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

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**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 creAd. 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 derepression 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 CysX2His3 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
these three enzymes is under the control of pathway-specific induction and carbon repression. Three arabinose-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 (Kulmberg *et al.*, 1993; Cubero & Scaccozzio, 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 (*cspAl*) was used for contour-clamped homogeneic 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 were plated on MM plates (see below) containing 1% (w/v) 4-dimethylaminobenzoic 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: *funA1* (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 0.2% (v/v) of a trace metal solution (Vishniac *et al.*, 1957) and appropriate carbon and nitrogen sources. Where necessary, media were supplemented with 4 μg biotin 1-1, 1 mg nicotinamide 1-1, 1-4 mg p-aminobenzoic acid 1-1, 200 mg histidine 1-1, 200 mg leucine 1-1, 365 mg lysine 1-1 and 1.22 g uridine 1-1. 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 TRZol (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 *PstI* fragment from plasmid pC2X1 (containing the C-terminal region of *abfA*; Flipphi *et al.*, 1993c); a 1.7 kb EcoRI-XhoI fragment from plasmid pB2 (containing the *abfB* full-length cDNA; Flipphi *et al.*, 1993a); a 1.1 kb EcoRI-XhoI fragment from plasmid pC2N4 (containing the *abnA* full-length cDNA; Flipphi *et al.*, 1993b); and a 0.9 kb EcoRI fragment from plasmid p2S (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 XhoI-EcoRI 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 previously described by Witteveen *et al.* (1989) using the bicinchoninic acid method (Sigma). ABF activity was determined by measuring hydrolysis of p-nitrophenyl-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 endoarabinase 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<sup>Δ</sup> 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.

<table>
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<th>Strain</th>
<th>Relevant genotype</th>
<th>Morphology*</th>
<th>GABA</th>
<th>GABA + D-glucose</th>
<th>L-Proline</th>
<th>L-Proline + D-glucose</th>
<th>L-Alanine</th>
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* Morphology classes: normal, white mycelium and good sporulation; type A, yellow mycelium and moderate sporulation; type B, white mycelium and poor sporulation.

Aspergillus niger creA mutants

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 (Sc滋zocchio 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

An 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-
Glucose  |  Glc+Ara  |  Arabinose
---|---|---
|  |  |  |
| abfA |  |  |
| abfB |  |  |
| abnA |  |  |
| 28S |  |  |

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<td>1 2.6 5.0 4.5 0.3</td>
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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.

Immunochromatographic analysis of the arabinase protein 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
Aspergillus niger creA mutants

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-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.

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.
DISCUSSION

Hyphal 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; Flippi 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 Flippi 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 (Flippi 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 Flipphi 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 (Fillingier & 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 MIIG1 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. V.) grant B102-CT93-0174 for research on wide-domain regulation.

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Received 22 April 1997; accepted 6 June 1997.