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KEYNOTE LECTURE

Mutant analysis, a key tool for the study of metabolism and development

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Introduction

This review is based on a keynote lecture, given to open the symposium 'The Use of Mutants to Study Plant Metabolism and Development', at the meeting of the Society for Experimental Biology held in April 1992. I suppose that the credentials which qualified me to give this opening address are that I have used mutants to study metabolism and plant development throughout my research career and still remain convinced of the central utility of this method of analysis.

The philosophy behind the use of mutants in the study of biological processes is straightforward; the isolation and characterization of mutants identifies the ways in which a process can go wrong, and so allows that way in which the process normally proceeds to be pieced together. Although this approach can now be regarded as classical, it still has considerable utility and provides the foundation for more detailed studies aimed at establishing the molecular mechanisms behind a process. The mutants also provide invaluable raw material for molecular studies. This classical approach has three stages, mutant isolation, genetic analysis of mutants, and phenotypic analysis of mutants. I do not wish to imply by this that these stages have to be sequential. While it is not, of course, possible to carry out mutant analysis without first having obtained mutants, it is certainly worth analysing a few mutants before starting to isolate a large sample.

Although I now study plant development using the moss *Physcomitrella patens*, and employ mutant isolation and analysis extensively in this system, I wish to illustrate the utility of mutants using examples from my earlier work on the assimilation of inorganic nitrogen, the reduction of nitrate to ammonium, in the fungus *Aspergillus nidulans*. These studies were heavily dependent on the study of mutants and illustrate many of the ways in which this sheds light on the workings of the wild-type. In my Ph.D thesis (Cove, 1963), I wrote that:

> "The reduction of nitrate to ammonium is thought to proceed according to the following scheme:-
>
> $$NO_3^- \rightarrow NO_2^- \rightarrow N_2O_2^- \rightarrow NH_2OH \rightarrow NH_4^+$$".

In view of the ascendancy of the one gene–one enzyme hypothesis at that time, it was natural for me to expect that there would be a number of genes required to specify this process and that there would therefore be a number of classes of mutants able to use ammonium as a source of nitrogen but not nitrate. This certainly turned out to be true, but the steps did not correspond in a simple way to the above scheme.

Mutant isolation

When faced with the task of isolating mutants affecting a process which one wishes to study, three questions arise:

- How should mutants be obtained?
- How should mutants be identified?
- How many mutants should be isolated?

Advice on how these questions should be answered is easy to give but, like much good advice, it is not always easy to follow. To obtain mutants, a number of different methods should be used; mutant should be induced by a variety of mutagens having different modes of action and the sample should ideally also include some spontaneous mutants. To identify mutants, a method should be employed which involves the minimum of preconceived ideas. Finally, as many mutants as possible should be isolated, or putting it another way, it is difficult to isolate too many mutants.

How to obtain mutants

The method used to obtain mutants cannot be considered without also considering how mutants are to be identified. If the identification of mutants is difficult or tedious, then it is obviously desirable to induce mutants at as high a frequency as possible. Mutagenesis using nitrosoguanidine (Adelberg *et al.*, 1965) generates principally

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point mutants at a high frequency. However, there are disadvantages in using such efficient mutagens, in that mutations will have been induced in more than one gene in many of the survivors, making both genotypic and phenotypical analysis difficult, a problem which is dealt with more fully below. If difficulties of mutant identification are not overriding, then a rather less harsh mutagenic treatment is favoured. Since different mutagens induce different spectra of mutants, and since spontaneous mutants are again different, isolating mutants in a variety of ways is always desirable (see Table 1).

How to identify mutants

To study nitrate assimilation, we wish to obtain mutants blocked in the reduction of nitrate to ammonium. The straightforward way to do this is to identify mutants which can use ammonium as a nitrogen source but do not use nitrate. This approach involves no selective procedure, and hence no preconceptions, but as a consequence it is tedious, at least if no simple technique for replica plating exists. When I initiated these studies, I therefore employed a procedure termed 'total isolation', although this may be a more apt description of the feelings of the experimenter than of the technique. Using hand inoculation, just over 30 000 mutagenised clones were screened for growth on nitrate and ammonium. Of these, 137 were unable to use nitrate but were able to use ammonium, and these were to prove the valuable raw materials for future studies. The haploidy of fungal tissue makes mutant isolation without selection much easier than a similar screen in which an M2 had to be bred.

There are now selective procedures available to isolate mutants abnormal in nitrate assimilation. Nitrate reductase, the first enzyme in nitrate utilization, also catalyzes the reduction of chlorate to chlorite, which is toxic (Cove, 1976a, 1976b). The wild-type, having nitrate reductase, is therefore killed when grown on a medium containing chlorate. Strains lacking nitrate reductase should therefore be resistant to chlorate, and this is generally true. However, reliance on chlorate selection to obtain mutants lacking nitrate reductase, illustrates the problems of using a procedure for mutant isolation which involves a preconception, since it turns out that not all mutants lacking nitrate reductase are chlorate resistant. It was also found that not all chlorate-resistant mutants lack nitrate reductase and so the use of both methods of isolation has proved valuable, but only when used critically. In *A. nidulans*, there is another more generally selective procedure for obtaining mutants unable to use a nitrogen source, which involves the use of a putrescine auxotroph (Herman and Clutterbuck, 1966). Although the basis of this procedure is still not understood, it generates a similar spectrum of mutants to total isolation. Table 1 gives the distribution of mutant types obtained by total isolation, selection for chlorate resistance and using putrescine selection.

How many mutants should be isolated?

This question does appear to be able to be answered logically. If it is assumed that all genes involved in the process to be studied mutate at the same frequency and that loss of function of the genes involved is required to impair the process, then it is expected that the distribution of mutants affected in the process will conform to a Poisson distribution and it is therefore easy to predict when saturation has been reached. The conditions stated are, however, seldom fulfilled. All genes do not mutate at the same rate, and a particular process may be impaired as a result of loss of function in some genes but only from particular alleles of others (see below). Because of this it is dangerous to conclude too soon that all the genes affecting a process have been identified by mutation.

Table 1. Relative frequency of *cnx* and *niaD* mutants in samples of mutants obtained by different methods

Mutagenic treatment	Selective procedure	Total sample size	Relative frequency (%)					
			niaD	*cnx*				
				A,B,C	E	F	G	H
UV	None	39	23	38	21	10	5	3
NTG	Putrescine starvation	48	42	23	21	6	0	8
NTG	Chlorate resistance	161	55	27	2	11	2	2
None	Chlorate resistance	204	92	4	0	3	1	0

Data from Cove (1963) and (1976a).

Genetic analysis

Classical genetic analysis can be used to establish the number of genes which can mutate to give a similar phenotype, whether a phenotype has a complex genetic basis and also the linkage relationships of the genes which affect the system.

The number of genes involved

The complementation test provides the simplest way of establishing whether mutants with similar phenotypes have arisen by mutation in the same or different genes. In *A. nidulans* there is a very simple procedure for testing for complementation among mutants unable to utilize nitrate as a nitrogen source, which exploits the heterokaryon stage. Complementation between two strains is tested simply by superimposing conidiospore inocula of the two strains on medium containing nitrate as the sole nitrogen source. On such a medium, mutants unable to utilize nitrate show very thin sparse growth. If two strains complement then they are able to form a heterokaryon which grows much stronger. Complementation testing therefore is simple, requiring only a single petri dish for each test.

Mutants of *A. nidulans* unable to use nitrate, can be divided readily into two phenotypic classes depending on whether they are able to use nitrite as a nitrogen source. When complementation tests are carried out on mutants of the class which is unable to use nitrate but able to use nitrite, it is found that they can be placed into eight groups, A to H (Cove and Pateman, 1963). Groups D to H are simple, members of a group fail to complement with other members of that group, but complement with members of all other groups, the classic situation expected if each mutant group is affected in a single gene. The complementation relationships between groups A, B and C are such that members of group A complement with members of group C and also with groups D to H, but not with other members of group A or with members of group B. Group C mutants likewise complement group A mutants, as well as groups D to H mutants, but not with A or B mutants. The basis of the relationship between mutant groups A, B and C has still not been established. Crosses between A, B and C mutants show that all are closely linked (Cove and Pateman, 1963). It may be that A and C represent two closely linked genes, B mutants being mutant in both genes, possible as a result of deletion. More likely, the mutants are affected in a gene coding for a bi-functional protein. Complementation relationships are commonly shown diagramatically using bars to represent groups of mutants, overlapping bars indicating an absence of complementation. Figure 1 summarizes the complementation of these relationships.

Complementation analysis is a very important procedure for establishing the number of genes which are involved in a process. In organisms where most tissues are haploid and in which heterokaryons are not formed naturally, complementation testing must rely on obtaining diploid tissue by such processes as apospory or somatic hybridization. Complementation testing in diploid organisms is straightforward providing cross-fertile homozygous mutant lines are available, since the F_1 between them is a complementation test, as was shown originally by Bateson *et al.* (1905), who found that F_1 from a cross between two different varieties of *Lathyrus odoratus*, the sweet pea, each of which was true breeding for white flowers, produced purple flowers. Where only heterozygous mutant lines are available, the situation is more complex and it is not easy to establish the number of complementary genes, particularly if some are linked.

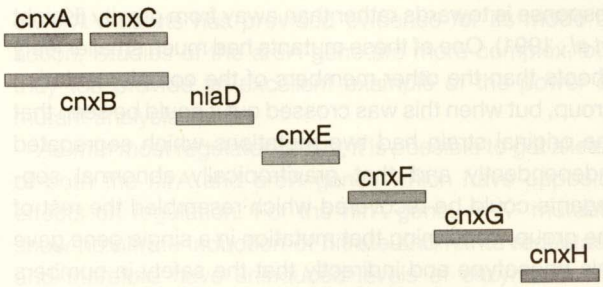

Figure 1. Complementation relationships between mutants of *A. nidulans*, unable to use nitrate, but able to use nitrite as a source of nitrogen. Each complementation group is represented by a bar. Bars which overlap vertically represent groups between which complementation does not occur.

Genetic complexity of phenotypes

It has already been mentioned that with powerful mutagens, there is the risk that a mutant phenotype may result from more than one mutation. The obvious way to determine whether this is true, is to outcross the mutant stock and to observe the phenotypes of segregants in a cross. However, sometimes this can be difficult and will always require time. A less rigourous, but simpler way of concluding that a phenotype is due to mutation in a single gene, is to observe a large sample of independently isolated mutants. If the majority of mutants in a single complementation group have the same phenotype, it is unlikely that this phenotype is due to mutation in more than one gene. It is difficult to advise on how large a sample is needed, since studies aimed at answering this question do not appear to have been carried out. Some more recent work of my research group on gravitropic mutants of *Physcomitrella patens* obtained by nitrosoguanidine mutagenesis, will serve as an example. One complementation group, represented by five strains, shows a gravitropic response in which the polarity of response was reversed, i.e. the

response is towards rather than away from gravity (Knight *et al.*, 1991). One of these mutants had much smaller leafy shoots than the other members of the complementation group, but when this was crossed out it could be seen that the original strain had two mutations which segregated independently and that gravitropically abnormal segregants could be recovered which resembled the rest of the group, confirming that mutation in a single gene gave this phenotype and indirectly that the safety in numbers approach, in this case only four independent strains, was reliable.

Linkage relationship

I will not review in detail here the genetics of nitrate assimilation in *A. nidulans* (see Cove, 1979, for such a review). Genetic analysis revealed that at least 15 genes can mutate to block nitrate assimilation, only two of which code directly for the enzymes involved, i.e. are classical structural genes. The *nia* gene codes for nitrate reductase and the *nii* gene for nitrite reductase. The roles of some of the other genes are dealt with below, but none of them shows close linkage to one another or to the structural genes. However, *nia* and *nii* are closely linked. This must surely be significant but the detailed significance at the molecular level is still being worked out. The genetic architecure of the region has been established by deletion mapping (Tomsett and Cove, 1979). This work indicated that there were no genes between the *nii* and *nia* genes since deletions from the *nia*D gene into the *nii*A gene, which therefore lacked the entire intergenic region, lead to the same phenotype as an *nii*A *nia*D double point mutant. This intergenic region has now been cloned and work is proceeding on establishing its function. The two genes appear to be divergently transcribed, possibly sharing common regulator elements. However, the possibility still exists that the two genes are linked to maximize their chances of co-segregation. Genetic analysis is therefore able to provide clues, but molecular analysis is required to fill in the details.

Phenotypic analysis

Detailed analysis of the phenotypes of both the wild-type and mutants affected in the process under investigation is essential and should usually be carried out in parallel. For metabolic systems, obvious analyses include establishing the ability to utilize suspected intermediates in a metabolic pathway, and it has already been stated that in the case of nitrate assimilation, testing for the ability to use nitrite, allowed nitrate non-utilizing mutants to be divided into two groups. However, advances in knowledge can come by less logical routes, and serendipity always needs to be an ally. I had put forward a number of explanations in my thesis, all I thought plausible, for the finding that mutants blocked between nitrate and nitrite fell into eight complementation groups. However, as my thesis was about to be submitted I discovered by chance that none of my explanations were correct, necessitating hasty revision. It turned out that quite unexpectedly, members of all but one of the complementation groups were impaired not only in nitrate utilization but also in the utilization of certain purines as a nitrogen source (Cove, 1963; Pateman *et al.*, 1964).

This discovery was made as a result of using an adenine auxotrophy as a mapping marker. It was noticed that when grown on nitrate, some nitrate non-utilizing mutants were able to utilize as a nitrogen source the low levels of adenine included to supplement the adenine auxotrophy, others were not. More detailed analysis showed that members of only one complementation group could use adenine as a nitrogen source (*nia*), members of the other seven groups could not. These mutants were unable to synthesize not only nitrate reductase, but also an enzyme of purine catabolism, purine hydrolase I (at the time called xanthine dehydrogenase). This led to the proposal that the genes involved coded for synthesis of a co-factor shared by nitrate reductase and purine hydrolase, and the genes were designated *cnx*. This was the first firm evidence for the existence of a molybdenum co-factor, which has now been characterized. However, there are still problems arising from the analysis of the phenotypes of *cnx* mutants which have not yet been resolved. A straightforward way of determining if a gene codes for a structural component of an enzyme is to isolate a temperature-sensitive allele of the gene, and investigate whether the enzyme produced by strains with this allele is more temperature-sensitive than the wild-type. We carried out such analyses (MacDonald and Cove, 1974), showing as a control that temperature-sensitive mutants in the *nia* structural gene did indeed produce a nitrate reductase more labile than normal. Most temperature-sensitive *cnx* strains produced, at the permissive temperature, a nitrate reductase indistinguishable from wild-type. This is the result which is to be expected if the genes concerned are involved in the biosynthesis of a co-factor. Temperature-sensitive *cnx*H mutants, however, produce a more labile enzyme than the wild-type, suggesting that the *cnx*H gene product may be a structural component of the nitrate reductase enzyme, and hence that the co-factor contains a peptide component. Here, there is no confirmation for the genetic prediction from molecular studies, but if the *A. nidulans* molybdenum co-factor does not contain a peptide, this finding is difficult to explain.

Of the remaining genes which can mutate to prevent growth on nitrate while not affecting the utilization of ammonium, four turn out to be involved in riboflavin synthesis (Cove, 1979). Not all alleles of such genes have this

Table 2. Nitrate and nitrite reductase levels in wild-type strains grown on different nitrogen sources

	Nitrogen source(s)		
	Urea	Urea + nitrate	Urea + nitrate + ammonium
Nitrate reductase	4	91	9
Nitrite reductase	1	151	10

Enzyme levels are specific activities (nanomoles of substrate reduced per milligram of soluble protein). Data from Cove (1979).

specific effect, the more usual phenotype is an absolute riboflavin requirement. However, mutants having alleles which only partially impair function (leaky mutants) synthesize enough riboflavin to allow growth on ammonium, but insufficient for growth on nitrate and nitrite where there is a high flavin requirement. This illustrates the point that the selection procedure used, in this case the inability to use nitrate, can result in getting a sub-class of mutant alleles for a gene and may exclude, as in this case, alleles leading to loss of function.

The synthesis of the enzymes of nitrate assimilation is subject to two types of control, being induced by nitrate and repressed by ammonium (see Table 2). Two of the genes which can mutate to affect nitrate assimilation, *nir*A and *are*A, are concerned with its regulation. The *nir*A gene codes for a pathway-specific regulator mediating the nitrate induction of nitrate and nitrite reductase (Pateman and Cove, 1967; Pateman *et al.*, 1967). The *are*A gene product is a multipathway regulator which is required for the expression of a number of genes which are subject to repression in the presence of ammonium (Arst and Cove, 1973). I will consider here only how the phenotype analysis of *nir*A mutants has provided evidence for its mode of action. Studies of the *are*A gene are more complex, but they too provide an excellent example of the power of mutant analysis.

As with most regulator genes, it is possible to get alleles of both the *nir*A and *are*A genes which have opposite effects on regulation. For the *nir*A gene, *nir*A$^-$ mutants show no nitrate induction of nitrate and nitrite reductase and therefore have uninduced levels of enzyme in the presence and in the absence of nitrate, while *nir*A^c mutants show constitutive synthesis of the enzymes, producing induced levels whether nitrate is present or absent (see Table 3). Classically, the analysis of dominance and recessivity of alleles of regulator genes has been used to establish their mode of action (Jacob and Monod, 1961). In the *Escherichia coli* lactose system, the recessivity of the constitutive *lac i*$^-$allele to the *lac i*$^+$ allele led Jacob and Monod to conclude that it represented a loss of function allele, and therefore that the wild-type gene product must act negatively, repressing the lactose operon except under inducing conditions. Such an interpretation of dominance and recessivity assumes that the regulator gene product is synthesized in excess, which is not a reliable assumption and in the case of the *nir*A gene is found not be be true. It is thus more reliable to establish the mode of action of a regulator gene, not by dominance tests, but instead by considering the relative rate of mutation to the alternative mutant phenotypes. It is a reasonable assumption, but not as I have already explained without potential pitfalls, that the most common result of a mutation leading to an observable phenotype will be loss of gene function. In the case of *nir*A, *nir*A$^-$ mutants are much more common than *nir*A^c mutants and so it may be concluded that loss of function leads to non-inducibility.

Table 3. Specific activities of nitrate and nitrite reductase in diploid wild-type and *nir*A strains grown under inducing and non-inducing conditions

	Nitrogen source(s):			
	Urea		Urea + nitrate	
	Nitrate reductase	Nitrite reductase	Nitrate reductase	Nitrite reductase
*nir*A$^+$/*nir*A$^+$	2.2 (0.7)	2.0 (0.7)	67.8 (6.1)	108.9 (7.7)
*nir*A$^-$/*nir*A$^-$	1.9	3.6	2.1	3.2
*nir*A^c/*nir*A^c	91.9 (8.6)	35.2 (7.7)	91.6 (8.1)	124.5 (15.8)
*nir*A$^+$/*nir*A^c	33.3 (5.2)	10.1 (2.4)	59.1 (6.3)	106.1 (10.2)
*nir*A$^+$/*nir*A$^-$	1.9 (0.2)	1.7 (0.5)	41.7 (2.5)	80.4 (5.4)
*nir*A^c/*nir*A$^-$	24.7 (3.5)	12.0 (3.3)	38.7 (4.4)	56.7 (7.7)

Except those for the *nir*A$^-$/*nir*A$^-$ diploid strain, values are means (and standard errors) for at least four independent experiments (data from Cove, 1969). The *nir*A$^-$/*nir*A$^-$ values are from a single experiment (Cove, unpublished data). Specific activities are expressed in enzyme units per milligram of soluble protein, where 1 enzyme unit brings about the conversion of 1 nM of substrate or the formation of 1 nM of product in 1 min.

Thus the *nir*A gene product is needed for the expression of the *nia*D and *nii*A genes, i.e. its product is an activator.

Dominance studies of *nir*A alleles (Cove, 1969; see Table 3) do not establish a clear pattern of dominance. Both *nir*A^{c}/*nir*A^{+} diploids grown in the absence of nitrate and *nir*A^{-}/*nir*A^{+} diploids grown in the presence of nitrate have enzyme levels significantly different from the corresponding homozygous diploid strains. This pattern is consistent with the *nir*A gene product being present in near-limiting quantities so that one wild-type copy of the *nir*A gene cannot make enough product to turn on two sets of structural genes (Cove, 1969). This limitation shows up in enzyme assays but does not show up in growth tests of diploids. *nir*A^{+}/*nir*A^{-} diploid strains grow at the same rate as *nir*A^{+}/*nir*A^{+} diploid strains, indicating that nitrate and nitrite reductases must be made in excess. In heterokaryons however, the limiting concentration of the *nir*A gene product can be demonstrated. A heterokaryon between a *nir*A^{-} *nia*D^{-} strain and a *nir*A^{+} *nia* D^{-} strain grows much more weakly than, for example, a heterokaryon between a *nir*A^{+} *cnx*A^{-} strain and a *nir*A^{-} *cnx*A^{+} strain. This not only demonstrates that the less ordered distribution of genomes within a heterokaryon leads to a more stringent requirement for *nir*A gene product, but also indicates that it is likely that the *cnx*A gene (and other *cnx* genes) do not require the *nir*A gene product for their expression.

I hope that in this review, I have been able to convey to you why I remain enthusiastic for the classical study of mutants. I am, however, equally enthusiastic about molecular genetic analysis. The two are clearly complementary, each made easier by the other.

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