Mutants affecting histidine utilization in *Aspergillus nidulans*

BY MERYL POLKINGHORNE AND M. J. HYNES

Department of Genetics, La Trobe University, Bundoora, Victoria 3083, Australia

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**SUMMARY**

Wild-type strains of *Aspergillus nidulans* grow poorly on L-histidine as a sole nitrogen source. The synthesis of the enzyme histidase (EC. 4.3.1.3) appears to be a limiting factor in the growth of the wild type, as strains carrying the mutant areA102 allele have elevated histidase levels and grow strongly on histidine as a sole nitrogen source. L-Histidine is an extremely weak sole carbon source for all strains.

Ammonium repression has an important role in the regulation of histidase synthesis and the relief of ammonium repression is dependent on the availability of a good carbon source. The level of histidase synthesis does not respond to the addition of exogenous substrate.

Mutants carrying lesions in the sarA or sarB loci (suppressor of areA102) have been isolated. The growth properties of these mutants on histidine as a sole nitrogen source correlate with the levels of histidase synthesized. Mutation at the sarA and sarB loci also reduces the utilization of a number of other nitrogen sources. The data suggest that these two genes may code for regulatory products involved in nitrogen catabolism. No histidase structural gene mutants were identified and possible explanations of this are discussed.

**INTRODUCTION**

Detailed studies of histidine utilization have been carried out in bacteria. It has been found that L-histidine can serve as a sole carbon and nitrogen source by virtue of its degradation to L-glutamate. The synthesis of the first enzyme in the catabolic pathway, histidase (EC. 4.3.1.3) is inducible and subject to catabolite repression in *Salmonella typhimurium* (Smith, Halpern & Magasanik, 1971), *Bacillus subtilis* (Chasin & Magasanik, 1968), *Klebsiella aerogenes* (Magasanik et al. 1965) and *Pseudomonas aeruginosa* (Lessie & Neidhart, 1967). In *K. aerogenes* and *P. aeruginosa* there is an additional control mechanism operating when nitrogen is limiting. This control mechanism allows the utilization of histidine as a sole nitrogen source in the presence of sources of strong catabolite repression. In *K. aerogenes* it has been directly demonstrated that glutamine synthetase (EC. 6.3.1.2) is involved in this control mechanism (Prival & Magasanik, 1971; Prival, Brenchley & Magasanik, 1973; Tyler, Deleo & Magasanik, 1974). Histidase activity has also been detected in a number of eukaryotes, including *Ustilago sphaerogena*.
L-Histidine is a very poor sole nitrogen source for wild-type strains of *Aspergillus nidulans* (Hynes, 1973a). However, strains containing the mutation *areA*102 (formerly called *amdT*102 – see Hynes, 1972, 1973a; Arst & Cove, 1973) can grow strongly on histidine as the sole nitrogen source (Hynes, 1973a). The *areA* gene is thought to play a fundamental role in the regulation of nitrogen catabolism (Arst & Cove, 1973). Therefore a study of histidine utilization in *A. nidulans* has been undertaken. This paper shows that regulation of histidase in *A. nidulans* differs from the bacterial systems and describes the properties of mutants altered in histidase regulation.

2. MATERIALS AND METHODS

(i) Strains

Table 1 lists the strains used in this study together with their genotypes. The genetic markers in the strains have been described by Clutterbuck (1973). The *areA*102 and *areA*19 mutations were originally designated *amdT*102 and *amdT*19 respectively (Hynes, 1972, 1973; Arst & Cove, 1973).

Table 1. List of genotypes of strains used

| Strain  | Genotype                                      |
|---------|-----------------------------------------------|
| *areA*102 | *biA*1; *areA*102; *niiA*4                   |
| *puA*2; *areA*102 | *biA*1; *puA*2; *areA*102                  |
| *areA*+  | *biA*1; *niiA*4                               |
| *areA*19 | *biA*1; *areA*19; *niiA*4                    |
| *areA*102; *sarA*3 | *biA*1; *areA*102; *sarA*3; *niiA*4        |
| *areA*102; *sarA*6 | *biA*1; *areA*102; *sarA*6; *niiA*4        |
| *areA*102; *sarA*10 | *biA*1; *areA*102; *sarA10; *niiA*4      |
| *areA*102; *sarA*31 | *biA*1; *areA*102; *sarA31; *niiA*4      |
| *areA*102; *sarA*5 | *biA*1; *areA*102; *sarA5; *niiA*4         |
| *areA*102; *sarB*7 | *biA*1; *areA*102; *sarB7; *niiA*4        |
| *areA*102; *sarA*18 | *biA*1; *areA*102; *sarA18; *niiA*4       |
| *areA*102; *sarB*33 | *biA*1; *areA*102; *sarB33; *niiA*4       |
| *areA*102; *sarA*36 | *biA*1; *areA*102; *sarA36; *niiA*4       |
| *areA*102; *sarA*40 | *biA*1; *areA*102; *sarA40; *niiA*4        |
| *areA*102; *sarA*42 | *biA*1; *areA*102; *sarA42; *niiA*4       |
| *areA*102; *sarA*44 | *biA*1; *areA*102; *sarA44; *niiA*4        |
| *areA*102; *sarA*47 | *biA*1; *areA*102; *sarA47; *niiA*4        |
| *areA*+; *sarA*3 | *yA*1; *Acr1; *galA1; *pyroA*4; *sB3; *sarA*3 |
| *areA*+; *sarA*31 | *yA*1; *galA1; *sarA31; *riboB2*          |
| *areA*+; *sarB*7 | *yA1; *galA1; *pyroA4; *sarB7; *riboB2*   |
| *areA*+; *sarB*33 | *yA1; *galA1; *pyroA4; *sarB33; *riboB2*  |

(ii) Isolation of mutants

The technique of Mackintosh & Pritchard (1963) was used for the isolation of mutants unable to grow strongly on L-histidine as a sole nitrogen source. Conidia of either *areA*102 or *puA*2;*areA*102 were mutagenized with *N*-methyl-*N*-nitro-nitrosoguanidine using the method described previously (Hynes & Pateman,
Histidine utilization in A. nidulans 1970, except that 250 μg of mutagen per ml was used. The conidia were then diluted as required and spread on glucose–10 mM ammonium minimal media containing deoxycholate (0.08 %) to induce micro colony formation. After 3 days incubation at 37 °C the master plates were velvet replicated to glucose–10 mM histidine minimal media. Colonies which grew normally on ammonium but grew poorly on the histidine medium were isolated.

(iii) Genetic analysis and media

The general methods for genetic analysis were based on those described by Pontecorvo et al. (1953). Heterokaryons and diploids were formed by the standard technique (Roper, 1952). Nitrogen-free glucose minimal media and supplements have been described by Cove (1966). Carbon-free medium was minimal nitrogen-free salts solution. Nitrogen sources were added to the basic media either as solids or from concentrated sterile solutions. All nitrogen sources were used at a concentration of 10 mM except for the growth of mycelium (20 mM ammonium) and milk protein. Milk protein was stored as a concentrated sterile solution of powdered skim milk and added to media to give a final concentration of 1 % when used as a nitrogen and/or carbon source.

(iv) Growth of mycelium (Hynes, 1970; 1972)

Thick conidial suspension, 3–5 ml, was added to 200 ml of glucose–ammonium (20 mM) liquid media in a 1 l, Ehrlenmeyer flask and shaken for 16 h at 30 °C in a Gallenkamp orbital incubator. The mycelium was then harvested by filtration through a sterile nylon net, washed in minimal media and transferred to the treatment media for 4 h. The mycelium was then harvested, blotted dry and stored at −15 °C for not more than 3 days before extraction.

(v) Extraction

Frozen mycelium was ground in 20 × (vol./wt.) cold orthophosphate buffer pH 8.0 with washed ground glass. The supernatant was centrifuged for 30 min at 29600 g in a Sorvall RC2B refrigerated centrifuge. The clear supernatant was retained for enzyme assays and protein determinations.

(vi) Protein determination

Soluble protein was measured by the method of Lowry et al. (1951). Serum albumin was used as a standard. The protein content of most extracts was 1–3 mg/ml.

(vii) Histidase assay

The method used to assay histidase activity was based on the method of Tabor & Mehler (1955) which follows the increase in absorbance at 277 nm as the product, urocanic acid, is formed. The reaction mixture usually contained 0.1 ml of cell-free extract and 2.8 ml of orthophosphate buffer, pH 8.0. The reaction was started with the addition of 0.1 ml of histidine solution (3 mg/ml). The histidase assay
was found to be linear with time and activities were proportional to the amount of extract added. The assay pH was changed from 9.2 (Tabor & Mehler, 1955) to 8.0, which is the pH optimum of the histidase enzyme of *A. nidulans*. Urocanase activity has not been detected in *A. nidulans*. Therefore a high assay pH was not necessary to prevent interference with the histidase assay. All histidase-specific activities are expressed as nanomoles of urocanic acid produced per minute per mg soluble protein. The histidase levels of *areA102* on glucose minimal media have been used as a reference value for expressing the histidase levels of all other strains. Time course studies on the appearance of histidase activity have shown that histidase levels begin to fluctuate between 3 and 4 h after transfer. The fluctuations introduce considerable variation in the histidase levels of a given strain and therefore *areA102* levels are used as a standard. The cause of the fluctuations in histidase values is presently being investigated.

3. RESULTS

(i) *Growth properties on histidine* (Table 2)

Wild-type strains of *A. nidulans* grow very poorly when L-histidine is the sole nitrogen source. This has been found for strains derived from Glasgow stocks as well as several strains from different sources (kindly provided by Dr James Croft, Genetics Department, University of Birmingham). However, the *areA102* mutation allows strong growth on histidine as a nitrogen source (Hynes, 1973a). The difference in the abilities of *areA*+ and *areA102* strains to utilize histidine is not affected by replacing glucose with other good carbon sources. L-Histidine is an extremely weak sole carbon source for both *areA102* and *areA*+ strains. Urocanate, the immediate product of histidase activity, is neither a carbon nor nitrogen source for *A. nidulans*. A second *areA* allele, *areA19* has been described previously (Hynes, 1972). Strains containing this lesion grow more poorly than *areA*+ strains on histidine as the sole nitrogen source. A number of other mutant strains with *areA* lesions have been isolated (Hynes, in press) and these show varying abilities to utilize histidine (Table 4).

| Table 2. Growth properties of *areA*+, *areA102* and *areA19* strains on L-histidine and urocanic acid |
|---------------------------------------------------|---------------------------------------------------|---------------------------------|---------------------------------|
| Carbon source | Nitrogen source | *areA*+ | *areA102* | *areA19* |
|-----------------|-----------------|---------|-----------|---------|
| Glucose         | Histidine       | ±       | +++       | −       |
| Sucrose         | Histidine       | ±       | +++       | −       |
| Histidine       | Ammonium        | −       | −         | −       |
| Histidine       | Histidine       | −       | −         | −       |
| Glucose         | Urocanic Acid   | 0       | 0         | 0       |
| Urocanic acid   | Ammonium        | 0       | 0         | 0       |

+ ++ +, Strong growth; ±, weak growth; −, very weak growth; 0, carbon- or nitrogen-free growth.

Available at [https://www.cambridge.org/core/terms](https://www.cambridge.org/core/terms).
(ii) Regulation of histidase activities with different growth conditions

Histidase catalyses the breakdown of histidine to urocanic acid and ammonium (Mehler & Tabor, 1953) and therefore is the most probable means of utilizing histidine as a nitrogen source. Histidase activity has been detected in crude extracts and an assay developed (see Methods and Materials). Table 3 shows the results of histidase determinations on areA+, areA102 and areA19 strains. For all strains, growth in the presence of ammonium leads to very low levels of histidase, suggesting that histidase, like many other enzymes involved in nitrogen catalolism, is subject to ammonium repression. Transfer of mycelium to medium

Table 3. Relative histidase levels of mycelium of areA+ areA102 and areA19 strains grown under a variety of conditions.

| Treatment media* | Histidase activities† |
|------------------|----------------------|
| Carbon source    | areA+    | areA102 | areA19 |
| Glucose          | <5       | <5      | <5     |
| Glucose          | 18       | 100     | 6      |
| Glucose          | 17       | 102     | 7      |
| Carbon-free      | <5       | <5      | <5     |
| Carbon-free      | <5       | <5      | <5     |
| Histidine        | <5       | <5      | <5     |

* Mycelium grown on glucose-ammonium (20 mM) medium for 16 h prior to transfer to treatment media for 4 h.
† Histidase activities calculated as nanomoles of urocanic acid produced per minute per mg soluble protein. Results expressed relative to the histidase levels of areA102 on glucose-minimal media.

Table 4. Growth properties and histidase activities of various areA strains

| Strain  | Growth properties* | Histidase activities† |
|---------|--------------------|-----------------------|
| areA+   | ±                  | <5                    | 12                    |
| areA102 | + + +              | <5                    | 100                   |
| areA19  | −                  | <5                    | 2                     |
| areA201 | + + +              | <5                    | 111                   |
| areA205 | + +                | <5                    | 71                    |
| areA211 | + +                | <5                    | 95                    |
| areA272 | + +                | <5                    | 66                    |
| areA200 | ±                  | <5                    | 8                     |
| areA256 | + +                | <5                    | 71                    |
| areA238 | + +                | <5                    | 67                    |
| areA241 | + + +              | <5                    | 115                   |
| areA217 | 0                  | <5                    | <5                    |
| areA209 | 0                  | <5                    | <5                    |

* Scored after 2 days incubation at 37 °C on glucose-histidine (10 mM) medium. + + +, Strong growth; + +, good growth; ±, weak growth; −, very weak growth; 0, nitrogen-free growth.
† Mycelium grown for 16 h on glucose-ammonium (20 mM) medium prior to transfer to treatment media for 4 h. Histidase activities expressed as described for Table 3.
lacking a nitrogen source results in increased histidase levels. However, \(areA102\) strains have approximately 10-fold higher histidase levels than the wild-type strain, while the \(areA19\) strain produces lower levels than the wild-type. Tables 3 and 4 show that all \(areA\) mutant strains investigated have histidase levels compatible with their ability to utilize histidine, and that all these \(areA\) strains are sensitive to ammonium repression. Cycloheximide (15 \(\mu\)g/ml) has been found to abolish the increase in histidase levels accompanying transfer to medium lacking a nitrogen source. It can be concluded that histidase is subject to ammonium repression and that \(areA\) mutations can greatly affect the ability of \(A.\ nidulans\) to synthesize this enzyme.

Histidase does not appear to require external induction by histidine (Table 3). Histidase activities are similar when mycelium is transferred either to medium lacking a nitrogen source or to medium containing histidine. Urocanate also does not appear to induce histidase (data not shown). It cannot be rigorously excluded that induction occurs by some inducer formed during nitrogen starvation. It has been found that at least three other enzymes of nitrogen source catabolism — extracellular protease (Cohen, 1973) and two amidase enzymes (Hynes, unpublished) — do not appear to require induction.

The effect of carbon starvation on histidase synthesis has also been investigated. Results in Table 3 show that histidase levels of mycelium transferred to carbon-free ammonium medium remain at repressed levels. In addition, histidase activities do not increase when mycelium is transferred to medium lacking both carbon and nitrogen sources or to medium in which histidine is the sole carbon and nitrogen source. Thus the presence of glucose in the medium seems to be necessary for derepression of histidase. A similar phenomenon may apply to nitrate reductase (Hynes, 1973b) and to an amidase enzyme (Hynes, unpublished). This contrasts with the situation for acetamidase (Hynes, 1970) and extracellular protease (Cohen, 1973) where carbon starvation results in increased enzyme levels. Unpublished data show that there is no evidence for rapid disappearance of histidase from carbon-starved mycelium and that an inhibitor of histidase activity is not present in carbon-starved mycelium.

(iii) Isolation of mutants with reduced growth on L-histidine

Conidia of \(areA102\) containing strains were mutagenized in an attempt to isolate mutants affected in their ability to use histidine as a sole nitrogen source (see Methods and Materials). \(areA102\)-containing strains were used as parent strains because the poor growth of the wild type on histidine reduces the selection efficiency. A number of mutants which showed the required phenotype were isolated and those which retained the \(areA102\) allele intact were used in this study (Table 1). The basis of the \(sarA\) and \(sarB\) designations will be described later in this paper.

An unusual feature of these (and subsequent) mutation studies is the absence of histidase structural gene lesions in the mutants isolated. This will be discussed later. The mutant strains which were isolated are listed in Table 5 with a summary
of their growth properties on a variety of media. The table shows that in addition to L-histidine, these strains also grow more poorly on L-leucine, L-lysine, L-methionine, L-cysteine, L-phenylalanine, L-citrulline and D,L-homoserine as sole nitrogen sources. The areA102; sarA and areA102; sarB strains grow normally on all other nitrogen sources tested including ammonium, acetamide, L-serine, hypoxanthine and L-proline. These strains are not affected in the utilization of glutamate, acetamide, arginine and proline as sole carbon and nitrogen sources or the utilization of glucose, sucrose, fructose, maltose, glycerol, galactose, ethanol or acetate as sole carbon sources.

Table 5. Growth properties of mutant strains on a variety of nitrogen sources

| Relevant genotype | Ammonium | Histidine | Leucine | Lysine | Methionine | Citrulline | Phenyalalanine | Cysteine | Homoserine | Tryptophan |
|------------------|----------|-----------|---------|--------|------------|------------|---------------|----------|------------|-----------|
| areA+            | ++       | +         | ±       | ±      | ±          | ±          | ±             | ±        | ±          | ±         |
| areA102          | +        | +         | +       | +      | +          | +          | +             | +        | +          | +         |
| areA102; sarA3   | +        | +         | +       | +      | +          | +          | +             | +        | +          | +         |
| areA102; sarA6   | +        | +         | +       | +      | +          | +          | +             | +        | +          | +         |
| areA102; sarA10  | +        | +         | +       | +      | +          | +          | +             | +        | +          | +         |
| areA102; sarA31  | +        | +         | +       | +      | +          | +          | +             | +        | +          | +         |
| areA102; sarA5   | +        | +         | +       | +      | +          | +          | +             | +        | +          | +         |
| areA102; sarB7   | +        | +         | +       | +      | +          | +          | +             | +        | +          | +         |
| areA102; sarA18  | +        | +         | +       | +      | +          | +          | +             | +        | +          | +         |
| areA102; sarB33  | +        | +         | +       | +      | +          | +          | +             | +        | +          | +         |
| areA102; sarA36  | +        | +         | +       | +      | +          | +          | +             | +        | +          | +         |
| areA102; sarA40  | +        | +         | +       | +      | +          | +          | +             | +        | +          | +         |
| areA102; sarA42  | +        | +         | +       | +      | +          | +          | +             | +        | +          | +         |
| areA102; sarA44  | +        | +         | +       | +      | +          | +          | +             | +        | +          | +         |
| areA102; sarA47  | +        | +         | +       | +      | +          | +          | +             | +        | +          | +         |

All nitrogen sources tested were added to glucose-minimal media at a final concentration of 10 mM. Plates were scored after 2 days incubation at 37 °C.

Growth of the mutant strains was scored relative to the growth of areA102 for each media. Therefore relative growth between media is not comparable. Growth symbols used have been described in Table 4 with ‘+’ designating growth intermediate between ‘+’ and ‘±’.

(iv) Genetic characterization of mutants

The pleiotropic properties of these strains are retained in the progeny of crosses with non-mutant strains indicating that the areA102; sarA and areA102; sarB strains each carry a single mutation which affects the utilization of a number of nitrogen sources. Recombination studies (Table 6) have shown that all the mutant strains, with the exception of areA102; sarB7 and areA102; sarB33 carry a mutation at a single locus provisionally designated sarA (suppressor of areA102). areA102; sarB7 and areA102; sarB33 carry allelic mutations at a second locus designated sarB. Complementation studies in diploids (to be described later) confirm the recombination data. Allelic mutations do not complement in diploids.
whereas diploids heterozygous for sarA and sarB mutations show complementation for the utilization of histidine as a nitrogen source. On the basis of haploidization analysis (McCully & Forbes, 1965), both the sarA and sarB loci have been assigned to linkage group VII. The two loci are unlinked and neither locus shows linkage to the other linkage group VII markers tested—nicB, cnxF, hxB, lysC, lysD, and choA.

Table 6. Genetic analysis of mutant strains

| Cross | No. of areA102-type recombinants* | No. of progeny scored |
|-------|----------------------------------|----------------------|
| areA102; sarA3 x areA102; sarA3   | 0                   | 533                  |
| areA102; sarA3 x areA102; sarA6   | 1                   | 1915                 |
| areA102; sarA3 x areA102; sarA10  | 0                   | 1574                 |
| areA102; sarA3 x areA102; sarA31  | 7                   | 2107                 |
| areA102; sarA3 x areA102; sarA5   | 0                   | 999                  |
| areA102; sarA3 x areA102; sarB7†  | 6                   | 20                   |
| areA102; sarA3 x areA102; sarA18  | 0                   | 1289                 |
| areA102; sarA3 x areA102; sarB33† | 6                   | 80                   |
| areA102; sarA3 x areA102; sarA36  | 0                   | 895                  |
| areA102; sarA3 x areA102; sarA40  | 0                   | 1340                 |
| areA102; sarA3 x areA102; sarA42  | 1                   | 866                  |
| areA102; sarA3 x areA102; sarA44  | 1                   | 961                  |
| areA102; sarA3 x areA102; sarA47  | 0                   | 963                  |
| areA102; sarB7 x areA102; sarB33  | 0                   | 504                  |

* Only areA102-type recombinants (sar+) were scored as double mutants could not be reliably distinguished from single mutants.
† Not allelic to the mutation in areA102; sarA3 which reduces growth on histidine as a sole nitrogen source.

(v) Histidase activities of the mutants

The results of histidase assays of sarA and sarB strains are given in Table 7. There is again a correlation between the levels of the histidase enzyme and the ability of the strain to use histidine as a sole nitrogen source. In sarA and sarB strains, histidase synthesis remains subject to ammonium repression, retains its requirement for a carbon source for the relief of ammonium repression and is not elevated by the addition of substrate. Without altering these three properties of histidase regulation, mutation at either the sarA or sarB locus results in a reduction in the maximal levels of histidase synthesized under optimal conditions.

(vi) Temperature-sensitive mutant phenotypes

Growth tests at 25, 37 and 43 °C were used to determine whether any of the sarA or sarB mutations showed temperature sensitivity in their affects on nitrogen source utilization (Table 8). areA102; sarA10 was found to have a temperature-sensitive phenotype. At 25 °C the growth of this strain on L-histidine is indistinguishable from the growth of its sarA+ parent strain. areA102; sarA18, the least extreme of the sarA strains, shows a slight temperature dependence for its utilization of histidine as a sole nitrogen source. Unlike areA102; sarA10, areA102; sarB33 is cold-sensitive for histidine utilization. This sarB mutant grows more strongly at 37 °C than at 25 °C, and at 42 °C the growth of areA102; sarB33 is
Histidine utilization in A. nidulans

equivalent to areA102 strains. The temperature-sensitive phenotypes of areA102; sarA10, areA102; sarA18 and areA102; sarB33, as described above, have also been observed on L-leucine, L-lysine and L-methionine as sole nitrogen sources.

areA102; sarB33 and areA102; sarA10 were also tested for temperature sensitivity on L-citrulline and D,L-homoserine. The temperature sensitive phenotypes of areA102; sarA10 on these nitrogen sources are similar to those described on histidine. areA102; sarB33 is cold-sensitive for the utilization of citrulline and D,L-homoserine, as it is for histidine utilization. However, at 42 °C areA102; sarB33 is not phenotypically equivalent to areA102 on citrulline and homoserine, but retains its leaky mutant phenotype.

Table 7. Histidase activities of sarA and sarB strains

| Carbon source | Nitrogen source | Treatment media* |
|---------------|----------------|-----------------|
| Glucose Ammonium (10 mm) | Glucose Nitrogen-free | Glucose Histidine (10 mm) | Carbon-free Nitrogen-free |
| areA102 | <5 | 100 | 102 | <5 |
| areA102; sarA3 | <5 | 9 | 16 | <5 |
| areA102; sarA31 | <5 | 8 | — | — |
| areA102; sarA5 | <5 | 8 | — | <5 |
| areA102; sarA18 | <5 | 63 | — | — |
| areA102; sarA36 | <5 | 7 | — | <5 |
| areA102; sarA40 | <5 | 10 | — | — |
| areA102; sarA44 | <5 | 11 | — | <5 |
| areA102; sarA47 | <5 | 6 | 7 | <5 |
| areA102; sarB7 | <5 | 47 | 54 | <5 |
| areA102; sarB33 | <5 | 65 | 38 | <5 |

* Mycelium grown for 16 h on glucose-ammonium (20 mm) medium prior to transfer to treatment media for 4 h. Histidase activities expressed as described for Table 3.

Temperature-sensitive studies have shown that a number of sarA and sarB alleles can affect the levels of extracellular protease, as judged by milk clearing (Cohen, 1972). The sarA and sarB loci were found to be involved in the utilization of milk protein as a sole nitrogen source as well as a sole carbon and nitrogen source (Table 8). areA102; sarA10 produces elevated protease levels under all growth conditions. areA102; sarA18 and areA102; sarB33 show reduced levels of the extracellular protease when grown on milk as a sole nitrogen source at 25 or 42 °C. areA102; sarB33 retains a similar temperature-sensitive phenotype on milk as the sole carbon and nitrogen source whereas the protease levels of areA102; sarA18 are reduced at all temperatures. The production of extracellular protease by areA102; sarB7 and areA102; sarA42 is sensitive to growth temperatures above 25 °C when milk is the sole source of nitrogen. However, this temperature dependence for protease production is altered when milk is present as both a carbon and nitrogen source.

areA+ and areA102 strains were found to be unable to produce a halo of milk...
Table 8. *Temperature-sensitive studies on sar mutants*

| Strain                | Histidine | Leucine* | Citrulline† | Milk‡ | Milk§ |
|-----------------------|-----------|----------|-------------|-------|-------|
|                       | 25 °C     | 37 °C    | 42 °C       | 25 °C | 37 °C | 42 °C | 25 °C | 37 °C | 42 °C | 25 °C | 37 °C | 42 °C |
| areA102               | + + +     | + + +    | + + +       | 6     | 6     | 6     | 6     | 6     |       |       |       |       |
| areA102; sarA3        | ±         | ±        | ±           | ±     | ±     | ±     | ±     | ±     |       |       |       |       |
| areA102; sarA6        |           |          |             | ±     | ±     | ±     | ±     | ±     |       |       |       |       |
| areA102; sarA10||| + + +     | +         | + + +       | +     | +     | +     | +     | +     |       |       |       |       |
| areA102; sarA36       | ±         | ±        | ±           | ±     | ±     | ±     | ±     | ±     |       |       |       |       |
| areA102; sarA40       | ±         | ±        | ±           | ±     | ±     | ±     | ±     | ±     |       |       |       |       |
| areA102; sarA42||| + + +     | +         | + + +       | +     | +     | +     | +     | +     |       |       |       |       |
| areA102; sarA44       | ±         | ±        | ±           | ±     | ±     | ±     | ±     | ±     |       |       |       |       |
| areA102; sarA47       |           |          |             | ±     | ±     | ±     | ±     | ±     |       |       |       |       |
| areA102; sarB7||| + + +     | +         | + + +       | +     | +     | +     | +     | +     |       |       |       |       |
| areA102; sarB33||| ±         | +         | + + +       | ±     | ±     | +     | +     | +     |       |       |       |       | 3     | 6     | 2     | 2     | 6     | 4     |

Plates scored after 2 days incubation at the required temperature. Mutant strains are scored relative to the growth or milk clearing of areA102 for each medium and temperature. Growth symbols are described in Table 5. Milk clearing is scored from 1 to 8, with higher numbers denoting greater clearing.

* Similar temperature-sensitive growth patterns observed on L-lysine and L-methionine.
† Similar temperature-sensitive growth patterns observed on D,L-homoserine.
‡ Milk protein (1%) present as the sole nitrogen source in glucose-minimal media.
§ Milk protein (1%) present as the sole carbon and nitrogen source in glucose-free minimal media.
|| Temperature-sensitive phenotype.
Table 9. Effect of sarA and sarB mutations in areA+ strains

| Relevant genotype | Histidine | Leucine* | Phenylalanine | Aspartic Acid | Alanine† | Tryptophan‡ | Isolucine | Serine | Urea | Acetamide§ |
|-------------------|-----------|----------|--------------|--------------|----------|-------------|-----------|--------|------|-----------|
| areA102           | + + +     | + + +    | + + +        | + + +        | + + +    | + + +       | + + +     | + + +  | + +  | + + +     |
| areA+             | ±         | ±        | +           | + + +        | + + +    | + + +       | + + +     | + + +  | +    | + + +     |
| areA102; sarA3    | ±         | ±        | +           | + + +        | + + +    | + + +       | + + +     | + + +  | +    | + + +     |
| areA+; sarA3      | ±         | ±        | +           | + + +        | + + +    | + + +       | + + +     | + + +  | +    | + + +     |
| areA102; sarA31   | ±         | ±        | +           | + + +        | + + +    | + + +       | + + +     | + + +  | +    | + + +     |
| areA+; sarA31     | ±         | ±        | +           | + + +        | + + +    | + + +       | + + +     | + + +  | +    | + + +     |
| areA102; sarB7    | + +       | + +      | +           | + + +        | + + +    | + + +       | + + +     | + + +  | +    | + + +     |
| areA+; sarB7      | ±         | ±        | +           | + + +        | + + +    | + + +       | + + +     | + + +  | +    | + + +     |
| areA102; sarB33   | + +       | + +      | +           | + + +        | + + +    | + + +       | + + +     | + + +  | +    | + + +     |
| areA+; sarB33     | ±         | ±        | +           | + + +        | + + +    | + + +       | + + +     | + + +  | +    | + + +     |

All nitrogen sources were tested at a concentration of 10 mM. Plates were scored after 2 days at 37 °C. Growth symbols are described in Table 5.

* Similar growth patterns observed on L-lysine, L-methionine, L-citrulline and D,L-homoserine.
† Similar growth patterns observed on L-tyrosine and L-valine.
‡ Similar growth patterns observed on L-arginine and L-glutamate.
§ Similar growth patterns observed on propionamide and butyramide.
clearing when ammonium is present, with either glucose or milk as the sole carbon source. All mutant strains tested were similar in this respect except areA102; sarA10 which produces detectable amounts of extracellular protease in the presence of ammonium when milk is the sole carbon source.

(vii) Properties of mutants in an areA+ background

All the above tests have been carried out on strains containing the areA102 mutation. A number of areA+;sarA and areA+;sarB strains have been isolated. It has been necessary to construct these strains initially by means of haploidization using markers on linkage group VII since distinguishing the phenotypes of areA+ strains on histidine is difficult because of the very poor growth on this medium. Growth tests summarized in Table 9 show that sarA and sarB lesions can affect nitrogen source utilization in areA+ strains. As noted above, these effects are not detectable on histidine, as the wild-type strains grow poorly. In addition, the sar mutations do not significantly affect the utilization of nitrate, nitrite, glutamine, asparagine, hypoxanthine and uric acid by areA+ strains. However, areA+ strains carrying sarA or sarB lesions do grow more poorly than wild-type strains on nitrogen sources such as phenylalanine and citrulline, which are also used more poorly by areA102 strains carrying these lesions. In addition, the combination of the sarA and sarB mutations with the areA+ allele results in slightly poorer growth on some nitrogen sources (e.g. acetamide, glutamate, valine, serine and urea) not detectably affected in the original cweA102-containing strains. Notably the cold-sensitive sarB33 lesion results in reduction in the utilization of acetamide, glutamate and arginine as nitrogen sources at 25 °C without affecting their utilization as sole carbon and nitrogen sources. The presence of sarA and sarB mutations were also found to affect the milk clearing ability of areA+ strains.

These studies clearly indicate that the sar loci affect the utilization of many nitrogen sources. The catabolism of those nitrogen sources such as histidine, leucine and lysine which are used poorly by wild-type strains are detectably affected by sar mutations in an areA102 background. The effects of sar mutations on the utilization of other nitrogen sources such as acetamide, serine and glutamate are only detectable in an areA+ background.

(viii) Dominance properties of the mutants

Dominance studies of the sarA and sarB mutations with respect to histidine utilization have been carried out in diploids. The growth properties and histidase levels of representative diploids are given in Table 10. The poor growth on histidine of diploids homozygous for either sarA or sarB lesions indicates the lack of complementation between allelic mutations. However, the heterozygous diploid sarA3 sarB+/sarA+ sarB33 grows more strongly on histidine than either haploid parent strain due to complementation between non-allelic mutations. The areA+/areA102 diploid grows more strongly on histidine than the homozygous areA+ diploid, but grows more poorly than the homozygous areA102 diploid. This indicates that the areA102 allele is co-dominant to its wild-type allele for histidine utilization.
Histidine utilization in A. nidulans

Co-dominance of the areA102 allele for acetamide utilization has been described previously (Hynes, 1972). Diploid studies have also shown that mutations at the sarA locus are co-dominant to the wild-type allele for histidine utilization. Diploids homozygous for the areA102 allele and heterozygous at the sarA locus grow more poorly on histidine than homozygous areA102 sarA+ diploids. These results suggest that both the areA102 and sarA+ products are necessary for the strong utilization of histidine as a nitrogen source and that these products are limiting in heterozygous diploids (see Cove, 1969).

Table 10. Dominance studies in diploids

| Relevant diploid genotype | Growth on histidine* (10 mT) | Histidase activity† |
|---------------------------|-------------------------------|---------------------|
| areA+/areA+               | ±                             | 6                   |
| areA102/areA102           | + +                           | 100                 |
| areA+/areA102             | + +                           | 61                  |
| areA102 sarA3/areA102 sarA31 | ±                             | 9                   |
| areA102 sarA3/areA102 sarA+ | + +                           | 55                  |
| areA102 sarA3/areA+ sarA+  | +                             | 23                  |
| areA102 sarB33/areA102 sarB7 | +                             | 26                  |
| * areA102 sarB33/areA102 sarB+ | + +                           | 108                 |
| areA102 sarB33/areA+ sarB+ | +                             | 14                  |
| areA102 sarA3/areA102 sarB33 | + +                           | 58                  |

* Growth was scored on glucose-minimal media with 10 mM histidine as the sole nitrogen after 2 days incubation at 37 °C. Growth symbols are described in Table 5.
† Histidase activities determined on mycelium grown for 16 h on glucose-ammonium (20 mM) medium then transferred to nitrogen-free conditions for 4 h. Histidase activities calculated as described for Table 3. Results expressed relative to histidase levels of the areA102/areA102 diploid.

The sarB mutations are completely recessive to the wild-type sarB+ allele. Homozygous sarB+ diploids and heterozygous sarB+/sarB diploids show identical growth properties on histidine as a sole nitrogen source. The results of histidase assays illustrate the effects of the areA, sarA and sarB loci on histidase synthesis in these diploid strains. The assay results correlate well with the observed growth properties of the diploids.

4. DISCUSSION

Our results suggest strongly that conversion of histidine to urocanic acid and ammonium by histidase is a major route of utilization of histidine as a nitrogen source by A. nidulans. All strains producing low levels of histidase grow poorly on histidine as the sole nitrogen source. Furthermore, areA102 strains containing the gdhAlO lesion which results in loss of the NADP-linked glutamate dehydrogenase (Arst & MacDonald, 1973) grow poorly on histidine as a nitrogen source. This indicates that histidine is metabolized mainly via ammonium rather than via glutamate (e.g. by transamination), since gdhA10 results in poor growth in the absence of a source of glutamate. Other routes of histidine catabolism (e.g. Emes &
Hassall, 1973) must either contribute insignificantly to histidine utilization or be affected in a very similar way to histidase in the strains studied.

Histidase is regulated by ammonium repression as shown by the very low levels of this enzyme in mycelium of all strains grown in the presence of ammonium. Many other enzymes involved in the utilization of nitrogen sources in fungi have been shown to be subject to ammonium repression. (Pateman & Cove, 1967; Scaccocchio & Darlington, 1968; Arst & Cove, 1973; Dubois, Grenson & Wiame, 1973).

Furthermore, mutations at the areA locus have striking effects on histidase levels and there is considerable evidence for the product of the areA gene being involved in ammonium repression. (Arst & Cove, 1973; Hynes, in press). Unlike the bacterial systems (Smith, Halpern & Magasanik, 1971; Chasin & Magasanik, 1968; Magasanik et al. 1965; Lessie & Neidhart, 1967) histidase does not seem to be subject to induction by externally added histidine or urocanic acid and is not subject to catabolite repression. In fact, histidase activities are extremely low when histidine is the sole carbon and nitrogen source. The extremely weak growth of A. nidulans observed on histidine as a carbon source may be due to very low levels of alternative catabolic pathways. Recent results (Polkinghorne & Hynes, unpublished) indicates that histidase is subject to weak repression during growth on L-glutamate and strong repression during growth on L-glutamine. This regulation is shared by a number of other enzymes of nitrogen catabolism and there is some evidence suggesting that these control mechanisms may be distinct from ammonium repression (Hynes, 1974). In summary, the data clearly implicates histidase as being involved in the utilization of histidine as a nitrogen source. No urocanase activity has been detected in A. nidulans and the metabolic fate of the urocanate produced by histidase is presently being investigated.

Two loci, provisionally designated sarA and sarB, have been described in this paper. The sar designation (suppressor of areA102) has been used because mutations at either of these loci can lead to suppression of the effects of areA102 on the utilization of histidine, leucine, lysine and a number of other nitrogen sources which are utilized poorly by areA+ strains. Replacing the areA102 allele with areA+ in sar mutants has revealed that these loci play a role in the utilization of other nitrogen sources such as acetamide, serine and glutamate which are not detectably affected in areA102; sar double mutants. Apparently the involvement of these loci in the utilization of these latter nitrogen sources is effectively masked by the effects of the areA102 allele, whereas for histidine, leucine, lysine, etc., utilization the activating effect of the areA102 product is unable to compensate for the effects of sarA or sarB mutations.

The properties of the mutants reported here strongly suggest that the sarA and sarB loci have regulatory functions. These loci are involved in the utilization of structurally unrelated nitrogen sources and affect histidase and extracellular protease levels, neither of which require induction. Thus it is unlikely that the loci code for components of a permease system. The wide range of pleiotropy exhibited by the sar mutants also makes it unlikely that they code for sub-units common to
all the respective catabolic enzymes. The heterogeneity of the pleiotropic effects of \textit{sar} lesions and the temperature-sensitive phenotypes (including cold-sensitive effects) support the proposal of a regulatory function for these loci.

The \textit{sarA} and \textit{sarB} loci appear to be involved primarily in nitrogen source utilization. Their effects resemble the pleiotropic effects of \textit{areA} mutants (Hynes, 1973; Arst & Cove, 1973). So far, the only carbon source which is affected by \textit{sar} mutants is milk protein. It has also been observed that some \textit{areA} mutants affect growth and milk clearing when milk is the sole carbon source (Hynes, unpublished). It seems likely therefore that these loci code for regulatory products involved in the control of the levels of some enzymes of nitrogen catabolism. If the possibility that repressions during growth on glutamate or on glutamine constitute separate regulatory mechanisms from ammonium repression (see above) is confirmed, these genes would be candidates for the regulatory genes involved.

The properties of the \textit{sar} mutants reported here suggest that there is a stringent dose requirement for the products of these genes as well as for the \textit{areA} product for maximal histidase levels. In wild-type strains the products of these genes are limited in their ability to activate enzyme synthesis. The \textit{areA102} lesion results in an increase in the capacity for histidase synthesis, but there is still a requirement for the wild-type products of the \textit{sar} genes. This is shown most clearly in the studies on heterozygous diploids. The stringency of the requirement for the products of these genes would appear to be similar for the utilization of lysine, leucine and some other nitrogen sources, but less for the utilization of acetamide, glutamate and serine and a number of other nitrogen sources.

Despite the isolation of many mutants showing reduced growth on histidine, there are none which contain lesions in the structural gene (or genes) for histidase. Our method of detection of mutants would appear to be very sensitive since strains with leaky growth on histidine were isolated. A somewhat similar situation has been observed in yeast where no apparent maltase structural genes have been identified (ten Berge, Zoutwelle & van der Poll, 1973). There are a number of possible explanations. Structural gene mutants might be lethal. It would seem unlikely that this could be due to loss of histidase activity, since strains producing low levels of histidase are readily obtained, but it is possible that even at low levels the enzyme protein has some essential function. Another possibility is that there is more than one pathway of histidine utilization, but this requires that the effects of the \textit{areA}, \textit{sarA} and \textit{sarB} mutations on both pathways are very similar (see above). A likely possibility is that there are multiple structural genes for the histidase. This has also been proposed for the maltase situation in yeast (ten Berge \textit{et al.} 1973). Very preliminary evidence, based on enzyme kinetic studies, suggests that there may be more than one histidase enzyme. If the existence of multiple structural genes proves to be the case, then histidine utilization by \textit{A. nidulans} provides an interesting paradox between structural and regulatory competence, since the regulatory activity of the \textit{areA} and possibly the \textit{sar} gene products is limiting histidase levels rather than the structural information available for expression.
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