysis. From cross NW142//N616, 58 out of 102 progeny carried *areA1* and these were tested for derepression of GABA utilization in the presence of D-

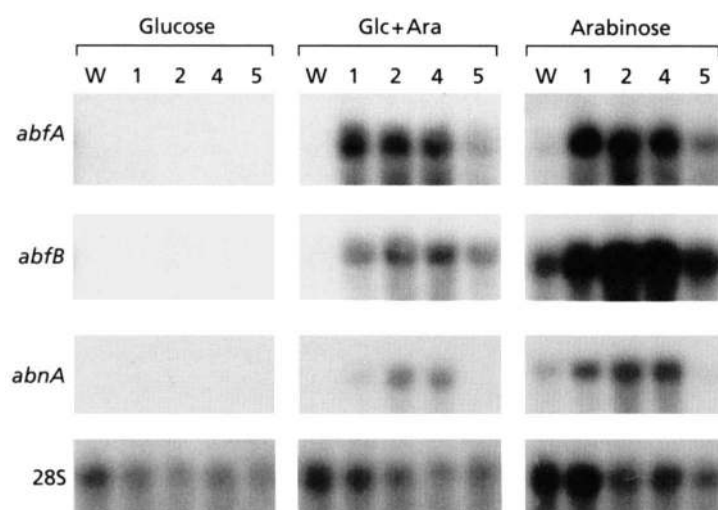


Fig. 2. Arabinase expression in *A. niger* wild-type and *creA* mutants. Mycelium was grown on sucrose, washed and transferred to D-glucose, D-glucose+L-arabinose (Glc+Ara) or L-arabinose as indicated in the figure. Northern blots were prepared as described in Methods and hybridized separately with *abfA*, *abfB*, *abnA* and 28S probes. RNA levels were quantified by laser densitometric scanning of autoradiograms. The 28S rRNA probe was used as an internal control. In the Table, the amounts of *abfA*, *abfB* and *abnA* mRNA relative to 28S rRNA are given for the growth conditions L-arabinose and D-glucose+L-arabinose. After growth on D-glucose, transcript levels were insignificant. In addition, for each of the three genes values were normalized to the induced wild-type level (i.e. NW141 grown on L-arabinose) which was set to 1. W, Wild-type; 1, *creA1*; 2, *creA2*; 4, *creA4*; 5, *creA5*.

	Glc+Ara					Arabinose				
	W	1	2	4	5	W	1	2	4	5
<i>abfA</i>	0.0	8.8	14.9	11.9	4.2	1	8.1	10.5	9.5	6.4
<i>abfB</i>	0.0	0.9	1.5	2.0	1.0	1	1.3	3.1	2.6	2.1
<i>abnA</i>	0.0	0.5	3.5	2.7	0.2	1	2.6	5.0	4.5	0.3

glucose. The recombination frequency between *creA1* and *leuA1* was 5.2%. All other markers gave recombination frequencies between 37 and 54%. Cross NW143//N616 gave comparable results. From a total progeny of 89, 33 recombinants contained *areA1*. Between *creA2* and *leuA1* 12.1% recombination was observed, while between *creA2* and the other markers recombination varied from 38 to 62%. This positioned *creA* on linkage group IV. Localization of the *creA* gene on chromosome IV was confirmed by CHEF analysis (Fig. 1).

Effects of *creA* mutations on expression of ABF and L-arabinose catabolic enzymes

The possible involvement of CREA in repression by D-glucose of arabinases and L-arabinose catabolic enzymes was studied in the *creA* mutants. Wild-type strain NW141 and *creA* mutants were grown for 26 h on MM with 2% sucrose, and mycelia were subsequently transferred to 1% L-arabinose, 1% L-arabinose+1% D-glucose and 1% D-glucose for 4 h. Samples were taken to analyse arabinase transcript levels, arabinase protein levels, activity of arabinases and L-arabinose catabolic enzymes and accumulation of the intermediates of L-arabinose metabolism, L-arabitol and xylitol.

Northern analysis using probes of *abfA* (encoding ABFA), *abfB* (encoding ABFB) and *abnA* (encoding ABNA) revealed that upon transfer to L-arabinose the wild-type strain expressed all three arabinases (Fig. 2).

Upon transfer of the wild-type strain to D-glucose or L-arabinose + D-glucose no expression of the three arabinase genes was detected. In the *creA* mutants, however, the arabinase genes were clearly expressed on L-arabinose + D-glucose. The strongest derepression was observed for mutants *creA2* and *creA4*, whereas moderate derepression was observed for *creA1* and *creA5*. On L-arabinose, a higher expression level of the arabinase genes was observed for all four *creA* mutants except for *abnA* in mutant *creA5*. Again the effect was most pronounced in the case of *creA2* and *creA4*. Relative to the induced wild-type levels, expression of *abfA* and *abnA* had increased more than *abfB*.

Arabinase activities were measured in the culture filtrates. During growth on L-arabinose + D-glucose, ABF activity, measured as PNP-A hydrolysis, was insignificant in a culture filtrate of the wild-type strain, but clearly present in the *creA* mutants (Fig. 3). ABF activity of mutants *creA2* and *creA4* even approached the induced wild-type level (i.e. comparable to NW141 grown on L-arabinose). On L-arabinose, ABF activity in *creA2*, *creA4* and *creA5* was 1.6-, 2.5- and 2.1-fold the wild-type level, respectively, whereas *creA1* was comparable to wild-type. ABNA activity remained too low to be accurately measured.

Immunochemical detection of the arabinase proteins by Western blotting qualitatively corroborated the results of the Northern analysis and the activity measurements (data not shown) and confirmed that of the two ABFs

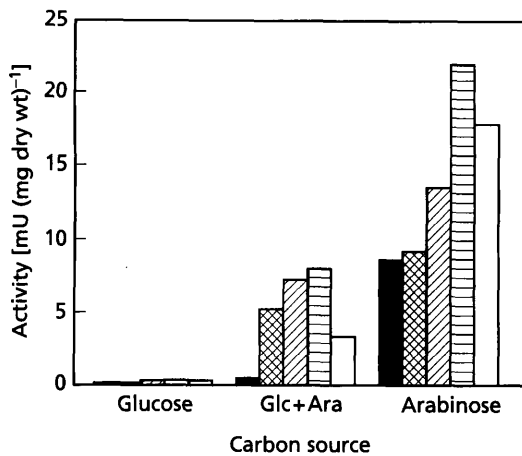


Fig. 3. ABF activity in culture filtrates of *A. niger* wild-type and *creA* mutants. Growth was as described in the legend of Fig. 2. ABF activity was determined by measuring hydrolysis of PNP-A. Activities are expressed as mU (mg dry wt)⁻¹ [equivalent to nmol PNP produced min⁻¹ (mg dry wt)⁻¹]. Data are the mean of two experiments. ■, Wild-type; ▨, *creA1*; ▩, *creA2*; ▪, *creA4*; □, *creA5*. Glc + Ara, D-glucose + L-arabinose.

present in wild-type *A. niger* (ABFA and ABFB), ABFB is the main activity (Van der Veen *et al.*, 1993). ABNA was not detected. Both ABFA and ABFB were clearly derepressed in the *creA* mutants grown on L-arabinose + D-glucose. On L-arabinose, the quantity of ABFB produced by the *creA* mutants was apparently comparable to that observed for the wild-type strain, but the ABFA levels of the *creA* mutants were higher than that of the wild-type. However, it should be remembered that Western blot analysis does not provide quantitative data.

Activities of the enzymes involved in catabolism of L-arabinose (L-arabinose reductase, L-arabitol dehydrogenase, L-xylulose reductase and xylitol dehydrogenase) were analysed in parallel. For all strains the activities of L-arabinose reductase and L-arabitol dehydrogenase were low during growth on D-glucose (Fig. 4). On D-glucose + L-arabinose, the L-arabinose reductase and L-arabitol dehydrogenase activities were still low in wild-type strain NW141, but were increased in the *creA* mutants. Similar results were obtained for L-xylulose reductase and xylitol dehydrogenase (data not shown). Derepression was most pronounced in the case of L-arabitol dehydrogenase, its activity in *creA1* and *creA4* being approximately fourfold the wild-type activity. In contrast to the results obtained for arabinases where *creA2* and *creA4* were most strongly derepressed, *creA1* and *creA4* were derepressed most for the L-arabinose catabolic enzymes. During growth on L-arabinose, the activities of L-arabinose reductase and L-arabitol dehydrogenase were up to twofold higher in the *creA* mutants than in wild-type strain NW141.

Derepression of the L-arabinose catabolic enzymes during growth on a combination of D-glucose and L-

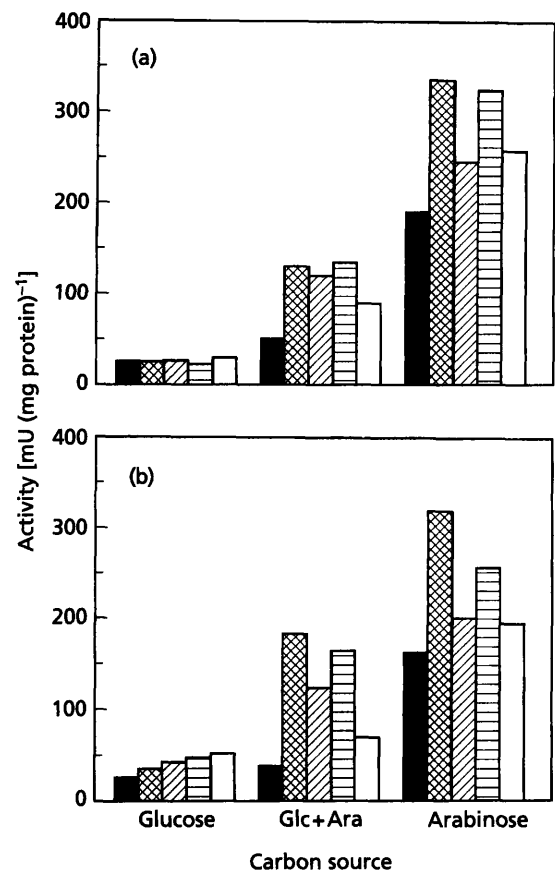


Fig. 4. Specific activity of L-arabinose reductase (a) and L-arabitol dehydrogenase (b) in cell extracts of *A. niger* wild-type and *creA* mutants. Mycelium was cultured as described in the legend of Fig. 2. Activities were measured as described in Methods. Data are the mean of two experiments. ■, Wild-type; ▨, *creA1*; ▩, *creA2*; ▪, *creA4*; □, *creA5*. Glc + Ara, D-glucose + L-arabinose.

arabinose might result in intracellular accumulation of the polyol compounds xylitol and L-arabitol, which are intermediates in L-arabinose degradation (Witteveen *et al.*, 1989). L-Arabitol levels cannot be determined unequivocally due to simultaneous formation of D-arabitol from the pentose phosphate pathway intermediate D-xylulose 5-phosphate under all conditions and the inability to distinguish between D- and L-arabitol by HPLC analysis. The appearance of xylitol, however, is diagnostic of the presence of L-arabitol. No xylitol was observed in any of the strains during growth on D-glucose, whereas a considerable amount of xylitol accumulated during growth on L-arabinose (Table 2). On D-glucose + L-arabinose, a very low level of xylitol was observed in the wild-type strain, but xylitol did accumulate in the *creA* mutants, indicative of derepression of L-arabinose catabolism. As expected, arabitol was found under all growth conditions, but the levels were relatively high during growth on L-arabinose, implying accumulation of L-arabitol.

Table 2. Intracellular polyol concentrations in *A. niger* wild-type and *creA* mutants during growth on D-glucose, D-glucose + L-arabinose and L-arabinose

Polyol concentrations are expressed in $\mu\text{mol (g dry wt)}^{-1}$. Data are the mean of two experiments.

Strain	Relevant genotype	D-Glucose		D-Glucose + L-arabinose		L-Arabinose	
		Xylitol	Arabitol	Xylitol	Arabitol	Xylitol	Arabitol
NW141	Wild-type	0	16	1	19	25	106
NW142	<i>creA1</i>	0	7	19	25	30	100
NW143	<i>creA2</i>	0	40	13	59	56	136
NW145	<i>creA4</i>	0	38	14	58	66	154
NW146	<i>creA5</i>	0	14	6	26	23	93

DISCUSSION

Hypal fungi seem to share the regulatory system responsible for carbon repression which was first described for *A. nidulans* on the basis of mutations in *creA* (Arst & Cove, 1973; Bailey & Arst, 1975; Hynes & Kelly, 1977) and further substantiated by the cloning of this gene (Dowzer & Kelly, 1989, 1991). The *A. niger creA* gene was found to be very similar to the *A. nidulans creA* gene and, moreover, was shown to be functional in *A. nidulans* (Drysdale *et al.*, 1993). However, thus far no *creA* mutants have been described for *A. niger*. In this report, we describe such mutants which have, as expected, a derepressed phenotype. The following results imply that the mutants we isolated are impaired in *creA*. The mutants were derepressed for a number of systems involved in carbon catabolism. In an *areA* background, the *creA* mutations allowed utilization of GABA, L-proline or L-alanine as nitrogen sources in the presence of D-glucose (Table 1). Whereas the parent showed repression of arabinases and L-arabinose catabolism in medium containing both L-arabinose and D-glucose, these functions were clearly derepressed in the *creA* mutants under the same conditions (Figs 2–4). Finally, transformation of the mutant strains with the *A. niger creA* gene resulted in a phenotype that was indistinguishable from the parental *areA* strain. The finding that the *A. niger creA* gene was able to complement the mutations is a strong indication that we have isolated *creA* mutants.

The different *A. niger creA* alleles also displayed non-hierarchical heterogeneity, observed with *A. nidulans creA* mutants as well (Arst & Bailey, 1977) and indicative of a direct effect of CREA on transcription. For example, whereas *creA2* and *creA4* were strongly derepressed for the use of GABA and L-alanine in the presence of D-glucose and less for L-proline, *creA5* showed exactly the opposite phenotype.

A. niger creA mutants further exhibited decreased growth rates and reduced sporulation. In addition, *A. niger creA1* produced a yellow pigment, which is probably a secondary metabolite whose biosynthesis is normally repressed by D-glucose.

From our results it appears that *creA2* and *creA4* are the most interesting alleles. These alleles are extremely useful for investigating the involvement of CREA in the control of other systems subject to carbon repression. Cloning and sequencing of the *creA* alleles may be useful for identifying domains in the protein, other than the zinc finger region, that are important for its function. This was recently done by Shroff *et al.* (1996) for a number of *A. nidulans creA* alleles. Three of the *A. nidulans creA* alleles analysed have missense mutations in the zinc finger domain whereas four other mutations result in truncations of CREA between the zinc finger domain and the C-terminus of the protein.

Analysis of arabinase expression in *A. nidulans creA* mutants has demonstrated that the arabinase system is suitable for investigating carbon repression in this fungus (Van der Veen *et al.*, 1994). The *A. niger* arabinases (ABFA, ABFB and ABNA) are induced by L-arabinose and L-arabitol, whereas addition of D-glucose prevents this induction (Van der Veen *et al.*, 1993; Flipphi *et al.*, 1994). Similarly, enzymes involved in catabolism of L-arabinose are expressed during growth on L-arabinose, but not on D-glucose (Witteveen *et al.*, 1989). These observations suggest repression of arabinases and L-arabinose catabolic enzymes by D-glucose. In this report, we show that this repression is in fact mediated by CREA. No expression of *abfA*, *abfB* and *abnA* was detected on a combination of L-arabinose + D-glucose in the wild-type strain. In the *creA* mutants, however, the three arabinase genes were clearly expressed under these conditions (Fig. 2). Biosynthesis and secretion of the arabinases was confirmed by Western blot analysis and activity in the case of ABFA and ABFB, but not for ABNA (Fig. 3). The absence of ABNA is explained by the delayed expression of *abnA* compared to *abfA* and *abfB*, which has been observed previously by Flipphi *et al.* (1994). L-Arabinose reductase and L-arabitol dehydrogenase, the enzymes involved in L-arabinose catabolism, were also derepressed in the *creA* mutants (Fig. 4). Several putative CREA-binding sites are present in the promoters of the three arabinase-encoding genes (Flipphi *et al.*, 1994) and it is most likely that CREA directly represses the genes encoding arabi-

nases and L-arabinose catabolic enzymes by binding to its cognate sequence(s) in the promoters of these genes. This is substantiated by the non-hierarchical heterogeneity amongst the different *creA* alleles. Thus, while *creA2* and *creA4* showed stronger derepression of *abnA* than of *abfB*, derepression of *abnA* was much less than that of *abfB* in *creA5*. Similarly, derepression of arabinase genes was more pronounced for *creA2* and *creA4* than for *creA1*, but *creA1* was more derepressed for L-arabinose reductase and L-arabitol dehydrogenase. Two other possible mechanisms of repression of genes encoding arabinases and L-arabinose catabolic enzymes are (1) a cascade mechanism, i.e. repression of a common transcription activator protein and (2) lack of inducer formation. A common transcription activator for arabinases has been proposed by Flippi *et al.* (1994) on the basis of the finding that extra gene copies of either *abfA* or *abfB* decreased expression of the other *abf* gene and, more obviously, of the more weakly expressed *abnA* gene. A cascade mechanism for repression operates in the case of the *alc* system in *A. nidulans*. The gene encoding the transcription activator of the *alc* system, *alcR*, is repressed by D-glucose, partially preventing induction of the *alc* system (Kulmburg *et al.*, 1993). However, most of the *alc* genes, including *alcA* which is the structural gene for alcohol dehydrogenase I, are also repressed directly by CREA (Fillinger & Felenbok, 1996; Kulmburg *et al.*, 1993). The second alternative for direct repression is lack of inducer formation. Arabinases are induced by L-arabitol, an intermediate of L-arabinose metabolism (Van der Veen *et al.*, 1993), and derepression of L-arabinose uptake and L-arabinose reductase, which could result in intracellular accumulation of L-arabitol, might be sufficient to induce expression of arabinases. Such a mechanism operates, for example, in the case of the *GAL* genes in *S. cerevisiae*, where MIG1 represses the expression of the D-galactose permease thereby reducing the level of functional inducer (Johnston *et al.*, 1994). Proper investigation of the relative contribution of the three repression mechanisms mentioned requires isolation of the genes encoding the putative arabinase transcription activator, the L-arabinose permease and the enzymes involved in L-arabinose catabolism.

ACKNOWLEDGEMENTS

The work described in this paper was financially supported in part by the Ministry of Economic Affairs, the Ministry of Education, Culture and Science, The Ministry of Agriculture, Nature Management and Fishery in the framework of an industrial relevant research programme of the Netherlands Association of Biotechnology Centres (ABON) and in part by the European Community (to J.V.; grant B102-CT93-0174) for research on wide-domain regulation.

REFERENCES

Arst, H. N., Jr & Bailey, C. R. (1977). The regulation of carbon metabolism in *Aspergillus nidulans*. In *Genetics and Physiology of Aspergillus nidulans*, pp. 131–146. Edited by J. E. Smith & J. A. Pateman. London: Academic Press.

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 in *Aspergillus nidulans* with an internally located *cis*-acting regulatory region. *Nature* **254**, 26–31.

Bailey, C. R. & Arst, H. N., Jr (1975). Carbon catabolite repression in *Aspergillus nidulans*. *Eur J Biochem* **51**, 573–577.

Bos, C. J., Debets, A. J. M., Swart, K., Huybers, A., Kobus, G. & Slakhorst, S. M. (1988). Genetic analysis and the construction of master strains for assignment of genes to six linkage groups in *Aspergillus niger*. *Curr Genet* **14**, 437–443.

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 *creA* 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.

Drysdale, M. R., Kolze, S. E. & Kelly, J. M. (1993). The *Aspergillus niger* carbon catabolite repressor gene, *creA*. *Gene* **130**, 241–245.

Fillinger, S. & Felenbok, B. (1996). A newly identified gene cluster in *Aspergillus nidulans* comprises five novel genes localized in the *alc* region that are controlled both by the specific transactivator *AlcR* and the general carbon-catabolite repressor *CreA*. *Mol Microbiol* **20**, 475–488.

Flippi, M. J. A., van Heuvel, M., Van der Veen, P., Visser, J. & de Graaff, L. H. (1993a). Cloning and characterization of the *abfB* gene coding for the major α -L-arabinofuranosidase (ABF B) of *Aspergillus niger*. *Curr Genet* **24**, 525–532.

Flippi, M. J. A., Panneman, H., Van der Veen, P., Visser, J. & de Graaff, L. H. (1993b). Molecular cloning, expression and structure of the endo-1,5- α -L-arabinase gene of *Aspergillus niger*. *Appl Microbiol Biotechnol* **40**, 318–326.

Flippi, M. J. A., Visser, J., Van der Veen, P. & de Graaff, L. H. (1993c). Cloning of the *Aspergillus niger* gene encoding α -L-arabinofuranosidase A. *Appl Microbiol Biotechnol* **39**, 335–340.

Flippi, M. J. A., Visser, J., Van der Veen, P. & de Graaff, L. H. (1994). Arabinase gene expression in *Aspergillus niger*: indications for coordinated regulation. *Microbiology* **140**, 2673–2682.

Goosen, T., Bloemheuvel, G., Gysler, C., de Bie, D. A., Van den Broek, H. W. J. & Swart, K. (1987). Transformation of *Aspergillus niger* using the homologous orotidine-5'-phosphate-decarboxylase gene. *Curr Genet* **11**, 499–503.

Hynes, M. J. & Kelly, J. M. (1977). Pleiotropic mutants of *Aspergillus nidulans* altered in carbon metabolism. *Mol Gen Genet* **150**, 193–204.

Johnston, M., Flick, J. S. & Pexton, T. (1994). Multiple mechanisms provide rapid and stringent glucose repression of *gal* gene expression in *Saccharomyces cerevisiae*. *Mol Cell Biol* **14**, 3834–3841.

Kelly, J. M. (1994). Carbon catabolite repression. *Prog Ind Microbiol* **29**, 355–367.

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.

Kusters-van Someren, M. A., Harmsen, J. A. M., Kester, H. C. M. & Visser, J. (1991). Structure of the *Aspergillus niger* *peIA* gene and its expression in *Aspergillus niger* and *Aspergillus nidulans*. *Curr Genet* **20**, 293–299.

- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Nehlin, J. O. & Ronne, H. (1990). Yeast MIG1 repressor is related to the mammalian early growth response and Wilms' tumour finger proteins. *EMBO J* **9**, 2891–2898.
- Nehlin, J. O., Carlberg, M. & Ronne, H. (1991). Control of yeast GAL genes by MIG1 repressor: a transcriptional cascade in the glucose response. *EMBO J* **10**, 3373–3377.
- Pontecorvo, G., Roper, J. A., Hemmons, L. M., MacDonald, K. D. & Bufton, A. W. J. (1953). The genetics of *Aspergillus nidulans*. *Adv Genet* **5**, 141–238.
- Ruijter, G. J. G. & Visser, J. (1997). Carbon repression in *Aspergilli*. *FEMS Microbiol Lett* **151**, 103–114.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Scazzocchio, C., Gavrias, V., Cubero, B., Panozzo, C., Mathieu, M. & Felenbok, B. (1995). Carbon catabolite repression in *Aspergillus nidulans*: a review. *Can J Bot* **73**, S160–S166.
- Schaap, P. J., Müller, Y., Baars, J. J. P., Op den Camp, H. J. M., Sonnenberg, A. S. M., Van Griensven, L. J. L. D. & Visser, J. (1996). Nucleotide sequence and expression of the gene encoding NADP⁺-dependent glutamate dehydrogenase (*gdhA*) from *Agaricus bisporus*. *Mol Gen Genet* **250**, 339–347.
- Shroff, R. A., Lockington, R. A. & Kelly, J. M. (1996). Analysis of mutations in the *creA* gene involved in carbon catabolite repression in *Aspergillus nidulans*. *Can J Microbiol* **42**, 950–959.
- Van der Veen, P., Flippi, 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., Flippi, M. J. A., Voragen, A. G. J. & Visser, J. (1993). Induction of extracellular arabinases on monomeric substrates in *Aspergillus niger*. *Arch Microbiol* **159**, 66–71.
- Van der Veen, P., Arst, H. N., Jr, Flippi, 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.
- Verdoes, J. C., Calil, M. R., Punt, P. J., Debets, F., Swart, K., Stouthamer, A. H. & Van den Hondel, C. A. J. J. (1994). The complete karyotype of *Aspergillus niger*: the use of introduced electrophoretic mobility variation of chromosomes for gene assignment studies. *Mol Gen Genet* **244**, 75–80.
- Vishniac, W. & Santer, M. (1957). The thiobacilli. *Bacteriol Rev* **21**, 195–213.
- Witteveen, C. F. B., Busink, R., Van de Vondervoort, P. J. I., Dijkema, C., Swart, K. & Visser, J. (1989). L-Arabinose and D-xylose catabolism in *Aspergillus niger*. *J Gen Microbiol* **135**, 2163–2171.
- Witteveen, C. F. B., Weber, F., Busink, R. & Visser, J. (1994). Isolation and characterisation of two xylitol dehydrogenases from *Aspergillus niger*. *Microbiology* **140**, 1679–1685.

Received 22 April 1997; accepted 6 June 1997.