

# Advances in MICROBIAL PHYSIOLOGY

*edited by*

A. H. ROSE

D. W. TEMPEST

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# Nitrogen Catabolite Repression in Yeasts and Filamentous Fungi

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## I. Introduction

Increasing interest in the study of gene expression and its regulation in fungi has been motivated by the desire to glimpse a critical step in the emergence of eukaryotes. Early work was conducted using concepts derived from characterization of prokaryotes and their viruses. Similarities which could be at least formally explained using terms such as repressors, activators, operators, and promoters have been described. Genetic identification of these components of regulatory circuits presents an opportunity to attempt a more direct molecular approach. At the same time, the need to select new kinds of mutants, identifying further regulatory components, remains a priority. Similarly, it is premature to sound the death knell for classical enzymology. Many enzymes and pathways resist characterization; others resist even recognition.

Fungi, particularly the ascomycetes, having been brought into the main stream of genetical research in the 1930s, 1940s, and 1950s, fostered a rationale in methodology that has proved extremely fruitful in the study of prokaryotes. The ascomycetes offer a wide array of physiological diversity. The obligately aerobic growth of a number of yeasts and filamentous fungi contrasts starkly with a preference for fermentation amongst *Saccharomyces* spp. and some other yeasts. Between these two extremes are a number of genera, such as *Kluyveromyces*, *Hansenula*, and *Debaryomyces*, with less pronounced preferences. Unfortunately, little work has been done on the genetics of these organisms which are well suited to both an aerobic and an anaerobic mode of life.

Amongst fungi, some well defined biochemical differences have been found in a variety of metabolic pathways. In some cases, it is precisely these pathways for which most information is available concerning regulatory processes, including pathways involved in utilization of nitrogenous compounds. In general, greater metabolic versatility accompanies a greater propensity for aerobiosis. The ability of filamentous fungi and aerobic yeasts to utilize nitrate and purines as nitrogenous nutrients is an example. For present purposes, it is important to note the frequent capability of aerobic fungi to utilize many nitrogenous compounds as sources of carbon as well as nitrogen. In contrast, *Saccharomyces* spp. are only able to utilize these compounds as sources of nitrogen. This imposes

restrictions for the study of catabolism in *Saccharomyces* spp., where fewer nitrogen catabolic enzymes have been identified. A possible advantage is that a study of regulation of nitrogen metabolism is not complicated by an overlap in regulatory domain with regulation of carbon metabolism. With increasing propensity for aerobiosis, the greater involvement of mitochondria in energy provision is apparently accompanied by differences in the use of compartmentation as a regulatory device, illustrated with particular clarity in the regulation of arginine metabolism in various yeasts.

Division of this review into separate sections dealing with filamentous fungi and the yeast *Saccharomyces cerevisiae* reflects our impressions of diversity in regulatory mechanisms involved in nutrition of nitrogenous compounds. At present, there seems little scope for broad generalizations on fungi. Glutamine is probably a key metabolite in regulation of nitrogen metabolism in fungi as well as in prokaryotes, reflecting its central position in the metabolism of both groups of organisms. However, the roles played by glutamine in the various organisms are probably very different. In filamentous fungi, glutamine apparently prevents activation of gene expression by a positive-acting regulator gene product mediating nitrogen metabolite repression.<sup>1</sup> In *Saccharomyces* spp., however, there is evidence only for negative regulation in the regulatory mechanism covering the domain of nitrogen nutrition, and other more localized regulatory processes are probably grafted as modulation on this more ubiquitous mechanism. Regulation of nitrogen metabolism in prokaryotes has been reviewed by Magasanik (1982) in a treatise that traces the intellectual development of the subject as well as summarizing the relevant experimental results and the conclusions that follow from them. It is now clear that the presence of additional, previously unrecognized, genes in an operon with the structural gene for glutamine synthetase was responsible for the now-discarded hypothesis for a major positive regulatory role for glutamine synthetases in enteric bacteria. However, a regulator protein that does play a major role in nitrogen regulation in *Escherichia coli* has been isolated (Reitzer and Magasanik, 1983). As one surveys what is presently known of the general regulation of nitrogen nutrition in fungi and prokaryotes, one is struck by the diversity and possibly led to speculate that variation in regulatory mechanisms might be responsible for a substantial portion of the diversity we see in Nature.

The section of this review dealing with yeasts was the responsibility of M. Grenson and J.-M. Wiame, that dealing with filamentous fungi was the responsibility of H. N. Arst, Jr.

<sup>1</sup> Throughout this review, the term *nitrogen catabolite repression* is used when referring to yeasts, whereas the term *nitrogen metabolite repression* is used when referring to filamentous fungi, in keeping with standard usage by workers with the two groups of organisms.

## II. Nitrogen Catabolite Repression in *Saccharomyces cerevisiae*

### A. METHODOLOGICAL CONSIDERATIONS: STRAINS AND CULTURES

#### 1. Strains

The ancestral use of yeasts in industry led to selection of many wild-type strains of *Saccharomyces cerevisiae*. Other fungi, primarily those of scientific interest such as *Neurospora crassa* and *Aspergillus nidulans*, are more homogeneous. Different strains of *S. cerevisiae* may differ in having or not having an enzyme; the finding of strains lacking asparaginase II is an example (Jones, 1977; Dunlop *et al.*, 1978). In addition, among strains belonging to an accepted taxonomical species, variable regulatory processes controlling a set of identical enzymes are frequently found. It is therefore not surprising that comparing non-isogenic strains, or crossing them, will lead to confusion. Differences in the regulation of prokaryotes are well known (*Escherichia coli* K-12, B, and W; Jacoby and Gorini, 1969). The need to use mutations that originate from different laboratories may introduce unintentional important differences. Many wild-type strains were originally diploids and show differences in their two haploid genomes. According to the chosen strain, one may not discover a given regulation. Such a case, which concerns the subject of this section of our review, is illustrated by comparing two haploid strains which were used a long time ago in Brussels. One strain is the 1705d previously used in the study of arginase–ornithine carbamoyltransferase (OCTase) interaction (Béchet and Wiame, 1965). The other is the classical  $\Sigma$ 1278b ( $\alpha$ ) strain from which the 3962c ( $\alpha$ ) mating-type mutant was derived precisely to ensure isogeny (Béchet *et al.*, 1970). Other mutants were obtained in these strains including some with auxotrophic markers for the same purpose. Collection markers were used only for determining allelism and, if necessary, were introduced by a number of backcrosses with the original  $\Sigma$ 1278b strain.

Arginase production under three conditions of exponential growth illustrates the difference between the strains (Table 1). Although accidental, the use of one instead of the other strain would have led to missing unrecognized regulatory processes in yeast. For example, using strain  $\Sigma$ 1278b instead of 1705d, because of poor arginase production when grown in the presence of arginine and ammonia, would not have shown arginase–ornithine carbamoyltransferase interaction *in situ*, whereas using strain 1705d instead of  $\Sigma$ 1278b would not have shown the ammonium effect.

TABLE 1. Arginase activity in two wild-type haploid strains of *S. cerevisiae*<sup>a</sup>

Strains	Nitrogen nutrient in the growth medium (3% glucose as carbon source)		
	Ammonia	Ammonia with arginine	Arginine
1705d <sup>b</sup>	17	210	247
Σ1278b <sup>c</sup>	5-8	20-24	240

<sup>a</sup> Arginase activity as  $\mu\text{mol hr}^{-1} \text{mg protein}^{-1}$  at 30°C.

<sup>b</sup> Unpublished results from C. Hennaut and J. M. Wiame.

<sup>c</sup> From Dubois *et al.* (1974).

Along the same line, Rytka (1975) showed that strain S288c differs from Σ1278b by one genetic character, which is designated *amc*<sup>+</sup> in Σ1278b and leads to a strong "ammonia effect" on the general amino-acid permease. Differences between strains 1705d and Σ1278b, as well as between the two haploid genomes of M25 used by T. G. Cooper and collaborators, are multigenic.

Although complementary markers are convenient for crosses, they introduce 50% of foreign genetic background at each cross. Crosses do not necessitate markers in strains; zygotes can be chosen by their morphological characters.

## 2. Growth

*Saccharomyces* spp. are very different from bacteria, although in vegetative growth they can be handled in a very similar way. This is probably the origin of the choice of these organisms when prokaryotic cellular physiologists became interested in eukaryotic organisms (Watson, 1975). The similarity in behaviour is due to their common unicellular nature, submerged cultivation, and almost absence of ageing. As a result, quantitative methods developed in the past for bacteria can be applied directly to yeasts (Monod, 1949, 1950; Monod *et al.*, 1952; Novick and Szilard, 1950; Cohen and Monod, 1957).

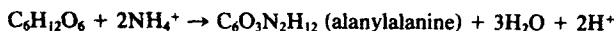
The most useful property is the prolonged exponential growth phase during which new cells resemble old ones. In other words, the growth is balanced. In a culture, if *a*, *b*, and *c* represent cell mass  $\text{ml}^{-1}$ , mass of protein  $\text{ml}^{-1}$ , and amount of a given enzyme  $\text{ml}^{-1}$ , respectively, the slopes

of  $\log a$  versus time, as well as  $\log b$  and  $\log c$ , are identical. A replot of  $a$  versus  $b$  or  $c$  in Monod co-ordinates ( $a \text{ ml}^{-1}$  versus  $b \text{ ml}^{-1}$ ) gives a straight line. The necessity to recall this elementary point is that, even today, one frequently finds an expression such as "enzyme activity after 2 hours of treatment" being used without any data expressing growth and, still worse, sometimes without the values for cell mass and enzyme activity before treatment.

The long balanced growth phase is a remarkable phenomenon. Cells use nutrients that are not in great excess: 1% glucose for *S. cerevisiae* (which grows mainly on the basis of fermentation even in aerobiosis) may be limiting at the end of the exponential phase of growth. This originates from a weak (or the absence of) Pasteur effect in species of the genus *Saccharomyces* (Ephrussi *et al.*, 1956; Vissers *et al.*, 1982). A large part of the ammonia usually present in minimal medium will be used up by the end of growth. Indeed, ammonia will be limiting when using less than the usual 20 mM concentration.

So, in spite of rapid rates of nutrient consumption, growth continues without extensive modification. One of the reasons is the occurrence of efficient transport systems. With a  $K_m$  value of  $1 \mu\text{M}$  for ammonia, this permease will remain saturated until the ammonia concentration is above  $10 \mu\text{M}$ .

Ethanol production remains below its toxic concentration. Hydrogen ion production is a more difficult question. Yeasts are known to be able to grow over a large pH range. The optimal pH value is usually between 4.5 and 6.5 but usually growth is not strongly modified between pH 3 and 8 (Rose, 1975). However, exchange of chemical compounds between growth medium and cells is under the control of pH value, especially with ionized compounds. Pyruvic acid is a good carbon source at pH 3.3, but not at pH 6.0. Oxaluric acid acts as a non-metabolizable inducer of urea degradation at pH 3.3, but it does not enter the yeast cell at neutral pH values. Thus, modifications in pH value may alter the influence of compounds used in the study of regulation, even if these pH modifications do not drastically modify growth. For example, during synthesis of a dipeptide from glucose and ammonia there is a concomitant production of one proton equivalent for each nitrogen atom assimilated:



Unbuffered media will change in pH value over an experiment. Oxalurate could be a poor inducer or a good one depending at what stage of the growth it is applied.

Commercial media are usually derived from the medium of Wickerham (1946). This medium is weakly buffered with phosphate. Phosphate with a

$pK_1$  value of 2.2 and a  $pK_2$  value of 7.2 does not ensure a constant pH value. Its initial pH is 4.5–5.0 and it is outside of the buffered regions which anyway are at the extremes of the useful pH scale. *Saccharomyces cerevisiae* does not use citric acid (Barnett and Kornberg, 1960) and so it can be used as a buffer, with the capacity to maintain a constant pH at the three useful values of 3.3, 4.7, and 6.0. Citric acid cannot be used as buffer for a number of other yeasts because they utilize it as a source of carbon (Lodder, 1971; Barnett *et al.*, 1983).

*a. Starvation.* Starvation for nitrogen has been often used to detect nitrogen catabolite de-repression. After being separated from growth medium, cells can be introduced in a medium lacking a nitrogenous nutrient. Usually this does not stop protein synthesis abruptly, since nitrogenous compounds present in vacuoles allow a limited synthesis. Vacuoles store basic amino acids very efficiently (Wiemken and Durr, 1974). After growth in a medium containing glucose and ammonia, starvation for ammonia led Middelhoven (1968) to observe a strong arginase synthesis and suggested nitrogen catabolite de-repression. Indeed, arginase is the key enzyme for use of arginine as a nitrogenous nutrient, and arginine is the most abundant amino acid in the vacuoles. Arginase synthesis may be under the control of induction rather than nitrogen catabolite repression when starved. We shall return to this subject later.

Starvation has also been observed as a signal for modification of enzyme activity as distinct from enzyme synthesis. The best known cases concern carbon catabolism, the very first being a modification of glycogen phosphorylase in animal tissues discovered by Cori and Cori under conditions of stress or hormonal treatment. Many other examples are known today, and they include bacterial and yeast enzymes. It is established that some of these modifications may occur by covalent modifications of enzymes catalysed by converting enzymes (adenylation and phosphorylation). Modification of enzyme activity usually is a reversible process which may help the cell to adapt to a new metabolic situation more quickly than enzyme dilution into new cells or by synthesis *de novo*.

Enzyme conversion can also occur in actively growing cells. This is so with glutamine synthetase in *E. coli* (Wulff *et al.*, 1967; Shapiro *et al.*, 1967; Stadtman, 1970). In the nitrogen catabolism of yeasts, glutamate dehydrogenase ( $NAD^+$ ) and glutamine synthetase are subject to modification of enzyme activity in addition to modification of synthesis (Hemmings, 1978). Modification of activity instead of synthesis is best analysed by the use of Monod co-ordinates during a shift from one growth condition to another (Béchet and Wiame, 1965; Legrain *et al.*, 1982; Grenson, 1983a,b).

To conclude, starvation should not be used to indicate occurrence of nitrogen catabolite repression without further control.

*b. Nitrogen limitation.* Growth of yeast with nitrogenous nutrients of different quality, but supporting exponential balanced growth, is the most usual way to modify the state of nitrogen catabolite repression. Exponential growth, however, does not exclude modifications of enzyme activity by processes distinct from synthesis. Indeed, the most immediate and frequent action *in vivo* is feedback inhibition, as well as activation, which operate as soon as the composition of the medium changes and which adjust the metabolic flux before the relative concentration of enzyme is regulated as the result of gene activity.

*c. Inducer exclusion.* Inhibition of activity of transport may lead to inducer exclusion. This can be misleading when modifications of nitrogenous nutrition are studied in the presence of an inducer. Amino acids and even ammonia may compete with inducer transport and may inactivate permeases. This has been well illustrated for different yeast permeases.

## B. ENZYMES AND GENES INVOLVED IN THE EARLY STEPS IN ASSIMILATION OF NITROGENOUS NUTRIENTS

### 1. Ammonia and Its Uptake

Ammonia is a very good nitrogenous nutrient. Its utilization is, however, greatly decreased by the presence of other very good nitrogen sources, such as asparagine and glutamine (Dubois *et al.*, 1974). Defects in ammonia transport have been obtained by selection of methylamine-resistant mutants. Roon and associates provided evidence for the existence of a transport system. Methylamine uptake is inhibited competitively by ammonia (Roon *et al.*, 1975a) and non-competitively by amino acids (Roon *et al.*, 1977). Transport of ammonia into the cell is mediated by at least two, and probably three, separate systems (Dubois and Grenson, 1979). A mutant with a double defect was selected by resistance to methylamine (100 mM) with proline as nitrogen source (C. Hennaut and J. M. Wiame, unpublished observations). This mutant bears two separate mutations: (1) a *mep1* mutation that leads to loss of the methylamine low-affinity transport ( $K_m$ , 2 mM;  $V_{max}$ , 50 nmol min<sup>-1</sup> mg protein<sup>-1</sup>) and (2) a *mep2* mutation leading to loss of a methylamine high-affinity transport ( $K_m$ , 250  $\mu$ M;  $V_{max}$ , 20  $\mu$ mol min<sup>-1</sup> mg protein<sup>-1</sup>). Ammonia inhibits competitively

both systems with  $K_i$  values of 20  $\mu\text{M}$  and 1  $\mu\text{M}$ , respectively. So the affinity for ammonia differs by a factor of 20. The generation times of the double mutant were respectively 10, 3.8, and 2 hours in the presence of 1, 4, and 20 mM ammonia. The growth rate with 20 mM ammonia indicates occurrence of an additional transport system (Dubois and Grenson, 1979). The identity of the systems described by Roon with *mep1* or *mep2* has not been established, but *mep1* could be the system first described by Roon. Regulation of these transport systems will be considered separately.

## 2. From Ammonia to Glutamate

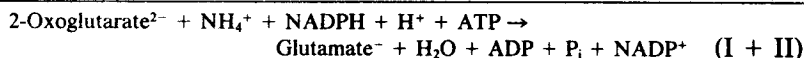
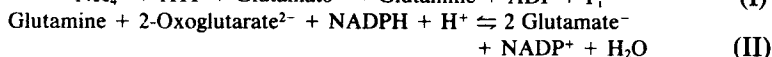
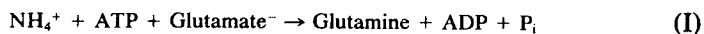
*a. Glutamate dehydrogenase and the  $gdhA^-$  mutations.* In *Saccharomyces cerevisiae* there are two glutamate dehydrogenases (GDHase), one specific for  $\text{NAD}^+$  and one for  $\text{NADP}^+$  (Holzer and Schneider, 1957). The role of these two enzymes was first deduced on the basis of their activities under different conditions of growth. There is a general agreement that the  $\text{NAD}^+$ -requiring enzyme is low in activity when cells are grown with ammonia or ammonia and glutamate (Hierholzer and Holzer, 1963), and that it is higher during growth on glutamate. As a result, this enzyme has been assumed to have a catabolic function.

Variations in the concentrations of the  $\text{NADP}^+$  enzyme are small or absent depending on the wild-type strain considered (Dubois *et al.*, 1974). The biosynthetic function of the  $\text{NADP}^+$ -GDHase, however, is shown by the  $gdhA^-$  mutants. The  $gdhA^-$  mutation that abolishes  $\text{NADP}^+$ -GDHase activity causes a generation time with ammonia of 240 minutes instead of 120 minutes as in wild type, whereas a normal rate of growth is attained by addition of glutamate to ammonia-containing medium (Grenson and Hou, 1972). The  $gdhA^-$  mutations are located in the structural gene for the enzyme (Grenson *et al.*, 1974). In the mutant,  $\text{NAD}^+$ -GDHase activity remains low when grown in the presence of ammonia or ammonia and glutamate. This raises the problem of the origin of the residual growth (generation time 240 minutes) of the  $gdhA^-$  mutant. Starting from a  $gdhA^-$  parent strain, a mutant with an obligate requirement for glutamate has been selected. It has an additional mutation designated as *ama*<sup>-</sup>. By itself, the *ama*<sup>-</sup> mutation does not affect growth rate with ammonia.

*b. Glutamate synthase and the  $ama^-$  and  $gdhCR^-$  mutations.* In a number of Gram-negative bacteria, glutamate can be formed from ammonia by a combination of the activity of glutamine synthetase and a new enzyme reaction discovered by Tempest and collaborators. It is usually desig-



nated as glutamate synthase (reaction I and reaction II) (Tempest *et al.*, 1970):



Enteric bacteria have only one NAD(P)<sup>+</sup>-specific glutamate dehydrogenase, and loss of this enzyme by mutation does not impair the capacity to grow on ammonia at normal rates when glutamate synthase is present (Brenchley and Magasanik, 1974). Glutamate synthase is also present in Gram-positive bacteria. Indeed, in some of them, glutamate dehydrogenase is absent (Elmerich and Aubert, 1971; Elmerich, 1972). The bacterial glutamate synthase allows the organism not only to assimilate ammonia but also to use it at a much lower concentration than is possible when the bacterium possesses only glutamate dehydrogenase. This is possible because of the expenditure of one molecule of ATP. In *S. cerevisiae*, there is also a glutamate synthase, but it is a NAD<sup>+</sup>-requiring enzyme (Roon *et al.*, 1974). The measured activity, ranging from 0.8 to 2  $\mu\text{mol hr}^{-1} \text{mg protein}^{-1}$  depending on the growth medium, is quite low compared with that of NADP<sup>+</sup>-GDHase (25–50  $\mu\text{mol hr}^{-1} \text{mg protein}^{-1}$ ) and of NAD<sup>+</sup>-GDHase (1–100  $\mu\text{mol}$ ). Results reported in Table 2 show that the *ama*<sup>−</sup> mutation leads to loss of glutamate synthase activity, and explain the absolute requirement for glutamate when *ama*<sup>−</sup> and *gdhA*<sup>−</sup> mutations are present together. This shows unambiguously that glutamate synthase may participate in ammonium assimilation. However, in contrast to bacteria, when the synthase is absent from a strain with a normal NADP<sup>+</sup>-GDHase, the presence of the *ama*<sup>−</sup> mutation does not modify growth rate even under conditions in which ammonia is limiting. Such conditions can be obtained with cytosine as the sole nitrogen source, the compound being slowly deaminated into uracil (see Table 2). So one cannot conclude that the NAD<sup>+</sup>-dependent glutamate synthase of *S. cerevisiae* helps the yeast to use ammonia at low concentration and, except for the artificial use of *gdhA*<sup>−</sup>, the physiological function of the glutamate synthase remains obscure. Thanks to the high affinity of the *mep2* ammonia transport, *S. cerevisiae* could dispense with a prokaryotic NADP<sup>+</sup>-specific glutamate synthase.

The double mutant *gdhA*<sup>−</sup>*ama*<sup>−</sup> (strain MG1694) has a normal NAD<sup>+</sup>-GDHase and this enzyme is normally regulated. Its level is low in the presence of ammonia, a situation that explains the requirement for glutamate by the MG1694 strain. From this strain, mutants were selected