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The Role of Genetics in the Effective Utilisation of Filamentous Fungi

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Filamentous fungi are of considerable economic and social significance. It is very likely that the future will see an expansion in the number of species which are commercially exploited. Equally, the future is likely to see filamentous fungi and their products being used for a growing range of processes and treatments. In further development of filamentous fungi and their products, genetic technology will play as significant a role as it already has in the realisation of current products and processes. This contribution to the symposium will examine a range of uses for genetic tools from the more classical whole cell mutagenic approach through to the more sophisticated methods comprising recombinant DNA technology. The role of genetics in improving a strain, once it has been identified as of potential commercial utility will be discussed. In addition the contribution will consider the potential for gene manipulation in the generation of diversity within species and compounds produced by them. Examples of work on antibiotic biosynthesis and enzyme production, from the author's laboratory and others, will be used to illustrate the principles discussed.

INTRODUCTION

Genetics has played a significant role in the successful exploitation of micro-organisms by industry. Whether the producing species is a prokaryote or eukaryote, whether the product is an enzyme or an antibiotic, strain improvement is critical in establishing a production process. Typically an organism isolated from the wild will produce, in commercial terms, low quantities of any detected desirable product. Whilst process design can lead to significant improvements in performance, genetic methods tend to bring about the most significant 'one step' enhancements (Ref. 1). It should be noted that although much of strain improvement is focused on improvements in product yield, genetic technology can also bring about other indirect improvements in the performance of a micro-organism. These include, for example, altering the spectrum of metabolites produced as well as altering the growth characteristics of a species. In addition micro-organisms can be made to produce novel products by combining genetic techniques with appropriate screening (Ref. 2).

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Amongst micro-organisms the filamentous fungi have, as a group, contributed much to biotechnology. They have been successfully used for the production of enzymes and antibiotics. They also have considerable potential for further exploitation as producers of new antibiotics and enzymes. In addition there is scope for the application of filamentous fungi to industries requiring a shift to more 'environmentally friendly' processes. It is worth noting that of the estimated 1,000,000 fungal species which exist, only a very small percentage have actually been tested for useful properties.

Traditionally, industrial strain improvement has relied largely on whole cell mutagenesis coupled to screening (Ref. 3). As an approach this is usually largely empirical with phenotypes being detected before the genetic change is determined at a molecular level. In addition the nature of mutagenic treatments is such that changes are not directed exclusively at loci which will generate beneficial change, necessitating the screening of large numbers of surviving strains for the desired phenotypes. In theory it is now possible to make genetic changes to an organisms' genetic 'make up' in an extraordinarily controlled manner. This is through the application of gene cloning techniques. However it is often forgotten that it is not always easy to apply these techniques to new and largely unknown species.

This contribution to the symposium will consider the application of genetic methods to species of filamentous fungi of commercial significance. It will start with an examination of traditional strain improvement via mutagenesis and recombination. It will go on to compare such methodology with the more 'modern' recombinant DNA techniques and end with some suggestions as to the best approach with any 'newly discovered' species of potential commercial value.

MUTAGENESIS AND SCREENING

As mentioned above most organisms produce products of interest at levels well below those needed for an economical commercial process. Industry has been remarkably successful at increasing yields of such products through the use of mutagenesis and screening. A classic example is that of penicillin production by *Penicillium chrysogenum* where yields from production strains are several thousands of times greater than those obtained from the strains used in early attempts to produce the antibiotic (Ref. 4).

With any new micro-organism it will usually be possible to isolate mutant strains of that organism which overproduce a product by treatment with chemical or physical mutagens. This pre-supposes that haploid spores of the fungus can be obtained and that an effective means of measuring the production capacity of large numbers of clones of the fungus is available. Even with the most effective of mutagens, the frequency of desirable phenotypes amongst the survivors is usually so low that large numbers need to be screened. Accordingly, to be successful, it is necessary to spend some time optimising mutation frequency and ensuring that any high throughput screening methodology does not generate too many false positives or negatives.

It is often assumed that with any micro-organism it is possible to apply a standard dosage of a mutagen and obtain a sufficient frequency of mutants amongst the survivors of the treatment. Usually standard dosage is expressed as one which reduces the population of cells or spores by a certain percentage. This 'rule of thumb' approach is fraught with danger. Not only do different species vary with respect to their responsiveness to mutagens but in addition many environmental

factors can affect the efficacy of a mutagenic treatment (Refs. 2, 3 and 5). Consequently, in order to ensure optimum frequency of mutants it is sensible to spend some time investigating the effectiveness of a mutagen with any new species. This can be done by using simple mutation assay systems to investigate genetic and environmental factors which affect mutation frequency. With such mutation assay systems it is possible to quickly optimise mutagenic treatments.

It has previously been shown that induced mutation frequency can be significantly enhanced in industrially used microbes, facilitating strain development Refs. 5, 6 and 7). The most significant enhancements usually arise when the DNA repair pathways in the species concerned are perturbed in such a way as to increase the proportion of damaged DNA repaired in an error prone fashion. Thus the isolation of DNA repair deficient mutants or the inhibition of certain types of repair by chemicals can lead to so called hypermutability.

Alongside the optimisation of mutagenic treatments it is important to develop a trustworthy screening process for the testing of survivors of mutagenesis (Refs. 8, 9 and 10). Very often it is not possible to use bioassay as a simple means of testing for levels of a product and therefore some chemical/biochemical assay will need to be developed (Refs. 11 and 12). With the utilisation of miniaturisation and automation it is usually possible to develop a screen of utility. However the degree of effort needed in this part of a strain improvement process should not be underestimated. The authors are aware of several strain development programmes which failed, not because of ineffective mutagenesis, but because of the inherent variability in poorly resourced screens.

As previously stated any screening procedure ideally needs to be able to test large numbers of samples rapidly. In addition it is very often the case that the target product must be detected and quantified within a complex mixture of metabolites and growth medium constituents. Recently Pyrolysis Mass Spectrometry has been assessed as a means to measure levels of different products in complex fermentation broths (Ref. 13). PYMS essentially involves pyrolysis of the sample followed by mass spectrophotometric analysis. The analysis is coupled to the use of neural networks which facilitate the removal from the spectrum of 'background noise'. One of the particular advantages of PYMS is that it allows the processing of solid samples which enables colony screening to be undertaken.

PROTOPLAST FUSION

The use of recombination in industrial strain improvement can be significant (Ref. 14), especially for the alteration of qualitative characteristics. In yield improvement programmes which rely on mutagenesis it is very often found that higher yielding strains acquire 'cryptic' mutations which reduce culture vigour. Relying on 'back crossing' and recombination then becomes a way of combining high yield features with the growth characteristics of ancestral strains. In early strain improvement programmes this type of genetic manipulation was limited due to the fact that the majority of industrially exploited fungi are imperfect species. This means that recombination must rely on the so called parasexual cycle which has allowed some rudimentary genetic maps to be constructed for industrially used fungi (Ref. 15). The diploid state can be difficult to achieve using fungal mycelium and the advent of protoplast fusion technology has facilitated this type of manipulation with industrial fungi, doing much to break down the cell wall barrier to recombination (Ref. 16, 17 and 18).

In addition to their application in strain 'breeding', protoplasts have a number of other proven uses with species of industrial significance. For example protoplast fusion has been used to generate novel products via intra species fusion (Ref. 19). However although the potential of this method for the generation of biochemical diversity has been proven little has been published about its application in screening programmes. Protoplasts are of course also significant in the application of gene cloning methodology to fungi. In order to use or effectively study a cloned gene it is usually necessary to be able to introduce the cloned gene into the organism from which it originates. With *Escherichia coli* this can be achieved using whole bacterial cells. However with fungi the cell wall again presents an impenetrable barrier thus requiring the preparation and utilisation of protoplasts (Ref. 20).

RECOMBINANT DNA TECHNOLOGY

Recombinant DNA techniques offer considerable control in the process of gene manipulation which naturally excites many biotechnologists. Whilst in theory cloning a gene is not difficult in practice this is only true when certain prerequisites are fulfilled. The extent to which recombinant DNA techniques can be applied is in direct proportion to the extent of fundamental knowledge of the species concerned. Thus with *Aspergillus nidulans* for example, genes can be cloned and studied against a background of knowledge of the biochemistry, physiology and formal genetics of the species. Sometimes it is possible to extrapolate some knowledge from such a model organism to a similar species (e.g. another species of *Aspergillus*) or to use the collection of characterised mutants of *A. nidulans* to test DNA from related strains.

With a new species, not related to those fungi which have been most intensively studied academically, it is considerably harder to immediately apply recombinant DNA techniques. Without detailed fundamental knowledge of the organism and its product or function of commercial interest, it is simply not possible to approach genetic engineering via recombinant DNA techniques in an informed and rational manner.

At the start of any strain development programme with a new species there is likely to be a dearth of information which would be of use in developing a strategy to clone genes. Fundamental knowledge of an organism's physiology or of the biosynthetic pathway of a complex product will obviously take time and effort to accumulate. Often there is simply not the scope to expend the staff time necessary to accumulate this knowledge and therefore a 'vicious circle' may arise from the desire to clone genes. Accordingly it is not surprising that even in the 1990s strain development still tends to lean heavily on the use of empirical mutagenesis and screening. However, there may be ways in which basic recombinant DNA techniques can be used with fungi in a largely empirical fashion.

EMPIRICAL APPLICATION OF RECOMBINANT DNA TECHNIQUES

As mentioned earlier the cloning of genes is usually so reliant on being able to transform a species that development of a transformation system/cloning vector is usually one of the first steps with any new species. Cloning vectors for fungi are different to vectors for bacteria in a number of ways (Ref. 21). Firstly they are generally not autonomously replicating structures and in most fungal transformation systems vectors persist in transformants by integrating at some location in

one of the chromosomes of the organism. Here they can be replicated as part of the chromosomal DNA and a number of laboratories have shown that such integrated vector sequences can be very stable indeed (Refs. 22 and 23). In addition it has been shown to be relatively common for such vectors to integrate in a largely random manner with each transformant retaining vector sequences at a different chromosomal location (see for example Ref. 24).

Integration of vector DNA into a chromosomal location will alter, by interruption, that sequence of chromosomal DNA. An analogy would be the insertional kind of mutations caused by transposons in bacteria and also some eukaryotes (Refs. 25, 26 and 27). Thus integrative vectors in fungi have the potential to act as a means of disrupting the function of other genes and indeed they have previously been used for this purpose by a number of workers. It is also possible however to hypothesise that integration of a vector sequence into certain locations could lead to changes in the levels or timing of expression of certain genes leading to perturbations in metabolism.

In the authors laboratory it has often been noted that the morphology and behaviour of individual transformants varies tremendously, lending support to the notion that integrative transformation might be a way to generate variability within a species for screening reasons. Figure 1 is a photograph of transformants of *Aspergillus nidulans* which have been transformed with an integrating plasmid containing DNA from another fungus.

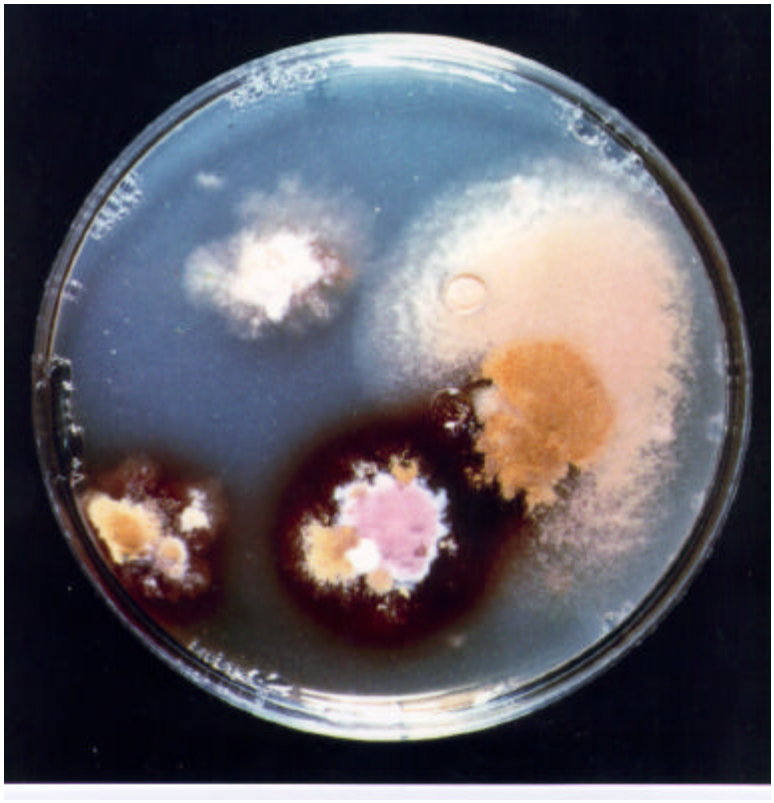

Fig. 1 Independent transformants of *Aspergillus nidulans* transformed with an integrating plasmid DNA.

As can be seen in the photograph above there is morphological variety among the transformants which is not seen in the untransformed strain. Several transformants (one shown in the photograph) were found to produce pigments not normally observed. Southern blotting analysis showed that in each transformant vector DNA was located at a different chromosomal location (Ref. 24). In other experiments an examination of the effect of random integration on penicillin yield by *Penicillium chrysogenum* has indicated that random integration can result in strains which either overproduce penicillin or no longer produce it at all (Renno, D. and Saunders, G. Unpublished data). Figure 2a shows the distribution of penicillin yields obtained from 50 separate clones of a wild type strain of *P. chrysogenum* (strain NRRL1951). This can be compared to the distribution observed for 50 independent transformants of strain NRRL 1951 (Fig.2b). Southern blotting analysis again revealed that the site of integration of vector sequences was different in each transformant.

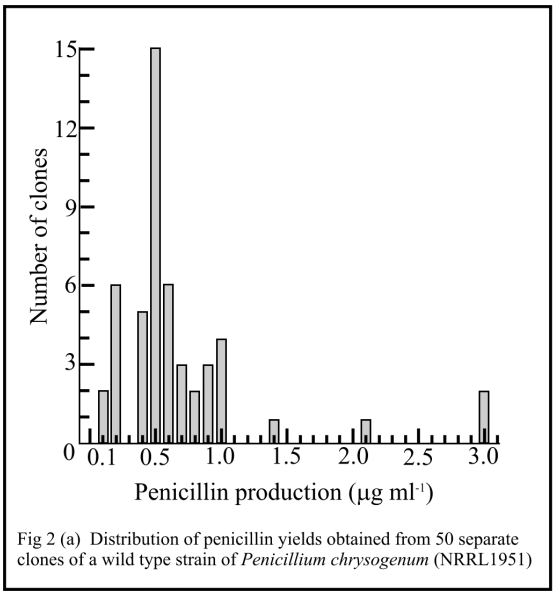

Fig 2 (a) Distribution of penicillin yields obtained from 50 separate clones of a wild type strain of *Penicillium chrysogenum* (NRRL1951)

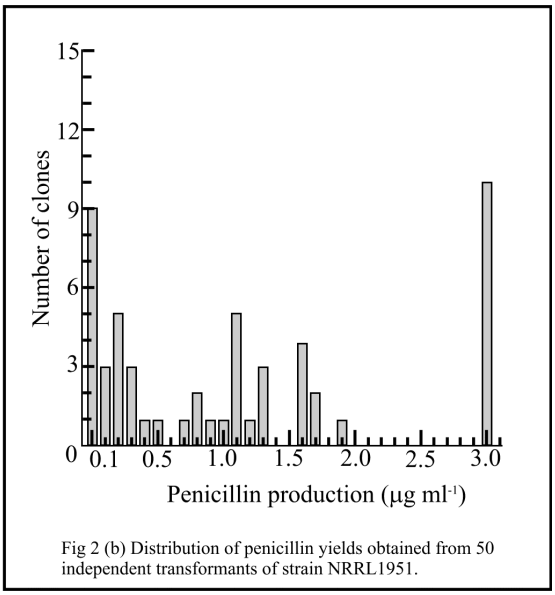

Fig 2 (b) Distribution of penicillin yields obtained from 50 independent transformants of strain NRRL1951.

In circumstances where a species not formerly subject to investigations is being developed for some potential commercial production of a pharmaceutical, it might be possible to use random integration as a means of initiating strain development. This would of course require that a transformation vector/system be developed for the species. Once available it might be possible to generate both over producers and non-producers of the metabolite in question. Over producers would obviously have direct significance but the generation of non-producers would also have potential value. It is possible to recover integrated vector sequences, plus flanking regions from transformant DNA (Ref. 21). This approach could therefore be an empirical way of marking and subsequently isolating sequences of DNA important in the production of the metabolite of interest.

As stated previously classical mutagenesis and screening remains the method of choice for those concerned with the development of a strain or species producing an identified metabolite of interest. Whilst gene cloning offers theoretical advantages in its rational application, this is only practically so if a great deal is known about both the producing organism and the biosynthesis of the metabolite concerned. However the empirical application of transformation may be an alternative worth considering and might well lead to the cloning of key genes. This would then permit the detailed studies which the cloning of genes facilitates. Obviously this kind of empirical transformation/cloning approach is most easily applied to a species related to those which have already been investigated as model species for a number of years.

INVESTIGATIVE USES OF RECOMBINANT DNA TECHNIQUES

Only a very few species of filamentous fungi have actually been exploited by industry. Even fewer have been studied in sufficient detail to represent a model species. The most intensively studied industrial filamentous fungal species are probably *Penicillium chrysogenum*, from the perspective of secondary metabolite production, and *Aspergillus niger* from the perspective of enzyme production. Technology, (for example transformation vectors and cloned gene sequences)

developed and determined for one species of *Aspergillus* or *Penicillium* can be used to manipulate related species, sometimes quite rapidly. A good example of this comes from the authors where a vector developed for one species of *Aspergillus* was used with another to rapidly generate enzyme overproducing strains (Ref. 28).

Work in a number of laboratories around the world has led to significant improvement in understanding of the regulation of penicillin biosynthesis. Two species, *P. chrysogenum* and *Aspergillus nidulans*, have been intensively studied in efforts to better understand the mechanisms which underpin formation of this secondary metabolite (Refs. 22 and 29). Those investigations have been accelerated in recent years by the prudent application of recombinant DNA techniques which made possible the cloning of the penicillin biosynthetic genes. Current understanding of the effect of carbon source and extracellular pH on regulation of the penicillin serves not only as a model for other systems but to adequately demonstrate how rapidly cloned genes, once available, can be used to help understand a complex biosynthetic process (Refs. 30, 31, 32, 33, 34 and 35).

In addition, the use of genetic manipulation techniques and an increased knowledge of how genes are expressed can provide a basis for improving production of important economic products. For example, the authors of this paper are also attempting to use an expression vector developed for *P. chrysogenum*, to express a laccase enzyme from *Pleurotus ostreatus*. Such an enzyme could have significant application in a wide range of industrial processes.

CONCLUSIONS

Industrial strain improvement, using genetic techniques, has and will continue to contribute significantly in the exploitation of filamentous fungi. Usually such strain improvement has been based on the use of largely empirical whole cell mutagenic methods coupled to high throughput screening methods. This approach, whilst having a record of success, is labour intensive and may not be easy for smaller companies to a exploit. Many would argue or presume that the rational methodology surrounding gene cloning is a better way to achieve significant strain developments. Whilst in ideal circumstances this is certainly the case the development of a 'new' species does not immediately provide the ideal circumstances needed.

In such circumstances the classical approach may still be the best way forward. However some consideration should be given to the more empirical application of recombinant DNA techniques both for the generation of novelty within species and for the isolation of strains with altered product yield. The isolation of product non producers by integration of vector sequences may allow the empirical isolation of DNA sequences important in the biosynthesis of the product.

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