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Studies on the Role of the *are*A Gene in the Regulation of Nitrogen Catabolism in *Aspergillus nidulans*

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Abstract

Mutants of *Apergillus nidulans* with lesions in a gene, *are*A (formerly called *amd*T), have been isolated by a variety of different selection methods. The *are*A mutants show a range of pleiotropic growth responses to a number of compounds as sole nitrogen sources, but are normal in utilization of carbon sources. The levels of two amidase enzymes as well as urease have been investigated in the mutants and have been shown to be affected by this gene. Most of the *are*A mutants have much lower amidase-specific activities when grown in ammonium-containing medium, compared with mycelium incubated in medium lacking a nitrogen source. Some of the *are*A mutants do not show derepression of urease upon relief of ammonium repression. The dominance relationships of *are*A alleles have been investigated in heterozygous diploids, and these studies lend support to the proposal that *are*A codes for a positively acting regulatory product. One of the new *are*A alleles is partially dominant to *are*A$^+$ and *are*A102. This may be a result of negative complementation or indicate that *are*A has an additional negative regulatory function. Investigation of various *amd*R ; *are*A double mutants has led to the conclusion that *amd*R and *are*A participate in independent regulatory circuits in the control of acetamide utilization. Studies on an *amd*R^c ; *are*A double mutant indicate that *are*A is involved in derepression of acetamidase upon relief of ammonium repression.

Introduction

It has been shown in *Aspergillus nidulans* that lesions in a gene, *amd*T, lead to pleiotropic effects on the utilization of many nitrogen sources (Hynes 1973*a*). Many of the uptake systems and enzymes involved in the utilization of nitrogen sources by *A. nidulans*, yeast and other fungi have been shown to be subject to repression by ammonium (Dubois *et al.* 1973; Pateman *et al.* 1973). The previously described *amd*T mutants did not show obvious alterations in their responses to ammonium repression of two amidase enzymes and of glutamate uptake (Hynes 1972, 1973*b*). The effects of carbon sources on these systems led to the suggestion that *amd*T was involved in the interaction between carbon and nitrogen metabolism (Hynes 1973*a*). However, it has been recently found that the *amd*T mutations are in the same gene as *are*A mutations, some of which can lead to loss of ammonium repression of a number of systems (Arst and Cove 1973). In addition, a strain, *xpr*D1, directly selected for loss of ammonium repression of extracellular proteases (Cohen 1972), has also been found to have a mutation in *are*A and to be derepressed for some other nitrogen-source utilization systems (Arst and Cove 1973). It has been suggested that the *are*A product is required for the synthesis of most systems of nitrogen catabolism and that ammonium repression involves an interference with the action of the *are*A product (Arst and Cove 1973). Carbon catabolite repression is proposed to be mediated by a separate regulatory system from ammonium repression.

This paper describes the isolation of new *are*A alleles and their properties. The pleiotropic effects of *amd*T102 (redesignated *are*A102) and *amd*T19 (redesignated *are*A19) have been used to isolate new alleles by selection using a variety of different methods. The properties of these new *are*A strains support the hypothesis that *are*A is a positively acting regulatory gene. Support for the involvement of *are*A in ammonium repression is provided by studies on urease and acetamidase levels in some of the mutants, but most of the *are*A alleles do not have large effects on responses to ammonium repression. The *amd*R gene appears to be involved only in the regulation of acetamide utilization (Hynes and Pateman 1970*a*, 1970*b*; Dunsmuir and Hynes 1973). Strains containing various *are*A and *amd*R alleles have been constructed and investigated. The results indicate that *are*A and *amd*R are independent of each other in their regulatory effects.

Table 1. Summary of the isolation of *are*A strains

*are*A$^+$ was formerly *amd*T$^+$; *are*A102 was formerly *amd*T102; *are*A19 was formerly *amd*T19

Strain	Parental genotype[A]	Origin	Method of selection
*are*A$^+$	*bi*A1 ; *nii*A4	—	—
*are*A102	*bi*A1 ; *nii*A4	NG-treated[B]	Growth on acrylamide
*are*A19	*bi*A1 ; *are*A102 ; *nii*A4	NG-treated	Resistance to fluoroacetamide
*are*A201	*bi*A1 ; *are*A19 ; *nii*A4	Spontaneous	Growth on histidine
*are*A205	*bi*A1 ; *are*A19 ; *nii*A4	Spontaneous	Growth on histidine
*are*A211	*bi*A1 ; *are*A19 ; *nii*A4	Spontaneous	Growth on glutamate
*are*A272	*bi*A1 ; *are*A19 ; *nic*B8	NG-treated	Growth on glutamate
*are*A200	*bi*A1 ; *are*A102 ; *nii*A4	Spontaneous	Growth on uric acid
*are*A256	*bi*A1 ; *are*A19 ; *nic*B8	NG-treated	Growth on histidine
*are*A238	*bi*A1 ; *are*A102 ; *nii*A4	NG-treated	Resistance to fluoroacetamide
*are*A241	*y*A1 ; *are*A217 ; *ribo*B2	NG-treated	Growth on formamide
*are*A217	*bi*A1 ; *pu*A2 ; *are*A102	NG-treated	Lack of growth on histidine
*are*A209	*bi*A1 ; *pu*A2 ; *are*A102	NG-treated	Lack of growth on histidine

[A] Full genotype of strain from which the new *are*A strain was obtained.
[B] NG-treated = *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treated.

Materials and Methods

Strains

Genetic markers in the strains are those in general use (Clutterbuck 1973). The isolation of the mutants, *amd*T102 and *amd*T19, and some of their properties have been previously described (Hynes and Pateman 1970*a*; Hynes 1972, 1973*a*). The finding that the *amd*T locus is almost certainly identical to the *are*A locus (Arst and Cove 1973) has led to the redesignation of these strains as *are*A102 and *are*A19 respectively as this locus name is the more general one. The pleiotropic effects of these lesions in the *are*A locus have allowed the isolation of strains with new *are*A alleles by many different methods. These are described in Table 1, and the methods used to select the mutants and their genetic characterization are outlined below. In each case the strain has been designated by the *are*A allele it contains. It should be noted that most of these new strains probably have more than one lesion in the *are*A gene as they are presumably derived by intracistronic mutational events.

Methods of Isolation of New Mutants (see Table 1)

Strain *are*A238 was isolated in the same experiment as *are*A19 (Hynes 1972); *are*A211 was isolated as a spontaneously occurring sector on glucose–glutamate medium. Other mutants, isolated by positive methods (i.e. selected for stronger growth on the particular nitrogen source), were obtained by spreading conidia (either treated with mutagen or untreated) on the relevant medium. Strains *are*A217 and *are*A209 were isolated by replica-plating, using the method of Mackintosh and Pritchard (1963).

Mutagen Treatment

N-methyl-*N'*-nitro-*N*-nitrosoguanidine (NG) was used as a mutagen. The method of treatment of conidia for the isolation of *are*A102, *are*A19 and *are*A238 has been described previously (Hynes and Pateman 1970*a*; Hynes 1972). For the isolation of *are*A217 and *are*A209 a dense conidial suspension in tris–maleate buffer, pH 6·0, was treated with 193 μg/ml of NG for 30 min at 37°C. In isolating *are*A272 and *are*A256, a similar treatment was employed except that 150 μg/ml NG was used at room temperature, while *are*A241 was obtained in a similar way except that 200 μg/ml NG was used for 20 min at room temperature.

Culture Conditions

Media have been described before (Hynes 1970, 1972). Growth tests on solid media at 37°C were performed as described previously (Hynes 1973*a*, 1973*b*). Mycelium for enzyme assays was grown in 200 ml of medium in 1-litre flasks in a Gallenkamp orbital incubator at 30°C. Mycelium was harvested by previously described methods (Hynes 1970, 1972).

Enzyme Assays

Amidase (EC 3.5.1.4) was assayed as before (Hynes 1970, 1972), with the modification that all crude extracts were made in 100 mM orthophosphate buffer at pH 7·2, and amide substrate solutions were also made in this buffer.

Urease (EC 3.5.1.5) was assayed by the method for amidase assays using 10 mM urea in 100 mM orthophosphate buffer, pH 7·2, as substrate.

Chemicals

These were the best commercial grades available; 2-thiouric acid was a generous gift of D. Phillipides, C. Scazzocchio and H. Arst, Genetics Department, Cambridge University.

Table 2. Results of crosses of *are*A mutant strains to wild-type strains (*are*A$^+$)

Mutant *are*A strains were crossed to *are*A$^+$ strains, and segregation of the original parent *are*A allele looked for. However, no cases of segregation of the original parent *are*A allele were observed in any cross

Mutant strain	No. of progeny examined	Mutant strain	No. of progeny examined
*are*A201	890	*are*A256	80
*are*A205	256	*are*A238	150
*are*A211	580	*are*A241	165
*are*A272	500	*are*A217	696
*are*A200	1700	*are*A209	96

Results

Genetic Characterization of are*A Strains*

It has been previously shown (Hynes 1972) that the *are*A19 lesion is very closely linked to the *are*A102 lesion and does not complement *are*A102 in growth on formamide. The new *are*A strains have been crossed to *are*A$^+$ strains and segregation of the original parent *are*A allele looked for (Table 2). The results indicate that the sites of the new lesions are close to the original lesions. In addition, in crosses between strains containing *are*A217 and *are*A19, no *are*A$^+$ wild-type recombinants have been observed (0/132). In heterozygous diploids, *are*A217 and *are*A209 do not complement *are*A102 for growth on formamide as sole nitrogen source, and *are*A200 and *are*A217 do not complement for growth on acetamide as sole nitrogen source. This evidence, together with the facts that the mutants were isolated by a variety of selective tech-

niques, include spontaneous mutants and have phenotypes related to those of the parent *are*A alleles and to those isolated by Arst and Cove (1973), indicates that all the strains have resulted from intracistronic events at the *are*A locus. Detailed fine structure mapping is necessary to determine the number and relative positions of the sites within the *are*A gene.

Table 3. Nitrogen-source utilization by *are*A mutants

All nitrogen sources were present at a concentration of 10 mM in glucose medium, except for urea, which was at a concentration of 5 mM. +++ Very strong growth. ++ Strong growth. + Growth. ± Very poor growth. — Background growth. Symbols on different media are not equivalent

Strain	Nitrogen source							
	NH_4^+	Urea	Formamide	Acetamide	Acrylamide	Glutamate	Histidine	Uric acid
*are*A$^+$	++	++	++	+	—	+	±	++
*are*A102	++	++	±	+++	+++	++	++	—
*are*A19	++	+	—	±	—	—	—	±
*are*A201	++	++	±	+++	++	++	++	—
*are*A205	++	+	±	+	+	±	+	±
*are*A211	++	++	±	+++	++	++	++	—
*are*A272	++	++	±	+	+	±	+	±
*are*A200	++	+	+	±	—	±	±	++
*are*A256	++	+	±	+	+	±	+	±
*are*A238	++	+	—	+	±	++	+	—
*are*A241	++	++	±	+++	++	++	++	—
*are*A217	++	±	—	—	—	—	—	—
*are*A209	++	±	—	—	—	—	—	—

Growth Properties of the are*A Mutants*

Table 3 summarizes the most important growth properties of the mutants. The utilization of most nitrogen sources is affected in the mutants, but the pattern of growth response is characteristic of the particular *are*A allele present. The strains derived from *are*A19 have regained most of the properties of *are*A102 to some extent, with *are*A201 and *are*A211 being only slightly weaker than *are*A102 on most nitrogen sources. Strain *are*A241 is indistinguishable from *are*A102. Strain *are*A238 has reduced ability to grow on acetamide and acrylamide and slightly reduced growth on most other nitrogen sources.

The most interesting new *are*A mutants are *are*A217, *are*A209 and *are*A200. Strains *are*A217 and *are*A209 show almost complete loss of ability to utilize all nitrogen sources, with the exception of ammonium. Strain *are*A200, isolated as a revertant of *are*A102 capable of growth on uric acid, was found to have reduced growth on some nitrogen sources. This strain was found to grow better than *are*A102 on formamide, and, unlike *are*A102, was sensitive to inhibition of green conidial colour by 2-thioxanthine and 2-thiouric acid (see Darlington and Scazzocchio 1967).

Utilization of a number of carbon sources is not altered in the *are*A mutants, including those (e.g. acetamide and proline) capable of acting as sources of both carbon and nitrogen. However, there is slightly reduced utilization of L-glutamate as a carbon source by *are*A217 and *are*A209, and utilization of milk protein as the sole carbon source is completely abolished in these two mutants.

Enzyme Activities of the are*A Mutants*

Table 4 shows the results of amidase determinations on *are*A strains pregrown in glucose–ammonium medium and then transferred to medium lacking a nitrogen source, while Table 5 shows the amidase results for *are*A strains grown in glucose–acetamide medium. As shown before (Hynes 1972, 1973*a*), *are*A102 has increased acetamidase activity compared with *are*A$^+$, while *are*A19 has low activity. The new *are*A strains have acetamidase levels consistent with growth responses on acetamide and acrylamide. Formamidase levels are lower than the wild type (*are*A$^+$) for all the new *are*A mutants, with the exception of *are*A200. It has been shown before that the formamidase levels of *are*A102 and *are*A19 are lower than for *are*A$^+$ (Hynes 1972). The extreme *are*A mutants, *are*A217 and *are*A209, have non-significant amidase levels in glucose medium lacking a nitrogen source (Table 4).

Table 4. Amidase activities of various *are*A strains

Mycelium was grown for 16 h in glucose–10 mM ammonium tartrate medium and then transferred to medium without a nitrogen source for 4 h before harvesting. n.s., No significant activity

Strain	Relative specific activities[A]			
	Acetamidase		Formamidase	
	Glucose present	Carbon absent	Glucose present	Carbon absent
*are*A$^+$	14	16	197	40
*are*A102	100	34	100	28
*are*A19	5	27	n.s.	50
*are*A201	81	35	131	66
*are*A205	26	43	60	114
*are*A211	72	36	77	57
*are*A272	18	25	20	100
*are*A200	6	22	127	43
*are*A256	58	23	54	57
*are*A238	29	41	43	69
*are*A241	117	46	120	34
*are*A217	n.s.	64	n.s.	57
*are*A209	n.s.	49	n.s.	74

[A] Expressed relative to the specific activity of *are*A102 in glucose medium.

All the new *are*A strains produce significant amidase levels in the absence of a carbon source (Table 4). It is important to know if the particular *are*A allele present in the strain affects the amidase level in the absence of a carbon source, as this would indicate whether the *are*A product is active during carbon starvation. Unfortunately, the data do not help in reaching a final decision on this point. Although the amidase levels of the *are*A strains vary under conditions of carbon starvation, these are not related to the enzyme level present in glucose-grown mycelium. This is shown best for *are*A217 and *are*A209 which produce insignificant acetamidase in the presence of glucose and yet have the highest levels of this enzyme in the absence of a carbon source. It cannot yet be decided whether this is due to indirect or direct effects of the *are*A product in response to carbon starvation. In addition, it should be noted

that enzyme levels under conditions of starvation for both carbon and nitrogen are rather variable. The reasons for this are not apparent at present.

All the *are*A strains grow equally well on acetamide as the sole source of carbon or as the sole source of carbon and nitrogen. It has been shown that *are*A$^+$, *are*A102 and *are*A19 strains produce similar acetamidase levels when mycelium is incubated in medium containing acetamide as the sole carbon and nitrogen source (Hynes 1972). Similar results have been obtained for *are*A217 (results not shown). This indicates that the *are*A product is not limiting under conditions of induction and no catabolite repression.

Table 5. Ammonium repression of amidase enzymes in various *are*A strains

Mycelium was grown for 16 h in glucose medium with the indicated nitrogen sources. Both NH_4^+ (as ammonium tartrate) and acetamide were present at a concentration of 20 mM. n.s., No significant activity

Strain	Relative specific activities[A]					
	Acetamidase			Formamidase		
	NH_4	Acetamide	Acetamide $+NH_4$	NH_4	Acetamide	Acetamide $+NH_4$
*are*A$^+$	n.s.	44	n.s.	n.s.	331	n.s.
*are*A102	5	107	11	n.s.	88	n.s.
*are*A19	n.s.	22[B]	n.s.	n.s.	n.s.	n.s.
*are*A201	n.s.	71	n.s.	n.s.	37	n.s.
*are*A205	n.s.	63[B]	n.s.	n.s.	37	n.s.
*are*A211	5	76	4	n.s.	46	n.s.
*are*A272	n.s.	49[B]	4	n.s.	26	n.s.
*are*A200	n.s.	36[B]	n.s.	n.s.	334	6
*are*A256	n.s.	73[B]	5	n.s.	46	9
*are*A238	n.s.	60[B]	n.s.	n.s.	31	n.s.
*are*A241	n.s.	93	8	n.s.	67	n.s.

[A] Expressed relative to the specific activity of *are*A102 in glucose medium (Table 3).
[B] Low yields of mycelium were obtained from these strains in this medium.

Substantial formamidase levels are found under conditions of carbon starvation (Table 4) even for *are*A217 and *are*A209. This has been found previously for *are*A19 (Hynes 1972). Since formamide is not a carbon source for *A. nidulans*, it is surprising that the formamidase levels of the extreme *are*A strains increase with carbon starvation.

Growth of mycelium in ammonium-containing medium leads to much lower amidase levels than those present in mycelium incubated in medium lacking a nitrogen source, for most *are*A strains (Tables 4 and 5). This is also found when the acetamidase inducer, acetamide, is present (Table 5). Several *are*A strains (e.g. *are*A102) are slightly insensitive to ammonium repression. The extreme negative *are*A strains (*are*A19, *are*A217 and *are*A209) have very low or non-significant amidase activities in ammonium-free medium (Table 4) and therefore the sensitivity of these strains to ammonium repression cannot be assessed. As discussed below, it can be shown, using double mutants of *are*A19 and *are*A217 with *amd*R^c lesions, that these *are*A lesions result in lack of derepression upon relief of ammonium repression. Therefore it can be concluded that most *are*A mutations do not result in complete insensitivity to ammonium repression of the amidases, but some effects on ammonium repression can be detected.

It has been shown that urease is only weakly repressed by ammonium in *A. nidulans* (Scazzocchio and Darlington 1968). However, uptake of thiourea and urea is subject to strong ammonium repression (Dunn and Pateman 1973). Therefore, the poor growth of various *are*A strains on urea does not necessarily reflect urease activities. Investigations of urease activities in the *are*A mutants have therefore been carried out (Table 6). Starvation for nitrogen was found to lead to approximately threefold derepression of urease in the *are*A^+ strain compared with ammonium-grown cultures. The *are*A102 strain produced slightly less urease than wild type and derepressed about twofold upon nitrogen starvation. Many other *are*A strains had lower urease levels than wild type, but derepressed similarly to wild type. The three extreme pleiotropic negative *are*A strains, *are*A19, *are*A217 and *are*A209, had urease levels about half that of the *are*A^+ strain in ammonium, and showed no response to nitrogen starvation. This provides strong evidence for *are*A being involved in ammonium regulation of urease.

Table 6. Urease activities of various *are*A strains

Mycelium was grown for 16 h in glucose–ammonium tartrate medium and then transferred to similar medium or to medium lacking a nitrogen source for 4 h before harvesting

Strain	Specific activity[A] when: NH_4 present	Nitrogen absent	Strain	Specific activity when: NH_4 present	Nitrogen absent
areA^+	186	453	*are*A200	122	384
*are*A102	149	298	*are*A256	97	272
*are*A19	84	83	*are*A238	98	200
*are*A201	81	235	*are*A241	173	301
*are*A205	86	213	*are*A217	88	70
*are*A211	147	298	*are*A209	84	58
*are*A272	101	194			

[A] Expressed as nmoles NH_4^+ produced per minute per milligram soluble protein.

Extracellular protease activity may be detected in plate tests by observing clearing of milk powder (Cohen 1972). On 1% milk powder as the sole nitrogen source and in the presence of glucose, *are*A102, *are*A238, *are*A241, *are*A201 and *are*A211 grow strongly and exhibit greater extracellular protease activity than *are*A^+, while *are*A200, *are*A256 and *are*A205 grow less well and have reduced extracellular protease compared with *are*A^+. The more extreme *are*A strains, *are*A19, *are*A217 and *are*A209, grow extremely poorly and have no detectable protease activity. Inclusion of ammonium in the medium allows ammonium repression of protease to be assessed. All the *are*A strains are subject to ammonium repression of extracellular protease, but *are*A238 is less sensitive than the other strains.

Properties of Diploids Heterozygous for are*A Alleles*

Various diploids, heterozygous for the new *are*A alleles with *are*A^+ and *are*A102, have been constructed and investigated. Strong growth on a particular nitrogen source is at least partially dominant to poor growth in nearly all cases. Formamidase and acetamidase determinations correlate with the growth tests. Similar results for *are*A19 have been observed previously (Hynes 1972, 1973*a*).

In contrast to the other *are*A alleles, however, *are*A200 is partially dominant to *are*A$^+$ in its effects on acetamide, acrylamide, urea, glutamate and milk protein utilization. Furthermore, *are*A200 is partially dominant to *are*A102, with respect to histidine and acrylamide utilization. Table 7 compares the properties of *are*A200 haploid and diploid strains with *are*A$^+$ and *are*A102 strains, with respect to acetamidase phenotype. These results show that *are*A200 has abnormal dominance properties compared with the other *are*A alleles, in that this is the only allele examined in which reduced growth on a particular nitrogen source is not recessive to *are*A$^+$ or *are*A102. Possible explanations for this are discussed below.

Table 7. Dominance properties of *are*A200 with respect to acetamidase activity
Mycelium was grown for 16 h in glucose–ammonium tartrate medium and then with no added nitrogen source for 4 h before harvesting. Activities are expressed relative to that of strain *are*A102. Media and symbols are as for Table 3

Strain	Growth properties on solid medium containing: Acetamide	Acrylamide	Relative acetamidase specific activities
*are*A$^+$	+	—	14
*are*A102	+++	+++	100
*are*A200	±	—	6
*are*A102/*are*A102	+++	+++	100
*are*A102/*are*A$^+$	++	+	48
*are*A200/*are*A$^+$	±	—	7
*are*A200/*are*A102	++	±	34

Properties of amd*R and* are*A Double Mutants*

Mutation in a gene, *amd*R, affects the regulation of acetamidase. It is thought that this gene codes for a positively acting regulatory protein involved in the specific regulation of acetamidase synthesis—possibly in the induction process, although definitive evidence for this is lacking (Hynes and Pateman 1970*a*, 1970*b*; Dunsmuir and Hynes 1973). *amd*R^c alleles lead to high acetamidase levels, while *amd*R$^-$ alleles lead to poor growth on acetamide and to reduced acetamidase levels (Hynes and Pateman 1970*a*, 1970*b*). Since *amd*R lesions affect growth on acetamide as the sole carbon source (Hynes and Pateman 1970*a*) and acetamidase is insensitive to ammonium repression when acetamide is the sole carbon source (Hynes 1970), it is unlikely that the *amd*R gene is involved in ammonium repression. Growth of *amd*R mutant strains in ammonium containing medium leads to lowered acetamidase levels (Hynes and Pateman 1970*a*).

Some revertants of *are*A19 and *are*A217, selected for growth on acetamide, have been found to show reversion only for the acetamide phenotype and to have *amd*R^c lesions (M. J. Hynes, unpublished data). *amd*R^{c}6 ; *are*A19 and *amd*R^{c}104 ; *are*A217 double mutants have been constructed, and these retain the pleiotropic phenotype of the *are*A alleles on other nitrogen sources, but are able to grow strongly on acetamide and weakly on acrylamide as sole nitrogen sources. Similar results have been found by Arst and Cove (1973).

Although growth of *amd*R$^-$44 strains is rather leaky on acetamide media, it is possible to score *amd*R$^-$44 relative to *amd*R$^+$, in contrast to the report of Arst and

Cove (1973). It is suggested that the leaky growth results from the action of other control genes (including *are*A). There is not a complete correlation between the acetamidase levels and the growth on acetamide medium of *amd*R⁻44 strains (Table 8). This may be due to the growth conditions employed for the acetamidase determinations not fully showing up the effects of the *amd*R⁻44 lesion. This may be resolved when more is known about the role of the *amd*R gene.

Table 8. Properties of *amd*R ; *are*A double mutants

Mycelium was grown for 16 h in glucose–ammonium tartrate and then transferred to glucose medium with no added nitrogen source for 4 h before harvesting. Activities are expressed relative to that of strain *are*A102. Media and symbols are as for Table 3

Relevant genotype	Growth on solid medium containing N as:		Relative acetamidase specific activities
	Acetamide	Acrylamide	
*amd*R⁺ ; *are*A⁺	+	—	14
*amd*R⁺ ; *are*A102	+++	++	100
*amd*R⁺ ; *are*A19	±	—	3
*amd*R⁻44 ; *are*A⁺	±	—	12
*amd*R⁻44 ; *are*A19	—	—	2
*amd*R⁻44 ; *are*A102	++	+	71
*amd*R^{c}6 ; *are*A⁺	++	++	152
*amd*R^{c}6 ; *are*A19	++	+	66
*amd*R^{c}2 ; *are*A102	++++	++	274
*amd*R^{c}2 ; *are*A⁺	++	++	140

In order to study the interaction of *amd*R and *are*A loci, the properties of various double mutants have been investigated (Table 8). It is clear that both genes affect acetamidase levels and their effects are superimposed. *amd*R^{c}6 results in increased acetamidase levels in the *amd*R^{c}6 ; *are*A19 double mutant, while *are*A102 largely compensates for *amd*R⁻44. Very low acetamidase levels are observed in the *amd*R⁻44 ; *are*A19 double mutant, while very high levels are observed in the *amd*R^{c}2 ; *are*A102 strain. Overall, these studies indicate that these two regulatory circuits may act independently in the regulation of acetamidase synthesis.

Studies on *amd*R^c ; *are*A double mutants provide an opportunity for testing the hypothesis that *are*A is involved in ammonium regulation of the acetamidase. If the *are*A product is required for acetamidase synthesis in response to relief of ammonium repression, then extreme negative *are*A alleles such as *are*A217 and *are*A19 should result in no response to relief of ammonium repression. These *are*A strains produce such low levels of acetamidase in an *amd*R⁺ background that such experiments are not possible. However, when an *amd*R^c lesion is present, acetamidase levels are elevated (see above), enabling responses to relief of ammonium repression to be measured. As shown in Fig. 1, the presence of *are*A217 in *amd*R^{c}104 strains abolished the four- to fivefold derepression resulting from nitrogen starvation of ammonium-grown cultures. Similar results have been found for the comparison between an *amd*R^{c}6 strain and the double mutant strain *amd*R^{c}6 ; *are*A19 (M. J. Hynes, unpublished data). This therefore provides support for a role for the *are*A product in ammonium regulation of the acetamidase. However, it might have been expected that the enzyme level in the *amd*R^{c}104 ; *are*A217 strain would be similar to that of the *amd*R^{c}104

strain grown in the presence of ammonium. In fact the *amd*R^{c}104 ; *are*A217 strain produced approximately twice the acetamidase level of the *amd*R^{c}104 strain grown in ammonium medium. One possible explanation (among others) for this result is that the *are*A product has, in addition to a positive regulatory function, a negative effect on enzyme synthesis. The *are*A217 lesion could then be proposed to abolish both positive and negative regulatory functions of the *are*A product. At present fully convincing evidence for a negative role for *are*A is lacking (see Discussion).

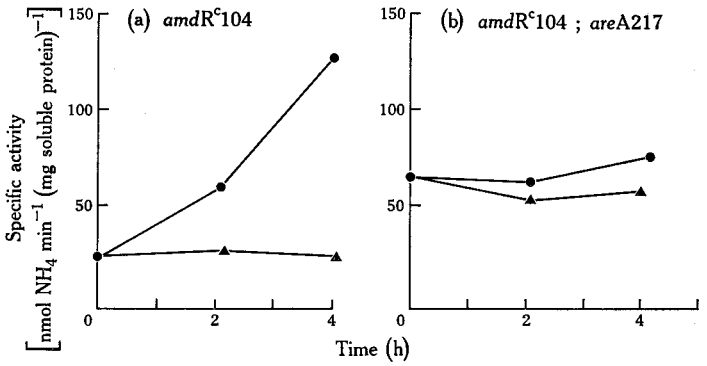

Fig. 1. Derepression of acetamidase in strains *amd*R^{c}104 (*a*) and *amd*R^{c}104 ; *are*A217 (*b*). Mycelium was grown for 16 h in glucose–10 mM ammonium tartrate medium; a sample of mycelium was harvested at this time and other samples transferred to glucose–10 mM ammonium tartrate medium (▲) or to medium lacking a nitrogen source (●). These were then harvested at 2 and 4 h after the transfer.

Discussion

The properties of *are*A102 and *are*A19 previously led to the suggestion that the *are*A product is involved in the interaction between carbon and nitrogen metabolism (Hynes 1973). However, the work of Arst and Cove (1973) indicates that the role of the *are*A product is related to the effects of ammonium on the catabolism of nitrogen sources. There is support for this from the studies of new *are*A alleles reported here. In particular, *are*A238 does show some derepression for extracellular protease activity, response of urease to derepression is altered in some of the mutants, and *are*A217 affects derepression of the acetamidase. More recent studies on the effect of a lesion in the *gdh*A gene (which is probably the structural gene for the NADP-linked glutamate dehydrogenase—Arst and MacDonald 1973; Kinghorn and Pateman 1973) on ammonium repression in *are*A102 ; *gdh*A double mutants have provided further evidence for an involvement of *are*A in ammonium repression (Hynes 1974). However, it seems that lesions in the *are*A gene commonly result in altered levels of the enzymes of nitrogen-source catabolism rather than greatly affecting responses to ammonium. For example, the altered *are*A product of *are*A102 results in increased capacity for acetamidase (Hynes and Pateman 1970*a*; Hynes 1972, 1973*a* and this paper) and histidase (Polkinghorne and Hynes 1975) synthesis without greatly affecting ammonium repression under the conditions employed. Where capacity for enzyme synthesis is altered, it is difficult to determine whether genuine derepression occurs.

The heterogeneous phenotypes of the new *are*A alleles, isolated by a variety of methods, support the proposal of a positive regulatory role for the *are*A product in nitrogen catabolism. A positive function for *are*A is also indicated by the dominance relationships of *are*A alleles, since loss of growth and low enzyme levels are recessive to strong growth and high enzyme levels in heterozygous diploids (with the exception of *are*A200—see below). In addition, the studies on urease in the various *are*A strains (Table 3) and on acetamidase in the *amd*R^{c}104 ; *are*A217 double mutant (Fig. 1) show that lesions in the *are*A gene can lead to an inability to respond to relief of ammonium repression by transferring mycelium to medium lacking a nitrogen source.

The proposal of a positive role for the *are*A gene product does not eliminate the possibility that, in addition, *are*A plays a negative (repressing) role in nitrogen catabolic regulation. The abnormal dominance relationships of *are*A200 in heterozygous diploids (see above and Table 4) could be explained by postulating that *are*A200 produces an altered product that results in some repression in the absence of ammonium. Equally well, however, the dominance properties of *are*A200 could be explained by the *are*A gene product being composed of more than one subunit with *are*A200 resulting in negative complementation. It has been noted above that the higher acetamidase levels of the *amd*R^{c}104 ; *are*A217 strain compared with *amd*R^{c}104 in ammonium medium could be explained by proposing that *are*A217 abolishes a negative function of the *are*A product.

The properties of *amd*R ; *are*A double mutants suggest that these two regulatory genes affect acetamidase synthesis independently. A full description of regulation of the acetamidase must await further work on the additional regulatory systems operating (see below). It seems that selection for growth on acrylamide selects for mutants producing acetamidase levels greater than those produced by fully induced wild type strains, and this is supported by earlier studies (Hynes and Pateman 1970*a*). This would explain why the replacement of *amd*R$^+$ with *amd*R^c results in suppression of the acetamide phenotype of *are*A19 and *are*A217, even though these strains grow poorly on acetamide as the sole nitrogen source. This is also supported by the earlier observations that substantial acetamidase activities are found in *amd*R^c strains grown in glucose–ammonium medium (Hynes and Pateman 1970*a* and this paper). This further emphasizes the point made earlier about the difficulty of showing altered response to repression where capacity for enzyme synthesis is altered.

Substantial amidase activities are observed in all *are*A strains under conditions of carbon starvation (Table 4), and all strains grow equally well on acetamide as the sole carbon source. Results concerning the effects of carbon sources on nitrogen source utilization by *are*A19 and *are*A102 (Hynes 1973*a*, 1973*b*) and similar results with the new *are*A strains (not reported here in detail) are compatible with those of Arst and Cove (1973) except that glycerol does give some reduction in acetamide utilization by extreme *are*A strains.

It now seems clear that the previous conclusion, that *are*A is involved in an interaction between carbon and nitrogen metabolite repression, resulted from the carbon starvation treatments employed in the study of *are*A102 and *are*A19 bringing into operation the additional regulatory circuit concerned with carbon catabolite repression, and the fact that both of these mutants do not obviously affect ammonium repression (Hynes 1972). It should now be possible to investigate the problems of how the

catabolism of compounds capable of acting as both carbon and nitrogen sources is regulated (Hynes 1970).

A number of partial revertants of *are*A217 have been isolated on sucrose–acetamide medium (M. J. Hynes, unpublished data). Some of these are of abnormal morphology, are able to grow on glucose–proline but not on glucose–glutamate medium and are due to mutation at loci distinct from *are*A. These have apparently similar properties to some of the *cre* mutants described by Arst and Cove (1973) and provide support of their suggestion that carbon catabolite repression is distinct from ammonium repression.

Additional suppressors of *are*A217 have been isolated that have normal morphology, do not map in *amd*R and do not suppress the effects of *are*A217 on proline utilization. One of these maps close to *amd*S (the probable structural gene for the acetamidase—Dunsmuir and Hynes 1973) and is *cis*-dominant with respect to *amd*S (Hynes 1975).

Mutation at two loci has been found to result in loss of ability of *are*A102 to grow strongly on histidine and a number of other nitrogen sources (Polkinghorne and Hynes 1975). This indicates further the complexity of the regulation of the catabolism of nitrogen sources in *A. nidulans*. Future work is aimed at determining whether the genetic complexity can be resolved into only a few fundamental regulatory elements.

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