Isolation and Characterization of Methylammonium-resistant Mutants of *Saccharomyces cerevisiae* with Relieved Nitrogen Metabolite Repression of Allantoinase, Arginase and Ornithine Transaminase Synthesis

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SUMMARY

The sensitivity of *Saccharomyces cerevisiae* α - Σ 1278b to methylammonium was dependent on the nature of the nitrogen source. Yeast strains carrying novel mutations, *allNmr* or *argNmr*, were isolated by selection for methylammonium resistance in media with allantoin or arginine as the sole nitrogen source. The mutations result in a considerable relief from nitrogen metabolite repression of allantoinase synthesis (*allNmr*), or of arginase and ornithine transaminase synthesis (*argNmr* and, to a lesser extent, *allNmr*). Both mutations were recessive to the wild type and were neither linked to each other, nor to the structural genes of allantoinase, arginase and ornithine transaminase. Hence they represent regulatory genes without operator characteristics. Experiments with ammonium-limited continuous cultures indicated that ammonium transport was not affected by the *allNmr* and *argNmr* mutations. Ammonium repression of catabolic NAD-dependent glutamate dehydrogenase was also unaffected. The *argNmr* locus, in cooperation with other loci, appears to control nitrogen metabolite repression of the arginine degradative pathway; it is probably pathway-specific. The *allNmr* locus may have a general function in nitrogen metabolite repression of various degradative pathways since the *allNmr* mutation causes resistance to methylammonium in the presence of several metabolically unrelated nitrogen sources.

INTRODUCTION

Mutants of *Aspergillus nidulans* with impaired repression by ammonium of many enzymes involved in the degradation of nitrogenous substances have been isolated by selection for resistance to methylammonium (Arst & Cove, 1969). Methylammonium is considered to be a structural analogue of ammonium. In mutants resistant to methylammonium, designated *meaR*, the repression of numerous enzymes by ammonium is relieved. Although a common nitrogen metabolite repressor for all of these enzymes was assumed, in a subsequent paper (Arst & Page, 1973) methylammonium resistance was attributed to impairment of the common permease for ammonium and methylammonium. Likewise, a recent paper on methylammonium transport in a strain of *Saccharomyces cerevisiae* (Roon *et al.*, 1975 *a*) reveals that methylammonium resistance is associated with defective transport of and growth on ammonium. In the present study it is demonstrated that selection for methylammonium resistance is a valuable tool in the isolation of yeast mutants with impaired nitrogen metabolite repression of enzymes involved in the degradation of allantoin and arginine.

Saccharomyces cerevisiae is able to utilize exogenous allantoin as the sole source of

nitrogen but is unable to produce allantoin by purine base catabolism (DiCarlo, Schultz & McManus, 1951; LaRue & Spencer, 1968). Allantoin is degraded in yeast to urea and glyoxylate by three successive hydrolytic enzyme reactions (DiCarlo, Schultz & Kent, 1953; Cooper & Lawther, 1973), the first being catalysed by allantoinase (allantoin amidohydrolase, EC 3.5.2.5). Allophanate, an intermediate of the urea degradative pathway in yeast (Roon & Levenberg, 1970; Whitney & Cooper, 1972), is considered to be the inducer of the whole pathway catalysing the conversion of allantoin into glyoxylate, ammonium and bicarbonate, since all of the enzymes appear to be uninducible in mutants unable to synthesize the compound (Cooper & Lawther, 1973; Whitney, Cooper & Magasanik, 1973).

Allantoinase synthesis in protoplasts of *S. carlsbergensis* is repressed by nitrogenous compounds like glutamine, asparagine and ammonium (van de Poll, 1970). Bossinger, Lawther & Cooper (1974) observed only a slight repression by ammonium but strong repression by glutamine, asparagine or serine when added singly, or by ammonium and alanine when added together, to a *S. cerevisiae* culture in glucose medium provided with a gratuitous inducer.

The pathway of arginine metabolism in *S. cerevisiae* is initiated by the successive actions of arginase (L-arginine amidohydrolase, EC 3.5.3.1 and ornithine transaminase (L-ornithine:2-oxoacid aminotransferase, EC 2.6.1.13) (Middelhoven, 1964). Yeast arginase is activated *in vivo* by ferrous ions; *in vitro* these can be replaced by other divalent cations. The enzyme is strongly competitively inhibited by ornithine (Middelhoven, 1969*a*; Middelhoven, de Waard & Mulder, 1969). The syntheses of arginase and ornithine transaminase are regulated by induction and repression. Arginine and ornithine serve as the inducers (Middelhoven, 1964, 1970). Fully induced enzyme activities are observed after growth on arginine as the sole nitrogen source. The addition of readily assimilable nitrogenous compounds like ammonium, asparagine, aspartate, glutamine or alanine results in much lower enzyme activities; however these are higher than those produced in the absence of inducer (Middelhoven, 1970). Nitrogen metabolite repression is indicated, in particular, by the strong derepression of both enzymes during nitrogen starvation in the absence of inducers (Middelhoven, 1969, 1970; Whitney & Magasanik, 1973). Myo-inositol probably plays a role in this process (Middelhoven, 1969*b*). No evidence was obtained for carbon catabolite repression or for inactivation repression (Middelhoven, 1969*c*, 1970).

Yeast mutants (*gdhA*) lacking anabolic NADP-specific glutamate dehydrogenase [L-glutamate:NADP⁺ oxidoreductase (deaminating), EC 1.4.1.4] and characterized by their slow growth in ammonium medium, were claimed to have lost nitrogen metabolite repression of a general amino-acid permease (Grenson & Hou, 1972), allantoinase and arginase but not of ornithine transaminase or catabolic NAD-specific glutamate dehydrogenase [L-glutamate:NAD⁺ oxidoreductase (deaminating), EC 1.4.1.2] (Dubois, Grenson & Wiame, 1973, 1974). Nitrogen metabolite repression was considered to result from the interference of the NADP-specific glutamate dehydrogenase molecule with the process of enzyme synthesis (Dubois *et al.*, 1974). The role of *gdhA*⁺ in nitrogen metabolite repression in yeast has been questioned by several authors (van de Poll, 1973; Bossinger & Cooper, 1975; Roon, Larimore & Levy, 1975*b*).

The mutation *gdhCR* has also been claimed to result in the mitigation of nitrogen metabolite repression of arginase and allantoinase (Dubois & Grenson, 1974). In *gdhCR* mutants the synthesis of catabolic NAD-specific glutamate dehydrogenase is also derepressed.

As in yeast, *gdhA* mutants of *A. nidulans* exhibit impaired ammonium repression of several enzymes (Kinghorn & Pateman, 1973; Arst & MacDonald, 1973), although the

Table 1. *Yeast strains used*

All strains are derived from the isonuclear wild-type strains α - Σ 1278b and a-3962c.

Strain	Genotype	
α -HP100	wild type	α -his, ura
a-LA100	wild type	a-leu, ade
D40E	wild type	α -his, ura, eth^R /a-leu, ade
DgdhA1	gdhA	α -ile, gdhA1/a-ade, gdhA1, eth^R
α -AllNmr-5	allNmr	α -his, ura, allNmr5
D252E	allNmr/wild type	α -leu, his, ura, eth^R , allNmr5/a-ade
D246E	allNmr	α -leu, his, ura, eth^R , allNmr5/a-ade, eth^R , allNmr5
a-ArgNmr-12	argNmr	α -leu, ade, argNmr12
D251E	argNmr/wild type	α -his, eth^R , argNmr12/a-leu, ade
D245E	argNmr	α -his, eth^R , argNmr12/a-leu, ade, eth^R , argNmr12

repression by glutamine and glutamate is retained (Hynes, 1974). Enzymes involved in arginine or allantoin catabolism were not included in these studies.

In the present study, the repression of allantoinase, arginase and ornithine transaminase by several nitrogenous compounds is investigated in the wild type, in a *gdhA* mutant and in two methylammonium-resistant mutants (*allNmr* and *argNmr*).

METHODS

Yeast strains and genetic techniques. All strains used (Table 1) were derived from two isonuclear wild-type strains of *S. cerevisiae*, α - Σ 1278b and a-3962c, kindly provided by Dr J. M. Wiame, Brussels. The origin of the strains has been described previously (Béchet, Grenson & Wiame, 1970). Auxotrophic mutants of these strains were selected by the method of Horn & Wilkie (1966). Mutants lacking NADP-specific glutamate dehydrogenase (*gdhA*), arginase, ornithine transaminase or allantoinase were isolated on phloxine B medium (Middelhoven, Broekhuizen & van Eijk, 1976b). Ethyl methanesulphonate (1%, v/v) was used for mutagenesis; excess reagent was removed from the cells by suspending them in 10% (w/v) sodium thiosulphate and subsequent washing (Parker & Mattoon, 1969). Mating of strains and sporulation of diploids were achieved by conventional techniques (Fink, 1970), except that glucose was replaced by maltose in the pre-sporulation medium. Ascospores were separated by ultrasonic treatment. Auxotrophic haploid segregants were recognized as described above (Horn & Wilkie, 1966) and were screened for their nutritional requirements by replica plating. Respiratory-deficient strains were identified by their inability to grow on yeast extract/ethanol/peptone agar and were discarded. The mating type of the segregants was deduced from their ability to complement other auxotrophic strains of different nutritional requirement and known mating type. Following this procedure, two auxotrophic segregants were obtained, α -HP100 (α -his, ura) and a-LA100 (a-leu, ade), which were used as parent strains in the isolation of methylammonium-resistant mutants. To obtain these mutants, cells of both strains were treated with ethyl methane-sulphonate and spread on glucose/methylammonium agar with allantoin or arginine as the nitrogen sources. The concentration of methylammonium chloride was 50 mM in glucose/allantoin agar and 500 mM in glucose/arginine agar; L-histidine, uracil, L-leucine and adenine were added at 200 mg l⁻¹ if required. Resistant mutants produced colonies in about 3 to 7 days and were streaked on medium of the same composition. Pure cultures were maintained on yeast extract/glucose/peptone agar.

Prototrophic diploid strains, homozygous with respect to the methylammonium

resistance marker, were obtained by mating a methylammonium-resistant mutant with a methylammonium-sensitive strain of complementary nutritional requirement, which had previously been mutated to resistance to 50 mg DL-ethionine l^{-1} . This resistance is recessive to wild type and is presumably due to the lack of specific methionine permease (Gits & Grenson, 1967) rather than of a general amino-acid permease since the wild-type sensitivity to 10 mg L-canavanine sulphate l^{-1} had been retained. After sporulation of the diploid and ultrasonic separation of the ascospores, the suspension was plated on glucose/ammonium agar supplemented with DL-ethionine (25 mg l^{-1}) and the nitrogenous substances required by the parent strains (each at 200 mg l^{-1}). Segregant strains were screened for methylammonium resistance (see below), nutritional requirements, respiratory ability and mating type. Diploids were constructed in the usual way from methylammonium-resistant segregants of opposite mating type and complementary nutritional requirements.

Growth conditions. The growth medium contained 40 g glucose l^{-1} ; mineral constituents, vitamins and buffer were added as described previously (Middelhoven, 1969c). Nitrogen sources were each added to a concentration of 40 mM-nitrogen. The medium, pH 5.5, was sterilized for 15 min at 120 °C; glucose was autoclaved separately and labile nitrogen sources were sterilized by Seitz filtration. Solid media contained 20 g agar l^{-1} .

Portions of medium (10 to 20 ml) in 100 ml conical flasks were inoculated with 0.1 ml of a pre-culture in the same medium and incubated in a rotary shaker at 30 °C until no more than half the maximum cell yield (on the basis of 40 mM-nitrogen) was obtained; this usually took about 16 h. Growth was monitored turbidimetrically. For some experiments larger amounts of yeast cells were grown in Kluyver flasks.

The substrate constant for ammonium and the maximum specific growth rate in ammonium-limited chemostat cultures were determined as described by Middelhoven *et al.* (1976a).

Resistance to methylammonium was observed in liquid media containing different nitrogen sources and variable concentrations of methylammonium chloride. Portions of medium (2 ml) in test tubes were inoculated with 1% (v/v) of a fully grown pre-culture in 10 mM-glutamate medium and observed visually at intervals over 3 days. Replica plating was not suitable for demonstrating methylammonium resistance.

Enzyme determinations. Preparation of cell-free extracts, protein determination, activation of arginase with $MnCl_2$ and determination of ornithine transaminase were as described by Middelhoven (1964). However, in the present study, the glycinate buffer was omitted from the incubation mixtures for the arginase assay because 100 mM-L-arginine pH 9.5 buffered the system sufficiently by itself, and urea was determined with *p*-dimethylaminobenzaldehyde by the method of Hagan & Dallam (1968). This latter method was found to be satisfactory provided that arginine was added to the urea standard series in amounts equal to those present in the samples. Incubation mixtures for the allantoinase assay contained (in a total volume of 1.0 ml): 0.2 ml cell-free extract (0.5 to 2 mg protein), 10 μ mol Na_2EDTA pH 7.2, 100 μ mol Tris/HCl buffer pH 7.2 and 30 μ mol allantoin, added in that order. After 1 h at 30 °C, the reaction was stopped by the addition of 1.0 ml 1 M-perchloric acid and 8.0 ml water. Samples of the supernatant fluid were heated for 10 min at 100 °C to convert the reaction product, allantoic acid, to glyoxylic acid which was then determined by the method of Vogels & van der Drift (1970). Conditions were such that no more than 2.5 μ mol allantoin was hydrolysed during the enzyme reaction. Glutamate dehydrogenases were determined according to Doherty (1970). All specific enzyme activities were expressed as μ mol substrate converted h^{-1} (mg protein) $^{-1}$ at 30 °C.

RESULTS

Methylammonium chloride inhibits growth of *S. cerevisiae* strain α - Σ 1278b when added to growth media containing arginine, allantoin or certain other nitrogenous compounds as the sole nitrogen source. Mutants resistant to methylammonium chloride were selected in two auxotrophic strains, α -HPI00 and a-LA100, by spreading cells treated with ethyl methanesulphonate on agar media containing the analogue and different nitrogen sources. Two hundred of the mutants obtained were screened for metabolite repression of the enzymes allantoinase, arginase and ornithine transaminase.

Dominance or recessivity of the resistance to methylammonium was recognized from the properties of heterozygous diploids obtained by mating mutants to wild-type strains. Complementation of the different mutations was studied in diploids obtained by mating methylammonium-resistant mutants of α -HPI00 with those of a-LA100. On the basis of these preliminary experiments, methylammonium-resistant yeast strains showing impaired nitrogen metabolite repression in allantoin or arginine catabolism were divided into two groups, designated *allNmr* and *argNmr*. Mutants belonging to the former group, isolated on the basis of methylammonium resistance in the presence of various nitrogen sources (DL-allantoin, urea, DL-2-aminobutyrate, L-arginine), were recessive to wild type and showed impaired nitrogen metabolite repression with regard to allantoinase synthesis and, to a lesser extent, with regard to arginine catabolism. *argNmr* mutants, exclusively isolated on methylammonium/arginine medium, were also recessive to wild type but showed impaired nitrogen metabolite repression only in arginine catabolism, allantoinase synthesis being controlled as in the wild-type strains. Mutants representative of the two groups, α -AllNmr-5 and a-ArgNmr-12, were crossed with ethionine-resistant wild-type strains. The resulting diploids were sporulated and segregant strains were isolated on the basis of ethionine resistance. Amongst these segregants there was a 1:1 distribution of wild and Nmr phenotypes, indicating that the mutations were of a nuclear type and not linked to the ethionine resistance marker. Moreover, a complete correlation was observed between methylammonium resistance and impaired nitrogen metabolite repression. For detailed physiological studies, prototrophic diploid strains, homozygous for the *Nmr* markers, were preferable to haploid auxotrophic strains, as the latter generally show lower maximum specific growth rates. In this way, any effect of growth rate or auxotrophy on nitrogen metabolite repression is precluded as far as possible.

Methylammonium resistance

A wild-type diploid yeast strain (D40E), diploid yeast strains homozygous with respect to the *gdhA*, *allNmr* and *argNmr* mutations, and heterozygotes *allNmr*/wild type and *argNmr*/wild type were grown at various methylammonium concentrations (0, 1, 2, 5, 10, 20, 50, 100, 200, 500, 750 and 1000 mM) in media with different nitrogen sources. The threshold concentrations of methylammonium toxicity, shown in Table 2, represent the lowest concentrations at which a decreased growth rate was observed. The sensitivity of the wild-type strain D40E to methylammonium varied greatly with the nitrogen source. In the presence of allantoin, growth was inhibited at 2 mM, whereas in the presence of ammonium or of nitrogen sources from which ammonium is readily generated (e.g. asparagine, glutamine) concentrations of 750 mM were tolerated. The methylammonium sensitivity of *DgdhA1* was about the same as that of the wild type except for slightly increased resistance in urea, allantoin and proline media and increased sensitivity in leucine and serine media. The *argNmr* diploid D245E showed marked resistance to methylammonium

Table 2. *Threshold concentrations of methylammonium toxicity*

The concentrations (mM) listed are the lowest at which inhibition of the growth rate was observed.

Yeast strain ... Genotype ...	D40E wild type	DgdhA1 gdhA	D245E argNmr	D246E allNmr	D251E argNmr/ wild type	D252E allNmr/ wild type
Nitrogen source						
Ammonium	> 1000	1000	1000	1000	—	—
DL-Alanine	750	> 1000	1000	1000	—	—
DL-Allantoin	2	5	1	100	1	1
DL-2-Aminobutyrate	50	100	200	500	50	100
4-Aminobutyrate	50	50	50	200	50	50
L-Arginine	200	200	1000	1000	50	100
L-Asparagine	1000	1000	1000	1000	—	—
L-Aspartate	500	500	500	500	500	500
L-Citrulline	100	100	100	100	50	50
L-Glutamate	50	50	100	200	50	50
L-Glutamine	1000	> 1000	> 1000	1000	—	—
L-Isoleucine	50	100	50	200	50	50
L-Leucine	500	50	1000	500	—	—
L-Ornithine	50	50	50	200	10	50
L-Proline	50	100	50	500	—	—
L-Serine	500	100	500	500	500	500
L-Threonine	50	100	50	200	50	50
L-Valine	200	100	50	500	50	50
Urea	50	200	50	500	50	50

in arginine medium and increased resistance in the presence of 2-aminobutyrate, glutamate and leucine. The *allNmr* diploid D246E showed increased resistance to methylammonium in the presence of a number of nitrogen sources (allantoin, 2-aminobutyrate, arginine, glutamate, isoleucine, ornithine, proline, threonine, valine and urea). Since many of these compounds are unrelated structurally and metabolically, the *allNmr* locus probably has a general function not restricted to allantoin catabolism. The heterozygous diploids D251E and D252E behaved in essentially the same way as the wild type. The *allNmr* and *argNmr* mutations are clearly recessive to wild type.

Allantoinase synthesis

The effects of the *allNmr*, *argNmr* and *gdhA* mutations on allantoinase synthesis are shown in Table 3. In the wild-type diploid D40E, allantoinase was produced at a basal level after growth in the presence of readily assimilable nitrogen sources such as ammonium, glutamine, asparagine, ammonium/glutamate and ammonium/aspartate. The same nitrogen sources efficiently repressed allantoinase synthesis when added together with allantoin, arginine or urea. Growth of the wild-type strain D40E in the presence of all other nitrogen sources tested resulted in higher specific allantoinase activities. It is not clear whether allantoinase synthesis requires the presence of the urea catabolite allophanate as an internal inducer, as was observed in another yeast strain (Cooper & Lawther, 1973). Specific allantoinase activities after growth on allophanate-generating nitrogen sources like allantoin, urea and arginine were lower than those after growth on DL-alanine, DL-2-aminobutyrate and other amino acids (e.g. valine, leucine and methionine; not shown). However, an essential role of allophanate in allantoinase synthesis cannot be ruled out on the basis of this experiment.

The diploid strain DgdhA1, lacking NADP-specific glutamate dehydrogenase, showed a

Table 3. *Specific allantoinase activities in diploid yeast strains grown in glucose media with different nitrogen sources*

The results are expressed as μmol allantoin hydrolysed h^{-1} (mg protein) $^{-1}$ under standard conditions and indicate the extremes of at least four determinations.

Yeast strain Genotype	... wild type	D40E	DgdhA1 gdhA	D246E allNmr	D252E allNmr/ wild type	D245E argNmr
Nitrogen source						
NH ₄ ⁺	0.2-0.6	1.4-1.6*	0.9-1.0	0.3-0.4	0.4-0.5	
L-Glutamate	1.0-2.2	1.1-2.1	1.2-1.3	1.2-1.5	1.0-1.5	
L-Glutamine	0.2-0.3	0.3-0.6	0.9-1.2	—	—	
L-Asparagine	0.3-0.4	0.3-0.7	0.8-0.9	0.1-0.2	—	
NH ₄ ⁺ +L-Glutamate	0.1-0.2	0.6-1.2	1.1-1.3	0.2-0.3	—	
NH ₄ ⁺ +L-Aspartate	0.1-0.2	0.2-0.3	0.7-1.3	—	—	
DL-Alanine	4.5-6.8	5.2-7.8	5.1-8.5	—	—	
+NH ₄ ⁺	0.5-1.0	2.3-2.9	3.5-8.9	—	—	
DL-2-Aminobutyrate	2.2-9.2*	3.1-5.4*	5.4-6.7*	—	—	
+NH ₄ ⁺	0.6-1.0	2.3-4.5	4.8-5.3	—	—	
+L-Asparagine	—	1.4-2.1	3.7-5.4	—	—	
DL-Allantoin	2.5-4.0	2.9-4.7*	1.9-2.6	2.7-3.1	3.0-3.2	
+NH ₄ ⁺	0.4-0.6	2.8-3.0*	1.5-1.9	0.5-0.8	0.4-0.7	
+L-Glutamate	1.8-1.9	1.1-4.2	1.4-2.0	1.5-1.9	1.0-2.2	
+L-Glutamine	0.3-0.4	0.3-0.7	1.5-1.6	—	—	
+L-Asparagine	0.3-0.4	0.3-0.7	0.9-1.1	0.1-0.2	0.1-0.2	
+NH ₄ ⁺ +L-Glutamate	0.1-0.4	0.6-1.6	1.4-1.5	0.2-0.3	0.1-0.2	
L-Arginine	2.2-3.2	1.8-3.6	1.6-4.4	2.6-2.8	0.6-1.3	
+NH ₄ ⁺	0.6-1.2	1.9-3.2	1.6-4.5	0.6-0.9	0.7-0.9	
+L-Glutamine	0.3-0.4	0.2-0.3	1.2-1.8	—	—	
+L-Asparagine	0.1-0.2	0.3-0.4	0.5-0.7	0.1-0.2	0.1-0.2	
+NH ₄ ⁺ +L-Glutamate	0.2-0.3	0.5-0.8	1.6-3.3	0.1-0.2	0.3-0.4	
Urea	2.7-3.4	2.4-2.7*	1.2-2.5	2.3-2.4	1.9-2.8	
+NH ₄ ⁺	1.3-3.1	3.1-3.5*	1.4-2.0	1.1-1.6	1.3-1.7	
+L-Glutamine	0.8-1.1	1.9-2.2	2.7-2.9	—	—	
+L-Asparagine	0.3-0.5	0.4-1.0	0.8-1.2	0.2-0.3	—	
+NH ₄ ⁺ +L-Glutamate	0.2-0.3	0.8-2.4	2.1-2.4	0.2-0.3	0.1-0.3	

* Specific growth rates, about 0.15 h^{-1} ; specific growth rates of other cultures, 0.35 to 0.41 h^{-1} .

different regulation of allantoinase synthesis. Repression by ammonium, which is slowly assimilated by this strain, was weak but repression by glutamine and asparagine was almost as strong as in the wild-type strain D40E (cf. van de Poll, 1973). However, in the *allNmr* diploid D246E, allantoinase synthesis during growth in the presence of glutamine, asparagine, ammonium/glutamate and ammonium/aspartate was considerable. Obviously the nitrogen metabolite repression of allantoinase synthesis was greatly relieved by the *allNmr* mutation. This was not the case with the *argNmr* mutation (D245E) which had no effect on allantoinase synthesis. It is concluded that the *allNmr* mutation is recessive to wild type since the heterozygous diploid D252E behaves like the wild-type diploid D40E.

Arginine catabolism

Average specific activities of arginase and ornithine transaminase in diploid strains grown with different nitrogen sources are given in Table 4. In all strains the presence of arginine or another inducer in the medium was required for synthesis of ornithine transaminase exceeding the basal level [i.e. 0.2 to $0.4 \mu\text{mol h}^{-1}$ (mg protein) $^{-1}$]. Arginase was also induced

Table 4. *Average specific activities of arginase and ornithine transaminase in diploid yeast strains grown with different nitrogen sources*

		Specific activities of arginase (A) and ornithine transaminase (OT) are expressed as μmol substrate converted h^{-1} ($\text{mg protein})^{-1}$.											
Yeast strain	Genotype	D40E wild type		<i>DgdhA1</i> <i>gdhA</i>		D245E <i>argNmr</i>		D251E <i>argNmr</i> / wild type		D246E <i>allNmr</i>		D252E <i>allNmr</i> / wild type	
Nitrogen source		A	OT	A	OT	A	OT	A	OT	A	OT	A	OT
Arginine		52	1.8	48	1.8	31	1.7	48	1.7	36	1.2	58	2.5
+ 40 mM- NH_4^+		24	0.4	52	1.1	29	1.7	25	0.4	31	1.0	20	0.5
+ 400 mM- NH_4^+		5	0.3	55	1.5	19	0.9	7	0.2	7	0.2	6	0.3
+ Glutamate		54	0.4	62	0.7	40	1.7	48	0.5	83	0.7	48	0.5
+ Glutamine		21	0.4	35	0.6	23	1.1	22	0.3	33	0.4	30	0.7
+ Asparagine		14	0.3	20	0.6	27	2.0	22	0.5	18	0.6	10	0.6
+ Serine		15	0.4	15	1.0	33	1.8	18	0.5	12	0.3	13	0.5
40 mM- NH_4^+		4	0.3	9*	0.2*	5	0.3	—	—	5	0.1	—	—
Asparagine		9	0.4	10	0.3	10	0.3	—	—	16	0.2	—	—
Glutamate		18	0.3	22	0.2	30	0.2	—	—	32	0.2	—	—

* Specific growth rates, 0.15 h^{-1} ; specific growth rates of other cultures, 0.35 to 0.41 h^{-1} .

in the wild-type α -E1278b and related yeast strains during growth on media with glutamate, leucine and some other amino acids (Dubois & Wiame, 1976), but not in another strain of *S. cerevisiae* (Middelhoven, 1970). The enzymes responded differently to nitrogen metabolite repression, ornithine transaminase synthesis being very sensitive and arginase synthesis being repressed completely only by very high (400 mM) concentrations of ammonium chloride and, to a lesser extent, by 40 mM-ammonium chloride, glutamine, asparagine and serine when added to glucose/arginine medium. In *DgdhA1* the induction of arginase by arginine was repressed only by asparagine and serine, whereas the induction of ornithine transaminase was also repressed by other nitrogenous compounds. Nitrogen metabolite repression in the *argNmr* diploid D245E was relieved to a greater extent, enzyme synthesis being partly inhibited only by 400 mM-ammonium chloride and glutamine. The *allNmr* mutation (D246E) relieved arginase and ornithine transaminase synthesis from repression by 40 mM-ammonium chloride; the repression by other nitrogenous compounds was almost the same as in the wild type. The behaviour of the heterozygous diploids D251E and D252E indicated that the *argNmr* and *allNmr* mutations were both recessive to wild type.

The maximum specific arginase activities in strains D245E and D246E, after growth in arginine medium, were lower than that in the wild type. For the *argNmr* strain D245E, this was an effect of growth conditions since the arginase activity in cells grown in Kluysver flasks was as high as that in the wild-type strain D40E. However, the arginase activity of strain D246E (*allNmr*) was always significantly lower than that of strain D40E, an effect attributable to the *allNmr* mutation.

Previously the derepression of arginase and ornithine transaminase during nitrogen starvation of yeast cells, grown in the absence of an inducer, has been a valuable tool in the study of nitrogen metabolite repression (Middelhoven, 1968, 1970; Whitney & Magasanik, 1973). However, strain α -E1278b and its derivatives did not show this phenomenon in a reproducible way, and so derepression during nitrogen starvation was not included in this study.

Table 5. Maximum specific growth rates and substrate constants for ammonium obtained for diploid yeast strains grown in ammonium-limited continuous cultures

Yeast strain	Genotype	Maximum specific growth rate (h^{-1})	Substrate constant for ammonium (μM)
D40E	wild type	0.41	5-11
D245E	<i>argNmr</i>	0.40	5-6
D246E	<i>allNmr</i>	0.42	9

Genetic characteristics

The recessivity of the *allNmr* and *argNmr* mutations to wild type and the 1:1 distribution of wild and Nmr phenotypes among segregants obtained by sporulation of heterozygous *Nmr*/wild type diploids have been described above. Sporulation of heterozygous diploids *allNmr/argNmr*, *allNmr/allantoinase-less*, *allNmr/gdhA*, *argNmr/gdhA*, *argNmr/arginase-less* and *argNmr/ornithine transaminase-less* resulted in a random segregation of the different markers (results not presented). Segregant strains bearing two markers could be readily isolated. These results imply that the *allNmr*, *argNmr* and *gdhA* mutations belong to different linkage groups and that the former two loci represent true regulatory genes without operator characteristics. If the *Nmr* loci were to have an operator function, linkage to structural genes would have been observed.

Ammonium assimilation

Roon *et al.* (1975a) demonstrated that, in their yeast strain, methylammonium resistance is caused by defective transport of methylammonium and ammonium into the cell. Arst & Page (1973) suggested that in *A. nidulans* methylammonium resistance is also accompanied by defective ammonium transport. Although the *allNmr* and *argNmr* mutants showed the same growth rates on ammonium medium as the wild-type strain, the maximum specific growth rates (μ_m) and the substrate constants for the ammonium ion were determined in ammonium-limited chemostat cultures (Middelhoven *et al.*, 1976a). No significant differences between *Nmr* diploids and the wild type were observed (Table 5). Thus the *Nmr* loci represent true regulatory genes having no effect on ammonium transport.

Glutamate dehydrogenases

Previously described yeast mutants with relieved nitrogen metabolite repression of arginase or allantoinase are either devoid of anabolic NADP-specific glutamate dehydrogenase (*gdhA*; Dubois *et al.*, 1973) or have derepressed levels of catabolic NAD-specific glutamate dehydrogenase (*gdhCR*; Dubois & Grenson, 1974). Identity of the *Nmr* mutations and the *gdhA* mutation has been ruled out on the basis of genetic experiments, methylammonium resistance and the rate of ammonium assimilation, but identity of one of the *Nmr* mutants and the *gdhCR* mutation (not included in this study) is possible. To investigate this possibility, both glutamate dehydrogenases were determined in yeast grown with ammonium, with ammonium/glutamate and with glutamate. In all strains NAD-specific glutamate dehydrogenase was induced only in glutamate medium without ammonium (Table 6). The specific activity of this enzyme in D246E (*allNmr*) grown in glutamate medium was somewhat lower than that in the other strains. The nitrogen source had little influence on NADP-specific glutamate dehydrogenase synthesis. Its activity in both *Nmr* diploids was somewhat higher than in the wild-strain α -21278b. These results indicate that the *Nmr* mutations are not identical with either the *gdhA* or the *gdhCR* mutations.

Table 6. *Average specific activities of NAD-specific and NADP-specific glutamate dehydrogenases in diploid yeast strains grown with different nitrogen sources*Specific activities are expressed as μmol substrate converted h^{-1} (mg protein) $^{-1}$.

Nitrogen source	α - Σ 1278b Genotype ... wild type		D245E <i>argNmr</i>		D246E <i>allNmr</i>		<i>DgdhA1</i> <i>gdhA</i>	
	NAD	NADP	NAD	NADP	NAD	NADP	NAD	NADP
NH_4^+	0.53	20.0	1.08	60.0	0.74	79.0	0.10*	0.2*
NH_4^+ + Glutamate	0.53	16.5	0.45	53.0	0.52	47.0	0.53	0.2
Glutamate	4.8	35.1	7.6	50.0	2.0	53.0	4.7	1.2

* Specific growth rates, about 0.15 h^{-1} ; specific growth rates of other cultures, 0.35 to 0.41 h^{-1} .*Other methylammonium-resistant mutants*

Besides mutants of the *allNmr* and *argNmr* types, a third genetic class of methylammonium-resistant mutants was frequently isolated on plates with various nitrogen sources (e.g. DL-allantoin, L-arginine, DL-2-aminobutyrate). In these mutants allantoinase synthesis was repressed by ammonium/glutamate, asparagine and glutamine but not by ammonium chloride. The mutation was semi-dominant with respect to the latter property. Heterozygous diploids with the wild type showed allantoinase activities halfway between those in the parent strains when grown on ammonium or on allantoin/ammonium medium. However, these mutants did not possess a true regulatory mutation, since the substrate constant for ammonium in them was 50 to $100 \mu\text{M}$. The latter was considerably higher than the values for wild-type and *Nmr* mutants (Table 5). Although growth rates in ammonium medium were normal, this class of allantoin/methylammonium-resistant mutants was probably defective in ammonium transport. It is therefore difficult to understand why repression by ammonium was relieved only in allantoinase synthesis and not in arginine catabolism.

DISCUSSION

This paper deals with two novel mutations, *allNmr* and *argNmr*, that cause impaired nitrogen metabolite repression of enzymes involved in allantoin and arginine catabolism. Yeast strains carrying these mutations were selected on the basis of their resistance to methylammonium chloride. The cause of the toxicity of this substance and the mechanism of its action are unknown. In the wild-type strain D40E sensitivity to methylammonium varied considerably with the nitrogen source (Table 2). Methylammonium resistance has been attributed to defective ammonium transport (Arst & MacDonald, 1973; Roon *et al.*, 1975a). Although some mutants with defective ammonium transport were found among allantoin/methylammonium-resistant mutants, isolated in the present study, ammonium transport by *allNmr* and *argNmr* strains was as efficient as in the wild type (Table 5).

The obligatory role of allophanate as an inducer of allantoinase synthesis (Cooper & Lawther, 1973) was not apparent in our experiments, as enzyme activities in cultures grown in alanine or 2-aminobutyrate medium were higher than those in cultures grown with the allophanate-generating nitrogen sources allantoin, urea and arginine. Nitrogen metabolite repression of allantoinase synthesis was little affected by the *gdhA* (cf. van de Poll, 1973) and *argNmr* mutations but was considerably relieved by the *allNmr* mutation.

Nitrogen metabolite repression of arginase synthesis in the wild-type strain D40E was less pronounced than in a yeast strain previously used (Middelhoven, 1970). Moreover,

the depression during nitrogen starvation of arginase and ornithine transaminase shown by yeast strain α - Σ 1278b and its derivatives was less significant and reproducible than in yeast strains used in previous studies (Middelhoven, 1968, 1970; Whitney & Magasanik, 1973). This suggests that considerable differences in regulation of enzyme synthesis may exist between different yeast strains. Nitrogen metabolite repression of arginase was greatly relieved by the *gdhA* mutation (cf. Dubois *et al.*, 1973, 1974) but was still exerted by serine and asparagine. The repression of ornithine transaminase by several nitrogenous substances, except ammonium, was hardly affected by the *gdhA* mutation (cf. Dubois *et al.*, 1973). Nitrogen metabolite repression of both arginase and ornithine transaminase synthesis was considerably relieved by the *argNmr* mutation, although the sensitivity to glutamine and to very high ammonium concentrations was partly retained. The *allNmr* mutation had only a small effect on nitrogen metabolite repression of arginase and ornithine transaminase: it relieved enzyme synthesis from repression by 40 mM-ammonium chloride but barely from that by other nitrogen sources.

Both the *allNmr* and the *argNmr* mutations are recessive to wild type with respect to all phenotypic properties tested. The genes are not linked to each other, nor to the *gdhA* locus, nor to the structural genes of allantoinase, arginase and ornithine transaminase. Hence they represent regulatory mutations without operator function, different from known mutations with impaired nitrogen metabolite repression (*gdhA* and *gdhCR*). The *gdhCR* mutation is characterized by derepression synthesis of catabolic NAD-specific glutamate dehydrogenase (Dubois & Grenson, 1974), a property not shown by the *allNmr* and *argNmr* mutants (Table 6).

The *argNmr* locus appears to function in the nitrogen metabolite repression of enzymes involved in arginine catabolism. It is probably pathway-specific as pleiotropic effects were not observed, except for an increased resistance to methylammonium in leucine and glutamate media and a slight derepression of NADP-specific glutamate dehydrogenase (Table 6). The *allNmr* locus, however, appears to have a more general function. Besides a considerable relief of allantoinase synthesis from nitrogen metabolite repression and the coincident methylammonium resistance in allantoin medium, several pleiotropic effects of the *allNmr* mutation were observed. The synthesis of arginase and ornithine transaminase in *allNmr* mutants was insensitive to repression by 40 mM-ammonium chloride (Table 4); maximum specific activities of arginase and NAD-specific glutamate dehydrogenase (Table 6) were lower than in the wild type, whereas NADP-specific glutamate dehydrogenase was slightly derepressed as in *argNmr* mutants. Moreover, the *allNmr* mutation led to methylammonium resistance in media with several amino acids as the sole nitrogen source (Table 2).

Our results support the conclusions of others (van de Poll, 1973; Bossinger & Cooper, 1975; Roon *et al.*, 1975b; Hynes, 1974) questioning the role of the *gdhA* gene in nitrogen metabolite repression (Dubois *et al.*, 1973, 1974). Neither allantoinase nor arginase synthesis is completely insensitive to repression by nitrogenous compounds in *gdhA* mutants.

The data presented in this paper shed some light on the mechanism of nitrogen metabolite repression in yeast. This regulatory device appears to be achieved by the cooperation of several genes, some of which (*argNmr*) have a pathway-specific function and some (*gdhCR*, *allNmr*) a more general function. The role of the *gdhA* mutation in nitrogen metabolite repression is questionable. The involvement in nitrogen metabolite repression of allantoin and arginine catabolism of other genes is to be expected as neither the *allNmr* nor *argNmr* mutations result in a complete relief of nitrogen metabolite repression. The role of other genes in this regulatory system is being investigated.

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