BIOCHEMICAL GENETICS OF CEPHALOSPORIN C PRODUCTION

by

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Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy

April 1988

CONTENTS

ACKNOWLEDGEMENTS

I would like to express my thanks to my supervisors, Prof. J. F. Peberdy and Dr. J. A. Birkett for their advice, support and encouragement during the course of this work and to Dr. G. Perez-Martinez and Dr. S. Harford for many helpful discussions.

Thanks are also due to Mr D. R. West, Mr. J. B. Green and Ms. L. Atkin for technical assistance and to Mr. B. V. Case for photographic work.

I wish also to acknowledge financial support from the SERC and Glaxochem Ltd.

In addition, I would like to thank my friends in the laboratory at Nottingham for making my stay so enjoyable.

E. Norman
April 1988

ABSTRACT

The biosynthetic pathway which leads, in Cephalosporium acremonium, to the production of the commercially important β-lactam antibiotic Cephalosporin C (CPC) has been the subject of extensive biochemical studies and is now well characterized. In contrast, genetic analysis in this organism was limited until the application of protoplast fusion techniques facilitated parasexual analysis and allowed a genetic map to be established. (Hamlyn 1982; Hamlyn et al 1985). Subsequently, work leading to our understanding of the genetic basis of the CPC biosynthetic pathway in C. acremonium began. (Perez-Martinez 1984; Perez-Martinez and Peberdy in preparation).

The studies described here were aimed at extending this understanding to a point at which individual genes implicated in the pathway could be identified and positioned on the linkage map. A programme of mutagenesis resulted in the production of a number of 'blocked' mutant strains of C. acremonium which were phenotypically unable to carry out particular steps of the CPC biosynthetic pathway. The segregation of several of these mutations

relative to other genetic markers was examined.

Crosses designed to detect complementation between mutations resulting in a 'blocked' phenotype were carried out and involved strains produced in other laboratories in addition to those characterized during this work. Complementation was shown between two mutations which apparently affected the same step in CPL biosynthesis (the conversion of penicillin N into deacetoxycephalosporin C) and evidence for the linkage of one of the mutations (cnp-6) to a mutation resulting in a requirement for inositol was obtained.

During the course of the complementation studies, it was noted that the haploid and heterozygous products obtained following C. acremonium protoplast fusion crosses did not always behave in the typical manner described previously. (Hamlyn 1984). The persistent heterogeneity of these fusion products and the possible implications of this are discussed.

To my
Parents

CHAPTER 1

INTRODUCTION

Cephalosporium acremonium

and the B-lactam antibiotic Cephalosporin C.

Cephalosporium acremonium (Acremonium strictum Gams) is a filamentous fungus of considerable importance being the organism used in the industrial production of the β-lactam antibiotic cephalosporin C (CPC) which forms the base for the chemical derivitization of a range of antibacterial agents. The organism belongs to the class Hyphomycetes of the Fungi Imperfecti and reproduces vegetatively by the production of ellipsoidal or spherical conidia. C. acremonium was transferred into the genus Acremonium following the taxonomical studies of Gams (1971) who also described further members of the group. The taxonomic descriptions of, and relationships between, many of these strains have been reviewed by Onions and Brady (1987). Some Cephalosporium species have been shown to have teleomorphic stages which have been classified into different orders of Ascomycetes. Both the anamorphic and teleomorphic stages of a number of these species produce compounds with antibacterial activity (Roberts 1952; Kavanagh et al 1958a ; Nara and Johnson 1959; Elander et al 1960; Cole and Rolinson 1961; Higgens et al 1974; Kitano et

al 1975). For example, the teleomorphic stage of Cephalosporium salmosynnematum which produces the antibiotic synnematin B has been classified into the genus Emericellopsis of the order Eurotiales (Groslags and Swift 1957). Species of the other related genera to Cephalosporium including Paecilomyces have also been observed to produce antibiotics related to others produced by C acremonium as have several species of Streptomyces (Heischman et al 1960; Pisano et al 1960; Kitano et al 1974, 1975, Kitano 1983).

Over past years a considerable effort has been directed towards developing an understanding of the mechanisms underlying the biosynthesis of CPC. A biosynthetic pathway has been established and a number of regulatory effects have been identified. These aspects together with work on the genetic basis of CPC production will be described. In comparison to studies on penicillin production by Aspergillus nidulans and Penicillium chrysogenum genetic studies on CPC biosynthesis are limited due to problems associated with the establishment of an amenable laboratory system for obtaining genetic recombination in C. acremonium.

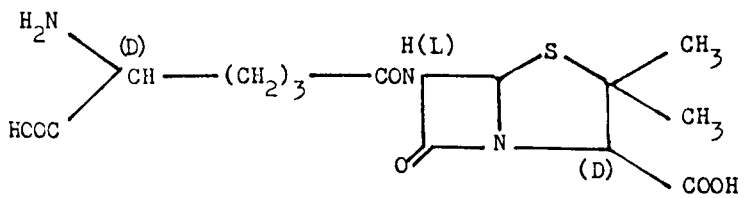
1.1 Antibiotic production by C. acremonium.

The C. acremonium strains used in the production of cephalosporin C based β-lactam antibiotics have all been obtained by mutagenesis and selection programmes from the original C. acremonium strain isolated by Brotzu (1948). The Brotzu isolate produced a number of antibiotic agents including five related compounds classified as the cephalosporin P group. These are acidic molecules, extractable in organic solvents, active against gram positive bacteria and resembling helvolic acid produced by Aspergillus fumigatus (Burton and Abraham 1951, Crawford et al 1952). However, most early interest centred on cephalosporin N which proved active against a wider spectrum of micro-organisms than did the P group of compounds. Cephalosporin N is hydrophobic and sensitive to inactivation by penicillinase enzymes. Structural analysis led to its classification as a penicillin having the B-lactam-thiazolidine ring structure shown in Fig. 1.1 (Burton and Abraham 1951; Crawford et al 1952; Abraham at 1953 and 1954; Newton and Abraham 1953 and 1954) Cephalosporin N, later renamed penicillin N (Pen N) is recognised as identical to synnematin B produced by Cephalosporium salmosynnematum (Olson et al 1952; Abraham et al 1955).

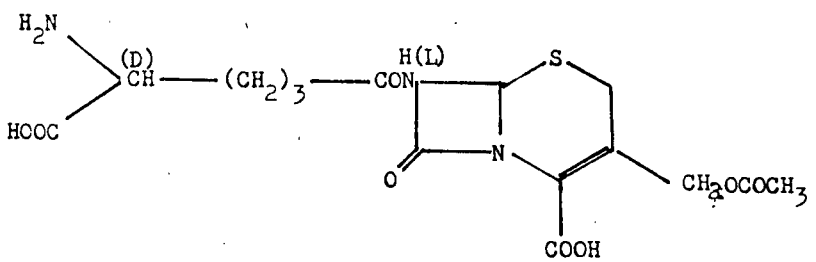
The presence of CPC as a contaminant in a crude

Fig. 1.1 The chemical structures of penicillin N and cephalosporin C.

Penicillin N



Cephalosporin C



preparation of Pen N was reported by Newton and Abraham (1955). Although this compound had a lower activity than Pen N against most micro-organisms tested, it was sensitive to penicillinase inactivation. On acid hydrolysis CPC yielded 7-aminocephalosporanic acid plus D-α-amino acid. The β-lactam dihydrothiazine ring structure shown in Fig 1.1 was consequently proposed for it (Abraham and Newton 1956 and 1961; Loder et al 1961)

1.2 Development of strains for the industrial production of CPC.

Strain M8650 which had been derived from the original CPC producing Brotzu isolate (Stauffer et al 1966) was chosen as the progenitor strain for programmes of strain improvement initiated from the 1950's onwards designed to produce strains capable of increased CPC production with growth characteristics suitable for industrial use. Spores were treated with a variety of physical and chemical mutagens and large numbers of survivors were screened for any increase in capability to produce CPC. As improved strains were isolated the fermentation conditions being used were gradually tailored to further optimise the level of production obtained. Of the mutagenic agents used, ultraviolet radiation was

shown to be the most effective in producing raised titre strains. However, since regions of genetic loci may exhibit differential sensitivities to mutagens at least one of the strain improvement programmes used a combination of chemical and physical treatments. Thus mutagenesis was carried out in rounds switching between the use of N-methyl-N-nitro-N-nitrosoguanidine, nitrous acid and ultraviolet radiation (Elander et al 1976). Strains subjected to multiple rounds of ultraviolet mutagenesis were found to become increasingly sensitive to the effects of ultraviolet light. After mutagenesis surviving populations had a decreased average ability to produce CPC, however, there was also an increase in the variability of titre produced. Therefore it was possible to select strains with increased production capacities in a stepwise manner through many rounds of mutagenesis (Brown and Elander 1966). During strain selection programmes it was noted that strains with improved CPC yields also tended to show altered growth characteristics. The growth rate appeared to have been lowered, giving a decrease in colony diameter on plates and a decrease in vegetative development during fermentation. The ability to sporulate was lowered and a tendency towards arthrospore formation was seen (Elander 1974). As the antibiotic titre of

strains increased it was found that the probability of obtaining even higher producing strains after mutagenesis decreased. This necessitated the improvement of the screening procedures being used to detect mutants with raised titres. At first the isolates obtained were screened for antibiotic production in shake flask fermentations but this limited the number which could be examined at any one time. Therefore a method of assaying colonies grown on agar plugs previously used for screening A. nidulans (Ditchburn et al 1974) was adapted for use with C. acremonium (Trilli et al 1978). Antibiotic production was monitored by placing the plugs on agar seeded with bacterial strains sensitive to the antibiotic in question and measuring the zones of inhibition produced on the plates. The relative production of antibiotic on plugs was found to correlate closely enough with production in shake flasks in order to make this method acceptable for use as a primary screen for raised titre mutants (Trilli et al 1978). Modifications of this method and screening techniques involving the calculation of a potency index, measured as the diameter of the colony divided by the diameter of the colony plus the inhibition zone produced by it, have been developed (Ball and McGonagle 1978; Freysoldt et al 1986).

In a review of mutagenesis in C. acremonium

Elander et al (1976) reported that auxotrophic and morphologically abnormal strains derived from mutagenised populations were frequently found to have reduced antibiotic titres. Such observations led to the proposal of a set of indirect selection parameters by which isolates could be recovered, on the basis of various characteristics not related specifically to CPC production for titre testing with an increased probability that they would be improved producers. These procedures are discussed in detail by Chang and Elander (1979) and also include the selection of mutants resistant to analogues of the amino acid precursors of the antibiotic. These mutants fall into two phenotypic classes those which may have the capacity to over-produce the precursors and those with altered permeability characteristics. Auxotrophic mutants obtained during the strain improvement programme were also reported occasionally to yield raised titre strains on reversion to prototrophy. Thus by observing the properties of colonies surviving muta- genesis for characters other than titre it might be possible to reduce the number which must be assayed before a strain with improved titre is obtained. These and other strategies used to improve the production of secondary metabolites have been reviewed by Calam (1970), Demain (1973) and Elander (1978). With the use of such strain

improvement programmes the titre of the M8650 progenitor strain has been increased considerably. The application of parasexual genetics to C. acremonium has also led to attempts to increase CPC titre by the use of breeding methods and these will be discussed in the following sections.

1.3 Genetic Studies

Originally the genetic manipulation of industrially important fungi was based on the empirical methods of mutagenesis and strain selection as discussed previously. There has, more recently, been considerable interest in the possibility of improving the performance of β-lactam producing fungi by the use both of planned breeding schemes and the techniques of molecular biology. Improvements which might be sought could relate either to an increase in titre or to the production of cultures with improved growth characteristics. The application of these approaches was delayed in C. acremonium due to the lack of amenable systems for achieving genetic recombination in the laboratory. However, the establishment and use of methods based on para-sexuality for achieving recombination firstly, in A. nidulans (Pontecorvo and Roper 1952; Roper 1952; Roper and Pritchard 1955; Pontecorvo et al 1953;

Forbes 1959) in which they were used alongside methods based on the sexual cycle of this organism and then in P. chrysogenum (Pontecorvo and Sermonti 1953 and 1954, Sermonti 1954, 1956, 1957 and 1961) have allowed the study of the genetics of penicillin production in these fungi to develop. The possible exploitation of the understanding gained from these studies has been discussed by Ball (1978 and 1980) and Rowlands (1983).

Parasexual genetic mechanisms are those by which genetic recombination may occur without the involvement of a standard sexual cycle. In the parasexual cycle hyphal anastomosis leads to the mixing of nuclei of two haploid strains in a common cytoplasm, thus forming an area of heterokaryotic growth. Nuclear fusion within such a heterokaryon results in the formation of a clone of diploid somatic cells which divide mitotically. Haploid segregants are eventually obtained from the diploid as a consequence of a lack of complete stability. Haploidisation occurs as chromosomes are sequentially lost as a result of mitotic nondisjunction events. This leads via a range of aneuploid states to the formation of haploid segregants often detectable as sectors growing out from the diploid. Haploidisation is random to the extent that for each chromosome either parental homologue may be lost at a

given nondisjunctional event. Therefore all the possible combinations of parental chromosomes should be detectable amongst the haploid segregants obtained from a diploid. As this process involves the redistribution of whole chromosomes, alleles linked together in one parent of a cross remain associated during segregation whilst alleles of unlinked genes show independent segregation. Therefore an examination of the segregation patterns amongst the progeny of a parasexual cross allows the establishment of linkage relationships. Mitotic recombination i.e. the recombination of genetic material between non-sister chromatids, may occur either before or during the process of haploidisation and lead to the recovery of segregants recombinant for genes positioned on the same linkage group and in some cases allows for the ordering of markers within linkage groups.

Pontecorvo (1956) estimated that unlike haploid nuclei in a heterokaryon fuse at frequencies in the range of 1 in 10^6 to 1 in 10^7 and that the frequencies of haploidisation and mitotic crossing over were of the order of 1 in 1000 and 1 in 500 respectively. Variations on the basic pattern of para-sexuality have been demonstrated in many fungi (for review see Caten 1981) with the timing and stability of the different stages involved and the

frequency with which nuclear fusion and mitotic recombination occur differing according to the fungus being studied.

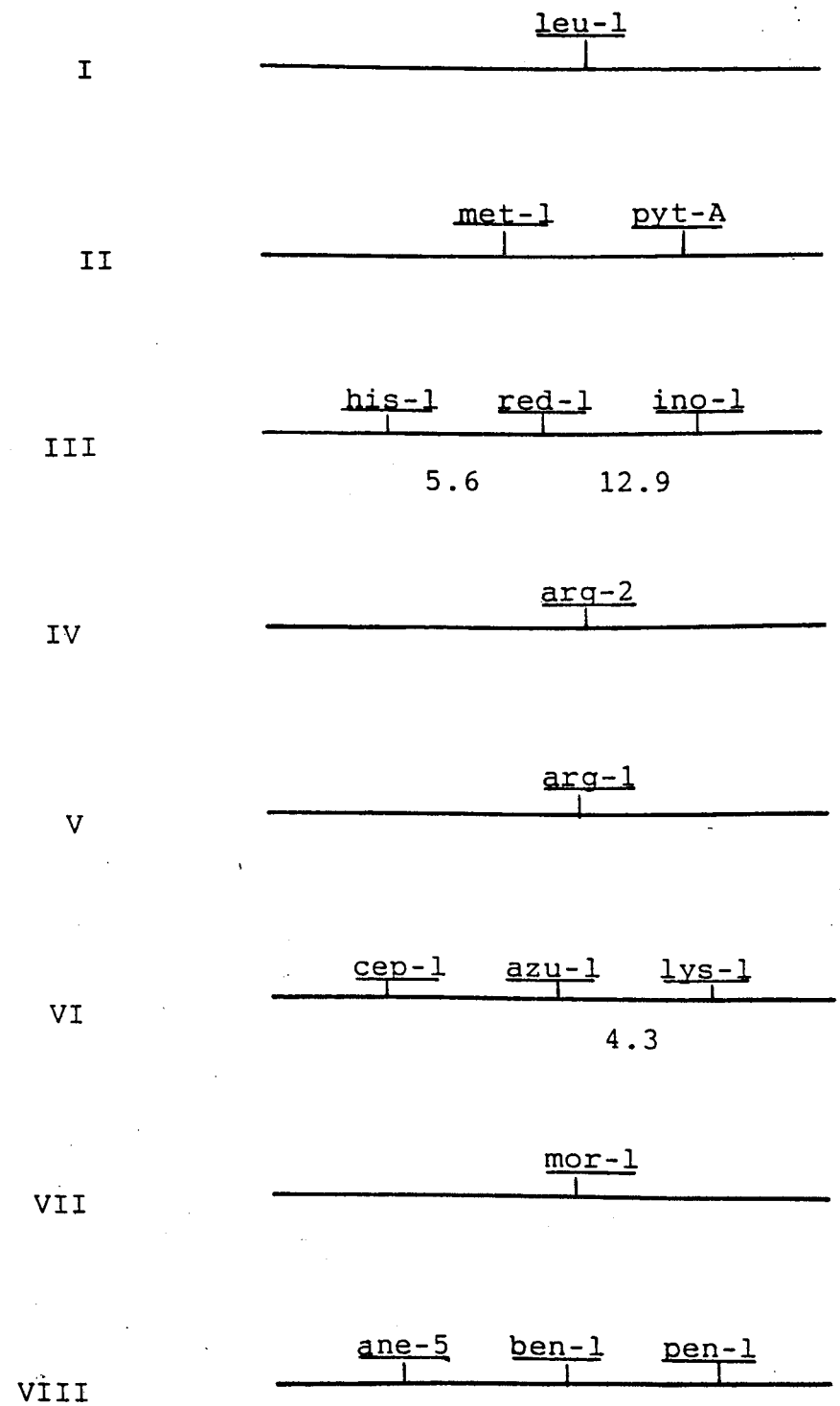
A limited investigation into the genetics of CPC production has been carried out in a teleomorphic stage of Cephalosporium sp.belonging to the genus Emericellopsis (Fantini and Olive 1960). However the majority of work has concentrated on the investigation of the genetics of the imperfect C. acremonium. Early attempts to establish parasexual crosses in C. acremonium were largely disappointing. The isolation of a stable diploid was described by Nuesch et al (1973). Hyphal cells of this strain were shown to be uninucleate but possessed nuclei approximately twice the size of those seen in haploid strains. Treatment of this putative diploid with agents known to induce mitotic segregation led to the isolation of haploid segregants with recombinant phenotypes suggesting that parasexual genetic analysis was in fact possible in C. acremonium. However, Elander et al (1974) and Elander et al (1974) reported unsuccessful attempts at constructing crosses by hyphal anastomoses and noted that it was difficult to obtain the formation of heterokaryons and diploids. This was thought to be due to the predominantly uninucleate nature of C. acremonium cells and the lack of nuclear migration which occurs

between them. Some recombination was seen in crosses between auxotrophs derived from raised titre lines. However, no clear evidence for the existence of diploids was found and the resulting recombinants were slow growing, had a low capacity for CPC production and were unstable in culture. Hamlyn and Ball (1979) reported the isolation of a number of stable prototrophic colonies following a cross, which were presumed to be haploid recombinants and of two unstable prototrophs which gave rise to a limited spectrum of auxotrophic segregants. However, such results were obtained from only ten out of forty crosses carried out and were therefore not particularly promising.

The technique of protoplast fusion has more recently been applied in order to bypass the difficulties found in establishing heterokaryon and diploid states in this organism. Following protoplast isolation and fusion, nuclear fusion could be observed in up to 1% of the treated protoplasts (Hamlyn and Ball 1979). Thus a system of parasexual analysis became feasible in C. acremonium. Regeneration and reversion of fused protoplasts yielded two types of progeny; stable and unstable. The stable prototrophs and recombinant auxotrophs were thought to be haploid products obtained by the rapid segregation of a transient diploid stage.

This led to the adoption of a method for the direct recovery of haploid recombinants by the plating of fused protoplasts directly onto selective media which prevented the growth of parental types but allowed the recovery of a range of recombinants. Unstable heterozygous colonies were also found; these segregated spontaneously to give haploid progeny with recombinant phenotypes. This work gave further support to the view that C. acremonium diploid nuclei may be unstable and showed that diploidization is frequently followed by the rapid segregation of chromosomes. The analysis of crosses between strains carrying auxotrophic and drug resistance markers has led to the establishment of a linkage map for C. acremonium (Hamlyn 1982; Hamlyn et al 1985; Perez-Martinez and Peberdy 1987), and subsequent work has examined aspects of the inheritance of antibiotic production in this fungus. The association of factors affecting Pen N and CPC production with linkage groups VIII and VI respectively has been shown, Fig. 1.2 (Perez-Martinez 1984). The possible usefulness of protoplast fusion as a technique to improve C. acremonium strains for β-lactam production was demonstrated by Hamlyn and Ball (1979) in a cross between two strains, one with favourable growth characteristics, the other with a raised titre phenotype. Amongst the progeny obtained at least

Fig. 1.2 Linkage map for C. acremonium.



one isolate had definitely improved production characteristics, giving rise to a yield of antibiotic at least 40% greater than that of the highest titre parent and also showing an improved growth rate and better sporulation. Minuth and Esser (1983) have also suggested that parasexual genetics may be of use in strain improvement and reported the transfer of genetic material during interspecific crosses between Cephalosporium spp.and intergeneric crosses between Cephalosporium spp.and Emericello psis spp. However transfer of genes other than those used as markers during protoplast fusion was rare.

It may be anticipated that the methods of recombinant DNA technology will be increasingly involved in both the study and the improvement of commercially important micro-organisms such as C. acremonium. The development of transformation systems in A. nidulans (Ballance et al 1983; Tilburn et al 1983; Yelton et al 1984; John and Peberdy 1984) and Neurospora crassa (Case et al 1978 Schweizer et al 1981; Akins and Lambowitz 1985) and the development of suitable cloning vectors for use with these fungi (Hughes et al 1983; Stohl and Lambowitz 1983; Ballance and Turner 1985; Vollmer and Yanofsky 1986), some four years ago paved the way for the use of these techniques in several fungi where formal genetic studies are less well developed. In 1984

the first report of transformation for C acremonium was described, in which resistance to hygromycin or G418 is used as a dominant selectable marker for the selection of transformants (Queener et al 1984; Penlava et al 1986; Skatrud et al 1987; Isogai et al 1987). More recently transformation of P. chrysogenum has also been reported (Cantorel et al 1987; Skatrud et al 1987; Stahl et al 1987).

Previously the existence of a native plasmid in C. acremonium has been reported (Minuth et al 1982; Esser et al 1983). However, this did not have the potential for development as a transformation vector. As in other species, attempts have been made to isolate DNA fragments which would promote transformation, these involve components of the mitochondrial genome (Minuth et al 1982) and in one study a 1.9kb fragment (Tudzynski and Esser 1982; Skatrud and Queener 1984; Oeser et al 1986) has been added as a construct to a transformation vector for C. acremonium (Queener et al 1984). The isolation of the C. acremonium DNA sequence encoding the enzyme isopenicillin N synthetase involved early in the CPC biosynthetic pathway has been reported (Samson et al 1985; Harford et al 1986) Expression of this sequence in E. coli (Samson et al 1985) led to the production of a protein which in crude cell extracts, catalysed the conversion of LLD-ACV tripeptide

into a penicillinase sensitive compound having properties consistent with isopenicillin N. Genes encoding isopenicillin N synthetase have also been isolated from P. chrysogenum (Carr et al 1986) and from Streptomyces clavuligerus (Jensen et al 1987). The predicted amino acid sequence derived from the S clavuligerus isopenicillin N synthetase gene sequence has been reported to show approximately 40% homology to the sequence of the enzyme from C. acremonium however, the degree of homology between the respective nucleotide sequences is lower. In P. chrysogenum the isopenicillin N synthetase gene sequence shows approximately 74% homology to the C. acremonium gene and the amino acid sequence predicted from this is approximately 73%. homologous to that predicted in C. acremonium (Carr et al 1986).

1.3.1 Genetic studies of β-lactam production in A. nidulans and P. chrysogenum.

In comparison to the limited study of the genetic bases of CPC biosynthesis in C. acremonium (Ball 1984; Perez-Martinez 1984) and Emericellopsis species (Fantini and Olive 1960; Fantini 1962) investigations into penicillin production in A. nidulans and P. chrysogenum (Ball 1973, 1984; MacDonald 1983; MacDonald and Holt 1976) have been more detailed and

may suggest approaches which could be of use in developing an understanding of CPC production at a genetical level.

A. nidulans was chosen as a model system since although producing only low levels of penicillin, extensive studies have been carried out on this organism making it a well documented system. Penicillin production was established to be under nuclear control (MacDonald et al 1963 a and b; Merrick and Caten 1975) and subsequently mutations affecting penicillin titre have been classified into at least seven complementation groups and assigned to linkage groups (Table 1.1). Four groups npe A, npe B, npe C and npe D were identified following the study of mutations reducing titre to below ten percent of the progenitor strain and the remaining three following examination of raised titre mutants. Interestingly when mutants with low titre were examined the majority isolated after mutagenesis were found to belong to the npe A group (Edwards et al 1974; Holt et al 1976). When naturally occurring non-producing strains were studied they were found to belong predominantly to two heterokaryon incompatibility groups but also to be mostly of the npe A type (Cole et al 1976). The effects of the titre increasing pen A1, pen B2 and pen C3 mutations in various combinations were investigated in

Table 1.1 Linkage group location of mutations affecting penicillin production in *A. nidulans*

Complementation Group	Linkage Group	Reference
npe A	VI	Edwards *et al* 1974
npe B	III	Makins *et al* 1983
npe C	IV	
npe D	II	
pen A	VIII	Ditchburn *et al* 1976
pen B	III	
pen C	IV	

heterozygous diploids and in haploid recombinants from crosses between the mutant strains. Whilst pen A1 was recessive, pen B2 was dominant and pen C3 semi-dominant. Both the pen A1 and pen C3 mutations were epistatic to pen B2 and pen A1 was additionally epistatic to pen C3 (Ditchburn et al 1976).

Crosses between A. nidulans strains with naturally different titres of penicillin followed by the quantitative study of progeny titres have shown antibiotic production to be poly-genically determined with many of the genes concerned having additive effects (Merrick and Caten 1975). Starting with a sexual cross between two producing strains derived from different heterokaryon incompatibility groups, and so with a high input of natural variation, Merrick has further shown (1976, 1975a) it is possible to obtain a substantial (60-160%) increase in titre over four or five generations of inbreeding by continually selecting the highest producing progeny to be the parents of subsequent crosses. This would indicate some potential for the use of breeding techniques in titre improvement providing fresh sources of variation were introduced periodically, for example by crossing between separate lines of strain selection (Merrick 1975b). However, the initial strains fed into the

hybridization and selection programme by Merrick were derived from a natural population rather than from existing strain improvement lines in which recurrent mutagenesis had previously been used to introduce titre improving mutations. Simpson and Caten (1980, 1981) attempted to improve titre by crossing strains derived via two different lines of mutagenesis. Ethylmethylsulphonate and near ultraviolet light plus 8 -methoxysporalen treatment were used as mutagens. Strains were crossed and the highest titre progeny selected as the parents for the next generation. Although some increase in titre was found, the presence of non-additive, unfavourable gene interactions appeared to limit the titre improvements possible. Whether the presence of genes with non-additive effects on production is a general characteristic of strains produced as a result of programmes of mutagenesis in contrast to the mainly additive effects of genes found in the strains used by Merrick is not certain but the poor titre improvement obtained by Simpson and Caten would suggest that the use of mutagenesis and hybridisation together as a means of titre improvement may meet with a variety of problems as anticipated by Sermonti (1961).

The study of penicillin production in P. chrysogenum has depended upon the use of parasexual

analysis. Early work established that heterozygous diploids could be formed from strains with complementary nutritional requirements; following segregation the diploids gave haploid recombinants (Pontecorvo and Sermonti 1954; Sermonti 1954). As in A. nidulans penicillin production was shown to be largely under nuclear control (MacDonald et al 1963a) and when mutants with reduced capacities to synthesise penicillin were isolated by Sermonti (1956) eight out of nine belonged to the same complementation group. The recessive nature of a number of non-producing mutants was shown by Caglioti and Sermonti (1956) and diploids formed from crosses between raised titre strains and their progenitors were generally found to be of low titre suggesting that the mutations leading to improved titre were recessive (MacDonald et al 1963b). When strains with improved penicillin titres were crossed the diploids formed had varied titres some much reduced compared to the parent strains but some comparable to those of the parents (MacDonald et al 1965; MacDonald 1966). In one cross two thirds of the diploids examined produced significantly less antibiotic than the parents and this was proposed to be the result of a dominant titre reducing mutation arising in one of the parental strains and having a selective advantage. Calam et al (1976) reported the

successful production of a diploid of high titre which had an improved ability to form spores compared to other high titre strains. This diploid was the result of both hybridisation and subsequent mutagenic treatment of the diploids produced. These workers also tested out various strategies to determine whether particular types of crosses and the use of particular mutagenic treatments might be of use in strain improvement. Their results, however, led to the conclusion that it would be difficult to predict which crosses would be most use for improving a complex system such as antibiotic production. Analysis of segregants from diploids revealed a difficulty in obtaining haploid segregants possessing alleles from both parental strains. MacDonald (1968) suggested the occurrence of this parental genome segregation could be due to the presence of chromosomal rearrangements in one or both of the parental strains leading to difficulties in recombination or to the selective advantage of parental type haploid recombinants. The use of chemical treatments to enhance the degree of segregation occurring in these situations was suggested (Macdonald 1971). This coupled with the careful choice of the parental strains largely enabled the problem of obtaining a good range of segregants to be overcome. By crossing closely

related 'sister' strains and using para-fluorophenylalanine to induce segregation Ball (1971) assigned twenty auxotrophic and drug resistance markers to positions on three linkage groups in a raised titre strain of P. chrysogenum so opening the way for the mapping of induced mutations having titre increasing effects to linkage groups I and II (Ball 1976).

Normansell et al (1979) have since examined twelve penicillin non-producing strains derived from relatively low-yielding P. chrysogenum lineage and allocated them to five complementation groups. Of the twelve mutations, seven belonged to one group (Y) which is reminiscent of the earlier work by Sermonti (1956). Three of the five complementation groups (Y, W and Z) were located on one linkage group with the other two being on separate linkage groups. Biochemical studies have suggested that mutants in groups X, Y and Z are unable to synthesise the tripeptide precursor of penicillin with those in group X additionally lacking the acyl exchange enzyme required later in the biosynthetic pathway. Mutants in group V were also deficient in the acyl exchange step. The npe A mutants of A. nidulans synthesise penicillin when supplied with ACV tripeptide suggesting a block at or before tripeptide synthesis (Makins and Holt 1982) and the functional equivalence

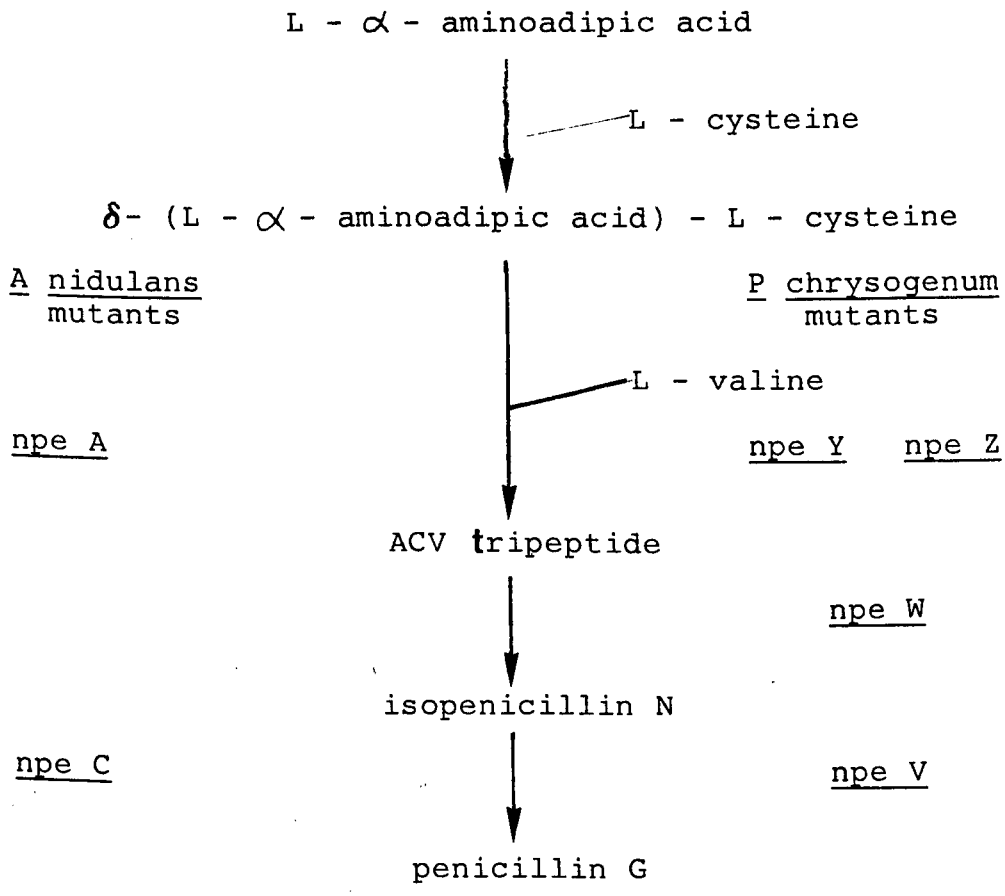
of npe A and npe Y in P. chrysogenum has been suggested following the co-fermentation of mycelium given gentle treatment with a lytic enzyme to weaken the cell wall (Makins et al 1981). The possible relationships of the mutations, studied in the two organisms, to the biosynthetic pathway leading to penicillin are shown in Fig. 1.3.

1.4 The biosynthesis of CPC.

In contrast to the limited genetic studies of the CPC production in C. acremonium, biochemical studies have produced a relatively detailed picture of the biosynthetic pathway involved.

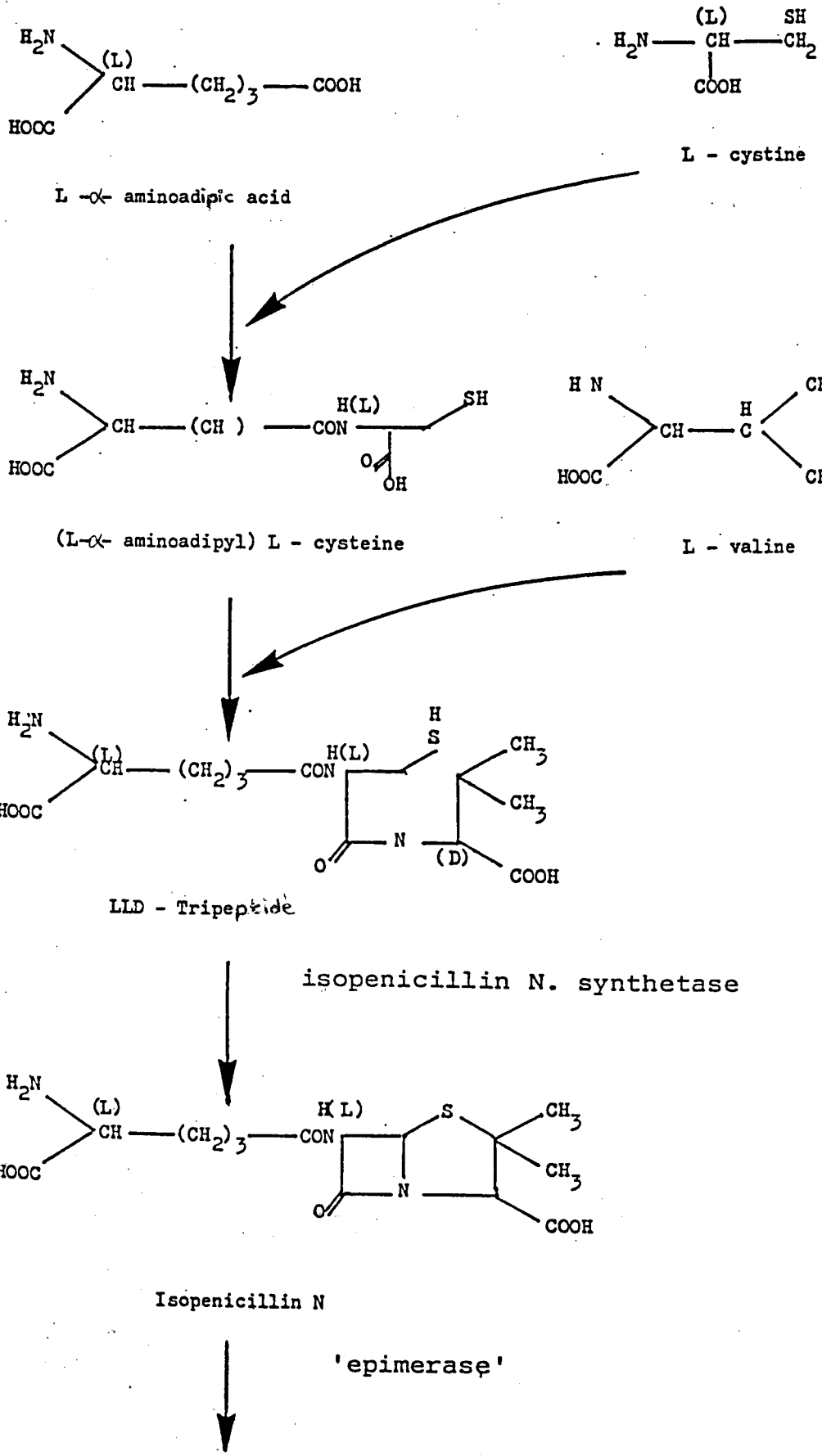
The B-lactam compounds present in C. acremonium fermentation broths are produced by a common biosynthetic pathway (Fig. 1.4) with Pen N being an intermediate in the formation of CPC. The pathway shows many similarities with those for the production of hydrophobic penicillins by Penicillium species (for review see Hersbach 1984) and for the synthesis of the cephalosporin based metabolites of Steptomyces species (for reviews see Elander 1974; Kanazaki and Fujisawa 1975). Variations in the biosynthetic pathway (which will be described below) do occur in both mutant strains of C. acremonium and

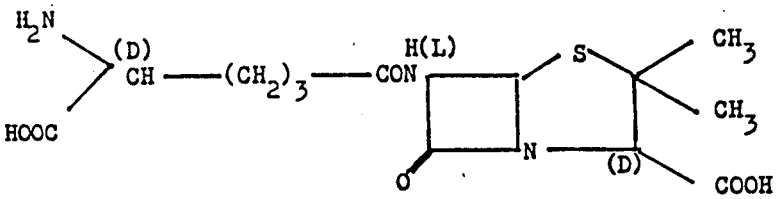
Fig. 1.3 **Mutations affecting steps in the antibiotic biosynthetic pathways in A. nidulans and P. chrysogenum.**



from MacDonald 1983

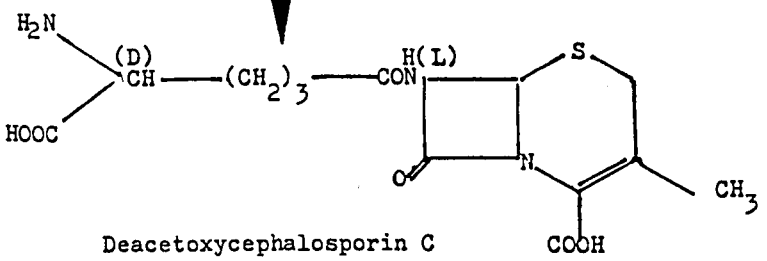
Fig. 1.4 The biosynthetic pathway leading to cephalosporin C.





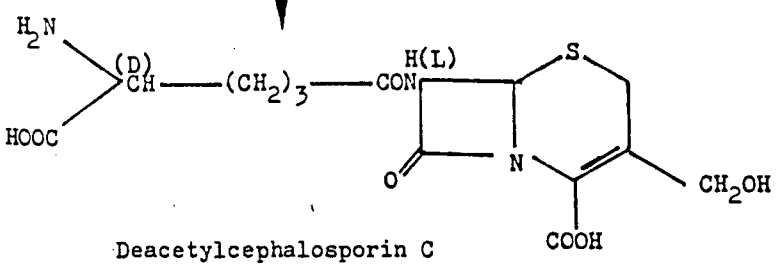
Penicillin N

'expandase'



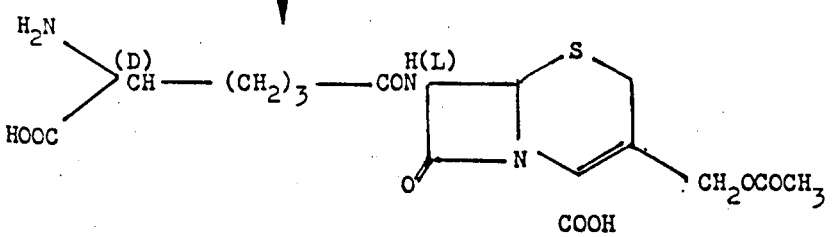
Deacetoxycephalosporin C

'hydroxylase'



Deacetylcephalosporin C

'deacetylcephalosporin C. acetyltransferase"



Cephalosporin C

in other related species. Many of the compounds produced as a result have been characterised and found to be modified versions of intermediates in CPC bio-synthesis e.g. RIT 2214 a penicillin with a L-S-carboxymethylcysteine in place of the α-aminoadipyl moiety (Troonen et al 1976), or to be the result of side reactions to the main pathway e.g. C-43-219, the product of the reaction of D-penicillamine and CPC (Kanazaki et al 1976). Other examples have been described by Kanazaki et al 1974, Fujisawa and Kanazaki 1975a, a Traxler et al 1975, Kitano et al 1976 and Kitano 1977 a and b.

1.4.1 The Arnstein tripeptide precursor.

CPC C biosynthesis begins with the formation of the same ACV tripeptide precursor molecule, the 'Arnstein tripeptide', δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine as that involved in penicillin production in P. chrysogenum. (Arnstein and Morris 1960; Loder and Abraham 1971 a and b; Fawcett et al 1976). This is the product of the non-ribosomal condensation of L-α-aminoadipic acid and L-cysteine followed by the addition of L-valine (Trown et al 1962). C. acremonium mycelium or broken cell preparations do not utilise exogenously supplied δ-(α-aminoadipyl)-L-cysteine as a precursor for

tripeptide biosynthesis. The molecule is first hydrolysed and the subunits are used for tripeptide synthesis (Loder et al 1969). The L-isomer of valine is preferentially incorporated and the final LLD configuration of the tripeptide necessary for its subsequent participation in the formation of isopenicillin N is produced during condensation with the D-(L-α-aminoadipyl)-L-cysteine dipeptide (Demain 1963). Since neither D-α-aminoadipic acid nor δ-(D-α-aminoadipyl)-L-cysteine are readily accepted as substrates it is apparent that the α-aminoadipic acid moiety is initially incorporated in the L configuration with the D configuration of the penicillin side chain being produced at a subsequent stage (Warren et al 1967; Loder and Abraham 1971 b, Fawcett and Abraham 1975). In addition to the ACV tripeptide two other peptides have been recovered in low concentrations from C. acremonium mycelium. Both resemble ACV but have an additional glycine moiety and in one case β-hydroxyvaline in place of valine (Loder and Abraham 1971 a and b).

1.4.2 Ring cyclization of the tripeptide.

The ACV tripeptide is converted by a cyclization reaction to form a β-lactam thiazolidine ring structure. The first experiments involving the use

of radio-labelled tripeptide failed to demonstrate the incorporation of the molecule into compounds with antibacterial activity. This was due to the impermeability of intact C. acremonium mycelium to the pre cursor. When protoplast lysates and other cell free systems were used tripeptide incorporation was obtained and more detailed studies have shown that the immediate product of cyclization is isopenicillin N with the α-aminoadipic acid side chain in the L configuration (Fawcett et al 1976; O'Sullivan et al 1979; Konomi et al 1979.

Isopenicillin N synthetase (IPNS), the enzyme catalysing the cyclization reaction requires ferrous ions, oxygen and the reduced form of the LLD tripeptide for activity. It has a pH optimum between 7.0 and 7.5, and a temperature optimum between 25 and 30°C (Sawada et al 1980a; Kupka et al 1983a). Purification of IPNS has led to estimates of its molecular weight ranging from approximately 31,000 (Abraham et al 1980; Kupka et al 1983b) to 41,000 +/- 1,000 (Hollander et al 1984). Pang et al (1984a) have noted similarities in the co-factor requirements of the C. acremonium enzyme and similar synthetases partially purified from P. chrysogenum and S. clavuligerus.

Several groups have examined the utility of analogues of the ACV tripeptide as substrates for

IPNS and so function as possible precursors for penicillin molecules with novel structures. Analogues showing some activity include the DLD form of ACV, δ-(L-carboxymethylcysteinyl)-L-cysteinyl-D-valine, phenoxyacety-L-cysteinyl-D-valine and phenylacetyl-L-cysteinyl-D-valine. However, in all these cases antibiotic formation is inefficient and conversion rates are low when compared to those with the natural substrate (Bahadur et al 1981; Baldwin et al 1984a and 1985; Wolfe et al 1984). Detailed investigations of the chemical mechanism and stereochemistry of the ring cyclization reaction and the subsequent stages of CPC biosynthesis are in progress but are beyond the scope of this discussion (Neuss et al 1973; Kluender et al 1973 and 1974; Huang et al 1975; Baldwin et al 1981a, 1984b; Pang et al 1984b).

1.4.3 The formation of Pen N.

Pen N, with the α-aminoadipyl side chain in the D configuration, is the product of an enzyme catalysed epimerisation of isopenicillin N. The activity of the epimerase can be demonstrated in protoplast lysates in the presence of $FeSO_4$, ascorbic acid and ATP. However, the enzyme is labile and prone to inactivation if stored in protoplast lysates

rather than as intact mycelium (Baldwin et al 1981b; Jayatilake et al 1981). An enzyme responsible for epimerase activity has been purified from the cephamycin producing S. clavuligerus and is more stable and therefore more amenable to study than the C. acremonium enzyme. It has a molecular weight of approximately 60,000 and to date no stimulatory cofactors have been identified (Jensen et al 1983; Lubbe et al 1986).

1.4.4 The relationship of Pen N to the cephalosporin compounds produced.

Evidence for the common pathway of Pen N and CPC biosynthesis came from two areas of study. Mutant strains of C. acremonium blocked in the synthesis of CPC were found to fall into two groups with respect to B-lactam formation (Pen N^+ CPC^-; Pen N^- CPC^-) with the production of CPC without Pen N not being observed. Strains blocked for CPC production accumulated other types of cephalosporin compounds identified as deacetoxycephalosporin C (DAOC) and deacetylcephalosporin C (DAC). The pattern of accumulation of these suggested a pathway converting Pen N first to DAOC then to DAC and finally to CPC (Fujisawa et al 1973; 1975a, 1975b; Queener et al 1974; Shirafuji et al 1979). In addition C.

acremonium cell free systems convert Pen N to a compound resistant to penicillinase but sensitive to cephalosporinase activity (Kohsaka and Demain 1976). The product of such reactions was shown to be DAOC (Yoshida et al 1978; Baldwin et al 1980, 1981b) thus strengthening the view that Pen N is an intermediate in the linear biosynthetic pathway leading to CPC.

1.4.5 Ring expansion to produce deacetoxycephalosporin C.

The enzyme catalysing the ring expansion of Pen N into DAOC has pH and temperature optima similar to those of the ring cyclization enzyme (Kupka et al 1983a) and estimates of its molecular weight have ranged from 28,000 to 35,000 (Kupka et al 1983b; Scheidegger et al 1984). For activity in cell free systems, α-ketoglutarate as a cofactor, ferrous ions, molecular oxygen and the presence of ascorbic acid are required. Low concentrations of ATP are stimulatory. This would suggest that the enzyme belongs to the class of α-ketoglutarate linked dioxygenases. A membrane bound location for the ring expansion enzyme within the cell was suggested but in the majority of cases the enzyme activity has

not been found to be associated with the cellular membranes (Kohsaka and Demain 1976; Hook et al 1979; Sawada et al 1979, 1980b; Baldwin et al 1980; Kupka et al 1983b). Investigations into substrate specificity have shown that penicillin G, ampicillin and carboxy-n-butyl-penicillin act as non-competitive inhibitors of the expandase, with no corresponding cephalosporin compounds being produced (Kohsaka and Demain 1976; Kupka et al 1983b). Expandase activity is reversibly lost during the storage of enzyme preparations in the absence of dithiothreitol suggesting a requirement for the sulphydryl groups present in the enzyme to be protected from oxidation. This could be indicative of the function of ascorbic acid in cell free assays of the expandase (Lubbe et al 1985a). In comparison to the ring cyclization reaction the rate of ring expansion is low suggesting that this step may be rate limiting (Kupka et al 1983a).

1.4.6 The formation of deacetylcephalosporin C.

Mutant strains of C. acremonium which fail to convert DAC into CPC generally produce the accumulated DAC by de novo synthesis from DAOC (Fujisawa and Kanazaki 1975b; Fujisawa et al 1975b). However, a mutant with unusual properties has been reported. During

fermentation this strain begins to accumulate CPC which is they hydrolysed to give DAC (Fujisawa et al 1973; Fujisawa and Kanazaki 1975c). Accumulation of DAC by this strain is due to the presence of an extracellular CPC acetylhydrolase enzyme not observed in the progenitor strain, which hydrolyses CPC at a rate greater than that of the non-enzymatic hydrolysis seen in some fermentations (Huber et al 1968; Fujisawa et al 1973, 1975c). Other extracellular acetylhydroxylase enzymes produced by C. acremonium have been reported (Hinnen and Nuesch 1976). The enzymatic conversion of DAOC to DAC requires the presence of α-ketoglutarate, ferrous ions, dithiothreitol, ascorbic acid and oxygen in cell free systems. The enzyme has a pH optimum of 7.0 and is highly substrate specific. Pre-incubation of cell extracts with the required cofactors before addition of DAOC substrate has been reported to cause an increase in the activity of the hydroxylase of up to ten-fold but these reports are not consistent (Turner et al 1984; Scheidegger et al 1984. The many similarities found between the hydroxylase and expandase enzymes suggests that DAC production is also catalysed by an α-ketoglutarate linked dioxygenase (Turner et al 1978; Felix et al 1980; Scheidegger et al 1984). Scheidegger et al (1984), during attempts to purify the two enzymes

found that both the ring expansion and hydroxylation activities correlated with a single protein fraction having a molecular weight of 33,000 +/- 2,000. They proposed that a single bifunctional enzyme might be responsible for the conversion of Pen N into DAC. Support for this view has come from Dotzlaf and Yeh (1987) and Baldwin et al (1987) who found that the two enzyme activities remained associated during purification and appeared to correlate with a single monomeric protein with a molecular weight of 41,000 +/- 2,000. In contrast the two corresponding activities present in extracts of S. clavuligerus have been separated, with the expandase having a molecular weight of 29,000 and that of the hydroxylase being 26,200 (Jensen et al 1985).

1.4.7 The formation of cephalosporin C.

The final stage of β-lactam biosynthesis in C. acremonium is the acetylation of DAC to form CPC. Felix et al (1980) showed the acetyltransferase enzyme to require acetylcoenzyme A, acetate and ATP for activity in ether permeabilized cells. Using cell free extracts Fujisawa and Kanzaki (1975 b) had previously shown a requirement for magnesium ions as a co- factor. The reaction has a pH optimum of pH 7.0 to 7.4 and acetyltransferase activity has been

shown to be absent in many mutant strains found accumulating DAC in preference to CPC.

1.5 The relationship between primary and secondary metabolism in C. acremonium : fermentation studies.

β-lactam antibiotics are produced as secondary metabolites by C. acremonium during fermentation. Production is largely associated with a phase in the life cycle in which little growth occurs. Pen N may begin to accumulate whilst cultures are still actively growing and utilizing rapidly metabolised carbon sources such as glucose but CPC production is delayed until a later stage characterised by a reduction in the rate of increase in cell mass and the use of other carbon sources. Antibiotic production continues until a short time after exhaustion of the carbon source. Following this there is a rise in both the pH and ammonium content of the culture and lytic effects are seen as both CPC titre and cell mass decrease; Nuesch et al 1973; Matsumura et al 1978). Although the basic pattern of antibiotic synthesis remains constant to a degree, the precise timing, rate of production and the maximum titres achieved vary between strains and between fermentation conditions. A comparison of the performance of a number of raised titre strains

under identical conditions has been made (Perez-Martinez 1984). Differences in growth pattern in relation to the carbon source supplied and in the level of phosphate required for sustained antibiotic production were seen as well as differing abilities to convert Pen N into CPC.

1.5.1 Carbon source regulation.

Carbon source regulation of antibiotic production has been examined using resting cell systems. Glucose, when present affects mainly the later stages of biosynthesis, thus lowering CPC but not Pen N production (Behmer and Demain 1983). Glycerol, maltose and sorbose have similar effects (Heim 1984). The ring expansion enzyme responsible for the conversion of Pen N into DAOC is subject to both glucose repression and inhibition whilst the cyclization reaction leading to the formation of Pen N is only slightly inhibited (Martin et al 1982; Behmer and Demain 1983; Scheidegger et al 1984). A decrease in Pen N production when glucose is present in very high concentration has been noted (Heim 1984) which might be indicative that an enzyme prior to IPNS in the pathway may have some sensitivity to carbon source control. Other slowly metabolized carbon sources, for example sucrose, galactose,

fructose and mannitol, or a tightly controlled supply of glucose tend to favour the production of high levels of CPC (Kennel and Demain 1978; Matsumura et al 1978). There is no clear evidence as to the mechanism underlying carbon catabolite repression of CPC biosynthesis in C. acremonium. Phosphorylated intermediates of glycolysis, for example glucose-6-phosphate and fructose-1, 6-bisphosphate, have been suggested as effectors mediating glucose control of cephamycin production in Streptomyces lactamdurans (Cortes et al 1986) and of the production of hydrophobic penicillins by P. chrysogenum (Revilla et al 1981). Control of cephamycin production appears to be via a decrease in ACV tripeptide formation and repression and inhibition of the ring expansion enzyme. In P. chrysogenum ACV tripeptide formation is reduced both by repression and by a lowering in the intracellular pool concentration of the precursor α-aminoadipic acid (Martin et al 1984; Revilla et al 1984; Alvarez et al 1987).

1.5.2 Regulation by phosphate.

In C. acremonium the effect of phosphate concentration on antibiotic production is linked to that of the carbon source supply. If the supply of carbon is tightly controlled to prevent excess carbon

accumulating then the phosphate level has little effect, however, in the presence of an excess of a readily used carbon source metabolism is directed towards an increase in cell mass rather than secondary metabolism. Should the supply of either carbon or phosphate become limiting a switch towards antibiotic production occurs (Kuenzi 1980). Studies of cell free systems showed levels of phosphate above 30 mM to repress Pen N formation. The ring expansion enzyme has also been shown to be sensitive to phosphate. Below 10 mM phosphate the enzyme is non-functional but at concentrations greater than 100 mM the enzyme is inhibited. Excess phosphate therefore tends to reduce the flow through the whole biosynthetic pathway in contrast to glucose which is more specific in its repression of the ring expansion enzyme (Martin *et al* 1982). Cephamycin production in *S. clavuligerus* appears to be regulated by phosphate repression and inhibition of ring expansion and inhibition of ring cyclization with repression accounting for the major effect (Aharonowitz and Demain 1977; Lubbe *et al* 1984, 1985b).

1.5.3 Regulation by nitrogen source.

Until recently information concerning the effect of different nitrogen sources on the CPC biosynthetic

pathway has been limited. Shen et al (1984) examined the effects of several inorganic and organic nitrogen sources and showed that ammonium sulphate, ammonium chloride, urea and two amino acids, L-arginine and L-asparagine could be used successfully to support antibiotic production. High concentrations of ammonium ions persisting in the culture to stages at which CPC biosynthesis occurred tended to decrease the yield of CPC with little effect on the yield of Pen N. In these conditions it appears that the ring expansion enzyme is considerably repressed by the excess ammonium ions with the ring cyclization enzyme being only slightly repressed. Addition of an ammonium trapping agent such as magnesium phosphate can reverse the effect. In Streptomyces species high concentrations of ammonium ions are thought to cause repression of both the ring cyclization and expansion enzymes and in contrast to the situation in C. acremonium, it appears that the major effect is exerted at the cyclization step (Brana et al 1985; Castro et al 1985).

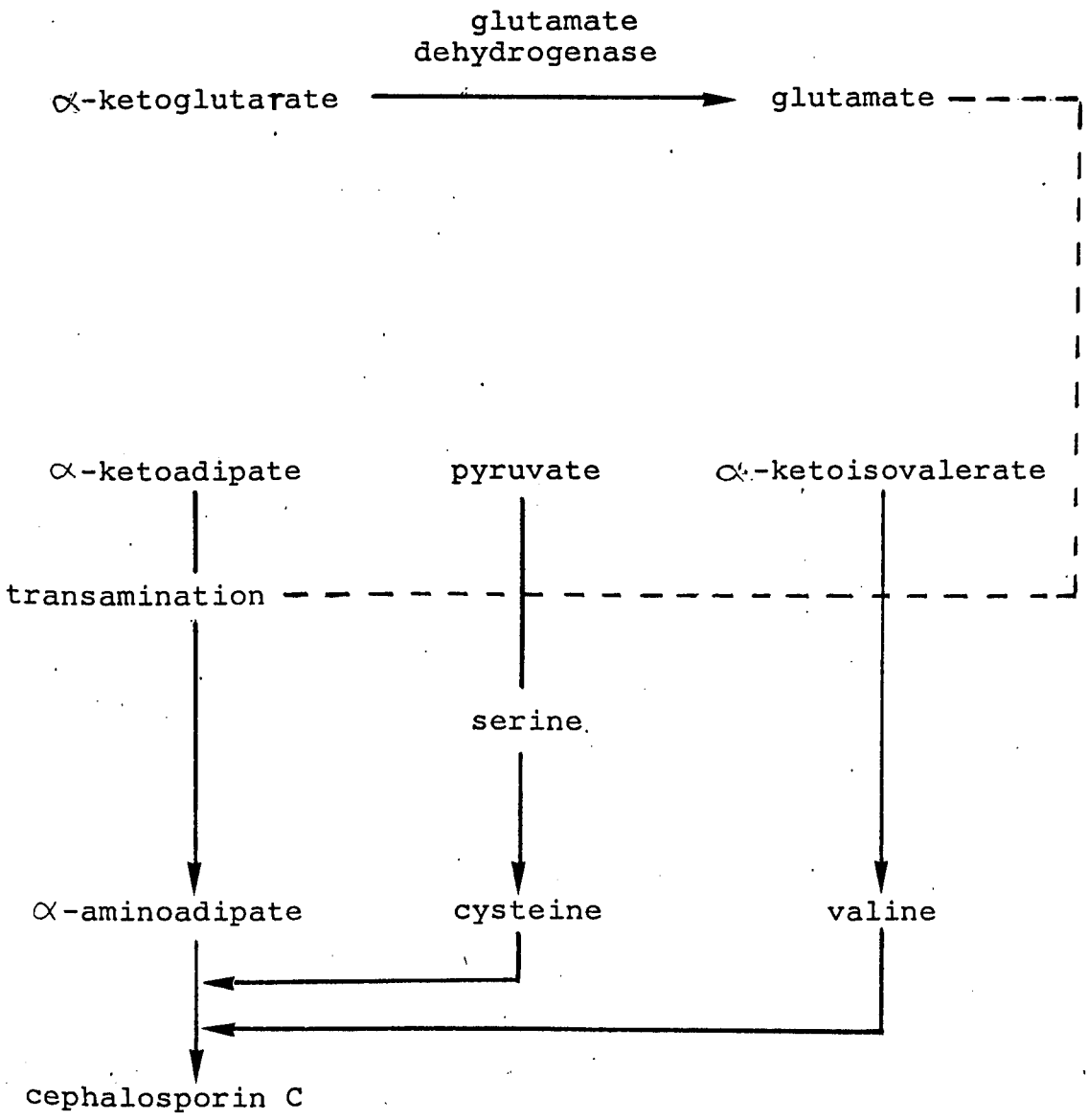
The activity of one particular enzyme involved in nitrogen metabolism has been correlated with CPC biosynthesis. Glutamate dehydrogenase is responsible for the production of L-glutamate from α-ketoglutarate and ammonium ions. Since both α-

ketoglutarate, as a precursor of α-aminiadipic acid, and L-glutamate, as a potential amino group donor in the transamination reactions leading to cysteine and valine formation, are required for the production of the precursors of CPC then the activity of glutamate dehydrogenase might be particularly relevant to antibiotic biosynthesis (Fig 1.5). A line of mutant strains of C. acremonium have been produced in which glutamate dehydrogenase, normally repressed once the growth phase of a fermentation is completed, is instead derepressed. Of this group of mutants, several had increased capacities to produce CPC, and it was suggested that in these cases that the altered regulation of the enzyme overcame a previous nitrogen limitation in antibiotic biosynthesis (Queener et al 1975; Aharonowitz 1980). Experiments with P. chrysogenum have suggested that a high intracellular pool of glutamate may cause the induction of enzymes required for penicillin synthesis (Lara et al 1982; Jaklitsch et al 1985). Other work though has suggested that it is the glutamine pool size which is important (Sanchez et al 1981).

1.5.4 The role of the non-sulphur containing precursors of CPC

The metabolic pathways leading to valine and α-

Fig. 1.5 The role of glutamate dehydrogenase.

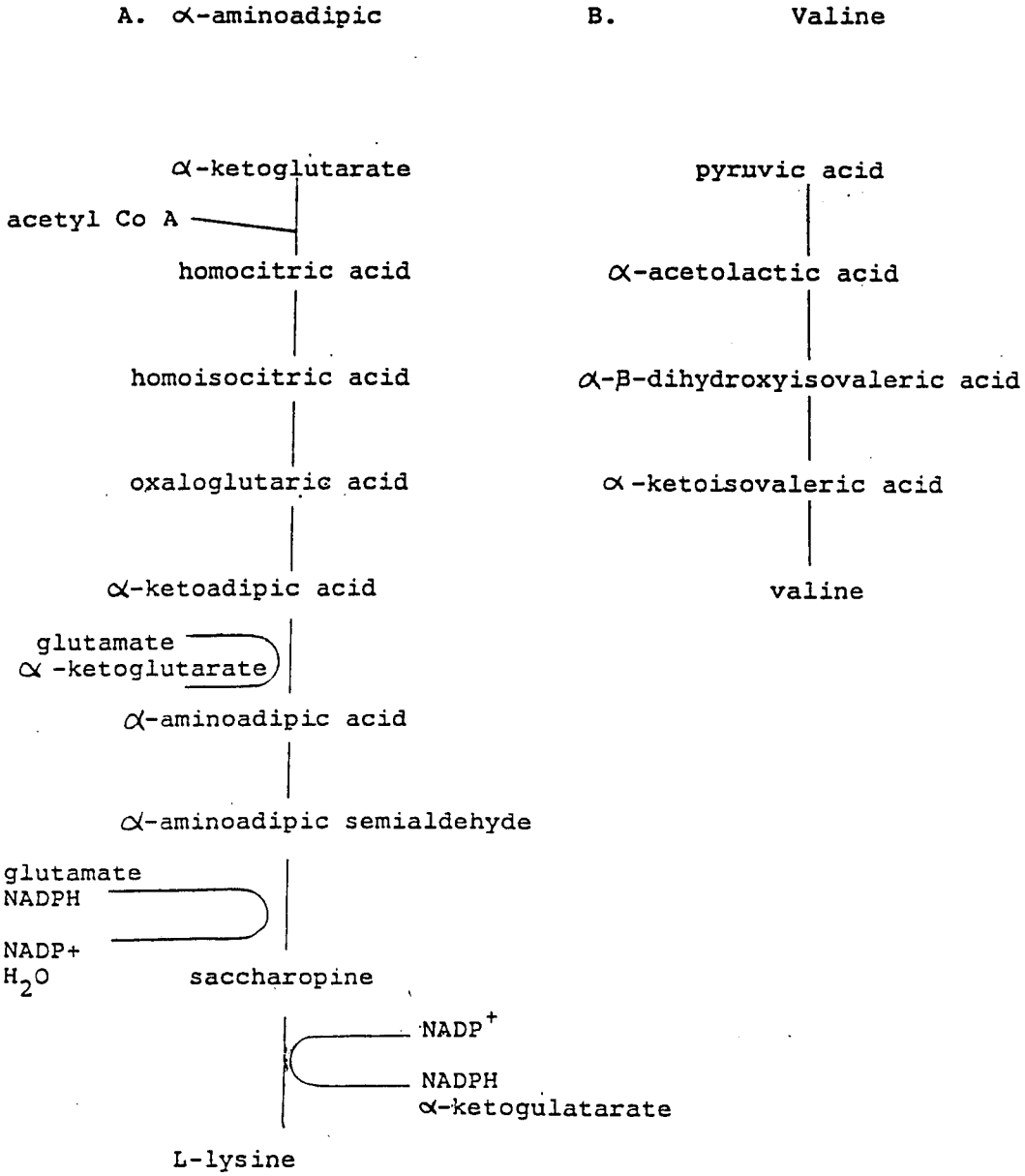


From: Queener et al 1975

aminoadipic acid are shown in Fig. 1.6. Since both these amino acids function as precursors of CPC alterations in their metabolism would be expected to affect the production of antibiotic. Lemke and Nash (1972) examined a lysine auxotroph of C. acremonium and found it to be deficient in antibiotic synthesis.

This was thought to be due to feedback inhibition of one of the early steps of lysine biosynthesis by exogenously supplied lysine. Such feedback would result in a decrease in the flow through the metabolic pathway common to both lysine and α-aminoadipic acid and so would decrease the availability of the latter for CPC synthesis. This was confirmed by the observation that supplying α-aminoadipic acid exogenously could restore the capacity for antibiotic production. A similar decrease in the ability to produce penicillin following lysine supplementation of cultures has been seen in P. chrysogenum (Demain 1957). Again addition of α-aminoadipic acid could reverse the effect (Somerson et al 1961). The more detailed studies on Penicillium have shown that lysine acts by inhibition and some repression to feedback regulate the first enzyme of its own biosynthesis, homocitrate synthase (Goulden and Chattaway 1968; Masurekar and Demain 1971 and 1974; Friedrich and Demain 1977 a and b; Luengo et al 1980). Lysine addition may both

Fig. 1.6 The biosynthetic pathways leading to α-aminoadipic acid and valine.



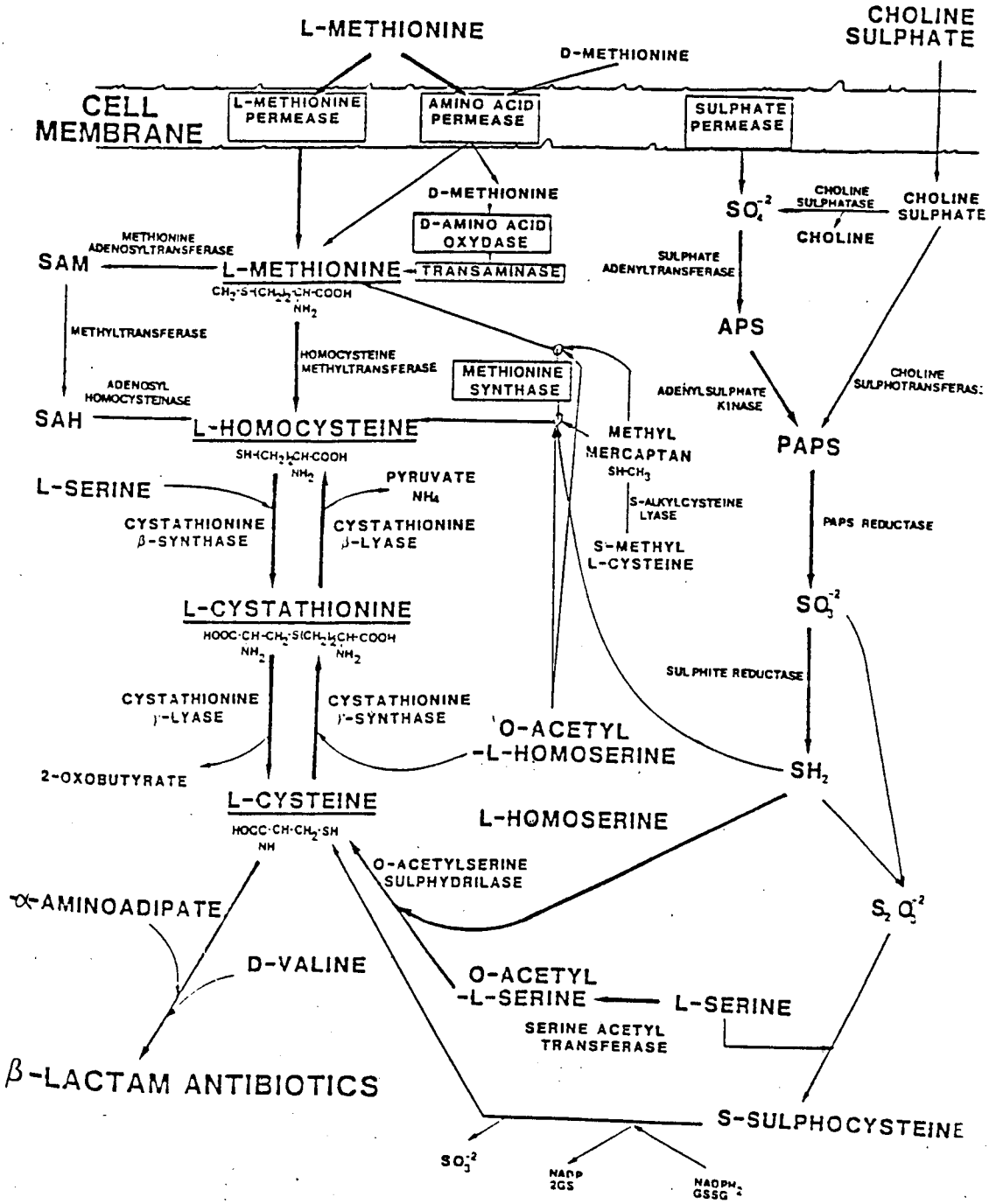
from Lehninger 1975

delay the onset of antibiotic synthesis and decrease its rate and there is some evidence that Penicillium strains selected as high producers of antibiotic may have an altered response to lysine so that the onset of synthesis is not delayed (Luengo et al 1979). Valine biosynthesis is also subject to feedback control. In both high and low producing strains of C. acremonium acetohydroxyacid synthetase is repressed and inhibited by valine, isoleucine and leucine with the enzyme being particularly sensitive to valine (Matsumura and Suzuki 1986).
This is in contrast to the report that valine acts by inhibition rather than by repression in P. chrysogenum and that high and low producing stains have altered responses to valine (Goulden and Chattaway 1969).

1.5.5 The role of sulphur metabolism in CPC production.

Sulphur metabolism, particularly with reference to the production of precursor cysteine for β-lactam biosynthesis, is of considerable importance in C. acremonium. The sulphur atom of cysteine may be derived from either inorganic sulphur via the sulphate reduction pathway or from methionine via reverse trans-sulphuration (Fig. 1.7). In C.

Fig. 1.7 Sulphur Metabolism.



From Perez-Martinez 1984.

acremonium methionine is generally preferred to inorganic sulphur for antibiotic production in contrast to the situation in P. chrysogenum (Tardew and Johnson 1958; Segal and Johnson 1961 and 1963; Caltrider and Niss 1966; Nuesch et al 1973). Niss and Nash (1973) proposed that the inferiority of sulphate for CPC biosynthesis in C. acremonium is due to the strict regulation of sulphate assimilation in this fungus. The preferential usage of methionine is reflected in the derepression of a sulphatase enzyme which occurs during antibiotic production. This enzyme is responsible for the mobilization of endogenous sulphur stored as choline sulphate and its conversion into sulphate for use in metabolism. Normally the sulphatase is repressed to different degrees by the presence of, for example, methionine, cysteine or sodium thiosulphate, presumably by the action of a common intermediate in their metabolism, possibly sulphate. During antibiotic production the sulphatase is derepressed in response to the additional requirement for sulphur generated by the diversion of methionine and cysteine towards CPC biosynthesis (Dennen and Carver 1969).

Although methionine is usually preferred by C. acremonium several mutants have been characterised which have an increased ability to use inorganic sulphate and so show similarity to P. chrysogenum.

In one such mutant the activity of L-serine sulphydrylase, involved in one of the two pathways for cysteine production from sulphate, had been doubled. This strain therefore maintained increased intercellular concentrations of cysteine and was sensitive to methionine. Addition of methionine above a certain level would raise the level of cysteine further so that the enzyme cysteine desulphydrase would be stimulated leading to the degradation of cysteine and a lowering of antibiotic production. (Komatsu et al 1975; Komatsu and Kordaria 1977). A second type of mutant resistant to DL-selenomethionine had an increased permeability to sulphate and a lowered sensitivity to feedback inhibition of sulphate assimilation by methionine (Matsumura et al 1980).

Methionine is thought to enter C. acremonium mycelium by two routes. The L-isomer appears to be taken up by a specific amino acid permease, to be quickly converted into the D-isomer and then deaminated producing ammonia as a nitrogen source and 2-keto-D-methiolbutyric acid which is excreted (Benz et al 1971). D-methionine is believed to enter the cell at a far slower rate probably via a non-specific permease and as such is less rapidly utilized and so may provide a longer lasting pool for antibiotic production. There is evidence that the uptake of methionine is considerably reduced by the presence of

sulphide in the cell (Benko et al 1967; Nuesch et al 1973, 1976; Demain 1974). Nuesch et al (1973) proposed that the production of cysteine from methionine via the action of cystathionine-γ-lyase was essential in order to provide 'active' cysteine molecules for antibiotic synthesis. This could explain the relative inefficiency with which exogenously supplied cysteine functions as a precursor of CPC.

Although methionine can act as a sulphur donor in CPC biosynthesis, proposals for an additional role were made following observations that its action was not solely consistent with that of a sulphur source in C. acremonium. Other fungal species which produce CPC are known to vary in their response to methionine (Kitano 1977c). From the early stages of investigation it was noted that the addition of methionine to fermentations, in constrast to other sulphur sources, caused a lowering of the growth rate as well as an increase in CPC production. The mycelium present also seemed to become swollen, irregular and fragmented, forming arthrospores (Kavanagh et al 1958; Caltrider et al 1968; Queener and Ellis 1975). Later work showed a possible correlation between the ability of a strain to produce high titres of antibiotic and the ability to form arthrospores and speculations have been made as to the

possible causal connections between methionine supply, differentiation and antibiotic production (Nash and Huber 1971; Drew et al 1976). In C. acremonium intermediates of the trans-sulphuration pathway between methionine and cysteine supplied to a fermentation in place of methionine do not promote the same stimulation of biosynthesis as seen with methionine itself. Some groups have shown that the non-sulphur containing methionine analogue, DL-norleucine may mimic the stimulatory effect of methionine and that S-methyl-cysteine in low concentrations will improve antibiotic production (Demain and Newkirk 1962; Demain et al 1963; Caltrider and Niss 1966; Drew and Demain 1973 and 1975a; Kitano 1977c). The stimulatory effect of methionine has also been found to be dependent upon its presence during the growth phase rather than the production phase of a fermentation (Demain et al 1963; Matsumura et al 1978) and to still occur even in mutants where trans-sulphuration is blocked between methionine and cysteine (Drew and Demain 1975b). Indeed a strain carrying a methionine auxotrophy was shown to require supplementation with methionine to a level above that required to allow growth if CPC was to be produced even if an excess of inorganic sulphur known to be able to support some antibiotic synthesis in the parental strain was provided (Drew and Demain 1975c).

Several theories have been proposed as to the precise mechanism of methionine stimulation. Drew and Demain (1973) suggested methionine might act by inhibiting cysteine desulphydrase and so having a sparing effect on the intracellular pool of cysteine available for antibiotic production. However, they were unable to demonstrate any effect on this or on any other enzyme relating to the use of cysteine in metabolism. Both methionine and norleucine have been shown to cause, probably by induction, an increase in the specific activity of the ring cyclization and expansion enzymes (Sawada et al 1980c). Matsumura et al (1980) observed that the concentration of methionine present in the intracellular pool of C. acremonium rises and peaks approximately ten hours before CPC production begins. This could indicate that methionine is in fact acting as a trigger or inducer of the enzymes responsible for CPC biosynthesis. It is interesting to note that for the intracellular methionine concentration to peak in fermentations with glucose as a carbon source, it is necessary for exogenous amino acid to remain after the depletion of glucose. The preferential usage of methionine for protein synthesis rather than β-lactam production whilst glucose remains in the culture medium has also been examined by Bartoshevich et al (1985). Methionine induction of secondary metabolite

biosynthesis if present in C. acremonium may operate in a similar manner to the proposed induction by tryptophan of ergot alkaloid production in strains of Claviceps (Bu'lock and Barr 1968; Robbers et al 1972). At present the exact mechanism of methionine stimulation of CPC production is not certain and awaits further investigation.

CHAPTER 2

Mutant Isolation and Characterisation

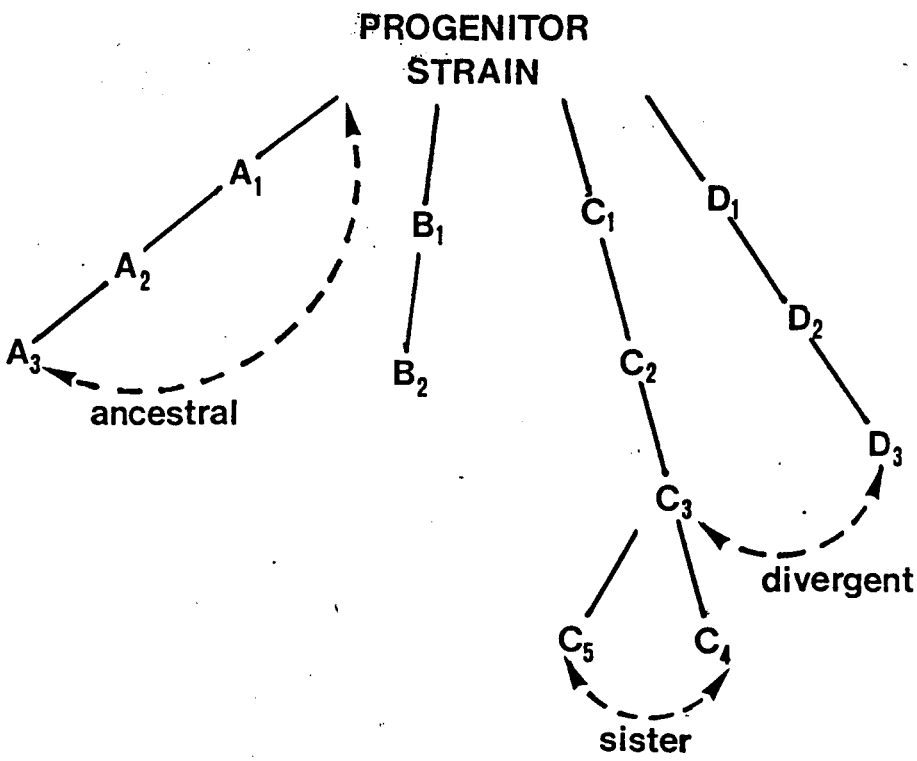
2.1 Introduction

The foundation for the study of the genetics of C. acremonium was laid by the application of protoplast fusion techniques to overcome problems associated with heterokaryon formation in this organism (Hamlyn and Ball, 1979). This made possible not only the development of a linkage map but also the examination of the inheritance of CPC titre.

Ball (1980) for simplicity of discussion, classified crosses into three main groups depending upon the degree to which the parental strains are related. Crosses can be ancestral, involving a progenitor strain from early in a selective lineage and a strain produced much later in the same selection programme; sister, where two closely related strains are crossed; or divergent and involving strains from different lines of mutagenesis and selection (Fig. 2.1). This system of classification has been applied to C. acremonium protoplast fusion crosses.

The first approach used in developing an understanding of the genetics of β -lactam production in C. acremonium mainly involved the use of ancestral and divergent crosses, although some work involved the use of two sister strains carrying mutations resulting in an impaired CPC biosynthetic pathway. The polygenic

Fig. 2.1 Classification of crosses between strains derived from a common progenitor strain.



nature of B-lactam production and the use of quite distantly related strains have meant that the data produced from these crosses has been largely quantitative with the segregation of genes having major effects on titre being quite difficult to observe over the background segregation of many alleles having pleiotropic effects on titre in the parental strains. Results have shown though, that it is possible to detect the association of effective factors influencing Pen N and CPC production with auxotrophic markers when the data are analysed using biometrical methods (Perez-Martinez 1984). However, it has not been possible to determine the linkage map location of any of the major genes coding for the enzymes directly involved in CPC biosynthesis.

The mapping of genes involved in the biosynthesis of penicillin in A. nidulans and P. chrysogenum has involved the construction of strains carrying mutations, which have major effects on antibiotic production, and the allocation of these mutations to complementation groups and linkage groups according to their segregation patterns (Edwards et al 1974; Ditchburn et al 1976; Normansell et al 1979; Makins et al 1983). To extend the information available concerning the genetics of CPC production, particularly with regard to the map location of the major genes involved, it was necessary to obtain

strains blocked in antibiotic production which were sufficiently isogenic to allow both recombination between them without interference due to phenomena such as parental genome segregation and the detection of the segregation patterns of the mutations involved with respect to each other and to other markers. To this end, the improved titre strain of C. acremonium, 20-2.1 (CO728), was chosen as the progenitor strain for the construction of a group of mutants blocked in CPC biosynthesis. Auxotrophic and drug resistance markers were also introduced where necessary to facilitate the use of these and other strains in the Nottingham collection in protoplast fusion crosses.

2.2 Materials and Methods

2.2.1 Strains

The strains used in this work had all been derived by mutagenesis and selection from M8650 C. acremonium progenitor strain. Strains 20-2.1 (CO728) and 20-2.9 an ultraviolet induced leucine auxotroph of 20-2.1 were kindly supplied by Glaxochem Ltd., Ulverston, Cumbria, U.K. Strain 20-2.1 (CO728) had been selected through six mutational steps to give an improved titre of CPC. Strain 20-3.1 (N-2) is a blocked mutant unable to convert the ACV tripeptide

precursor of CPC into isopenicillin N and was kindly supplied by Prof. Y Fujisawa (Takeda Research Laboratories, Japan). The arginine auxotroph of strain 20-2.1 and the leucine auxotroph of strain 20-3.1 were isolated following ultraviolet mutagenesis by Perez-Martinez (1984) and have been designated to be strains 20-2.3 and 20-3.4 respectively.

2.2.2 Culture Conditions

For long term storage cultures were resuspended in a medium containing three parts horse serum to one part nutrient broth (2.5% w/v) in 7.5% (w/v) glucose, lyophilized and kept at 4°C. Once revived cultures were maintained on slants of Sabouraud medium at 25°C and when necessary stored at 4°C. The composition of the media routinely used during the isolation and characterisation of mutant strains of C. acremonium is given in Table 2.1.

2.2.3 Ultraviolet (U.V.) Mutagenesis

Mutagenesis was carried out according to a protocol adapted from that of Hamlyn (1982) for the far ultraviolet (U.V.) induction of auxotrophy in C. acremonium. Cultures were grown on YEA medium to encourage sporulation. Since the production of

Table 2.1. Media Composition

Minimal medium (MM)

	gl^{-1}
Glycerol	24 ml
$Na\ NO_3$	2
$KH_2\ PO_4$	1
$Mg\ SO_4.7H_2O$	0.5
KCl	0.5
$Fe\ SO_4.7H_2O$	0.01
Agar (Difco Bacto)	20

pH adjusted to 6.8

Autoclaved 121°C, 20 min.

Osmotically stabilised minimal medium (OSMM) was MM supplemented with 40.9 g NaCl l^{-1} to give a final concentration of 0.7 M NaCl.

Sabouraud Medium

	gl^{-1}
Malt extract	24
Bacteriological Peptone	10
Maltose	40
Agar (Sigma)	20

pH adjusted to 7.5

Autoclaved 121°C, 20 min.

Yeast Extract Plus Casamino Acids Medium (YEA/Cas)

	gl^{-1}
Yeast extract	12
Casamino acids	4
Agar (Sigma)	20

Autoclaved 121°C, 20 min.

CSL medium

	gl^{-1}
Corn steep liquor	24 ml
Sucrose	20
Ammonium acetate	4.5
Ca SO_4	0.5
Mg $SO_4.7H_2O$	0.5

pH adjusted to 7.2

Autoclaved 121°C, 30 min.

Fermentation medium (fm)

	gl^{-1}
Soyabean flour	32
Sucrose	30
D-L Methionine	5
Calcium carbonate	1.5

pH adjusted to 6.8

Autoclaved 121°C, 30 min.

Plug assay fermentation media (pfm)

	gl^{-1}
Lab Lemco powder (Oxoid)	8
Soya bean flour	5
Corn steep liquor	5 ml
Ammonium acetate	2
Sucrose	36
Glucose	9
D-L Methionine	3
Agar (Sigma)	30

pH adjusted to 7.0

Autoclaved 121^{o}C, 30 min.

Where necessary media supplemented with aminoacids at 100 μg. ml^{-1} and vitamins at 10 μg. ml^{-1}.

conidia from strains with improved β-lactam titres was often poor, mutagenesis was frequently carried out on suspensions carrying a mixture of small mycelial fragments and conidia. Suspensions in water were adjusted to a density of between 10^6 and 10^7 colony forming units per ml. and exposed to U.V. radiation (2.4 joules $m.^{-2}$ sec^{-1}) for 80 sec with constant agitation, to give a theoretical survival level of between 1 and 10%. Serial dilutions of irradiated suspensions were then plated onto a recovery medium (YEA/Casamino acids) and incubated for 7-14 days at 25°C. Colonies developing were then transferred onto master plates of the recovery medium.

2.2.4 Screening and Characterisation of Mutagenised Populations.

2.2.4.1 Selection of Auxotrophic Mutants.

Colonies were replicated from YEA/Casamino acids master plates onto minimal medium (MM) using sterile cocktail sticks. Those which failed to grow were then tested on a range of MM plates supplemented with different combinations of amino acids, vitamins and nucleic acids after the method of Holliday (1956), in order to identify their growth requirements. The antibiotic production characteristics of auxotrophic

mutants were determined by the methods described in sections 2.2.4.3 and 2.2.4.4.

2.2.4.2 Selection of Drug Resistance Mutants.

Where the recovery of drug resistance mutants was required the mutagenised suspensions were normally plated, at a density of 10^6 to 10^7 colony forming units per plate, onto MM containing a toxic level of the drug in question and the growth requirements of the progenitor strain. Colonies which grew under these conditions were purified and their phenotypes confirmed.

2.2.4.3 Selection of Mutants Impaired in CPC Biosynthesis.

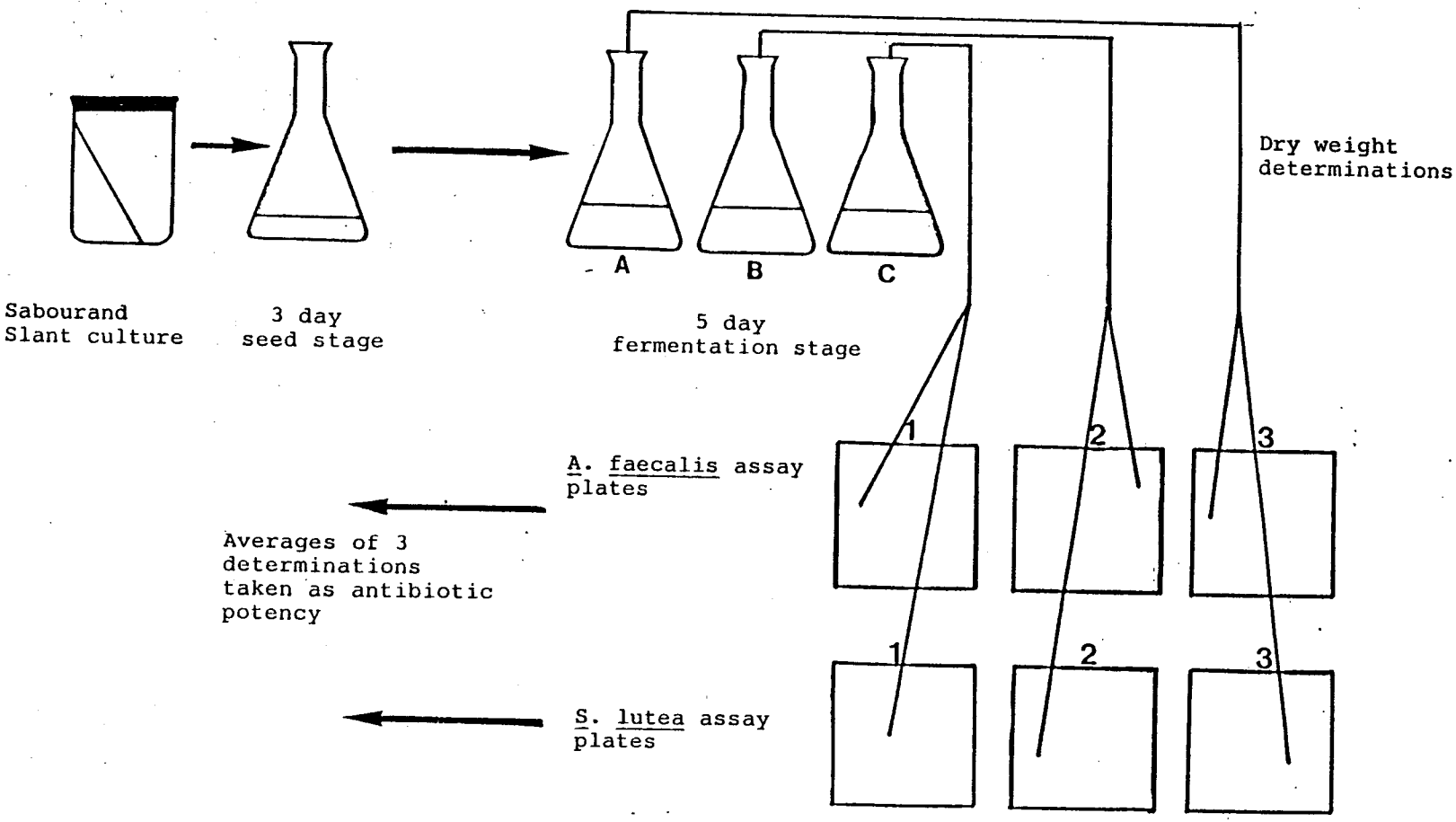
Colonies were replicated with sterile cocktail sticks from master plates onto 7 mm diameter plugs of a solidified form of a complex fermentation medium (PFM). Two plugs were inoculated for each colony to be tested. Following incubation in a humid atmosphere, at 25°C for 5 days, the plugs were assigned positions on assay plates according to a semi-random arrangement which was designed to minimise the effects of plug positioning on the results. The assay plates consisted of two layers of nutrient agar

in 30.5 cm square glass dishes the top layer of which was seeded with 0.4-0.7 ml of a fresh nutrient broth culture of Alcaligenes faecalis ATCC 8750, a bacterium sensitive to CPC but resistant to Pen N. After overnight incubation at 28°C the diameters of the zones of inhibition of bacterial growth around the plugs, due to the diffusion of CPC present in the plug cultures into the surrounding agar, were measured with callipers. Colonies which gave negative results from both plugs tested and a number of colonies giving only small zones of inhibition compared to those produced by control non-mutagenised colonies of strain 20-2.1 (CO728) were selected for further investigation.

2.2.4.4 Fermentation Analysis of Possible Blocked Mutants.

Fermentation analysis of colonies suspected of being impaired in CPC biosynthesis was carried out according to the protocol outlined in Fig. 2.2. Sabouraud slant cultures were grown for 7-14 days at 25°C before being used to inoculate the seed stage culture which was grown in 20 ml of CSL medium in 250 ml Erlenmeyer flasks. After 3 days incubation on a rotary shaker (184 rev. $min.^{-1}$ at 28°C.) three 4 ml aliquots of each culture were used to inoculate triplicate flasks containing 40 ml of a complex

Fig. 2.2 Protocol for the analysis of β-lactam production by C. acremonium



fermentation medium (FM). The flasks were incubated with shaking for a further 5 days before harvesting. Two 5 ml samples were taken from each flask. One sample was filtered through a dried and pre-weighed filter paper (Whatman No. 1, 7 cm diameter). The filter paper plus mycelium was then dried (95°C for 24 h), cooled and reweighed to determine mycelial dry weight as a measure of culture growth. The second sample was centrifuged for 15 min at 4,000 rev. min^{-1} in a bench centrifuge to pellet the mycelium and yield a clear supernatant which could be used for antibiotic determination. Bioassay plates were prepared as described previously except that two sets were made, one seeded with Alcaligenes faecalis ATCC 8750 the other with Sarcina lutea ATCC 9341 which is sensitive to Pen N and resistant to CPC. A 6 x 6 or 7 x 7 arrangement of 7 mm diameter wells were cut in the plates and 50 µl samples of the fermentation broth supernatants were placed in them. For each shake flask fermented two 50 µl samples were assayed, one against each of the two bacterial strains. Before assay for Pen N the supernatants were diluted 1 part to 3 to prevent excessively large zones of inhibition being produced. Samples were assigned to well positions according to a semi-random array designed to minimize edge effects on the assay plates. Standard solutions of Pen N and CPC (kindly supplied by Glaxo

Group Research, Greenford, Middlesex, U.K.) were freshly prepared at concentrations of 500, 200 and 50 $\mu g.ml^{-1}$ and 50 ul aliquots of these were also included in 9 wells on each of the assay plates. After overnight incubation at 28°C the diameters of the zones of inhibition around the sample wells were measured using callipers. A regression line relating the zone diameter produced to the logarithm of the antibiotic concentration was established using the results from the Pen N and CPC standard solutions and used to determine the concentrations of the antibiotics present in the fermentation broths. These concentrations were then expressed either as volumetric potencies, i.e. µg of antibiotic ml^{-1} of fermentation broth or as absolute potencies, i.e. µg. antibiotic mg^{-1} dry cell weight.

2.2.4.5 High Performance Liquid Chromatography (HPLC)

To obtain more precise information concerning the operation of the CPC biosynthetic pathway in mutants showing impaired β-lactam production samples of the centrifuged fermentation broths were examined by HPLC. The HPLC system described by Miller and Neuss (1976), was used with a Beckman model 114M pump, a model 160 U.V. detector and a mobile phase of 78.4% water, 12%

acetonitrile, 8% methanol and 1.6% glacial acetic acid. Absorbance at 254 nm was monitored and standard solutions of Pen N, deacetoxycephalosporin C (DAOC), deacetylycephalosporin C (DAC) and CPC were used to facilitate the calibration of the chromatograms with a Shimadzu model CR3A Chromatopac integrator.

HPLC separations were typically performed at a flow rate of 2 ml min^{-1} at a sensitivity of 0.05 Auf and chart speed of 1 mm min^{-1}.

2.2.4.6 Biochemical Assays of Enzymes Involved in CPC Biosynthesis.

The activities of two enzymes involved in the CPC biosynthetic pathway were examined in cell free extracts prepared from C. acremonium cultures grown under fermentation conditions.

2.2.4.6.1 Preparation of Cell Free Extracts.

Mycelium samples were recovered over a period of several hours from shake flask fermentations in FM medium, by centrifugation at 3,000 rev. $min.^{-1}$ for 10 min. The mycelium was washed in 50 mN Tris.HCL, 5 mM EDTA pH 8.0 buffer and again centrifuged to pellet. The mycelial samples were frozen in a dry ice/ethanol

bath and stored at -15°C. until use. Crude cell extracts were prepared by grinding the frozen material with sand, resuspending in 100 mM Tris.HCl pH 8.0 and centrifuging (15,000 rev.min.$^{-1}$, 5 min.) to pellet the cell debris. Supernatants were taken as cell extracts and stored on ice.

2.2.4.6.2 Assay of Isopenicillin N Synthetase Activity.

Isopenicillin N synthetase (IPNS) catalyses the conversion of the ACV tripeptide precursor molecule of CPC to isopenicillin N. Its activity may be monitored by virtue of the fact that the end product of the reaction, isopenicillin N, will react with a mixture of imidazole and mercuric chloride to give a chromophore the presence of which may be detected as an increase in optical density at 320 nm of a reaction mixture when compared to a blank sample. The protocol used to assay IPNS activity was kindly supplied by Glaxo Group Research Ltd., Greenford, Middlexex, U.K. . Assays were carried out in a total volume of 1 ml of reaction mixture as described in Table 2.2. Blanks were included for each assay with 500 µl of 0.5 M Tris.HCL pH 8.0 replacing the ACV tripeptide substrate. Reaction mixture were incubated at 30°C for 10 min. and were stopped by the

Table 2.2

Isopenicillin N synthetase assay reaction mixture

Component	Stock Solution mM	Volume Added µl	Final Conc.
ACV monomer	2 mM (in 0.5M Tris HCl) pH8.0	500 µl	1mM
Ascorbic acid	20 mM	10 µl	0.2mM
$FeSO_4$ .7 H_2O	5 mM	10 µl	50 µM
B-mecaptoethanol	100 mM	10 µl	1 mM
Cell extract	in 100mM Tris. HCl pH 8.0	25-400 µl	
Tris HCl pH 8.0	100 mM	to 1 ml	

addition of 5 ml of imidazole/mercuric chloride reagent. The reagent was prepared by combining solutions A (8.55g imidazole dissolved in approximately 70 ml of water and adjusted with concentrated HCl to give a pH of 65.8) and B (0.027g of mercuric chloride dissolved in approximately 20 ml of water) and adjusting the volume to 100 ml with water. After standing at room temperature for between 30 and 50 min. the A_{320} of the reaction mixtures were then read against the respective blanks. A standard curve relating the A_{320} to penicillin concentration was prepared using reaction mixtures containing a range of concentrations of penicillin G in place of the ACT tripeptide substrate and enzyme extract. Penicillin G was used to construct the standard curve due to difficulties in obtaining pure preparations of Pen N.

2.2.4.6.3 Assay of AcetylCoA:deacetylcephalosporin C O-acetyltransferase Activity.

The final step of CPC biosynthesis involves the acetylation of DAC. A method for the assay of the enzyme responsible for this reaction was adapted from that described by Sch eidegger et al (1985). Cell extracts were added to the reaction mix described in Table 2.3 and incubated at 30°C. for 30 min. Rather

Table 2.3

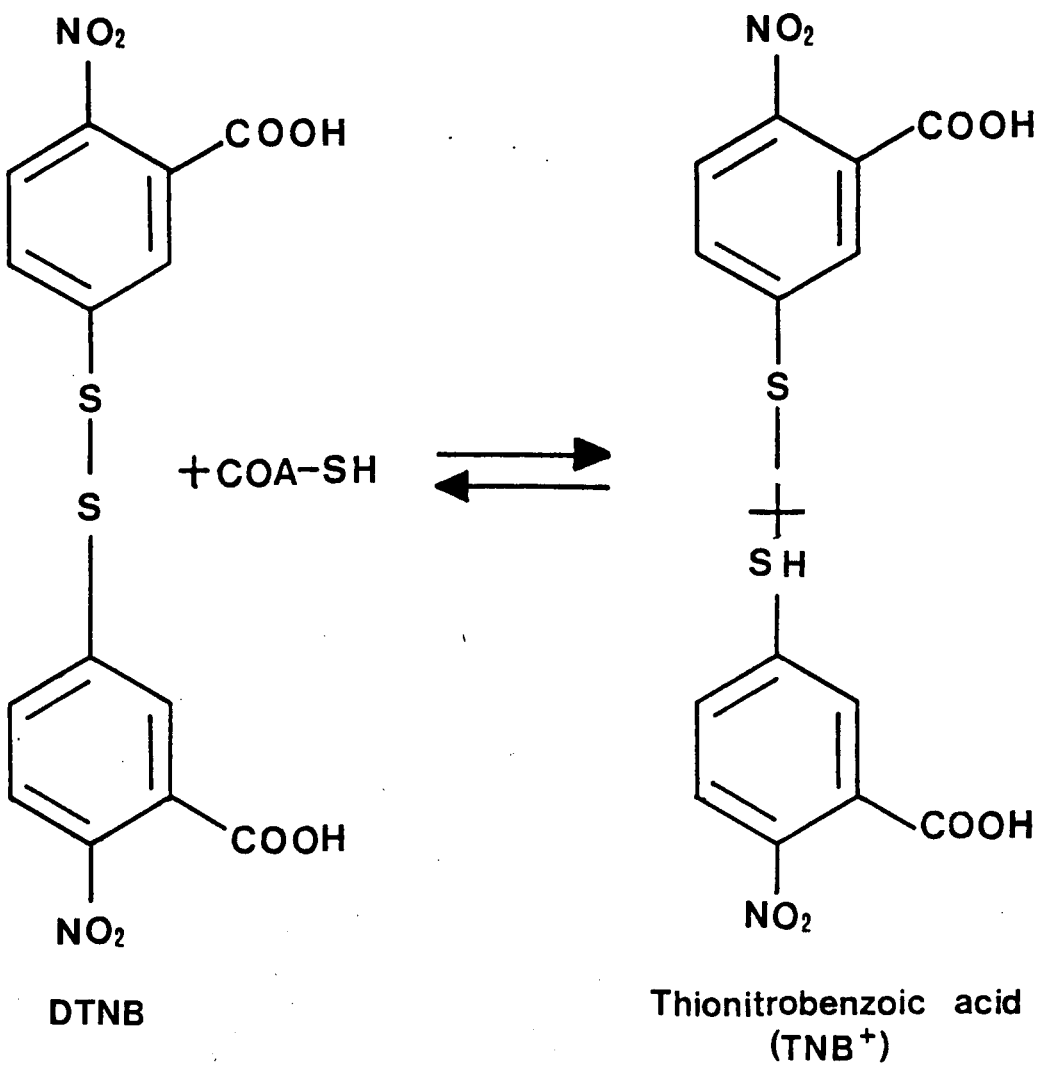
Acetyl CoA: deacetylcephalosporin C O-acetyltransferase assay reaction mixture

Component	Stock Solution mM	Volume Added µl	Final Conc
DAC	100 mM	20 µl	2 mM
AcetylCoenzyme A	10 mM	20 µl	200 µM
Cell Extract	in 100 mM TRis. HCl pH 8.0	20-500 µl	
Tris. HCl)	100 mM)		
$MgCl_2$ $6H_2O$)	5 mM) pH 8.0	to 1ml	

than monitor the formation of CPC by HPLC as in the published method, the possibility of using a spectrophotometric assay was investigated. When DAC is acetylated to yield CPC the acetylCoA used as the acetyl group donor yields free CoA-SH. This will react with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) to give CoAS-TNB plus thionitrobenzoic acid (TNB+) (Fig. 2.3). The liberated TNB+ gives a yellow colouration to the reaction mixture of pH 8.0 which may be quantified in terms of absorbance at 412nm. Since the yield should be in proportion to the amount of CPC produced addition of DTNB to the reaction mixture at the end of the assay and monitoring the A_{412} of the mixtures should give a measure of the acetyl CoA:deacetylcephalosporin C O-acetyltransferase activity in cell free extracts. Therefore after the 30 min incubation DTNB was added to the reaction mixtures to a final concentration of 200uM and the A_{412} monitored. Three control reactions were carried out for each assay in which either the DAC, cell extract of DAC plus cell extract portions of the reaction mixture were replaced by buffer. These allowed the monitoring of background non-DAC specific acetyltransferase activities.

The activity of citrate synthase which catalyses the condensation of acetylCoA with oxaloacetate to give citrate and free CoA-SH in the cell extracts was

Fig. 2.3 Scheme for the reaction of 5', 5' - dithiobis (2-nitrobenzoic acid) (DTNB) with CoA-SH



From: Lehninger 1975

also monitored. Oxaloacetate was added to a final concentration of 20μM in the reaction mixture in place of the DAC substrate. This reaction served as an internal standard ensuring that cell breakage and enzyme release had occurred during the preparation of the cell extracts tested.

2.2.4.6.4 Preparation of Acetyl Coenzyme A.

Acetyl CoA was prepared from CoASH by the method described by Ochoa (1955). CoASH (Sigma) 50 mg was dissolved in 4 ml water and a 10ul sample removed for the control test. $NaHCO_3$ (0.4 ml of a 1M solution) plus 7μl of acetic anhydride were added with vigorous mixing and the mixture incubated on ice for 20 min before a further 10μl sample was removed. To test for the removal of the sulphydryl groups from CoASH 20μl of a stock solution of DTNB plus 900μl of 500mM Tris.HCl pH 8.0 were added to the two 10μl samples saved during the preparation. The first sample would produce a yellow colouration due to the presence of CoASH and the second sample would only produce a yellow colouration if some CoASH remained after reaction with acetic anhydride. As some CoASH remained the pH of the solution was adjusted to approximately pH 7.5 and further acetic anhydride was added until no reaction with DTNB was seen. The pH

of the acetyl CoA solution was then adjusted to pH 6.5 with 0. 1 N HCl and the solution stored at -20°C until use.

2.2.4.6.5 Protein Determinations

The protein content of cell free preparations was routinely determined using the Bio Rad Protein Assay reagent (BioRad Laboratories) according to the manufacturer's instructions, with bovine serum albumin as a standard.

2.3 Results

2.3.1 Induction and Characterisation of Auxotrophic Mutations.

U.V. mutagenesis of strains 20-2.1 (CO782) and two other strains derived from it, 20-2.9 and 20-2.3 carrying requirements for leucine and arginine respectively, resulted in the isolation of a number of strains carrying new auxotrophic requirements. Table 2.4 shows the identification of these and their antibiotic titres, where available. Auxotrophic requirements were also introduced into several of the blocked mutants derived from 20-2.1 and the resulting strains are listed in Table 2.5. Mutagenesis of the

Table 2.4 Strains derived by ultraviolet mutagenesis from 20-2.1 (CO728), 20-2.9 (leu-5) and 20-2.3 (arg-8)

Strain Number	Auxotrophic markers		Antibiotic Titre ($\mu g.mg^{-1}$ drg weight)	
			Pen N	CPC
20-2.10	leu-5	lys-2	0	0
20-2.11	leu-5	met-8	119.3	27.7
20-2.12	arg-8	met-9	10.6	1.6
20-2.13	arg-8	leu-6	6.0	1.7
20-2.14	arg-8	leu-7	trace?	0
20-2.15	arg-8	met-11	-	-
20-2.16	arg-8	met-10	10.2	0.7
20-2.17	arg-8	ade-1	4.72	0
20-2.18	cys-1		-	-
20-2.19	cys-2		-	-

Table 2.5 Auxotrophic requirements introduced into strains impaired in the biosynthesis of CPC.

Strain Number	Progenitor Strain	Markers		
20-2.41	20-2.32	cnp-5	met-12	
20-2.42	20-2.32	cnp-5	orn-1	
20-2.43	20-2.35	cnp-6	met-13	
20-2.44	20-2.26	cnp-1	ino-2	
20-2.45	20-2.26	cnp-1	arg-9	
20-2.46	20-2.26	cnp-1	leu-8	
20-2.47	20-2.27	cnp-2	met-14	
20-3.11*	20-3.4	cnp-7	leu-4	his-2
20-3.12	20-3.4	cnp-7	leu-4	arg-10

* required YEA+Casamino acids medium to be supplemented with histidine

blocked mutants was carried out using cultures grown from single colony isolates, the antibiotic production characteristics of which had been verified, for strains 20-2.27, 20-2.35 and 20-2.26 by bioassay of FM plug cultures and for strain 20-2.32 by the biochemical assay of IPNS activity. However, despite this the auxotrophic strains derived from 20-2.32 when assayed produced a small amount of activity against Sarcina lutea ATCC 9341 revealing the production of low amounts of Pen N.

2.3.2 Induction and Characterisation of Drug Resistance Markers.

Resistances to 6-azauracil and 2-(4′-thiazoly) benzimidazole (thiabendazole TBZ) were introduced into a number of strains for possible future use as either selectable or secondary markers in protoplast fusion crosses. Table 2.6 details the strains obtained. Where these were to be used in crosses their antibiotic titres were confirmed by plug assay. Strains selected for thiabendazole resistance showed varying levels of resistance to this compound and Table 2.7 compares the resistance of a number of isolates of 20-2.11 after U.V. mutagenesis and selection on 25μM TBZ.

Table 2.6 Drug resistance markers introduced into strains on the 20-2.1 (CO728) and 20-3.1 (N-2) lineages

Strain Number	Progenitor Strain	Markers				Isolation Procedure	Selective Concentration of Fungicide
20-2.22	20-2.1	tbz-1				U.V.	20 µM
20-2.20	20-2.17	arg-8	ade-1	azu-2		Spontaneous	1 mg. ml^{-1}
20-2.33	20-2.11	leu-5	met-8	tbz-2		U.V.	25 µM
20-2.34	20.2.11	leu-5	met-8	azu-3		U.V.	1 mg. ml^{-1}
20-2.36	20-2.33	leu-5	met-8	tbz-2	azu-4	Spontaneous	1 mg. ml^{-1}
20-2.37	20-2.28	cnp-3	tbz-3			U.V.	20 µM
20-2.38	20-2.29	cnp-4	azu-5			U.V.	1 mg. ml^{-1}
20-2.39	20-2.32	cnp-5	tbz-4			U.V.	30 µM*
20-2.40	20-2.35	cnp-6	tbz-5			U.V.	30 µM*
20-2.48	20-2.42	cnp-5	orn-1	tbz-8		U.V.	30 µM
20-2.49	20-2.43	cnp-6	met-13	tbz-9		U.V.	30 µM
20-3.10	20-3.4	cnp-7	leu-4	tbz-6		U.V.	30 µM*
20-3.13	20-3.11	cnp-7	leu-4	his-2	tbz-7	U.V.	30 µM

N.B. * The frequency of spontaneous resistance to thiabendozole was 1 in 1 x 10^8 in YEA/Casamino acids medium

Table 2.7 Varying levels of resistance amongst isolates selected on 25 µM thiabendazole. following ultraviolet mutagenesis.

Strain		TBZ (µM) 0	0.1	1	5	10	25	50	100	1000
20-2.1 (Co 728)		+	+	+	+	+/-	-	-	-	-
20-2.11		+	+	+	+	+/-	-	-	-	-
20-2.11	isolate 3	+	+	+	+	+	+	+	+	+/-
After UV	isolate 5	+	+	+	+	+	+	-/+	-	-
and selection										
as TB2	isolate 9	+	+	+	+	+	+	-	-	-
	isolate 10	+	+	+	+	+	+	+	+	-/+
	isolate 11*	+	+	+	+	+	+	+	+	-/+

\+ normal growth

+/- reduced growth

-/+ poor growth

\- no growth

* isolate 11 was selected as strain 20-2.33

2.3.3 Isolation of Mutants Impaired in CPC Biosynthesis.

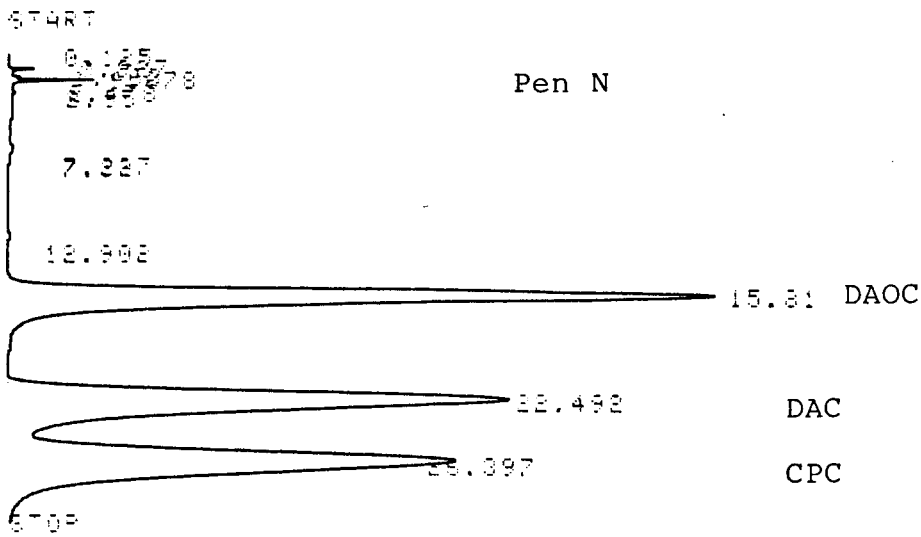
U.V. irradiation of spores and small hyphal fragments from the raised titre strain 20-2.1 led to the isolation of a number of strains with reduced capacities to produce CPC. The surviving populations from two mutagenesis experiments were screened by plug assay. Of 491 isolates screened following the first mutagenesis 17 were chosen as potential mutants and further examined in shake flask cultures. From the second mutagenesis 18 out of 550 isolates were selected. All of these were prototrophic. Since the aim was to produce blocked mutants, the phenotypes of which could be readily observed segregating amongst the progeny from crosses, the first group of mutants examined closely were those showing a complete absence of CPC production when shake flask fermentation tests were carried out. Of the four strains falling into this class, isolate 19/5, which at first appeared to be a non-producer of Pen N, when subsequently fermented did produce substantial titres of Pen N. Over two fermentations this isolate gave an average of 65.4% of the Pen N titre and 20.6%. of the CPC titre of the control 20-2.1 producing strain. As a result of this observation isolate 19/5 was not considered desirable for use as a blocked mutant in future

crosses although its reduced titre phenotype might warrant further investigation. Isolate 129/13 on fermentation appeared blocked before Pen N in the biosynthetic pathway and isolates 10/2 and 18/16 produced some Pen N but no CPC. Examination of a fresh culture of isolate 18/11 showed that this too had similar characteristics to isolates 10/2 and 18/16 and it was added to the group of mutants for further study. In addition, two isolates, 136/9 and 142/9 were selected as examples of strains which though still competent for the whole of the CPC biosynthetic pathway, produced lowered B-lactam titres.

2.3.4 HPLC Characterisation of Mutants Impaired in CPC Production.

Fermentation broths of the six mutants selected were examined by HPLC. The system used, allowed the detection of the intermediates involved in CPC biosynthesis subsequent to the formation of the ACV tripeptide, with the exception that it was not possible to distinguish between isopenicillin N and Pen N. The separation of standard samples of isopenicillin N, Pen N, DAOC, DAC and CPC which could be obtained is shown in Fig.2.4. The performance of four of the mutants in one five day fermentation experiment compared to that of a control 20.2.1

Fig. 2.4 **H.P.L.C. separation of Pen N/ Isopenicillin N, DAOC, DAC and CPC standards.**



Footnote Sample volume was 20 µl containing 5 µg of each standard compound.
Retention times are shown in minutes.

fermentation is detailed in Table 2.8. The presence of ACV tripeptide could be observed but the low absorbance at 254 nm of this compound coupled with its occurrence as a dimer in the broths complicates its detection so that the values given are only approximate. Pen N titre values calculated from bioassay data are also included in Table 2.8 and show a discrepancy with the values obtained by HPLC. Constituents of the fermentation broths, not related to the CPC pathway resulted in peaks of absorbance at 254 nm early in the chromatographic separation. The most likely explanation for the abnormally high isopenicillin N/Pen N HPLC results would appear to be the co-elution of one of these with the antibiotic peak. Chromatograms of fermentation broths from 20-2.1 (CO728) and mutants 18/11 (20-2.35) cnp-6 and 18/16 (20-2.26) cnp-1 are shown in Fig. 2.5. Strain 20-2.35 produced a 614.9 $\mu g.ml^{-1}$ Pen N and strain 20-2.26 173.7 $\mu g.ml^{-1}$. Neither of these strains produced any intermediate on bioassay subsequent to Pen N in the pathway.

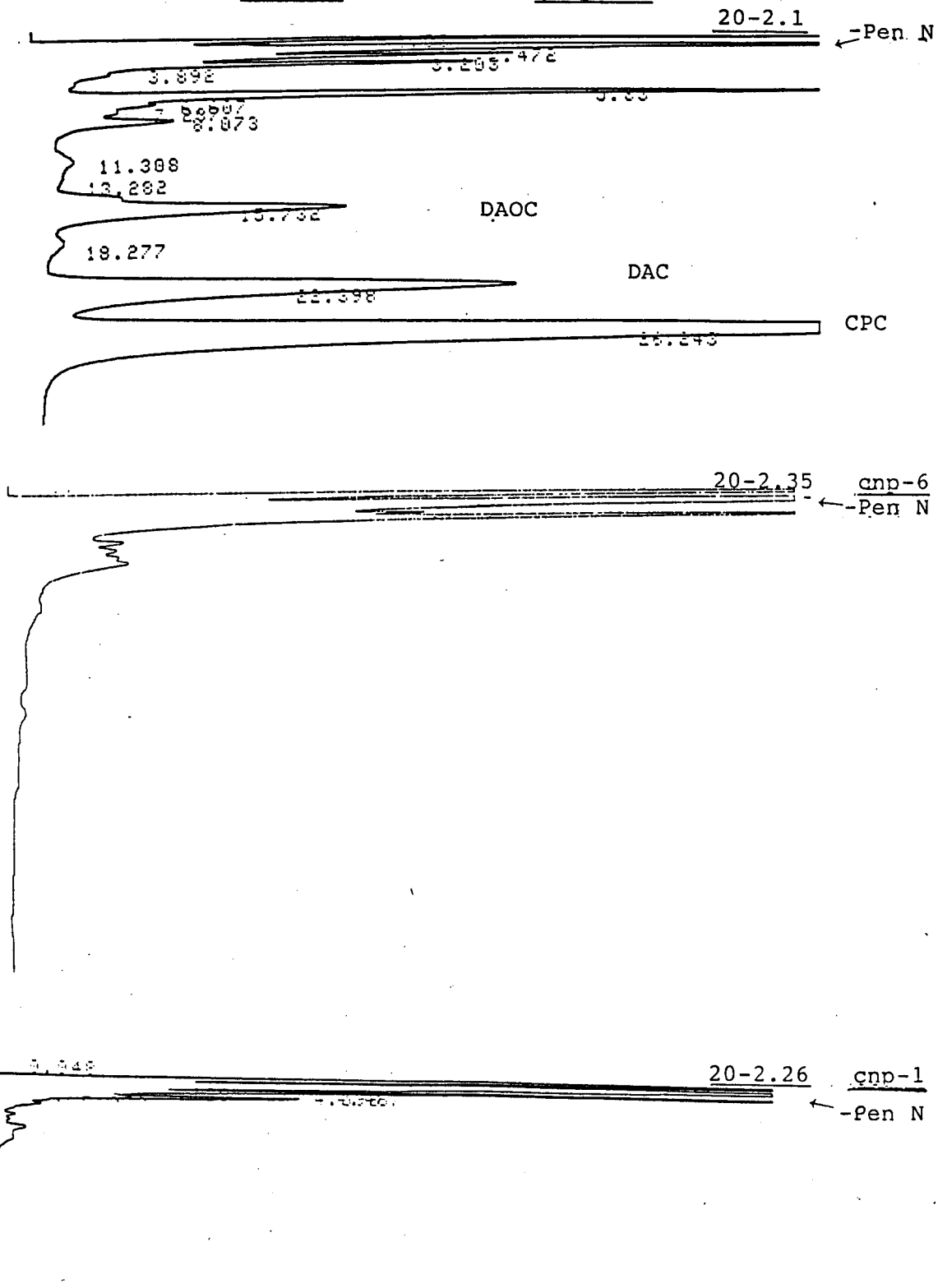
2.3.5 Isopenicillin N Synthetase (IPNS) Assay

The level of IPNS activity present in cell extracts of three strains was examined. Strain 20-2.1 was compared to both 20-3.1 cnp-7 known to be deficient in

Table 2.8 Production of intermediates and CPC by cnp mutants derived from strain 20-2.1 (CO728)

Isolate	Strain Number	Marker	ACV % of 20-2.1 (CO728)	Pen N/ Isopen N on HPLC %20-2.1 (CO728)	Pen N bioassay data $\mu g.mg^{-1}$	Pen N bioassay data % 20-2. (CO728)	DAOC		DAC		CPC	
							HPCC data $\mu g.mg^{-2}$	% of 20-2.1 (CO782)	HPLC data $\mu g.mg^{-1}$	% of 20-2.1 (CO728)	HPLCC data $\mu g.mg^{-1}$	% of 20-2.1 (CO728)
CO728	20-2.1		100	100	446.2	100	125	100	280	100	775	100
10/2	20-2.27	cnp-2	-	199.3	131	29.4	0	0	0	0	0	0
129/13	20-2.32	cnp-5	55.1	127.3	0	0	0	0	0	0	0	0
136/9	20-2.28	cnp-3	15.2	162.7	314.6	70.5	40	22.8	50	17.8	45	5.8
142/9	20-2.29	cnp-4	4.0	64.9	228.6	51.2	15	86	25	8.9	45	5.8

Fig. 2.5 H.P.L.C. separation of fermentation broths from strains 20-2.1 (CO728), 20-2.35 (cnp-6) and 20-2.26 (cnp-1)*



* results are from separate fermentation experiments.

the production of isopenicillin N and to 20-2.32 (cnp-5) the U.V. induced blocked mutant suspected of resembling 20-3.1 in lacking IPNS activity. As Pen N formation commences quite early in C. acremonium fermentations cell extracts were prepared from 72h. shake flask fermentation cultures in the first instance. Assays of extracts made at later stages of the fermentation showed that IPNS activity in 20-2.1 declines after 72 h. (Table 2.9). Extracts from a 72h. culture of strain 20-3.1 as expected showed negligable IPNS activity, the level being below the limits of detection (Table 2.10). On comparison of the IPNS activity present in 20-2.32 with that in 20-2.1 it was found that whilst the mutant had a much reduced capacity to synthesise isopenicillin N, showing only 24.3% of the IPNS activity present in the control, some synthesis was occurring (Table 2.11). This prompted a closer examination of strain 20-2.32. Bioassay of single colony isolates grown on plugs of FM revealed that several of the colonies tested produced a small zone of inhibition on plates seeded with Alcaligenes faecalis ATCC 8750. Therefore, the original culture was suspected to have been mixed and a non-producing isolate of strain 20-2.32 was selected and the IPNS assay repeated this time showing the absence of IPNS activity (Table 2.12).

Table 2.9 Time course of IPNS activity in the latter stages of the fermentation of 20-2.1

Hours of fermentation	Volume of extract assayed	A320-blank	µg Pen G equivalent	Protein conc$_1$ mg ml^{-1}	Specific Activity µg isopenN mg protein^{-1}
72	400µl	0.108	7.5	0.87	21.5
96	400µl	0.065	2.5	0.58	10.7
120	400µl	0.025	<	0.4	<

Table 2.10 A comparison of the IPNS activity present in 20-2.1 and 20-3.1 after 72 h. fermentation.

Strain	Volume of extract assayed	A320-blank	µg Pen G equivalent	Protein conc$_1$ mg ml^{-1}	Specific Activity µg$_{-1}$ isopenN mg protein^{-1}
20-2.1	25 µl	0.025	2.5		108.7
20-2.1	100 µl	0.09	10	0.92	108.7
20-2.1	400 µl	0.255	28.5		77.4
20-3.1	400 µl	0.005	<	1.11	<

Table 2-11 A comparison of IPNS activity in 20-2.1 and 20-2.32 after 72 h. fermentation

Strain	Volume of extract assayed	A320-blank	µg Pen B equivalent	Protein conc $mg.ml^{-1}$	Specific Activity μg_{-1} isopenN mg^{-1} protein $^{-1}$
20-2.1	400 µl	0.21	20	1.07	46
20-2.32	400 µl	0.113	11	2.45	11.2

Table 2-12 A comparison of IPNS activity in 20-2.1 and a single colony isolate of 20-2.32 after 72 h. fermentation.

Strain	Volume of extract assayed	A320-blank	µg Pen B equivalent	Protein conc $mg.ml^{-1}$	Specific Activity μg_{-1} isopenN mg^{-1} protein $^{-1}$
20-2.1	400 µl	0.1	10	1.27	19.7
20-2.32	400 µl	0.03	<	1.47	<

2.3.6 AcetylCoA:deacetylcephalosporin C O-acetyltransferase Assay.

Attempts to adapt the assay method of Scheidegger et al 1985 for use with crude cell preparations were unsuccessful. The use of DTNB and a spectrophotometric assay of activity, rather than HPLC analysis, to detect the amount of CPC product formed appeared to be feasible. Assays of citrate synthase activity using this system were successful in demonstrating that cell breakage had occurred with the DTNB reaction allowing the monitoring of the activity. However, no release of TNB+ above that occurring in the blank controls was seen when DAC acted as substrate. HPLC analysis of reaction mixtures revealed that there was no CPC formation occurring and a variety of changes were made to the assay protocol to attempt to remedy the situation. For example, cell extracts from 96 h. cultures were tested to check for the presence of enzyme activity later in the fermentation than expected, but no activity was seen. Addition of phenylmethylsulfonyl fluoride (PMSF) as a protease inhibitor to the cell extracts and the purification of extracts by either dialysis or ammonium sulphate precipitation also failed to improve the assay. A similar result was obtained when a variety of concentrations of freshly prepared

acetyl CoA were used. It is possible that the failure of the enzyme assay was due to insufficient purification of the crude cell extracts as the protocol from which the assay was adapted did involve more sophisticated protein purification techniques but no further attempts to purify the enzyme were made.

2.3.7 Strain Stability

During examination of the phenotypes of the blocked mutants derived from strain 20-2.1, it was noticeable that after sub-culturing there was a tendency for cultures previously showing a complete lack of ability to produce one or more of the β-lactams of interest on fermentation, to produce a low titre of the compound(s). When single colony isolates of these cultures were examined by plug assay, it was usually found that a small number produced the B-lactam concerned whilst most remained negative. The use of single colony isolates with confirmed titre characteristics allowed work to progress. However, the problem was more acute with strain 20-2.32. Biochemical assay of IPNS activity allowed the selection of an isolate showing no detectable activity for this enzyme. Auxotrophic markers were then introduced, however, when the marked strains were assayed for β-lactam production a small amount of Pen

N was detected.

2.4 Discussion

To facilitate future investigations into the genetic basis of CPC biosynthesis, a programme of mutagenesis was initiated. This covered two main areas.

A group of strains carrying mutations affecting the CPC biosynthetic pathway were derived by U.V. mutagenesis from the improved titre strain 20-2.1. The mutations induced were characterised with regard to the effect they exerted on the production of intermediate compounds in the CPC biosynthetic pathway. The apparent blocks in biosynthesis found may be an indication that the mutations affected either structural or regulatory genes concerned with CPC biosynthesis and further biochemical and genetical studies would be required to clarify this. HPLC analysis coupled with Pen N determinations by bioassay showed strains 20-2.27, 20-2.35 and 20-2.26 to be deficient in the conversion of Pen N into DAOC, i.e. in the ring expansion step of CPC biosynthesis. However, whilst 20-2.35 produced high levels of Pen N the other two strains produced lower levels of Pen N than the pro-genitor strain 20-2.1. This could indicate a reduction in the flow of intermediates

before Pen N in the pathway, although it might be expected that some conversion of Pen N into DAOC would be seen if the ring expansion enzyme was functional. Strains 20-2.28 and 20-2.29 both appear to be examples of mutants with a reduction in the flow of intermediates through the whole pathway. The remaining strain 20-2.32 appeared unable to synthesise isopenicillin N and as such resembled the Takeda IPNS mutant 20-3.1. It is not known whether the low level of Pen N/isopenicillin N detected in fermentation broths of 20-2.32 represented a true production of these compounds at a level below that detectable by the other assay methods employed or was purely the result of the co-elution of medium constituents. The use of other HPLC systems designed to separate Pen N and isopenicillin N from other substances present in fermentation broths would clarify this. It is notable that strains blocked between DAOC and DAC or between DAC and CPC were not obtained. Scheidegger et al (1984) and Dotzlaf and Yeh 1987 have proposed that one protein is responsible for both the ring expansion of Pen N to form DAOC and the hydroxylation of DAOC to give DAC in C. acremonium and this could have implications with respect to the types of mutants isolated.

The lack of detection of mutants deficient in the conversion of DAC into CPC could be the consequence of

the selection procedure adopted. If such mutants were leaky, allowing some CPC production as in the case of the Takeda mutant M40 (20-3.2) then a more detailed examination of strains with reduced CPC titres needs to be carried out in order to detect those accumulating DAC. Further mutagenesis to introduce auxo-trophic and drug resistance markers was carried out on the strains blocked in CPC production to enable their use in protoplast fusion crosses. As noted in section 2.3.7 the auxotrophically marked strains derived from 20-2.32 were found to produce a small but detectable amount of Pen N on bioassay.

Problems associated with variable antibiotic production characteristics had been encountered previously during the isolation of the blocked strains. The use of mixtures of uninucleate spores and small hyphal fragments which could have contained more than one nucleus per colony forming unit in the mutagenesis procedure may have led to the original isolation of mixed cultures following mutagenesis. Heterogeneous cultures may also have been isolated if U.V. mutagenesis in C. acremonium gave rise to mosaics as in yeast (Nasim and Auerbach 1966). These problems appeared to be overcome by the purification of single colony isolates although it was possible that some heterogeneity may have remained.

In the case of strain 20-2.32, however, the IPNS

deficient nature of a single colony isolate had been biochemically verified before the introduction of auxotrophic markers, and the reason for the restoration of a low level of Pen N production appears unclear. It is possible that the variation in antibiotic production by these mutants was not due to the isolation of mixed cultures but to the occurrence of a second mutation which modified or suppressed the effects of the first.

A detailed genetic analysis of the strain(s) in question would be required to investigate this possibility.

The second main area covered by the programme of mutagenesis was the construction of marked strains suitable for use in studies aimed at mapping mutations affecting the CPC biosynthetic pathway. Auxotrophically marked strains were already available for mapping studies following the work of Hamlyn (1982) and Perez-Martinez (1984) and included one strain 20-1.4 (azu-1 arg-1 mor-1 leu-1 red-1 pyt-1 ben-1) which covered markers on 7 of the 8 established C. acremonium linkage groups. However, the use of such strains in crosses designed to map mutations reducing CPC titre presents two problems. Firstly, the auxotrophically marked strains were derived from the low CPC titre M8560 progenitor strain and consequently the segregation of genes affecting

Figure 2.6 *C acremonium* strains of the 20-2.1 (CC728) lineage

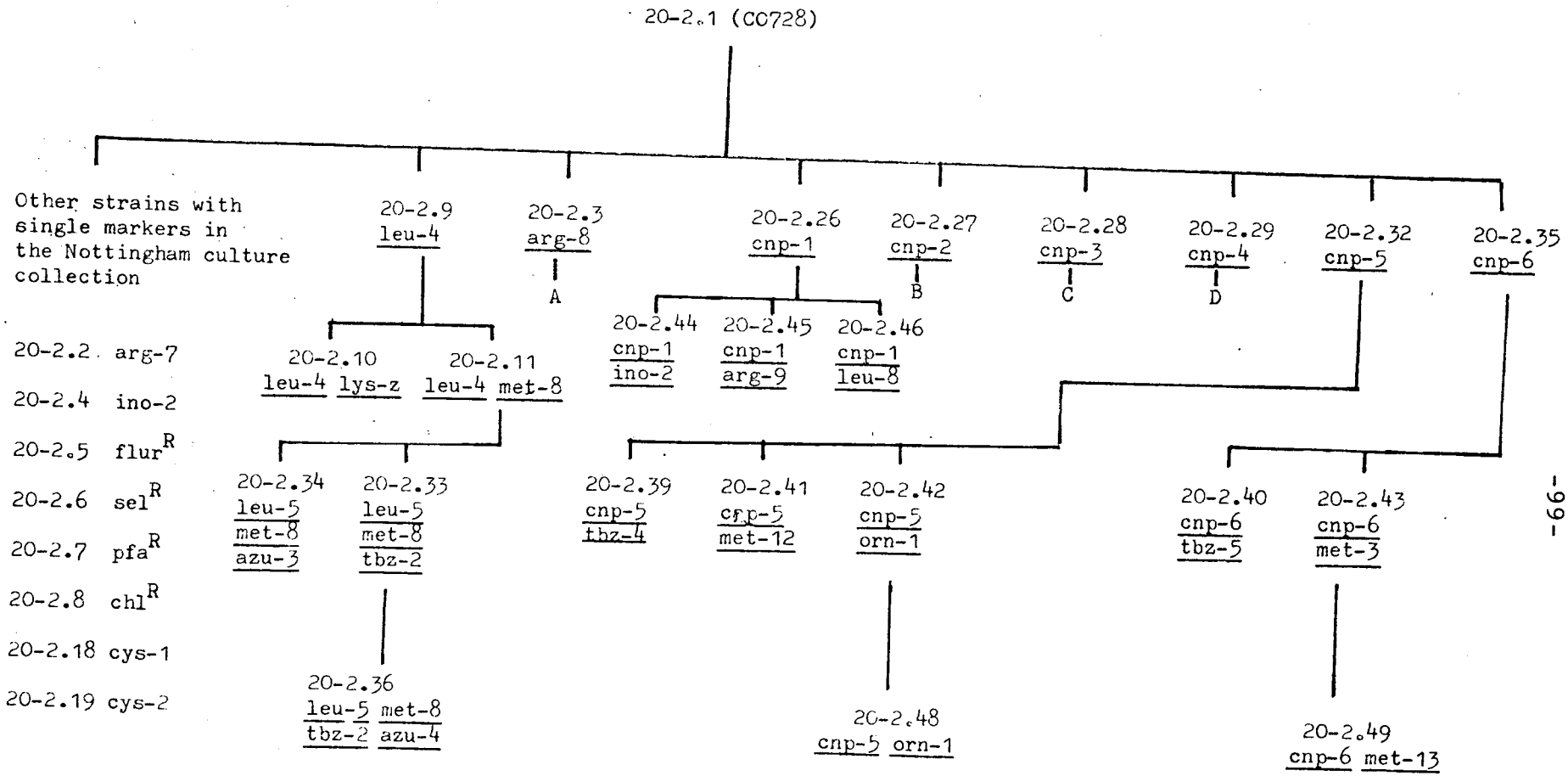
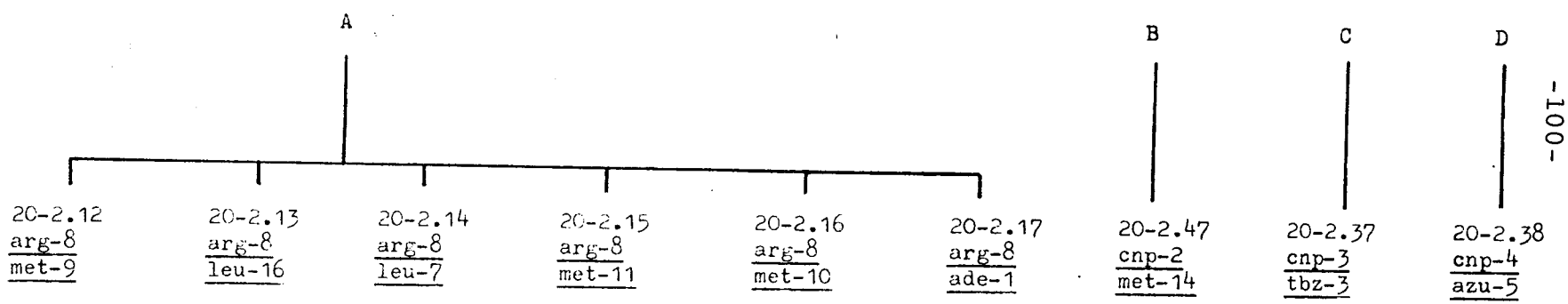


Figure 2.6 (continued) C acremonium strains of the 20-2.1 (C0728) lineage



titre might be anticipated to be difficult in crosses involving such strains. Secondly, the divergent nature of the M8650 strains and the 20-2.1 derived blocked mutants could suggest that barriers to recombination would be encountered in crosses between the two lineages. Therefore, it was considered desirable to develop the marked derivatives of strain 20-2.1 shown in Fig. 2.6 for use in future mapping work. A number of markers were also introduced into the Takeda IPNS deficient strain (20-3.4) to facilitate its use in crosses.

The introduction of auxotrophic markers into antibiotic producing P. chrysogenum strains is reported to occasionally result in a loss of or reduction in titre (MacDonald 1963) as has the continued subculturing of strains (MacDonald 1968). Following the introduction of markers into strain 20-2.1 and the subculturing necessarily involved in this procedure, many of the marked strains produced have greatly reduced titres. Strain 20-2.33, however, showed little reduction in titre making this a promising strain for future mapping work, although the inclusion of further markers, e.g spontaneous resistance markers, would enhance its value even more. Fig. 2.6 shows the 20-2.1 derived strains available, at the conclusion of this programme of mutagenesis, for use in the genetic studies described here.

CHAPTER 3

Genetic Analysis in C. acremonium Strain 20-2.1

3.1 Introduction

Previous work with the protoplast fusion system developed for use in C. acremonium resulted in the establishment of a linkage map (Hamlyn, 1982; Perez-Martinez, 1984). The markers positioned on this map were all induced in isogenic strains derived from the M8650 low CPC titre progenitor strain. Crosses between M 8650 marked strains and strains derived from improved titre lines illustrated the complex nature of the genetic basis of B-lactam production in this organism, with many alleles affecting titre segregating in each cross. Two effective factors, one for Pen N and one for CPC production were located on linkage groups VIII and VI respectively (Perez-Martinez, 1984).

To extend the understanding of the genetic basis of CPC biosynthesis further crosses aimed at relating mutations affecting titre to linkage groups, defined by auxotrophic and drug resistance markers, are necessary. As the segregation of alleles influencing titre may be more easily observed when strains from lineages of higher titre than the M8650 line are used the work described here has made use of the strains derived from 20-2.1 discussed in the previous Chapter.

Two groups of protoplast fusion crosses were performed. The first were between auxotrophically marked strains and designed to give information concerning the linkage group relationships between several of the markers available in the 20-2.1 series. The second group involved were crosses between a marked 20-2.1 strain which had retained a raised titre of CPC and blocked mutants produced from 20-2.1.

3.2 Materials and Methods

3.2.1 Strains and Culture Conditions

The strains used were as described in Chapter 2. Cultures were maintained on slants of Sabouraud's medium. Following fusion protoplasts were cultured on selective media based on a minimal medium (MM) osmotically stabilised by the addition of 0.7M NaCl (40.9 $g.l^{-1}$). The other media used are described in Table 2.1.

3.2.2 Protoplast Isolation and Fusion

The methods for protoplast isolation and fusion were based on those used by Hamlyn (1982) and Perez-Martinez (1984). Shake flask cultures (20ml CSL medium supplemented with the appropriate growth

requirements) were inoculated with mycelium scraped from Sabouraud slant cultures and incubated on a rotary shaker (184 rev. min. $^{-1}$ at 28°C) for 48 h to provide a seed culture. Four ml of this was used as inoculum to shake flasks containing 40 ml CSL medium (after the removal of the sediment present in the medium) plus growth requirements, and the incubation continued for a further 24 h. Mycelium was harvested through sintered glass filters (porosity 1), washed with water followed by buffer solution A (McIlvaine's phosphate-citrate buffer, 0.2M $NaHPO_4$, 0.1M citric acid, pH 7.3) and transf erred to 100 ml conical flasks. Buffer A (20 ml) supplemented with 0.02M β-mercaptoethanol was added and the mycelium incubated with gentle shaking for 10 min at 28°C. Following filtration and washing with water, the mycelium was recovered on discs of filter paper, transferred to the lytic digestion mixture in a ratio of 1 to 10 (weight to volume) and incubated with gentle shaking at 28°C for 3 h. The digestion mixture was prepared by dissolving 10 mg.ml^{-1} Cellulase CP (John E Sturge Ltd., Selby, N. Yorks. U.K.) plus 5 mg. ml^{-1} Novozym 234 (Novo Enzymes, Farnham U.K.) in buffer B (0.2M $Na2HPO_4$, 0.1M citric acid, 0.7M NaCl pH 4.0). The enzyme solution was centrifuged, 18,000 rev.min^{-1} for 30 min at 4°C to pellet any contaminating debris. Where necessary the enzyme mixture was stored frozen

at -20°C or for periods of up to 24 h at 4°C.

After incubation mycelial debris was removed from the lytic mixture by slow centrifugation (1,000 rev.min^{-1} for 3-5 mins). Protoplasts were then pelleted from the supernatant (2,000-3,000 rev. min^{-1} for 5 min), re-suspended and washed twice with osmotic stabilizer (0.7M NaCl). An equal number of protoplasts from the strains to be fused were mixed together and control platings were made on plates of the selective media used in the recovery of fusion products. The protoplasts were then pelleted and re-suspended in 1.5-2.0ml polyethylene glycol solution (30% PEG molecular weight, 8,000 in 0.05M glycine, 0.01M $CaCl_2$ pH 7.5). After incubation at room temperature for 10 min approximately 8 ml osmotic stabilizer were added and the protoplasts pelleted (2,000-3,000 rev.min^{-1} for 5 min). The pellet was then washed twice with osmotic stabilizer by resuspension and centrifugation to remove any remaining PEG. The protoplasts were resuspended in a suitable volume of osmotic stabilizer before plating and incubation at 25°C.

3.2.3 Control Platings and the Selection of Fusion Products.

Two types of selectant were recovered following

protoplast fusion in C. acremonium; quickly growing haploid recombinants arising from the rapid degeneration of a transient diploid stage and slower growing heterozygous colonies, assumed to be aneuploid for one or more linkage groups and which on subculture yield sectors of haploid recombinant growth. Two selection strategies were employed to allow the separation of the products of protoplast fusion from the parental strains used. One was dependent upon the presence of non-allelic auxotrophic markers in each parental strain and the other allowed fusions in which one of the parental strains was prototrophic to be carried out. After PEG treatment protoplasts were initially plated on cellophane discs overlaid on appropriately supplemented OSMM. After incubation for a period of time to allow for the regeneration of cell walls the cellophane discs were transferred onto appropriately supplemented MM. The technique of cellophane transfer was developed by Hamlyn (1982) to lessen problems encountered due to the syntrophic growth of parental strains on selective media. Plating a mixture of the two parental protoplast suspensions before treatment with PEG on the selective media to be used in a given fusion gave a measure of the background syntrophic growth which would still occur and also acted as a control for marker reversion. Protoplasts of the parental strains were

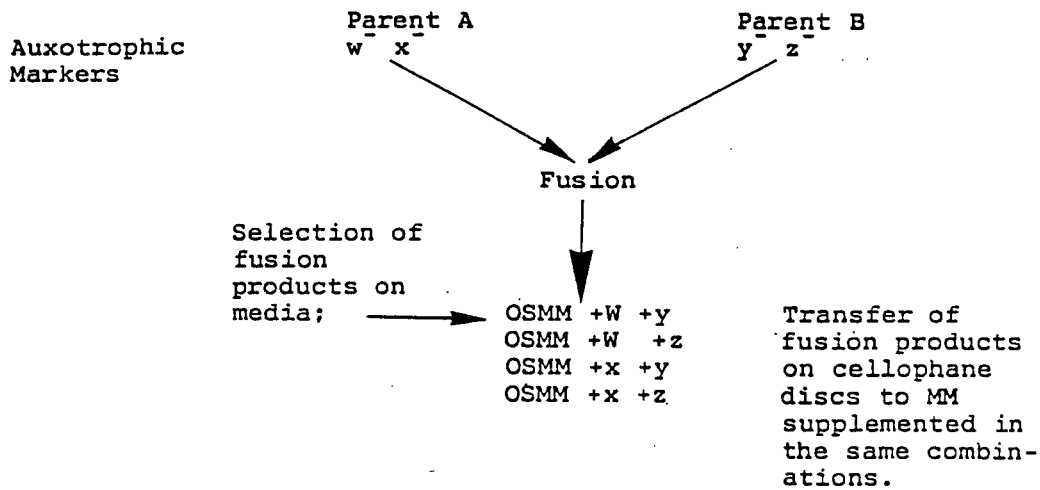
also plated separately on the selective media or on a series of media each lacking one of the strain growth requirements to detect any reversion of mutations or leaky growth which might occur. Control platings on media supplemented with the parental growth requirements were made to check the viability of the parental strains both before and after treat-ment with PEG. Protoplast suspensions appropriately diluted in water were plated out to determine the degree of contamination of the suspensions with osmotically stable hyphal fragments.

3.2.3.1 Selection Based on Auxotrophy

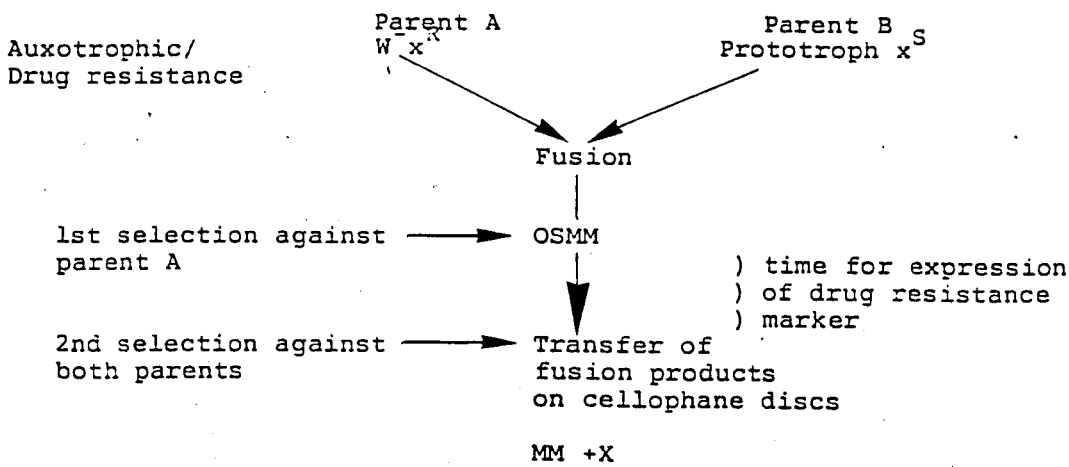
Where the parental strains to be used in an experiment each had one or more auxotrophic markers, products of the cross were selected by plating the fused suspensions on cellophane discs on a range of media supplemented in such a way that each lacked one of the growth requirements of each parental strain (Fig. 3.1a). After 4 days the cellophane discs were transferred as described and incubated for a further 14 to 21 days. Putative haploid recombinants were purified by plating mycelial fragments, resuspended in water, on a second plate of the selective medium from which they had been isolated. Colonies developing on these plates were transferred to master plates and

Fig. 3.1 Methods for the selection of fusion products.

a) Selection based on auxotrophy



b) Selection based on auxotrophy and drug resistance.



replicated onto a range of diagnostic media to determine their phenotypes with respect to any non-selective markers present. Presumptive heterozygous colonies were streaked out on plates of YEA/Casamino acids medium to observe the development of recombinant sectors which could then be purified by plating resuspended mycelial frag-ments on the same medium to give single colony isolates for genetic analysis .

3.2.3.2 Selection Based on Auxotrophy and Drug Resistance.

A selection system similar to that used by Bradshaw and Peberdy (1984) and Perez-Martinez (1984) was adapted to allow the selection of recombinant progeny following the fusion of two strains, one prototrophic and the other having auxotrophic markers and a drug resistance marker. Selection against the auxotrophic parent was achieved by plating on media lacking one of the growth requirements of the strain and selection against the prototrophic parent was by transferring the fusion mixture, plated on cellophane discs to MM supplemented with a toxic level of the drug to which the auxotrophic parent was resistant Fig. 3.1b. A period of time prior to exposure to the toxicant was necessary to allow expression of the resistance gene before selection could be effected. Trial transfers

of protoplasts inoculated on cellophane discs to media supplemented with a range of concentrations of 6-azauracil or 2-(4'-thiazoly)benzimidazole (thiabendazole) allowed the determination of the most suitable time of transfer and concentration of drug to be used. Fusion products recovered using this selection system were purified and analysed according to the methods described for those obtained purely by auxotrophic selection.

3.2.4 Assessment of Antibiotic Production.

The Pen N and CPC titres of progeny from the crosses were deter-mined by the plug assay or shake flask fermentation methods described previously (2.2.4.3, 2.2.4.4 and 2.2.4.5). Where shake flask fermentations were used, a Sabouraud slant culture of each strain was grown and used to inoculate a seed culture which in turn provided inoculum for three replica fermentation flasks. When possible the number of separate fermentation experiments necessary to examine all of the progeny from a cross was minimised to lessen the level of interference in the results due to environmental variation. Control fermentations of the parental strains were carried out simultaneously.

After bioassay of fermentation broths, and dry

weight determinations on the mycelium, specific potency (μg.antibiotic mg^{-1} dry weight) values were calculated for the progeny. A standard analysis of variance (The Open University 1978) was performed to determine to what degree the variation found between the specific potencies of the progeny was due to genetic differences rather than being due to environmental effects. Where the number of progeny obtained permitted, an estimation of the number of effective factors (k_1) segregating and influencing Pen N or CPC titre was made according to the formulae given in Table 3.1. Two methods of calculation are possible and differ as to whether the parental mean titres or the progeny extreme titres are taken into account. The second of these alternatives is considered to be the more reliable although it may give a bias towards a high value for k_1. The mean contribution ($\bar{d}$) of the effective factors could also be determined. Where the alleles segregating in a cross show additivity of action the mean titre of the progeny should correspond with the mid-parental titre. An expression of the divergence of the two means could be calculated as i (Table 3.1) and this provided a means for the estimation of the degree of occurrence of epistatic interactions. If there is no epistasis the value of i should be zero, any deviation from zero not accountable for by the limits of error of the

Table 3.1 Statistical methods

p_1 = mean titre of parent 1

p_2 = mean titre of parent 2

V_G = genetic variance

f_1 = progeny mean

Methods for the estimation of the numbers of effective factors (k_1) segregating and affecting titre in a cross.

A $$K_1 = \frac{(p_1 - p_2)^2}{4V_G}$$

B $$K_1 = \frac{(\text{highest-lowest progeny titre})^2}{4V_G}$$

Methods for estimation of the mean contribution (d) of effective factors

A $$d = \frac{p_1 - p_2}{2K_1}$$

B $$d = \frac{\text{Highest-lowest progeny titre}}{2K_1}$$

Method for calculation of i, an estimate of epistatic interaction

$$i = \frac{p_1 + p_2}{} - f_1$$

to indicate epistasis i must be different from zero either positively or negatively.

From: Burnett 1975

calculations of the means, is indicative of epistatic interactions.

3.2.5 Assessment of Titre Variation Amongst Single Protoplast Isolates of Strain 20-2.1.

The amount of natural variability with respect to titre, present in a culture of an antibiotic producing strain and contributing to the variation in titre amongst the progeny recovered following protoplast fusion was examined. A protoplast suspension was prepared from strain 20-2.1 and treated with PEG according to the fusion protocol. After plating and regeneration, a number of colonies were grown as slant cultures. These were used to inoculate shake flask fermentations and the absolute potencies of a number of isolates of 20-2.1 were determined.

3.3 Results.

A number of protoplast fusion crosses were successfully carried out as described, and provided progeny for analysis; however, several problems were encountered. Where one or more of the parental auxotrophic requirements used for selection purposes was leaky then a background of poorly growing colonies could sometimes be seen on fusion plates lacking such

requirements. This could also aggravate problems caused by cross-feeding of parental colonies resulting in syntrophic growth. In such cases it was more difficult to purify haploid selectants and to identify possible heterozygous colonies. In the crosses described below, only haploid-type fusion products were studied.

3.3.1 Mapping Crosses for Auxotrophic Markers in Strain 20-2.1.

Haploid recombinants were obtained on all of the possible selective media in cross 1, 20-2.10 (leu-5 lys-2) x 20-2.17 (arg-8 ade-1) and cross 2, 20-2.11 (leu-5 met-8) x 20-2.17. Genetic analysis results for these crosses are shown in Tables 3.2 and 3.3 and show the free assortment of the markers involved except for leu-5 and met-8 which would appear to be linked according to the data from cross 2. Examination of the control plates suggested that the ade-1 marker was somewhat leaky and that some reversion of the met-8 marker may have occurred, although only isolated revertants were seen.

During the purification of putative haploid recombinants from both crosses it was noticed that where colonies were taken from media lacking adenine the purification plates occasionally showed a range of

Table 3.2 **Genetic analysis of haploid recombinants recovered on selective media from cross 1**

20-2.10 (CO728 leu-5 lys-2) x 20-2.17 (CO728 arg-8 ade-1)

A. Pairwise Tables

			leu +	leu −	ade +	ade −	lys +	lys −	arg +	arg −	
MM +Leucine +Adenine	leu	+			(23)	—	23	—	(8)	15	MM +Leucine +Arginine
		−			13	—	(13)	—	11	(2)	
	ade	+	(19)	12			(36)	—	19	(17)	
		−	20	(3)			—	—	—	—	
	lys	+	39	(15)	(31)	23			(19)	17	
		−	—	—	—	—			—	—	
	arg	+	(39)	15	31	(23)	(54)	—			
		−	—	—	—	—	—	—			

			leu +	leu −	ade +	ade −	lys +	lys −	arg +	arg −	
MM +Lysine +Adenine	leu	+			(11)	—	6	(5)	(9)	2	MM +Lysine +Arginine
		−			—	—	—	—	—	—	
	ade	+	(43)	—			(6)	5	9	(2)	
		−	8	—			—	—	—	—	
	lys	+	21	—	(14)	7			(4)	2	
		−	(30)	—	29	(1)			5	(0)	
	arg	+	(51)	—	43	(8)	(21)	30			
		−	—	—	—	—	—	—			

( ) indicates recombinant phenotypes

Table 3.2 Continued

B. Phenotype analysis

Phenotypes				Number on selective media				
leu	lys	arg	ade	Leu Arg	Leu Ade	Lys Arg	Lys Ade	Total
+	+	+	+	8	19	4	14	45
-	+	+	+	11	12			23
+	+	-	+	15				15
-	+	-	+	2				2
+	+	+	-		20		7	27
-	+	+	-		3			3
+	-	+	+			5	29	34
+	+	-	+			2		2
+	-	+	-				1	1

Table 3.3 Genetic analysis of haploid recombinants recovered on selective media from cross 2 20-2.11 (CO728 leu-5 met-8) x 20-2.17 (CO728 arg-8 ade-1)

A. Pairwise Tables

MM +Leucine +Arginine

		leu +	leu −	ade +	ade −	met +	met −	arg +	arg −
leu	+			(39)	3	42	−	(42)	−
	−			2	(0)	(2)	−	2	−
ade	+	(23)	2			(41)	−	41	−
	−	−	−			3	−	(3)	−
met	+	23	(2)	(25)	−			(44)	−
	−	−	−	−	−			−	−
arg	+	(17)	2	19	−	(19)	−		
	−	6	(0)	(6)	−	6	−		

MM +Leucine +Adenine

		leu +	leu −	ade +	ade −	met +	met −	arg +	arg −
leu	+			(12)	−	11	(1)	(8)	4
	−			−	−	−	−	−	−
ade	+	(11)	−			(11)	1	8	(4)
	−	0	−			−	−	−	−
met	+	11	−	(11)	0			(8)	3
	−	(0)	−	0	(0)			0	(1)
arg	+	(11)	−	11	(0)	(11)	0		
	−	−	−	−	−	−	−		

MM +Methionine +Arginine

MM +Methionine +Adenine

( ) indicates recombinant phenotypes

Table 3.3 Continued

B. Phenotype analysis

Phenotypes				Number on selective media				
leu	met	arg	ade	Leu Arg	Leu Ade	Met Ade	Met Arg	Total
+	+	+	+	17	39	11	8	75
-	+	+	+	2	2			4
+	+	-	+	6			3	9
+	+	+	-		3			3
+	-	-	+				1	1

colony types from large, quickly growing haploid types to small, slower growing colonies reminiscent of the heterozygous colonies seen on fusion plates. On further investigation most of these appeared to be the results of the leaky growth of arg-8 ade-1 auxotrophs. An isolated case was found where after fragmentation and plating onto YEA/Casamino acids medium a colony gave rise to unstable colonies showing a range of phenotypes with abnormal morphologies and possible areas of sectoring. This might suggest that the original colony whilst resembling a haploid recombinant was in fact heterozygous.

3.3.2 Sensitivity of Protoplasts to 6-azauracil and thiabendazole.

Protoplasts prepared from strain 20-2.1 were plated (10^6 per plate, with a viability of 4.7%) on cellophane discs on OSMM, and incubated. After periods of between 1 and 4 days the cellophane discs were transferred onto a range of non-stabilized plates which contained various concentrations of 6-azauracil or thiabendazole, and further incubated. The degree of regeneration and growth occurring under these conditions is shown in Table 3.4. The transfer of cellophane discs after 2 days to media containing either 1 $mg.ml^{-1}$ 6-azauracil or 30μM thiabendazole

Table 3.4 Inhibition of protoplast regeneration and growth by 6 azauracil (AZU) and thiabendazole (TBZ)

(a) 6-azauracil

Conc. of 6-AZA $mg.ml^{-1}$ in H_2O	Degree of growth occurring following Cellophane transfer on day:			
	1	2	3	4
0.25	3(1)	3(2)	3(1)	4 (0.5)
0.5	2(0.5)	3(0.5)	3	3(3.5)
1.0	2	2	3	3

(b) Thiabendazole

Conc. of TBZ (μm) in ethanol	Degree of growth occurring following Cellophane Transfer Day;			
	1	2	3	4
10	6	6	6	6
20	3	3	3	4
30	1	2	3	3

1 → 6 represents the change from no growth visible on the plates through a stage of heavy background growth with few if any normally growing colonies to confluent growth. Numbers in parenthesis indicate the average number of any colonies occurring on the plates.

were chosen as the most suitable conditions for use for future selection purposes. Parental growth was reduced to a faint background and did not interfere with the detection and purification of fusion products. The regeneration of protoplasts prepared from strains 20-2.33 (leu-5 met-8 tbz-2) and 20-2.34 (leu-5 met-8 azu-3) under these conditions was confirmed.

3.3.3 Crosses Between Blocked Mutants and a Related CPC Producing Strain.

Crosses were carried out between mutants impaired in CPC bio-synthesis derived from strain 20-2.1 and a marked strain 20-2.33 (leu-5 met-8 tbz-2) which produced a good titre of CPC and had been derived from the same progenitor strain as the blocked mutants. The unavailability of auxotrophic markers in the blocked strains, at the time of these experiments, led to the use of thiabendazole as a selective agent against the prototrophic blocked strains and selection against the 20-2.33 parent by the omission of either leucine or methionine from the regeneration plates. Fig. 3.2 shows the effectiveness of this as a selection system.

Crosses involving strains 20-2.29 (cnp-4) and 20-2.27 (cnp-2) were successful in producing haploid

Fig. 3.2 Selection against parental strains in a protoplast fusion cross by the use of auxotrophic and drug resistance markers.

A. Regeneration of 10^6 protoplasts of strain 20-2.33 (leu-5, met-8, tbz-2) on medium supplemented with leucine methionine plus 30 m thiabendazole.

B. Regeneration of 10^6 protoplasts of strain 20-2.27 (cnp-2) on minimal medium.

C. Selection against 10^6 protoplasts of strain 20-2.33 and strain 20-2.27 by plating on medium supplemented with methionine plus 30 m thiabendazole.

recombinant progeny (Table 3.5). Genetic analysis revealed that the vast majority of the progeny recovered on media supplemented with either leucine or methionine were prototropic, thus supporting the evidence in section 3.3.1 for the linkage of the leu-5 and met-8 genes (Table 3.6). The recovery of progeny on either of the selective media, given the linkage of these markers, is indicative of the non-linkage of the tbz-2 marker to the two auxotrophic genes.

A preliminary screen of the antibiotic titres of a number of the progeny from these crosses was carried out using the plug assay system. Six PFM agar plugs were inoculated for each strain, 3 to be assayed for Pen N and 3 for CPC production. Fig. 3.3 shows the presence amongst the progeny of strains resembling both of the parental types for cross 3 as well as strains producing higher or lower titres of antibiotic, and Fig. 3.4 shows the preliminary results for cross 4. Shake flask fermentations of the progeny allowed a more accurate determination of antibiotic titre and gave the results shown in Figs. 3.5 and 3.6. Analysis of variance shows that a considerable portion of the variation seen amongst the progeny from both crosses, was genetic in origin (Tables 3.7 and 3.8).

The recovery in both crosses of progeny showing blocked and unimpaired CPC biosynthesis suggests that

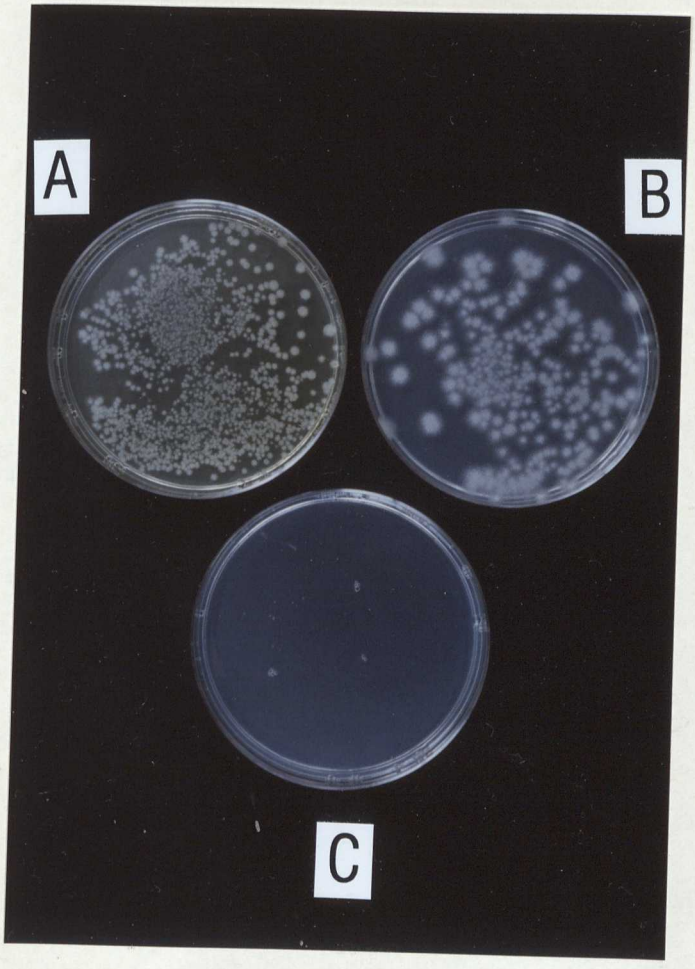

A
B
C

Table 3.5 Progeny recovered on selective media following crosses 3 and 4.

Cross Number	Parents	Number of progeny recovered		
		MM+ leucine +TBZ	MM+ met +TBZ	Total
3	20-2.33 (leu-5 met-8 tbz-2) × 20-2.29 (cnp-4)	31	25	56
4	20-2.33 (leu-5 met-8 tbz-2) × 20-2.27 cnp-2)	11	-	11

Table 3.6 Phenotype analysis of progeny recovered following crosses 3 and 4.

A) Cross 3

20-2.33 (*leu-5 met-8 tbz-2*) x 20-2.29 (*cnp-4*)

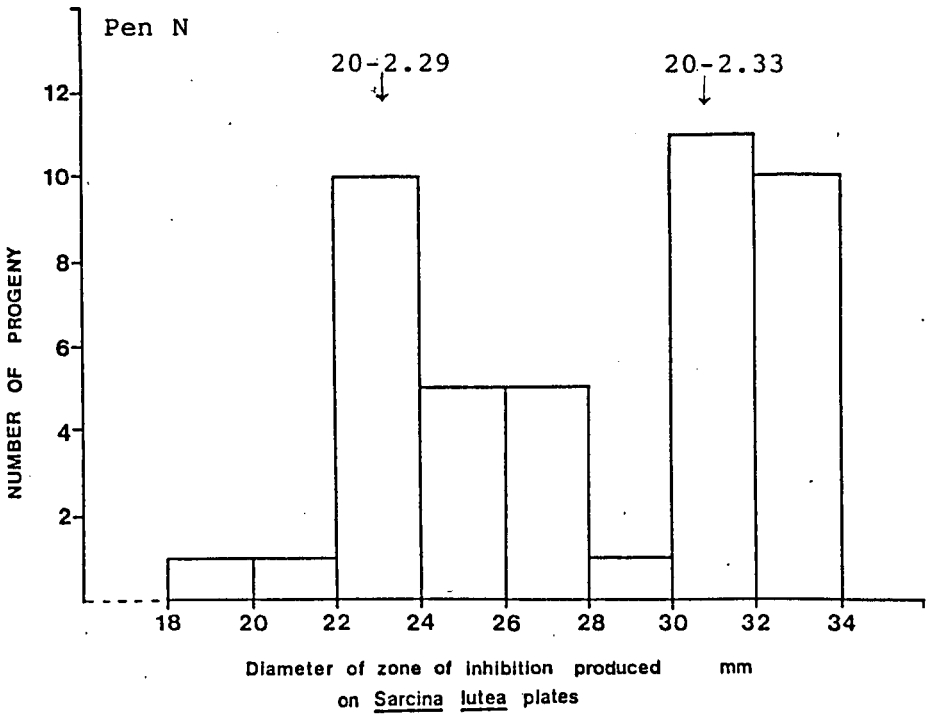
Phenotype		Number of progeny recovered		
met	leu	MM +leucine +TBZ	MM methionine +TBZ	Total
+	+	28	24	52
+	-	3		3
-	+		1	1

b) Cross 4

20-2.33 (*leu-5 met-8 tbz-2*) x 20-2.27 (*cnp-2*)

Phenotype		Number of progeny recovered		
met	leu	MM + leucine + TBZ	MM + methionine + TBZ	Total
+	+	10		10
+	-	1		1

Fig. 3.3 Distribution of antibiotic titres of progeny from cross 3 as determined by plug assay.



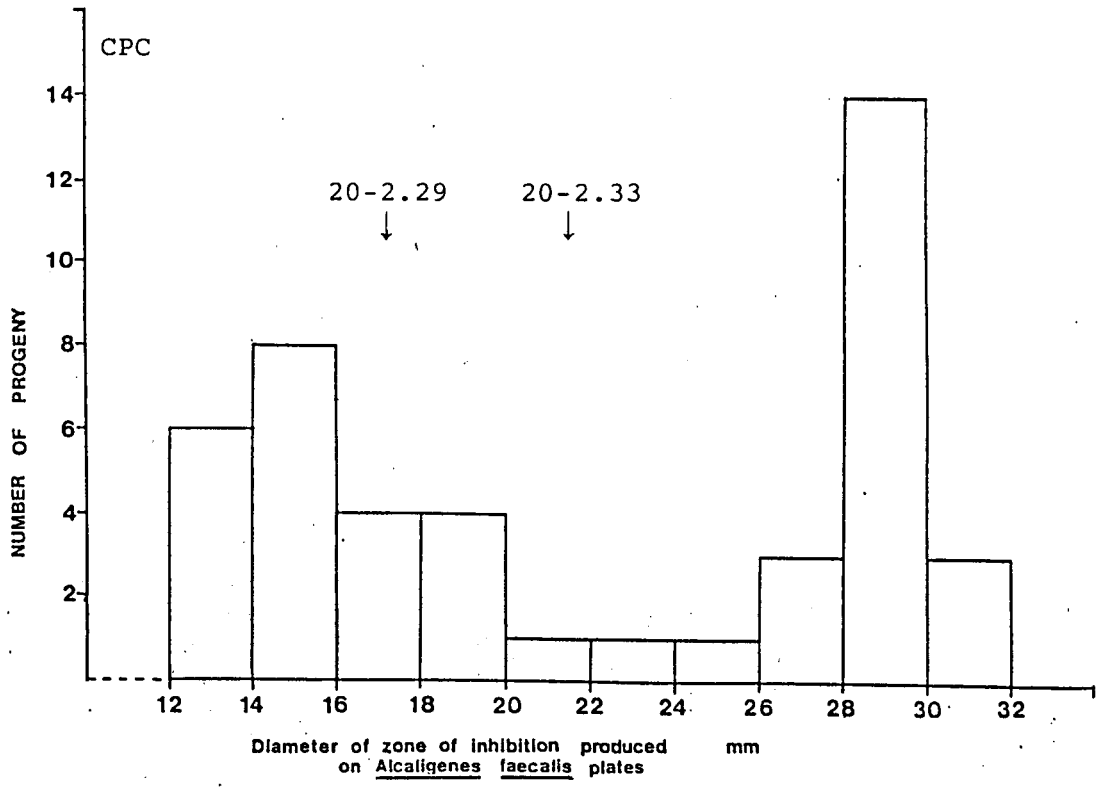
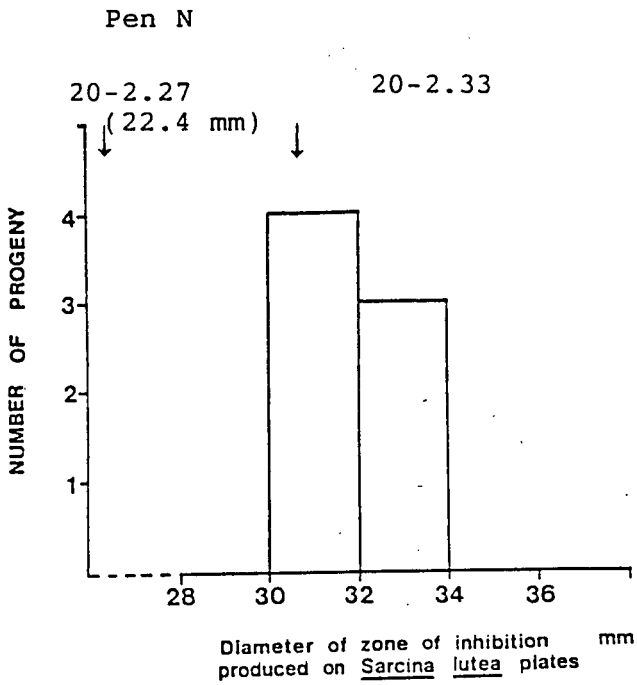


Fig. 3.4 Distribution of antibiotic titres of progeny from cross 4 as determined by plug assay.



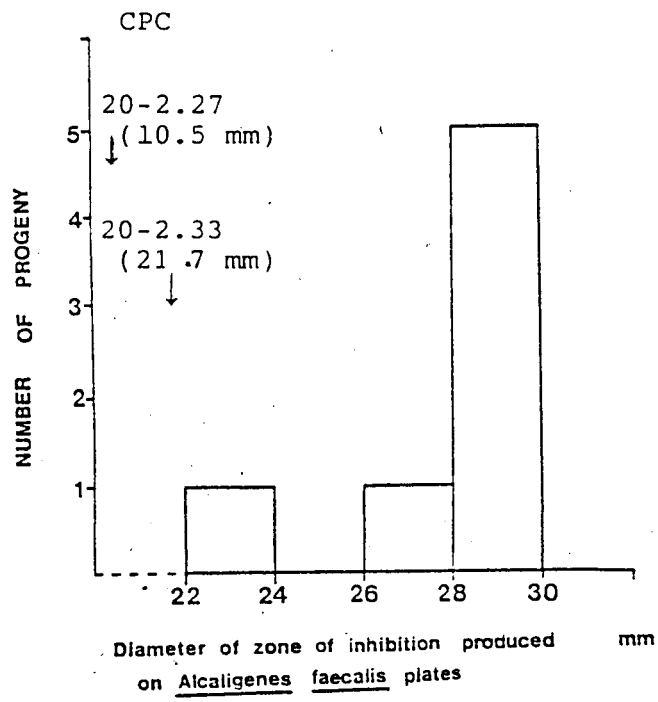
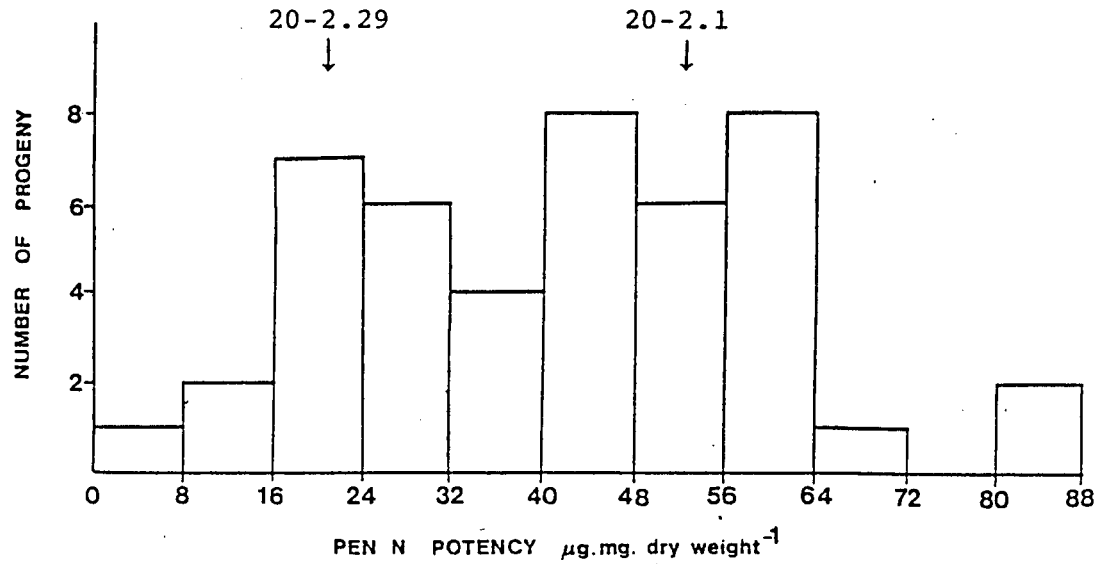


Fig. 3.5 Distribution of antibiotic titres of the progeny from cross 3. Shake flask fermentation and bioassay data.



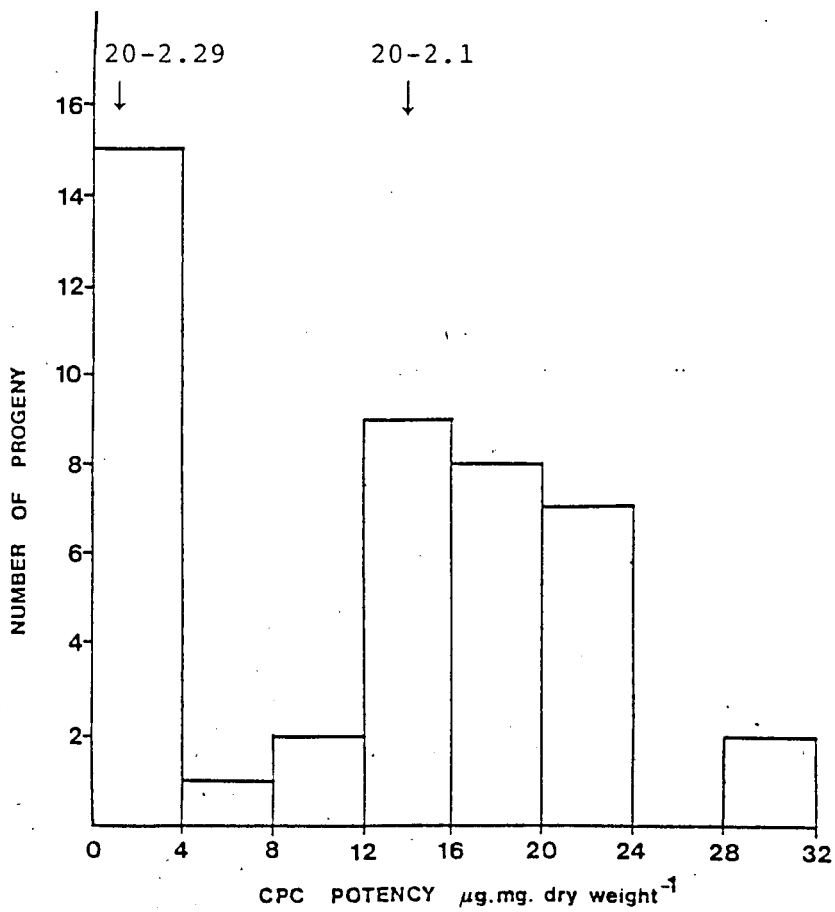
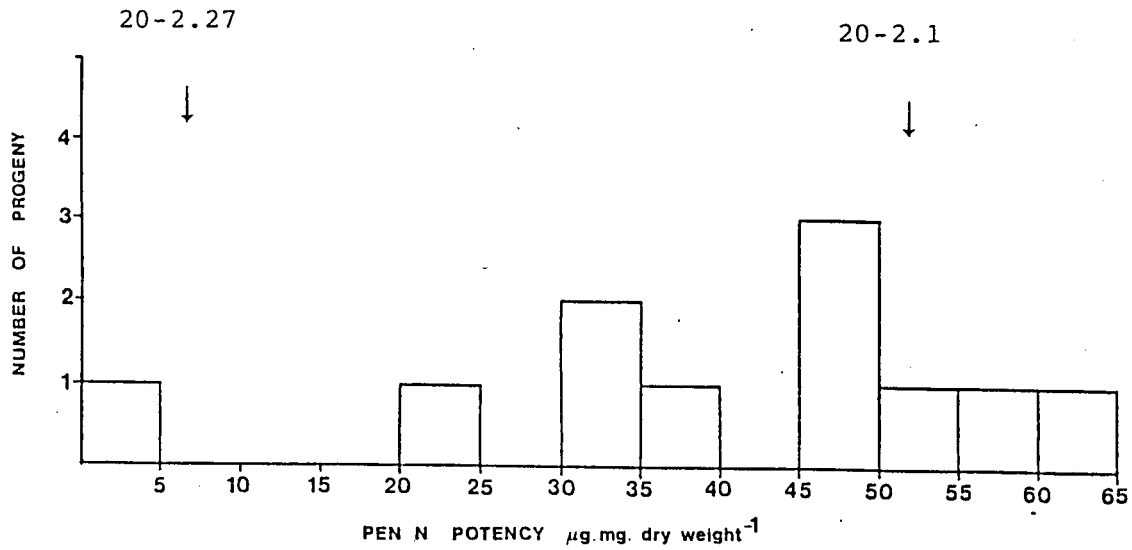


Fig. 3.6 Distribution of antibiotic titres of the progeny from cross 4: Shake flask fermentation and bioassay data



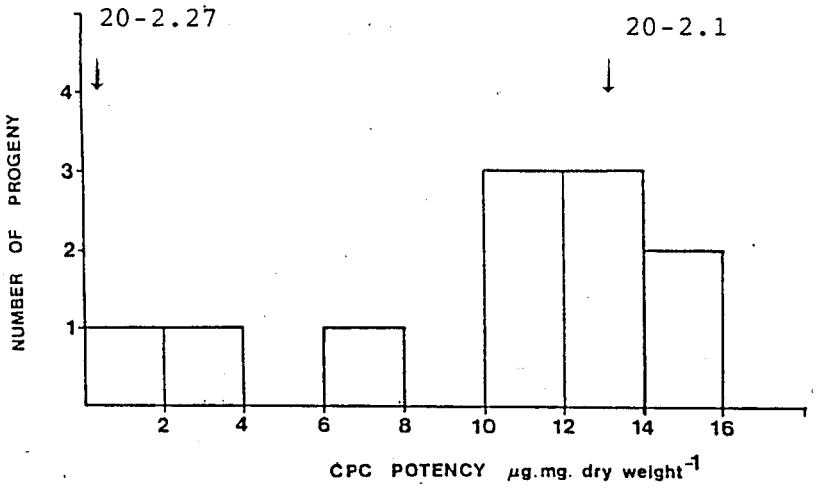


Table 3.7 Analysis of variance for cross 3.

	Variation Source	Degrees of Freedom	Mean Squares	Variance Ratio
Pen N	Total	137	510.84	
	Between Progeny	45	1206.34	
	Between Replicas	92	170.65	7.07*
CPC	Total	137	90.32	
	Between Progeny	45	229.20	
	Between Replicas	92	22.39	10.24*

* significant at $\alpha = 0.01$

Pen N Genetic Variance (V_G) = 510.84 - 170.65 = 340.19
= 66.6 % of total variance

CPC Genetic Variance (V_G) = 90.32 - 22.39 = 67.93
= 75.2 % of total variance

Table 3.8 Analysis of variance for cross 4.

	Variation Source	Degrees of Freedom	Mean Squares	Variance Ratio
Pen N	Total	32	44951	
	Between progency	10	974.11	4.62*
	Between replicas	22	211.06	
CPC	Total	32	26.49	
	Between progency	10	66.81	8.17*
	Between replicas	22	817	

*Significant at α = 0.01

Pen N Genetic Variance (VG) = 44951-211.06 = 238.45

= 53.05% of total variance.

CPC Genetic Variance (VG) = 26.49 - 8.17 = 18.32

= 69.15% of total variance.

there is no linkage between the cnp-2 and cnp-4 mutations induced in strains 20-2.27 and 20-2.29 and the leu-5, met-8 and tbz-2 markers. The small number of progeny recovered from the cross between 20-2.33 and 20-2.27 would render further statistical analysis unreliable, but estimations of the degree of epistasis occurring and of the number and mean contributions of the effective factors segregating in the cross involving 20-2.29 were made (Table 3.9). Comparing the parental and progeny mean titres for the cross between 20-2.33 and 20-2.29 it appeared that the alleles segregating with respect to Pen N production were mainly additive in effect. However, where CPC production was concerned some epistatic interactions are apparent with the progeny mean titre being somewhat higher than that of the parental strains.

When values of K_1, the estimated number of effective factors affecting antibiotic production, are calculated four assumptions are made about the titre-influencing alleles segregating in the cross. Thus the alleles are (i) unlinked, (ii) of equal and additive effect, (iii) not affected by epistatic interactions and (iv) distributed such that all having a positive effect occur in one parent and those having a negative effect in the other. For this cross the parents were deliberately chosen to minimize the difference in their genetic backgrounds, i.e. only one

Table 3.9 Estimation of the degree of epistasis (i), the number of effective factors (K_1) their mean contributions ($\bar{d}$) cross 3.

	Parental Means $\mu g.mg^{-1}$				Progeny Extremes $\mu g.mg^{-1}$		Progeny Means		i
	20-2.33 Mean (p_1)	SE	20-2.29 Mean (p_2)	SE	High	Low	Mean (f_1)	SE	
Pen N	52.57	4.73	21.52	3.88	85.95	0	40.29	1.92	-3.24
CPC	13.88	2.61	1.73	0.28	31.06	0	11.91	0.81	-4.10*

	Genetic Variance (V_G)	p_1-p_2	k_1	$\bar{d}$	Difference between progeny extremes	k_1	$\bar{d}$
Pen N	340.19	31.05	0.71	21.87	85.94	5.43	7.91
CPC	67.93	12.15	0.54	11.25	31.06	3.55	4.37

* different to 0 when the limits of error are considered.

p_1 = mean titre of parent 1
p_2 = mean titre of parent 2
V_G = genetic variance
SE = standard error

of the mutations introduced was thought to significantly affect titre. In view of this the calculation of the K_1 for Pen N would appear to be valid.

Where CPC production is concerned the presence of epistatic inter-actions complicates the situation although the values of K_1 are low suggesting the presence of a small number of genetic differences between the parental strains with regard to titre.

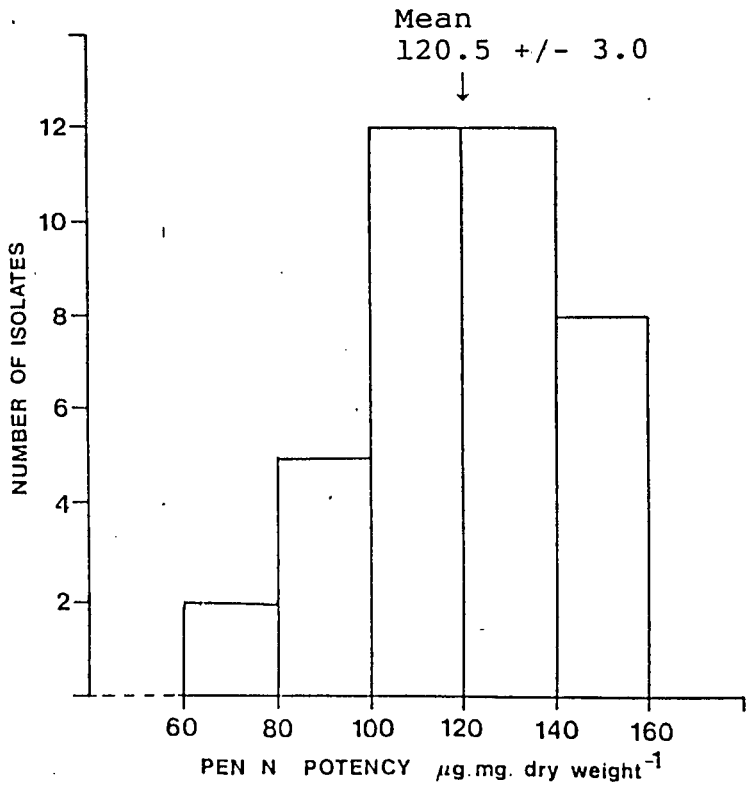
3.3.4 Titre Variation Amongst Single Protoplast Isolates of Strain 20-2.1.

Protoplasts of strain 20-2.1 were taken through the procedures involved in the PEG mediated fusion of C. acremonium protoplasts and then allowed to regenerate. Thirty nine isolates were then assayed by shake flask fermentation to determine their absolute Pen N and CPC potencies. Fig. 3.7 shows the spread of titres found amongst the isolates and Table 3.10 gives the results of an analysis of variance calculation on the data obtained, and shows that the majority of the observed variation was attributable to environmental effects.

3.4. Discussion

Protoplast fusion crosses between the CPC producing

Fig. 3.7 Distribution of antibiotic titres of single protoplast isolates of strain 20-2.1



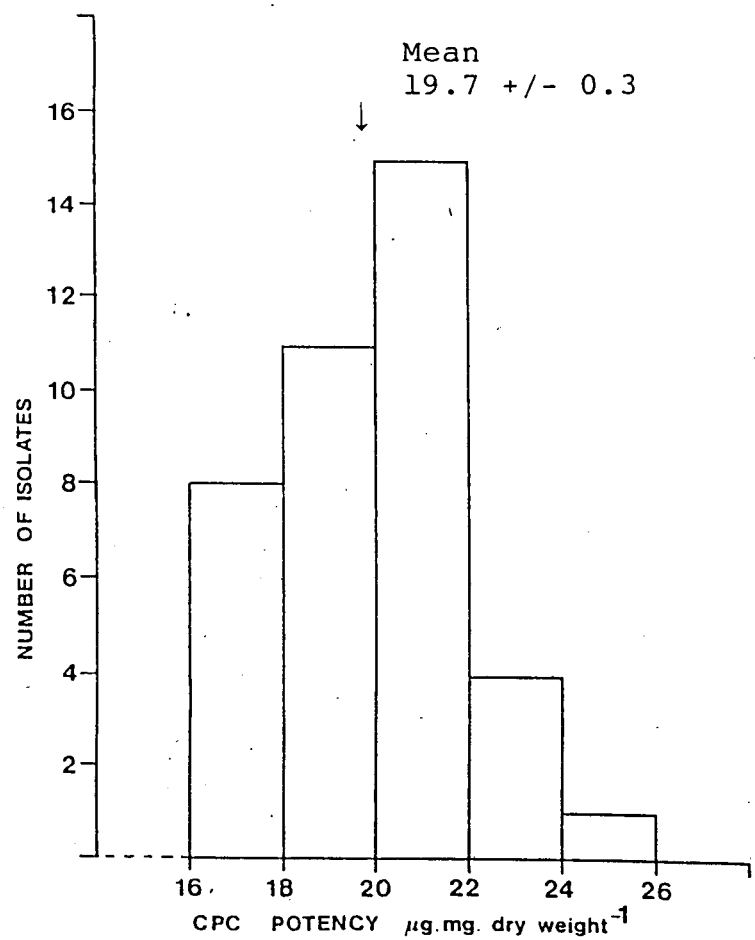


Table 3.10 Analysis of variance for the antibiotic titres of single protoplast isolates of strain 20-2.1

	Variation Source	Degrees of Freedom	Mean Squares	Variance Ratio
Pen N	Total	116	1061.64	
	Between isolates	38	1509.39	1.79
	Between replicas	78	843.50	
CPC	Total	116	11.25	
	Between isolates	38	11.53	1.04
	Between replicas	78	11.12	

Neither variance ratio is significant at $\alpha = 0.001$

Pen N Genetic Variance (V_G) = 1061.64 - 843.50 = 218.14

= 20.5 % of total variance

Environmental Variance = 79.4 % of total variance

CPC Genetic Variance (V_G) = 11.25 - 11.12 = 0.13

= 1.195 % of total variance

Environmental variance = 92.8 % of total variance

strain 20-2.33 and two strains blocked in CPC production have allowed the segregation of the mutant alleles involved to be monitored. The free assortment of these alleles with the markers used for selection was found. In comparison to the situation found with single protoplast isolates of the progenitor strain 20-2.1 a substantial proportion of the variation present amongst the progeny from both these crosses with regard to titre could be ascribed to genetic effects. The production of a trace level of CPC by the blocked strain 20-2.27, previously shown to be CPC negative, suggests though that it would have been advisable for the variation in titre amongst the progeny form both these crosses to have been compared with that amongst single protoplast isolates of the immediate parents to verify that the spread of titres was pre-dominantly the result of recombination rather than of strain variability.

Cross 3 involving 20-2.29 revealed the presence of epistatic inter-actions with the progeny mean CPC titre exceeding the mean parental value. The absence of significant numbers of progeny carrying the leu-5 and met-8 alleles makes it impossible to determine whether the increased CPC titre amongst the progeny is due to the removal of epistatic titre reducing effects resulting from the auxotrophies or other factors associated with the linkage group carrying them.

These crosses also served to investigate the potential use of drug resistance as a selectable marker and the plug assay system as a means of determining the β-lactam production characteristics of progeny without recourse to large scale shake flask fermentation experiments. A comparison of the data obtained by plug assay with that from shake flask experiments illustrates that both methods reveal the same basic trends found amongst the progeny. The data from plug assays is less accurate, with no determination of growth being taken into account and with inoculum size being standardised only by eye. However, the method has the advantage of allowing large numbers of progeny to be screened relatively rapidly and would be particularly useful in assaying progeny from crosses designed to assess the presence or absence of complementation between blocked strains.

The advantage of a selection strategy involving drug resistance markers may be seen in the use of prototrophic strains in crosses. Since repeated mutagenesis may in some cases lead to chromosomal rearrangements and problems in obtaining recombination between strains (MacDonald 1968) and since auxotrophic markers may have pleiotropic effects on titre, (MacDonald 1963), any means of reducing the number of mutagenic steps prior to the use of a strain in a fusion could prove of use.

Suitable conditions for the application of either 6-azauracil or thiabendazole in selection were also found, however, the level of spontaneous resistance to the former led to the preferential use of thiabendazole. Selection with 6-azauracil could be feasible providing the level of spontaneous resistance arising in the parental strains was very carefully monitored.

Thiabendazole is a benzimidazole fungicide which, in a similar manner to benomyl, interferes with the process of mitosis by binding to tubulin and affecting microtubule assembly and so may be used to induce the genetic segregation of diploids. Studies have suggested that segregation is induced primarily as a result of the non-disjunction of chromosomes rather than by a chromosome breakage and deletion mechanism or by increasing mitotic crossing over (Kappas et al 1974; Kappas 1978; Davidse 1983).

The possibility that the frequency of mitotic crossing over is increased, cannot be excluded and a comparison of the spectrum of progeny types obtained from an C. acremonium fusion following selection with thiabendazole with that obtained from the same fusion following auxotrophic selection would be useful in detecting any bias in the genetic analysis as a result of the use of the thiabendazole system. It would also be of interest to know the degree of dominance

shown by the tbz-2 gene following nuclear fusion. Van Tuyl (1977) discussing the dominance of similar mutations in A. nidulans, suggests that the degree of dominance may vary considerably between different resistance mutations. As the diploid stage of C. acremonium is only transient (Hamlyn 1982) it is not possible to determine the phenotypes of diploids heterozygous or homozygous for tbz-2. However, the successful selection of progeny from two crosses on the basis of resistance to thiabendazole would suggest that a sufficient expression of the tbz-2 marker was occurring at the time of transfer of the fusion products to thiabendazole to allow both survival and mitotic division.

Crosses designed to examine linkage relationships between auxo- trophic markers present in strains derived from 20-2.1 revealed the linkage of the leu-5 and met-8 markers in strain 20-2.33. The absence of particularly strong, pleiotropic titre reducing effects as a result of these auxotrophies had previously led to this strain being selected as the most suitably marked parent strain for use in crosses with the blocked mutants. However, the linkage of the two auxotrophic markers presents a problem. Selection of fusion products on media lacking either leucine or methionine will result in the loss of the 20-2.33 linkage group concerned in the

vast majority of the progeny and consequently in the loss of any alleles affecting titre positioned on the same linkage group. Therefore were future mapping work to be carried out, it would be beneficial for a third unlinked auxotrophy to be introduced for use as a selectable marker.

CHAPTER 4

Complementation and Mapping Studies with Strains Blocked in CPC Biosynthesis.

4.1. Introduction

The programme of mutagenesis described in Chapter 2 provided a number of mutant strains of C. acremonium blocked for CPC bio-synthesis. In addition to these several blocked mutants produced in other laboratories from divergent strains of C. acremonium were available in the Nottingham culture collection.

It was proposed to assess the complementation relationships between the genes affected as a first step towards the mapping of the mutations involved. As heterokaryon formation is infrequent in C. acremonium and the diploid stage is usually considered to be transient, (Nuesch et al, 1973), complementation between genes concerned cannot be assessed by determining the CPC biosynthetic capacity of diploids formed between pairs of the mutants. Therefore, the examination of the CPC titre of haploid segregants obtained following protoplast fusion crosses is necessary for complementation analysis. In addition to providing data allowing the allocation of mutations affecting titre to complementation groups such studies would necessarily also provide information regarding the presence or absence of

linkage between the titre affecting mutations and the selectable markers introduced into the parental strains as a pre-requisite for protoplast fusion crosses.

4.2 Materials and Methods.

Descriptions of the strains used, with two exceptions can be found in Chapter 2. Strain 20-3.5 (nic-1) was derived by U.V. mutagenesis from strain 20-3.2 (M-40) a blocked mutant deficient in the conversion of DAC into CPC and was kindly provided by Takeda Research Laboratories, Japan. Strain 20-5.2 (M8650-S^--M26-127a/2) required a reduced or organic source of sulphur for growth, carried a leaky mutation blocking the hydroxylation of DAOC to DAC and was kindly supplied by Dr. H. J. Treichler of Ciba Geigy, Basel, Switzerland.

The procedures used for the isolation and fusion of protoplasts and for the selection of recombinant progeny were generally as described in Chapter 3. Following crosses 9-12 a number of progeny were recovered after the fusion mix, diluted 1 part to 3 with osmotic stabilizer, had been plated directly onto cellophane discs on OSMM plates without the prior removal of the PEG present. Titre analysis of the progeny was carried out either by the plug assay or

the shake flask fermentation methods described in Chapter 2.

4.3 Results

Eight crosses between pairs of mutants were carried out (Table 4.1). Recombinant progeny were recovered from all of the crosses. Fusions 8, 9 and 10 yielded somewhat unusual fusion products which, whilst resembling haploid recombinants in their appearance, on purification behaved in a manner reminiscent of that of heterozygous fusion products. A more stringent purification system was adopted in order to obtain stable haploid progeny from these crosses.

4.3.1 Cross 5. 20-3.4 (*cnp-7 leu-4*) x 20-5.2 (*S⁻ cnp-8*)

A number of haploid progeny were obtained from this cross by selection on MM. As one of the parental strains (20-3.4) produced the yellow pigment, chrysogenin, the progeny were scored for its production as a non-selective marker. Chrysogenin production did not show linkage to either the *S⁻* or the *leu-4* markers with 29 out of 59 progeny producing the pigment. Bioassay of shake flask fermentation broths showed the range of Pen N titres occurring

Table 4.1 Recovery of progeny from crosses between pairs of strains impaired in CPC biosynthesis.

CROSS No.	PARENTAL STRAINS	NUMBER OF PROGENY RECOVERED				
		MM	MM +tbz	MM +leu	MM +his	MM +ino +tbz
5	20-3.4 (N-2 cnp-7 leu-4)) 20-5.2 (M8650-S M26-127. cnp-8))	59				59
6	20-3.4 (N-2 cnp-7 leu-4)) 20-3.5 (M-40 cnp-9 nic-1))	9				9
7	20-3.5 (M40 cnp-9 nic-1)) 20-5.2 (M8560-S M26-127a/2 cnp-8))	4				4
8	20-3.10 (N-2 cnp-7 leu-4 tbz-6)) 20-2.32 (CO728 cnp-5))		9			9
9	20-3.11 (N-2 cnp-7 leu-4 his-2)) 20-2.42 (CO728 cnp-5 orn-1))			77	56	133
10	20-3.11 (N-2 cnp-7 leu-4 his-2)) 20-2.43 (CO728 cnp-6 met-13))			31	59	90
11	20-2.43 (CO728 cnp-6 met-13)) 20-2.42 (CO728 cnp-5 orn-1))	57				57
12	20-2.49 (CO728 cnp-6 met-13 tbz-9)) 20-2.44 (CO728 cnp-1, ino-z))	82			105	188

amongst the progeny (Fig. 4.1) and an analysis of variance demonstrated the genetic as opposed to environmental basis of the differences in titre seen (Table 4.2). Strain 20-5.2 was reputed to produce approximately 900 $\mu g.ml^{-1}$ of Pen N and 100-300 $ug.ml^{-1}$ of DAOC but under the conditions used in this work it gave a titre averaging only 84 $\mu g.ml^{-1}$ Pen N despite showing good growth characteristics. HPLC analysis of fermentation broths demonstrated the presence of both parental types with regard to titre amongst the progeny, suggesting that the leu-2, cnp-7 and S^-, cnp-8 pairs of loci are not linked. Isolated strains with increased production of Pen N were also obtained but none of the progeny showed the ability to produce CPC. Recombinant progeny carrying both the mutant cnp-7 and cnp-8 alleles if present would be expected to be indistinguishable on the grounds of titre, from progeny carrying the cnp-7 allele alone. Recombinants wild-type for both the alleles, would on the other hand be expected to be recovered as CPC producers since the parental strains were impaired in different steps of the CPC biosynthetic pathway. A variety of explanations are possible as to the absence of CPC producing recombinants amongst the progeny. The parental strains involved had been derived from divergent strain improvement lines and could therefore have carried chromosomal rearrangements which may have

Fig. 4.1 Distribution of penicillin N titres amongst progeny recovered from Cross 5.

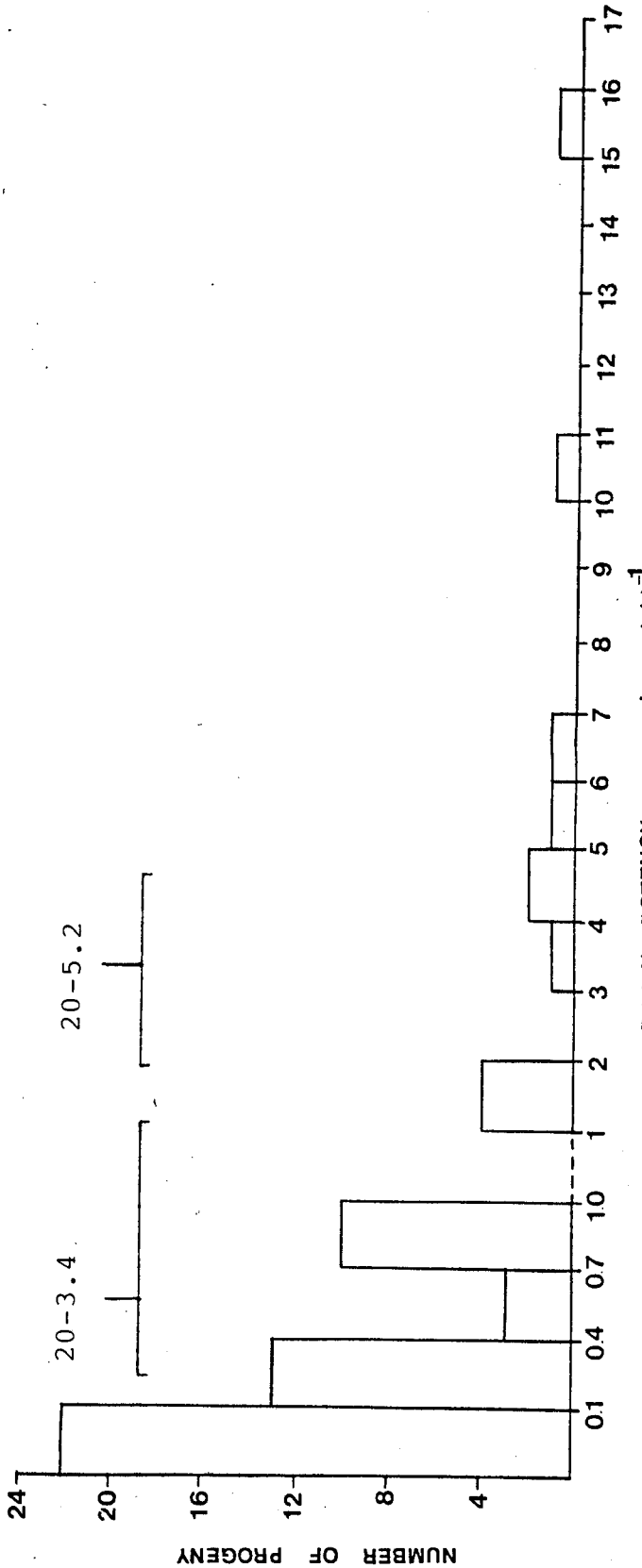


Table 4.2 Analysis of variance for Pen N titre of progeny from cross 5

Variation Source	Degrees of Freedom	Mean	Variance Ratio
Total	176	7.46	
Between progeny	58	22.02	73.21*
Between replicas	118	0.3007	

Genetic Variance (V_G) = 95.97% of total variation

* Significant at α = 0.01

given rise to difficulties in recombination between them and resulted in a loss of viability of or selection against some recombinant types. Alternatively, if cnp-7 and cnp-8 are linked, then mitotic recombination would have been necessary to yield strains producing CPC. However, the number of progeny obtained could have precluded the detection of such events. Linkage between the cnp-7 and S^- or the cnp-8 and leu-4 loci would also give rise to progeny predominantly of parental types with respect to titre. Further investigation of these possibilities would require the introduction of selectable markers into the strains to allow progeny carrying the linkage groups marked by the leu-4 and S^- alleles to be recovered.

4.3.2 Cross 6. 20-3.4 (cnp-7 leu-4) x 20.3.5 (cnp-9 nic-1)

The leaky nature of the nicotinic acid requirement of strain 20-3.5 led to problems in the selection and recovery of progeny from this sister strain cross. Shake flask fermentations of the 9 prototrophs obtained showed 5 to be completely negative for β-lactam production, and 2 to resemble the 20-3.5 parent in producing a high Pen N but low CPC titre. Strain 20-3.4 whilst classified as negative for Pen N

production, always gave fermentation broths which produced very small inhibition zones when assayed against Sarcina lutea ATCC 9341. It is notable that the 5 non-producing progeny did not produce even this slight inhibition and so were not completely identical to strain 20-3.4 in phenotype. The remaining 2 progeny produced a lowered total B-lactam yield than strain 20-3.5 but showed a higher ratio of CPC:Pen N production (Fig. 4.2) HPLC analysis of fermentation broths revealed that these latter strains did not accumulate DAC to the same degree as the parent 20-3.5 (Fig. 4.3), suggesting that some restoration of acetyl CoA deacetylcephalosporin C O-acetyltransferase activity may have occurred. The recovery of strains which yielded parental type titres and of strains with improved abilities to convert DAC into CPC might suggest that the cnp-7 and cnp-9 alleles were segregating freely in this cross, with respect to each other and to the leu-4 and nic-1 markers.

4.3.3 Cross 7. 20-3.5 (cnp-9 nic-1) x 20-5.2 (S^- cnp-8).

Problems associated with the leaky growth of strain 20-3.5 on fusion plates led to the recovery of only 4 prototrophic progeny from this cross. These had the antibiotic titre characteristics shown in Fig. 4.4.

Fig. 4.2 Distribution of antibiotic titres amongst progeny recovered from cross 6.

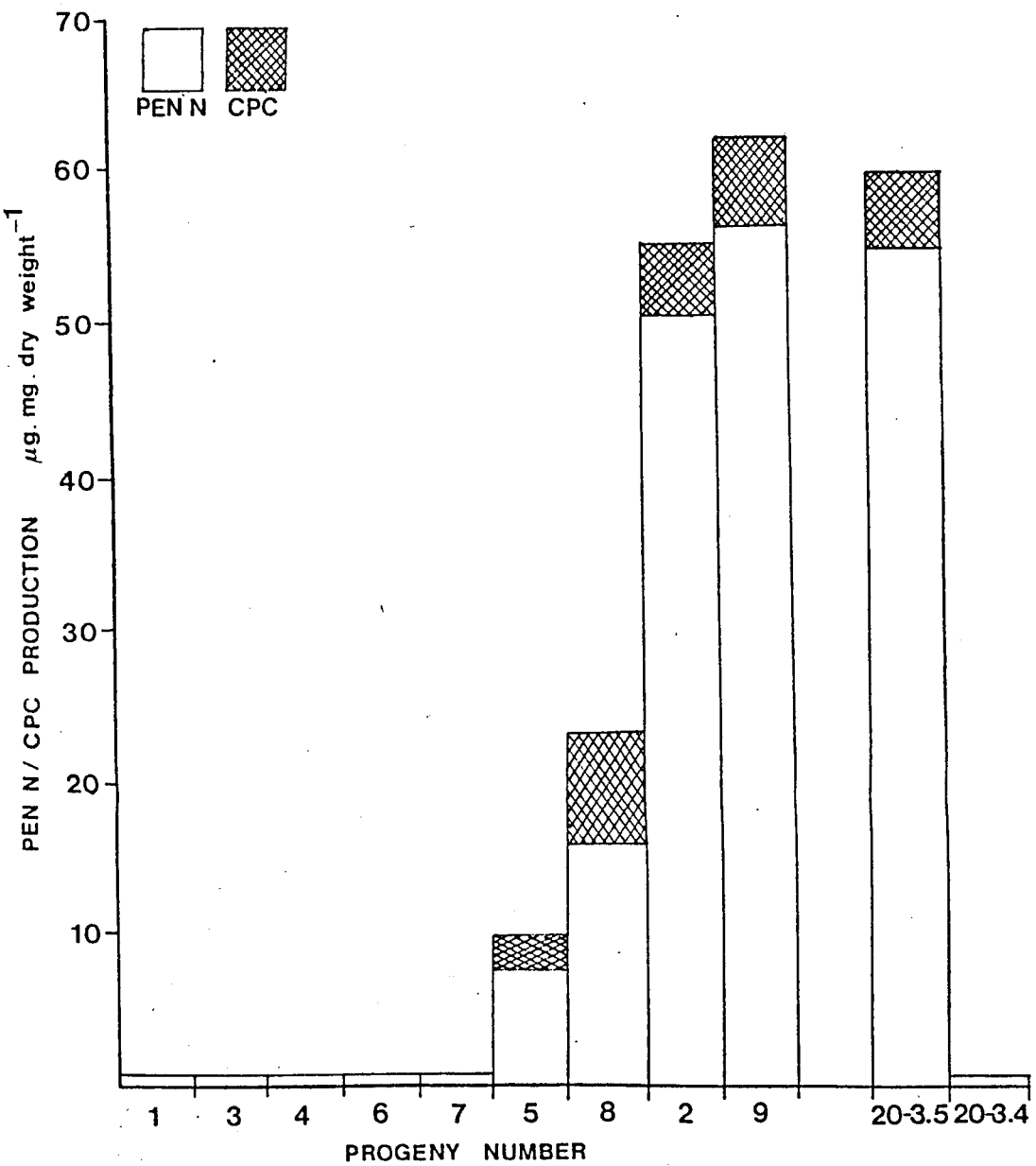


Fig. 4.3 H.P.L.C. analysis of cross 6, 20-3.4 (cnp-7) (leu-4) x 20-3.5 (cnp-9 nic=-1)

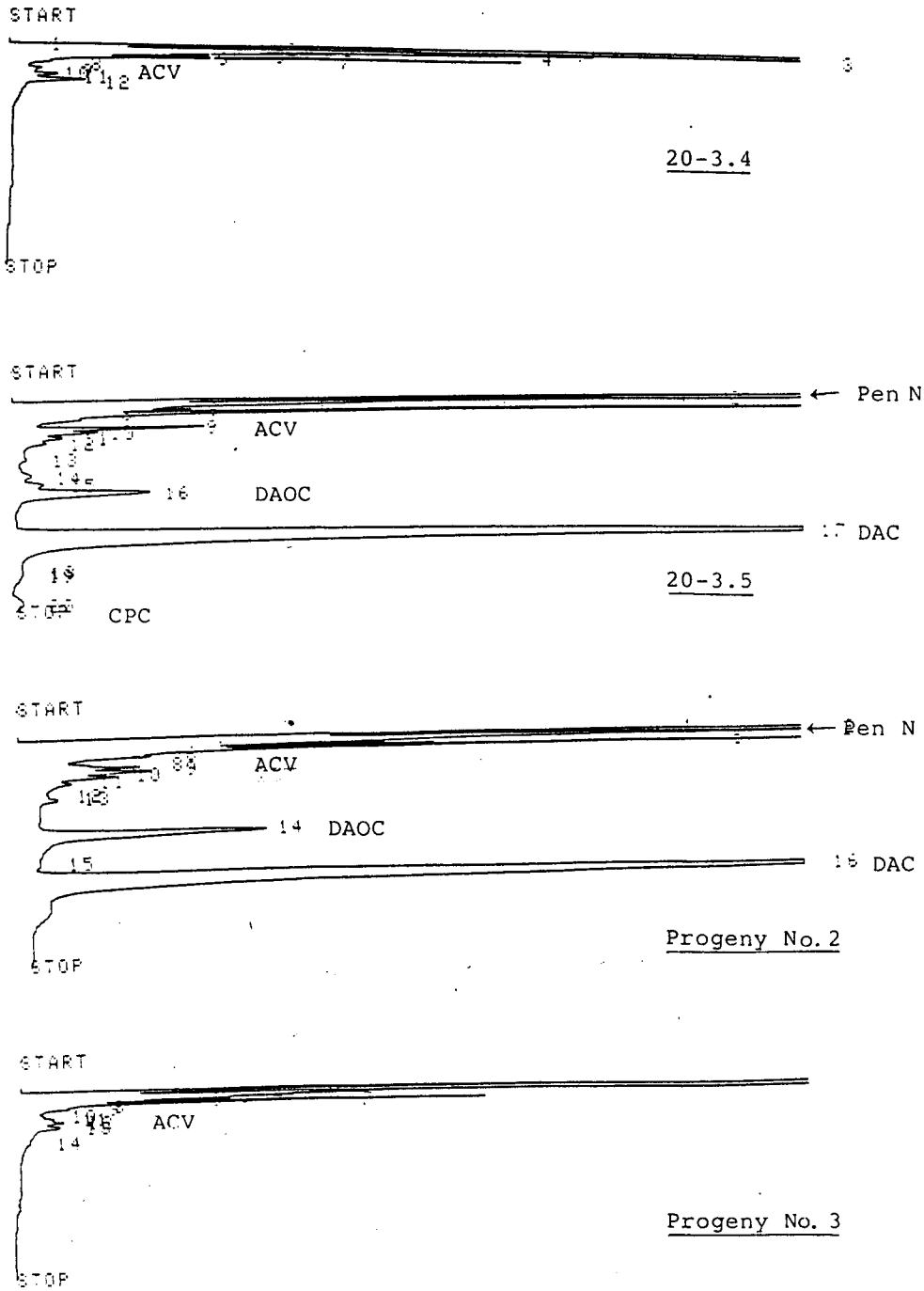
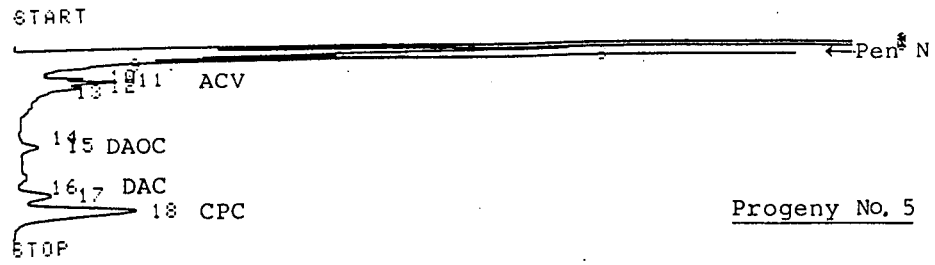
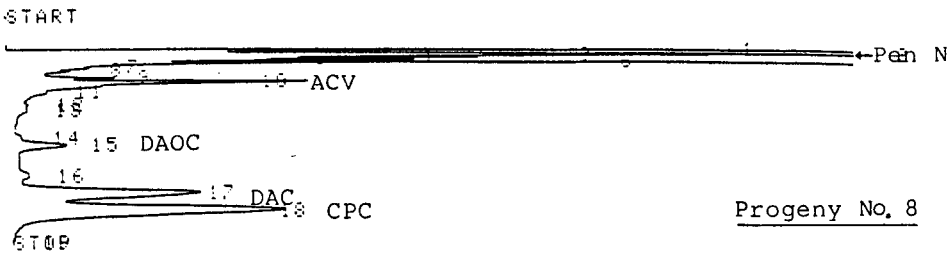


Fig. 4. 3 (cont) **Distribution of antibiotic titres amongst progeny recovered from cross 7.**

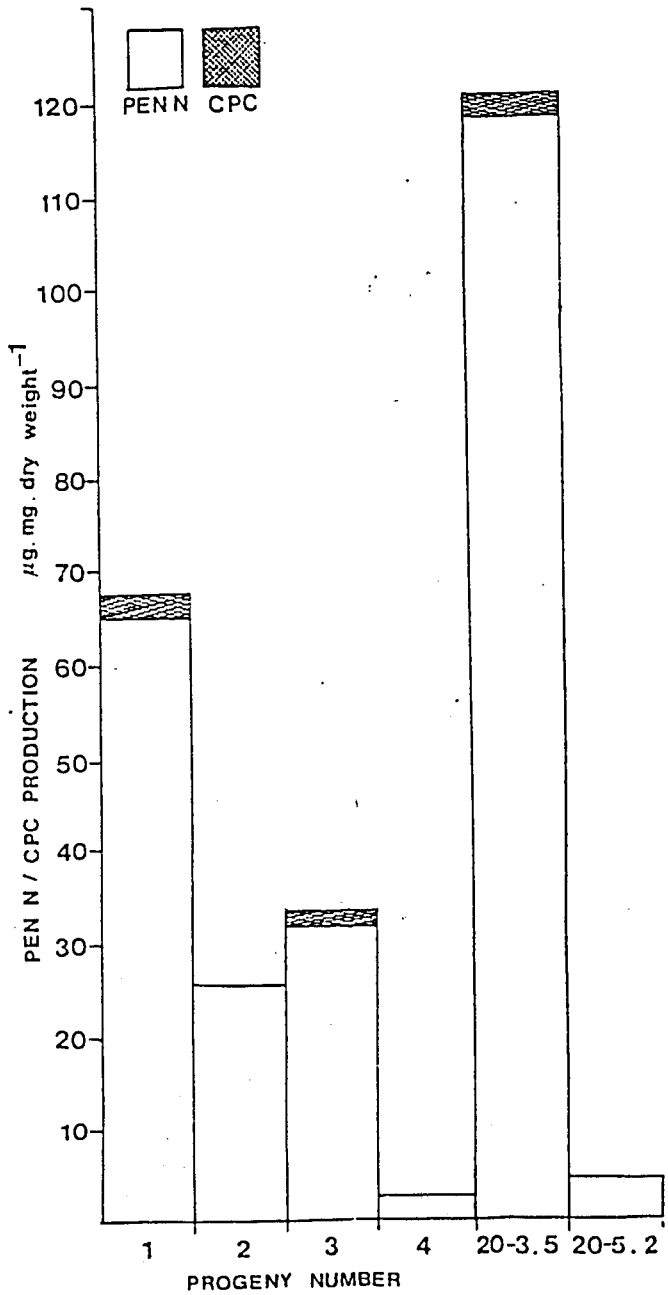


Progeny No. 5



Progeny No. 8

Fig. 4.4 Distribution of antibiotic titres amongst progeny recovered from cross 7.

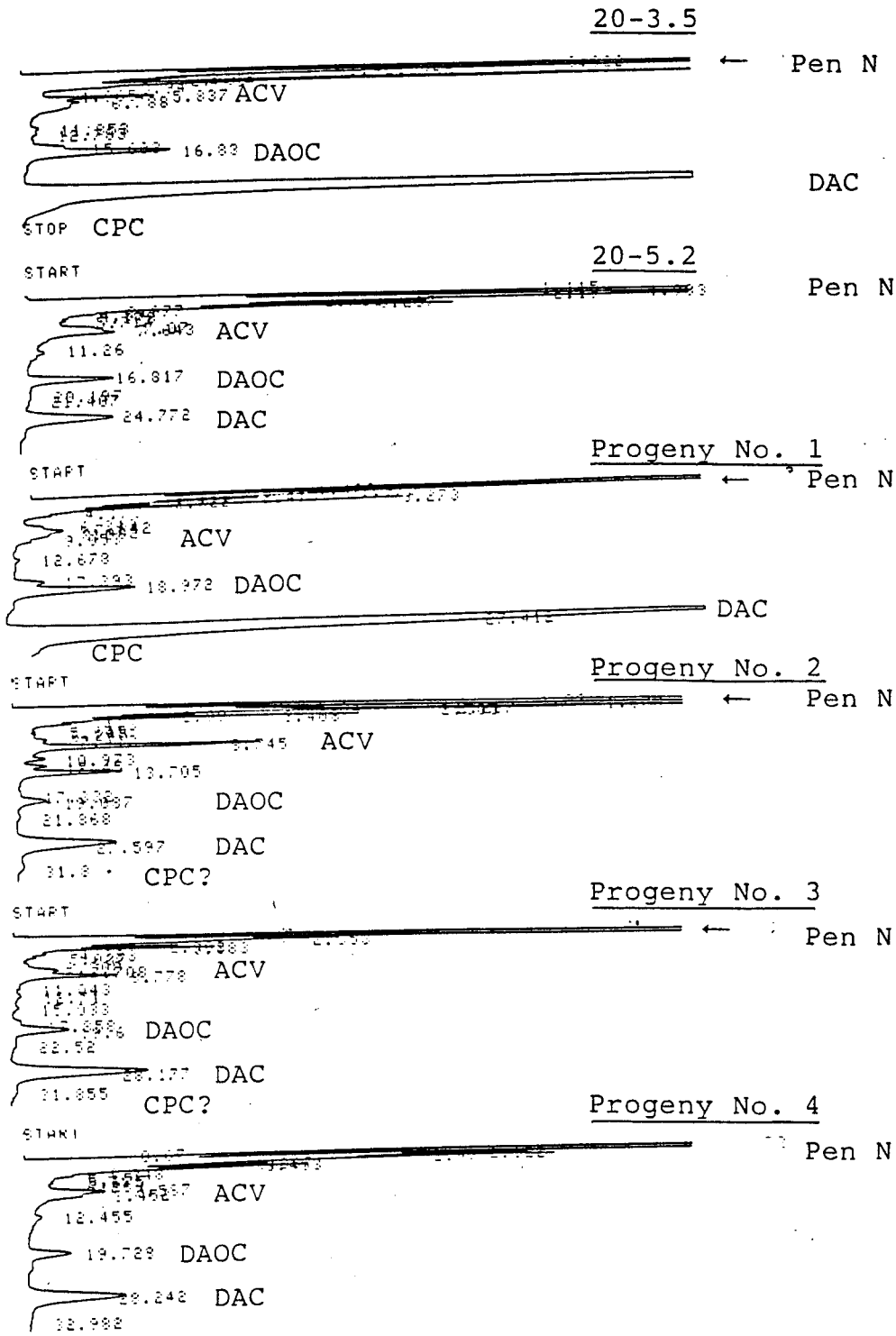


Two progeny strains (numbers 1 and 3) resembled the 20-3.5 parent strain in producing low levels of CPC, however, their Pen N titres were reduced in comparison to the parent. The remaining two strains both had titre characteristics similar to the other parent, 20-5.2. However, one of these (number 2 in Fig. 4.4) showed enhanced Pen N production. HPLC profiles of the strains were compared (Fig. 4.5) and revealed that where the progeny appeared to resemble the parental strains on the basis of the bioassay data, the pattern of accumulation of CPC biosynthetic intermediates could vary, hence progeny numbers 2 and 4 showed the accumulation of DAC apparently at the expense of DAOC. Taken as a whole the results are indicative of the occurrence of some recombination with respect to genes affecting β-lactam production. The extent to which this particularly concerned the cnp-8 and cnp-9 alleles cannot be determined. The divergent nature of the parental strains may have contributed a considerable level of background variation in titre to the cross.

4.3.4 Cross 8 20-3.10 (cnp-7 leu-4 tbz-6) x 20-2.32 (cnp-5), Cross 9 20-3.11 (cnp-7 leu-4 his-2)x 20-2.42 (cnp-5 orn-1)

Crosses 8 and 9 were carried out to test for

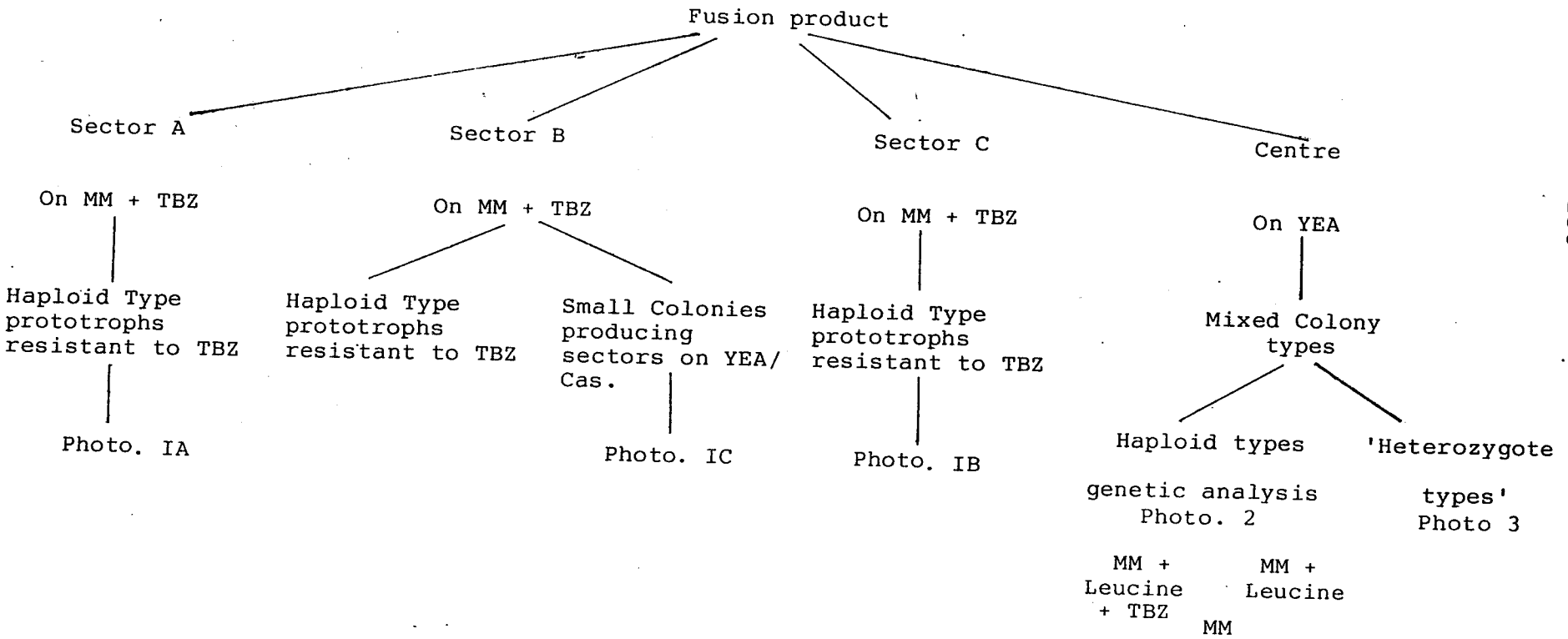
Fig. 4.5 **H.P.L.C. analysis of cross 7.**
20-3.5 (cnp-9 nic-1) x 20-5.2 (S⁻ cnp-8)



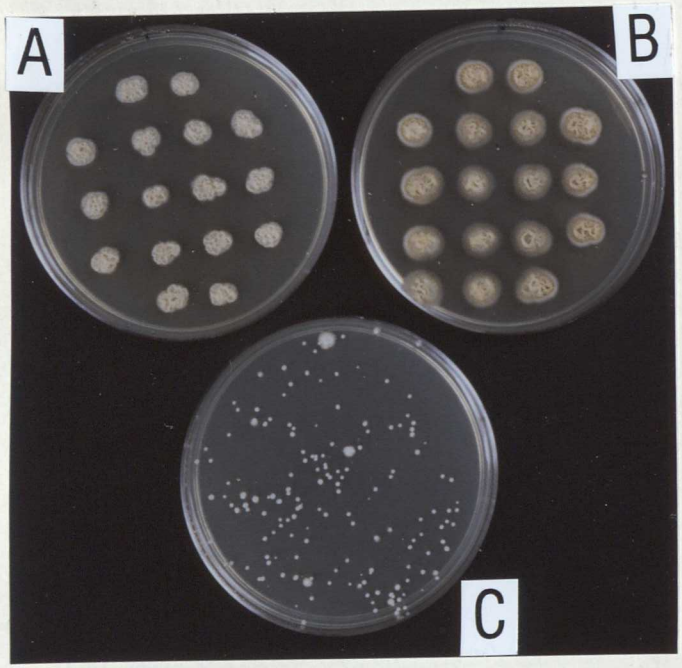
complementation between cnp-5 and cnp-7. Biochemically these mutations result in a decrease in the activity of isopenicillin N synthetase (IPNS). Both strain 20-3.1, the progenitor of strain 20-3.10, and a single colony isolate of strain 20-2.32 showed negligible IPNS activity on assay. However, the phenotype of strain 20-2.32 would appear to be not as stable as that of the other parental strain and on subculturing some B-lactam production occurred, so that when isolated following mutagenesis of strain 20-2.32, strain 20-2.42 was found to produce Pen N.

Progeny were selected following cross 8 on the basis of prototrophy and resistance to 30 uM thiabendazole. Only a small number of colonies were recovered from the fusion plates and these only after prolonged incubation. Seven of the isolates resembled haploid recombinants in their behaviour on purification and showed segregation with respect to the presence of chrysogenin, which is produced by 20-3.10 but not by the other parental strain. A further 3 colonies were examined which on purification gave a mixture of colony types. In two cases these had shown the presence of sectors on the original fusion plates. The results of the analysis of one of these putative fusion products are given in Fig. 4.6 and suggest that the original sectoring colony did possess areas of growth of a recombinant phenotype although

Fig. 4.6 Analysis of sectoring fusion product recovered following cross 8



1



2

3

the 20-3.10 parental phenotype could still be recovered from the central region of the colony.

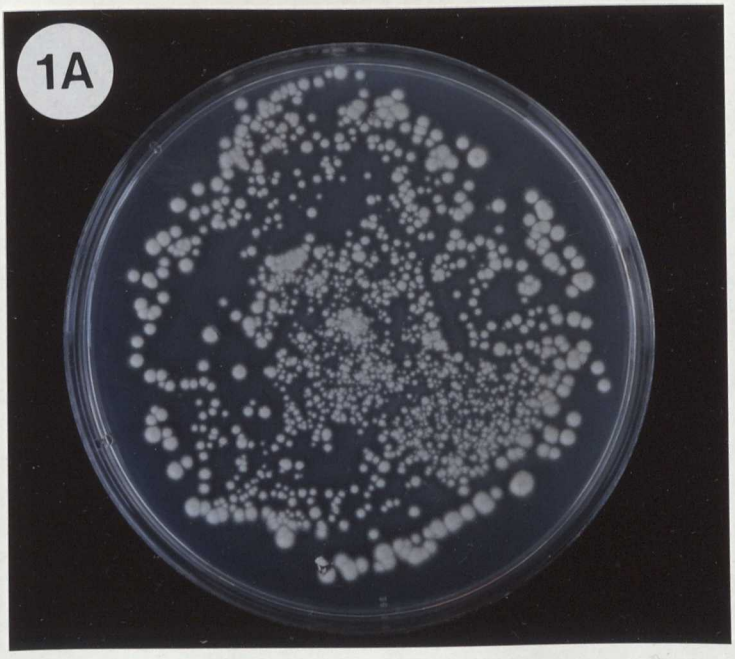
Further analysis of this cross was not undertaken partly because of the unusual length of time taken for the growth of the fusion products but mainly because the low number of progeny obtained indicated the necessity of repeating the cross in order to obtain sufficient numbers of recombinants for the complementation analysis of cnp-5 and cnp-7.

Following the introduction of additional auxotrophic markers into the two strains, used in cross 8 (see Fig. 2.5) to give strains 20-3.11 and 20-2.42, a further cross (cross 9) was performed. Presumptive haploid fusion products were recovered on media supplemented with either leucine or histidine, Fig. 4.7. On purification many of the progeny yielded heterogeneous populations of colonies which varied with respect to morphology and to chrysogenin production and which in turn gave further heterogeneous populations on purification (Fig. 4.8). It would appear that certainly for a proportion of the colonies the process of non-disjunction thought to follow relatively rapidly after diploid formation in C. acremonium had not been completed to give solely haploid recombinants. To assist in the recovery of progeny from the incompletely broken down fusion products a strategy of further purification was

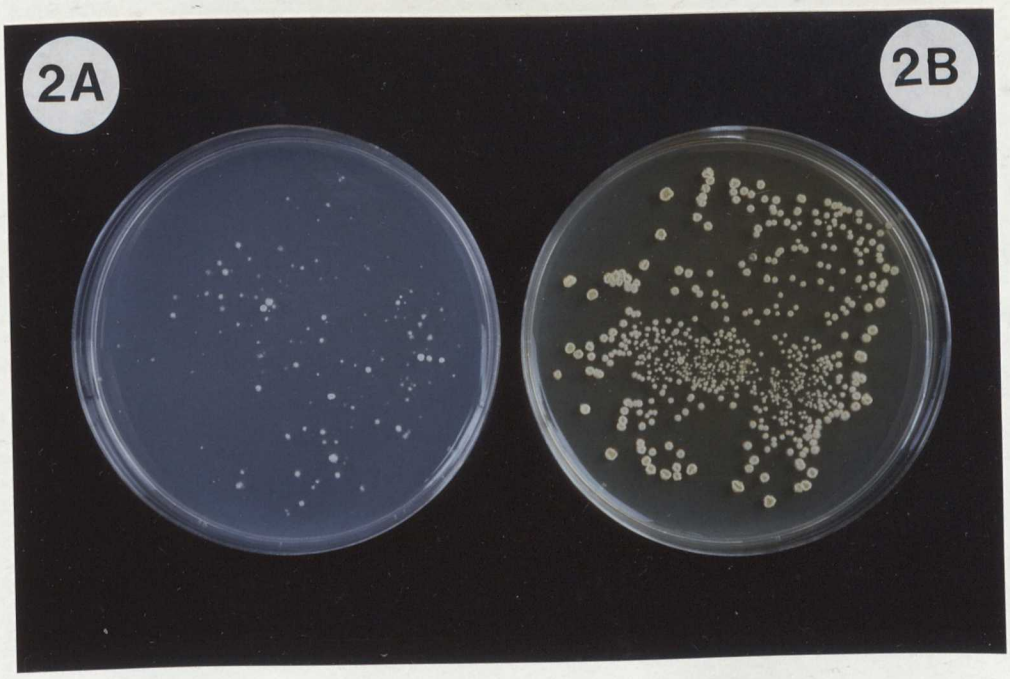
Fig. 4.7 Fusion products recovered from Cross 9.

Fig. 4.8 Examples of the heterogeneity observed during the purification of fusion products recovered from Cross 9 on MM + histidine

1A & B	Homogeneous and heterogeneous MM + histidine purification plates
2A & B	Heterogeneous and homogeneous MM + histidine + thiabendazole purification plates
2C	Heterogeneous colonies from 2A magnified approx x 7


1A


1B


2A
2B


2C

employed according to the scheme shown in Fig. 4.9. In order to avoid clonal effects, only one randomly chosen representative colony per original fusion product was selected following each round of purification. A low level of the fungicide thiabendazole (15-20μM) was included in the second stage purification plates but did not appear to result in a dramatic increase in the numbers of homogeneous colony types isolated in subsequent stages. After two rounds of purification on selective media to eliminate any syntrophically growing parental colonies the purification was continued on YEA/Casamino acids medium to allow the recovery of samples of all recombinant types arising from the 'heterozygous' fusion products. During purification many aberrant morphological types were seen (Fig. 4.10). These colonies were often still unstable and gave mixed populations on purification. In some cases these colonies (Fig. 4.11) resembled in appearance the 'stable heterozygote' described by Perez-Martinez (1984).

By comparison with A. nidulans where colonies aneuploid for specific linkage groups show specific morphologies (Upshall, 1971) it might be suggested that the range of aberrant morphologies observed amongst the products of this cross could represent specific aneuploid states in C. acremonium, however,

Fig. 4.9 Purification scheme for progeny isolated following cross 9

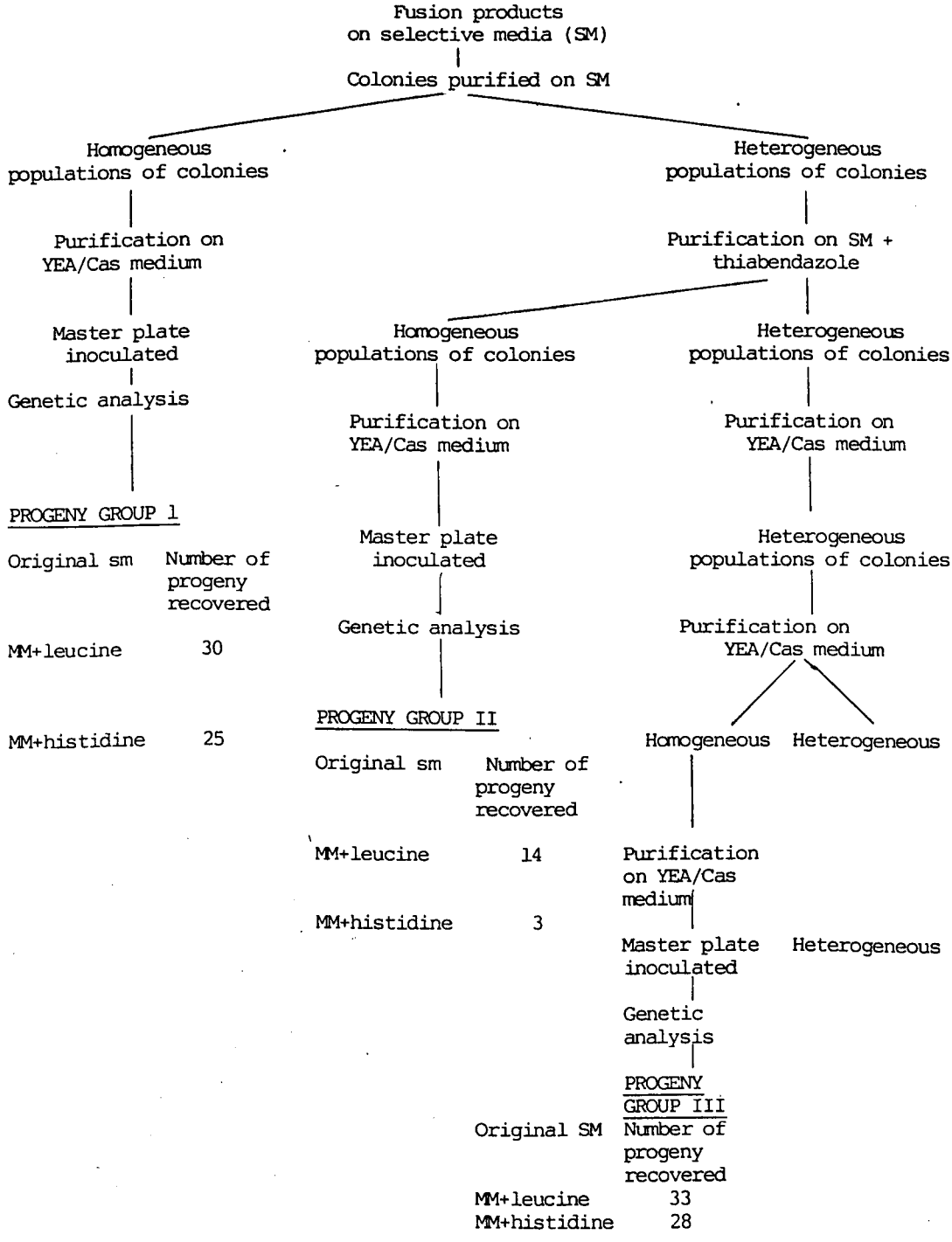


Fig. 4.10 Example of aberrant morphological colony types observed during purification of progeny from cross 9 on YEA/Cas medium.

Fig. 4.11 Colony resembling the 'stable heterozygote' described by Perez-Martinez (1984)

the lack of sufficient genetic markers in the parental strains did not permit this possibility to be investigated.

The unusual persistence of colonies which segregated to give mixed populations on purification and the requirement for as large a number of haploid progeny as possible from cross 9 led to the necessity of defining at which stage colonies would be accepted as being stable haploids suitable for phenotype analysis. The use of DNA estimations to determine ploidy was not feasible due to the large numbers of colonies which would have required screening and the difficulty frequently found in obtaining sufficient numbers of conidia. It is also unlikely that such estimations of ploidy would have been completely reliable in distinguishing haploids from colonies aneuploid for a small numnber of linkage groups. Therefore throughout the purification procedure isolates which gave morphologically homogeneous populations of colonies, with no visible evidence of sectoring on two consecutive purifications were taken as stable haploids and their phenotypes analysed. This approach though did not guarantee the haploid nature of the colonies selected. In many cases the colonies reaching this stage resembled morphologically one or other of the parental strains.

The number of progeny originating from the two

selective media which were taken for analysis from the different stages of the purification procedure is shown in Fig. 4.9. In view of their different origins the strains were designated into groups I, II and III. During analysis the progeny from these three groups were considered separately to allow the detection of any changes in the phenotypes being selected at the different stages of purification. Pairwise data for the groups can be found in Table 4.3, with Table 4.4 showing a summary of the distribution of the phenotypes found.

As the selection pressure was relaxed by allowing haploidization to continue on YEA/Casamino acids medium the recovery of alleles previously selected against shows their persistence amongst the fusion products through several selection stages. The poor recovery of the leu-4 wildtype allele on MM supplemented with leucine could be indicative of a selective advantage associated with the leu-4 allele under the given circumstances. There is little clear and consistent evidence for linkage between the auxotrophic markers involved in the cross. Data on progeny derived from MM supplemented with leucine shows the possibility of linkage between leu-4 and orn-1 but this is not apparent amongst the progeny derived from MM supplemented with histidine. Likewise data for group III isolates from MM plus leucine

Table 4.3
Pairwise analysis of progeny recovered following cross 9

(A) progeny originally recovered on MM + leucine

Group I Purified on MM + leucine

		orn		his		CPC		Chy	
		+	-	+	-	+	-	+	-
leu	+	(0)	-	0	-	0	0	0	0
	-	30	-	(30)	-	4	26	5	25
chy	+	5	-	5	-	0	5		
	-	26	-	26	-	4	21		

Group II Purified on MM + leucine + thiabendazole

		orn		his		CPC		Chy	
		+	-	+	-	+	-	+	-
leu	+	(0)	-	0	-	0	0	0	0
	-	14	-	14	-	3	11	6	(8)
Chy	+	6	-	6	-	0	6		
	-	8	-	8	-	3	5		

Group III Purified on MM + leucine + thiabendazole and subsequently on YEA/Cas

		orn		his		leu		chy		CPC*	
		+	-	+	-	+	-	+	-	+	-
orn	+			(3)	30	(3)	30	6	(27)	(5)	26
	-			0	(0)	0	(0)	(0)	0	(0)	0
his	+					3	(0)	(0)	3	(0)	3
	-					(0)	30	6	(24)	(5)	23
leu	+							(0)	3	(0)	3
	-							6	(25)	(5)	23
chy	+									(0)	6
	-									(5)	20

*Antibiotic titre was assessed for 31 of the 33 progeny recovered.

Table 4.3 continued

(B) Progeny Originally Recovered on MM+ Histidine

Group I Purified on MM + histidine

		leu		orn		CPC		chy	
		+	-	+	-	+	-	+	-
his	+	23	-	(23)	-	(19)	4	(0)	23
	-	(2)	-	2	-	(0)	2	0	(2)
chy	+	0	-	0	-	0	0		
	-	25	-	25	-	19	6		

Group II Purified on MM + histidine + thiabendazole

		leu		orn		CPC		chy	
		+	-	+	-	+	-	+	-
his	+	0	-	(0)	-	(0)	0	(0)	0
	-	(3)	-	3	-	(0)	3	0	(3)
chy	+	0	-	0	-	0	0		
	-	3	-	3	-	0	3		

Group III Purified on MM + histidine + thiabendazole and subsequently on YEA/Cas

		orn		his		leu		chy		CPC*	
		+	-	+	-	+	-	+	-	+	-
orn	+			(5)	21	(12)	14	4	(22)	(2)	20
	-			0	(2)	1	(1)	(0)	2	(0)	1
his	+					5	(0)	(1)	4	(2)	3
	-					(8)	15	3	(20)	(0)	18
leu	+							(2)	11	(2)	8
	-							2	(13)	(0)	13
chy	+									(1)	2
	-									(1)	19

( ) denotes recombinants
* Antibiotic titre was assessed for 23 of the 28 progeny recovered.

Table 4.4 Phenotype analysis for cross 9

A. Phenotype analysis of progeny originally selected on MM supplemented with leucine

Phenotype					Distribution Amongst progeny groups			
leu	chy*	his	orn	CPC**	I	II	III	Total
-	-	+	+	-	21	5		26
-	-	+	+	+	4	3		7
-	+	+	+	-	5	6		11
-	-	-	+	-			17	17
-	+	-	+	-			6	6
-	-	-	+	+			5	5
+	-	+	+	-			3	3

Overall CPC +/- ratio = 12:63

B. Phenotype analysis of progeny originally selected in MM supplemented with histidine

Phenotype					Distribution amongst progeny groups			
leu	chy*	his	orn	CPC**	I	II	III	Total
+	-	+	+	+	19		1	20
+	-	+	+	-	4		3	7
+	-	-	+	-	2	3	5	10
-	-	-	+	-			10	10
-	+	-	+	-			2	2
-	-	-	-	-			1	1
+	+	+	+	+			1	1

Overall CPC +/- ratio = 21:29

* =/- denotes presence/absence of chrysogenin
**+/- denotes production/non-production of CPC

suggests linkage of leu-4 and his-2 but this is not consistent throughout all of the data available.

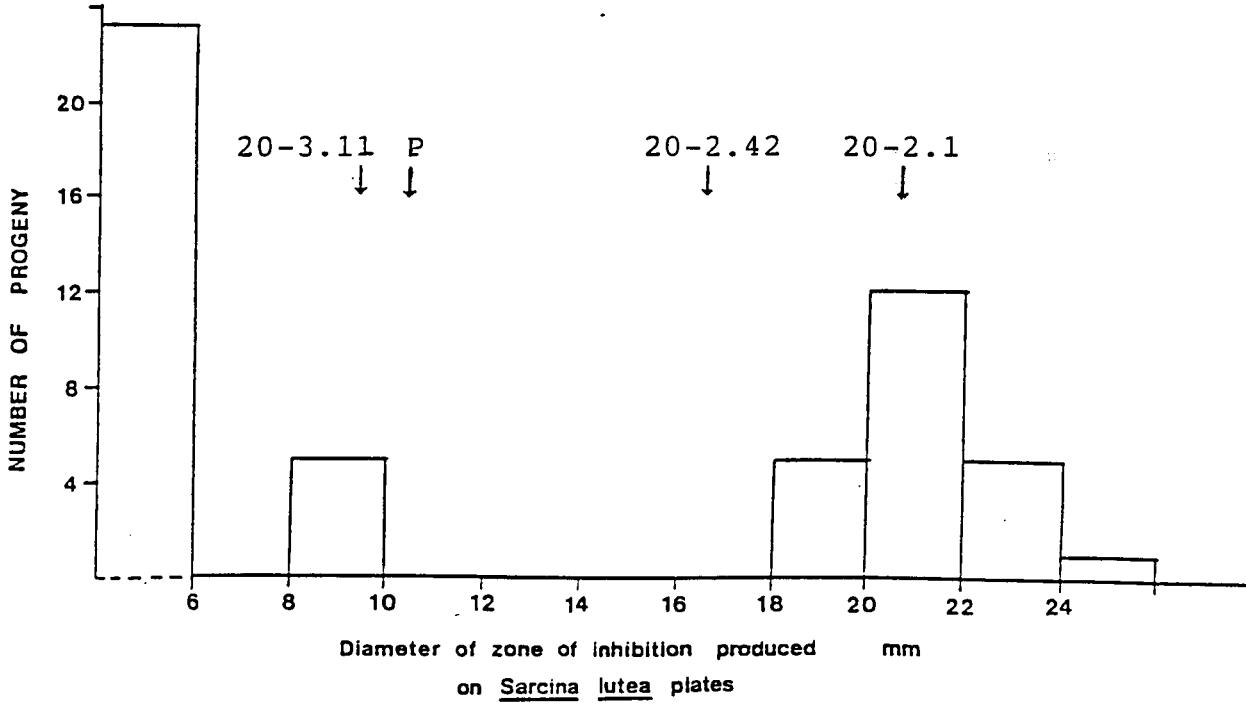
Progeny were tested for antibiotic production by plug assay, two plugs being assayed for Pen N and two for CPC production per isolate. A number of regenerant colonies from proto-plasts of the parental strains were assayed as controls. In a small number of cases progeny failed to give consistent assay results and consequently were omitted from the data. Plug assay results were considered primarily of being indicative of either CPC production or non-production. Evidence for the complementation of cnp-5 and cnp-7 was found amongst the progeny from both selective media used (Table 4.4). The numbers of CPC producing progeny recovered, suggest that is unlikely that cnp-5 and cnp-7 are allelic. Two non-allelic gene differences resulting in impaired CPC biosynthesis would be expected on segregation to give progeny producing CPC in a ratio of 1:3 producers to non-producers providing that there was no linkage between the genes and the selectable markers involved in the cross. This ratio is not found amongst the progeny isolated from either selective media. Progeny from MM plus histidine show a ratio close to 1:1 regarding CPC production contrasting with the situation amongst the progeny obtained from MM supplemented with leucine where an excess of CPC non-producers is seen. The

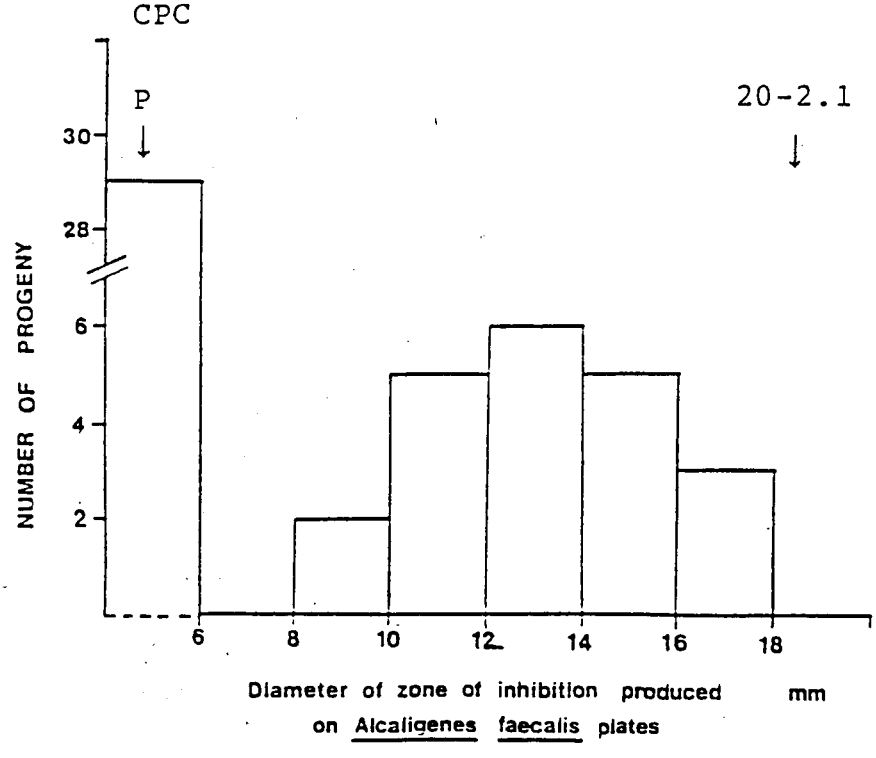
approximately 1:1 ratio on MM plus histidine could be explained by the linkage of either cnp-5 and orn-1 or cnp-7 and leu-4 resulting in the selective loss of one of the alleles adversely affecting CPC biosynthesis. Neither of these linkages are consistent with the data derived from progeny selected on MM plus leucine and the significance of the different ratios found with the two selective media remains unclear.

The distribution of Pen N and CPC titre amongst the progeny was also considered. Fig 4.12 shows a summary of progeny titres assessed in terms of the mean diameter of the zones of inhibition which were produced on bioassay. A breakdown of the titre distributions showing the contribution made by the different groups of progeny is shown in Appendix II. Detailed statistical analysis of the variability in titre shown was not carried out due to the lack of more accurate shake flask fermentation data. Progeny with cnp phenotypes resembling both parents were obtained in addition to those which show restored CPC production. A further group failed to produce even the small zone of inhibition typical of parent 20-3.11, when assayed against Sarcina lutea ATCC 9341. The majority of progeny falling into this category were isolated in group III after subculturing on YEA/Casamino acids medium and prolonged purification.

Fig. 4.12 Distribution of antibiotic titres amongst progeny recovered from cross 9

A. Pen N Progeny recovered on MM + histidine

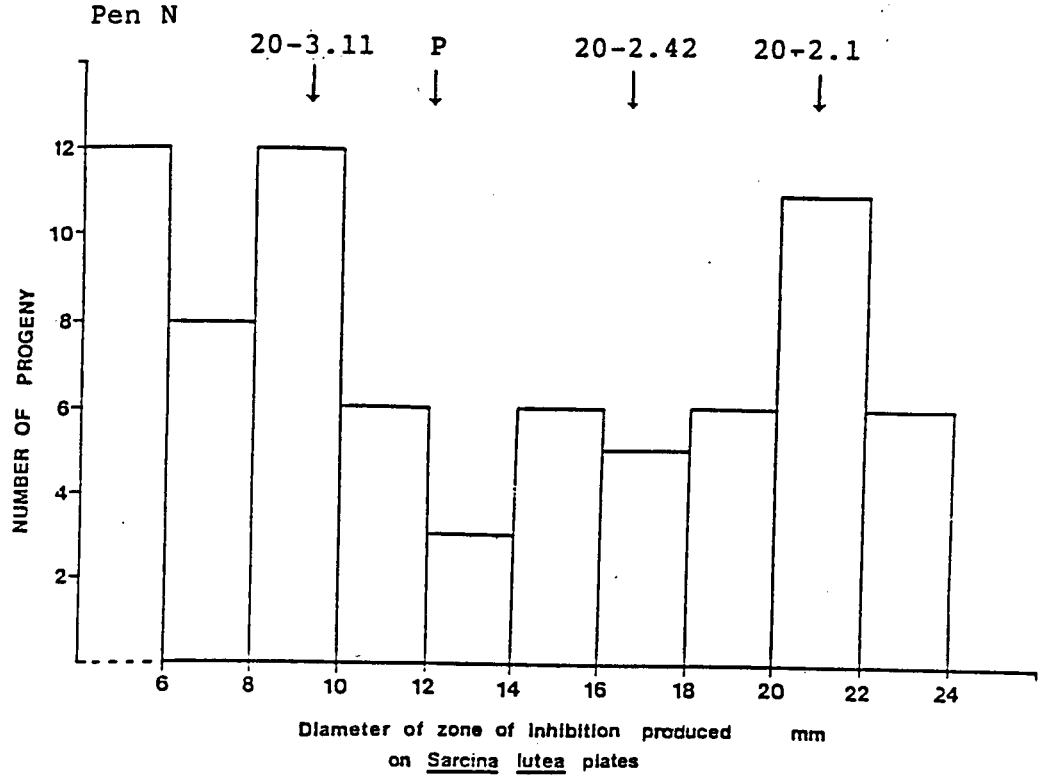


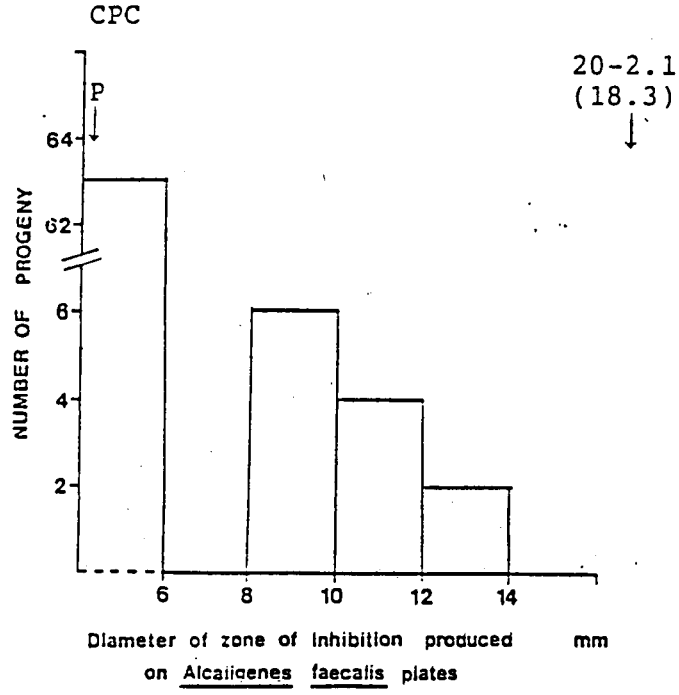


P = Progeny mean.

Fig. 4.12 Continued

B. Progeny recovered on MM + leucine





P = Progeny mean.

4.3.5 Cross 10 20-3.11(*cnp-7 leu-4 his-2*) x 20-2.43 (*cnp-6 met-13*).

The strains concerned in this cross differed phenotypically with respect to CPC biosynthesis; 20-3.11 being deficient in the production of isopenicillin N and strain 20-2.43 appearing to be impaired in the conversion of Pen N into DAOC. Fusion products were selected on MM supplemented with either leucine or histidine. On purification the behaviour of the presumptive haploid fusion products resembled those recovered from cross 9 in producing heterogeneous populations of colonies. These were purified according to the protocol described previously and the numbers of progeny selected after the different stages of purification are shown in Fig. 4.13. Phenotype analysis was carried out, again keeping the results from the different groups of progeny separate and using the plug assay method to determine antibiotic titres.

Table 4.5 shows the pairwise analysis for this cross with Table 4.6 showing a summary of the phenotypes recovered at the different stages of the purification procedure. Much of the data suggests free assortment between the auxotrophic and the chrysogenin markers, although some indication of linkage between *his-2* and chrysogenin production is

Fig. 4.13 Purification scheme for progeny isolated following cross 10

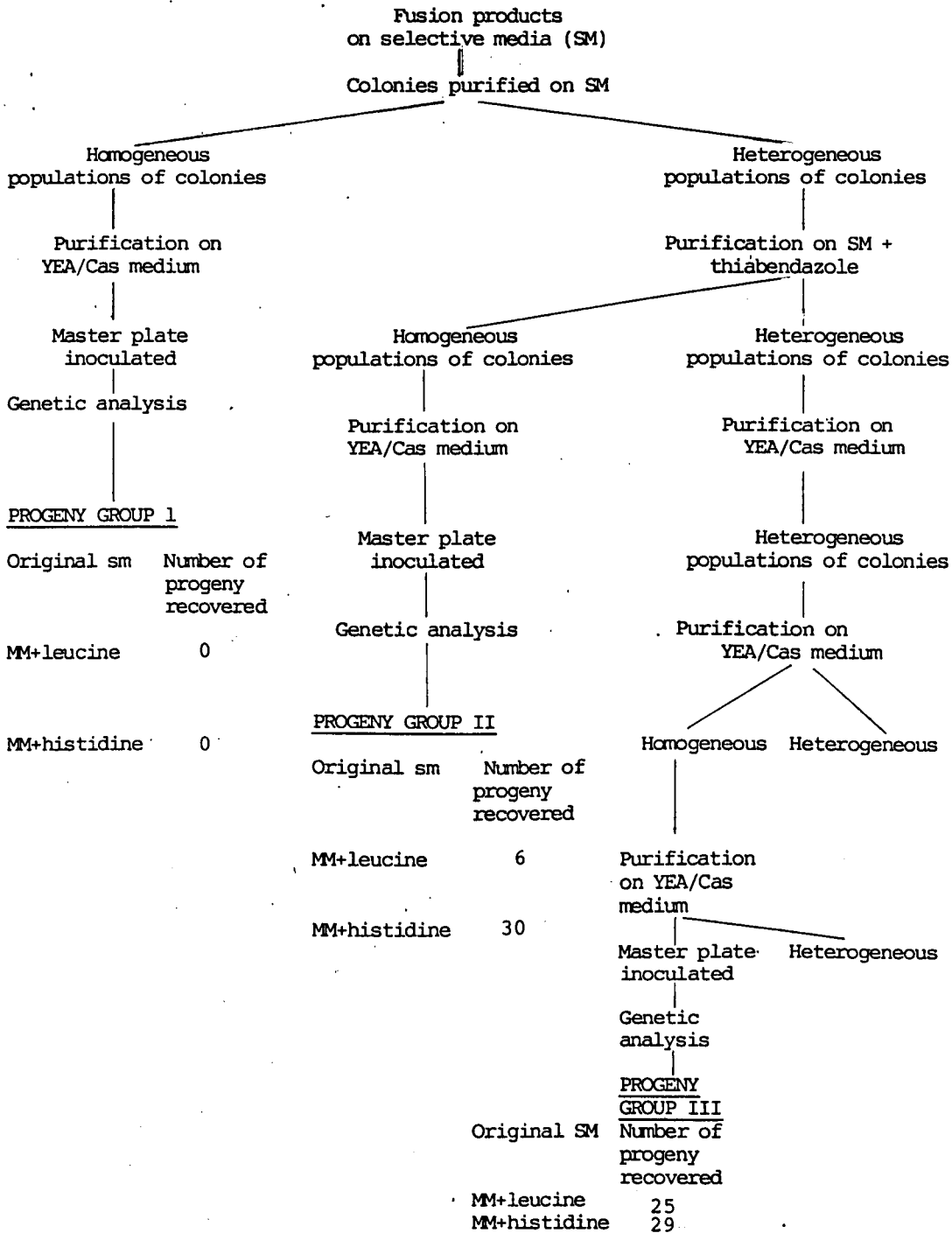


Table 4.5 Pairwise analysis of progeny recovered following cross 10

(A) Progeny originally recovered on MM + leucine

Group II Purified on MM + leucine + thiabendazole

		CPC		chy		his		met	
		+	-	+	-	+	-	+	-
leu	+	1	1	(0)	2	2	-	(2)	-
	-	0	4	4	(0)	(4)	-	4	-

Group III Purified on MM + leucine + thiabendazole and subsequently on YEA/Cas

		leu		his		met		chy		CPC*	
		+	-	+	-	+	-	+	-	+	-
leu	+			11	(3)	(13)	1	(2)	12	4	7
	-			(8)	3	10	(1)	1	(10)	0	8
his	+					(19)	0	(2)	17	4	12
	-					4	(2)	1	(5)	0	3
met	+							2	(21)	4	14
	-							(1)	1	0	1
chy	+									1	2
	-									3	13

( ) denotes recombinants
* Antibiotic titre was assessed for 19 of the 25 progeny recovered

Table 4.5 Continued

(B) Progeny originally recovered on MM + histidine

Group II Purified on MM + histidine + thiabendazole

		leu		his		met		chy		CPC*	
		+	-	+	-	+	-	+	-	+	-
leu	+			19	(11)	(30)	0	(10)	20	2	21
	-			-	-	-	-	-	-	-	-
his	+					(19)	-	(0)	19	2	12
	-					11	-	10	(1)	0	9
met	+							10	(20)	2	21
	-							-	-	-	-
chy	+									0	8
	-									2	13

Group III Purified on MM + histidine + thiabendazole and subsequently on YEA/Cas

		leu		his		met		chy		CPC**	
		+	-	+	-	+	-	+	-	+	-
leu	+			14	(8)	(20)	2	(3)	19	3	12
	-			(3)	4	7	(0)	1	(6)	1	6
his	+					(17)	0	(3)	14	4	12
	-					10	(2)	1	(11)	0	6
met	+							4	(23)	4	18
	-							(0)	2	0	0
chy	+									3	1
	-									1	17

* antibiotic titre was assessed for 23 of the 30 progeny recovered

** antibiotic titre was assessed for 22 of the 29 progeny recovered

Table 4.6 Phenotype analysis for cross 10

A. Phenotype analysis of progeny originally selected on MM supplemented with leucine

Phenotype					Group I	Group II	GroupIII	
leu	his	met	chy	CPC	MM+leu	MM+leu TBZ	MM+ leu TBZ YCH	Total
+	+	+	-	+		1	3	4
+	+	+	-	-		1	5	6
-	+	+	+	-		4	1	5
+	+	+	+	+			1	1
+	-	-	+	-			1	1
-	+	+	-	-			7	7
-	-	+	-	-			1	1

Overall CPC +/- ratio = 5 : 20

B. Phenotype analysis of progeny originally selected on MM supplemented with histidine

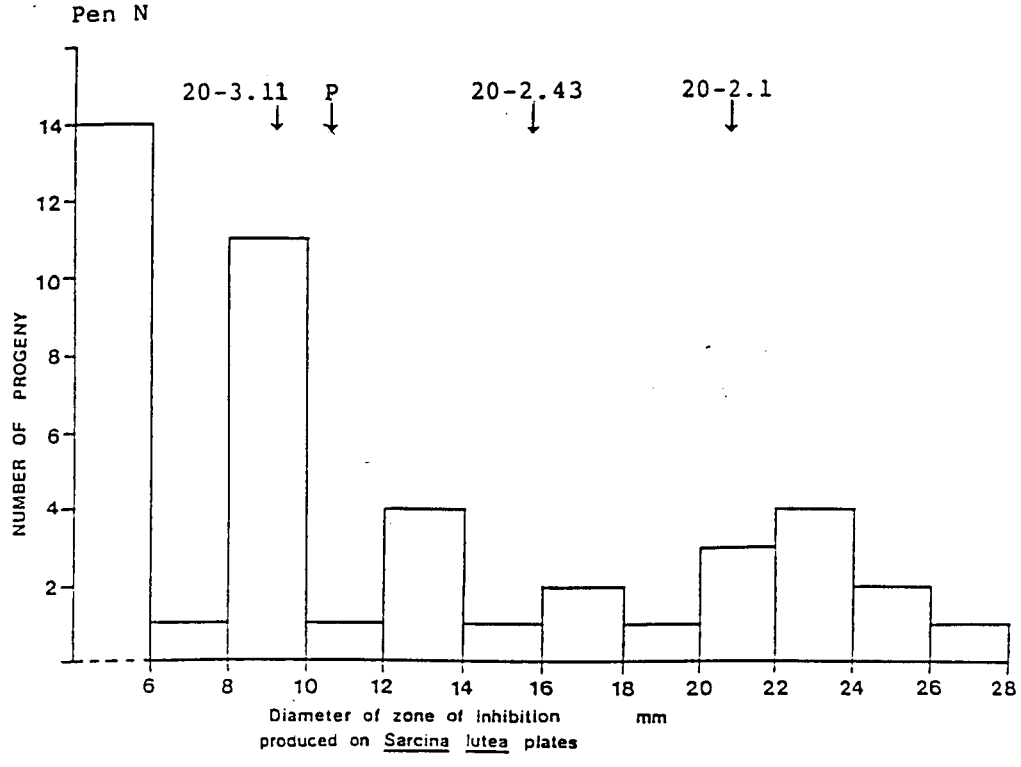
Phenotype					MM+his GroupI	MM+his TBZ GroupII	MM+his TBZ YCH GroupIII	Total
leu	his	met	chy	CPC				
+	+	+	-	-		12	10	22
+	+	+	-	+		2		2
+	-	+	+	-		8		8
+	-	+	-	-		1	2	3
+	+	+	+	+			3	3
-	-	+	-	-			3	3
-	+	+	-	-			2	2
-	-	+	+	-			1	1
-	+	+	-	+			1	1

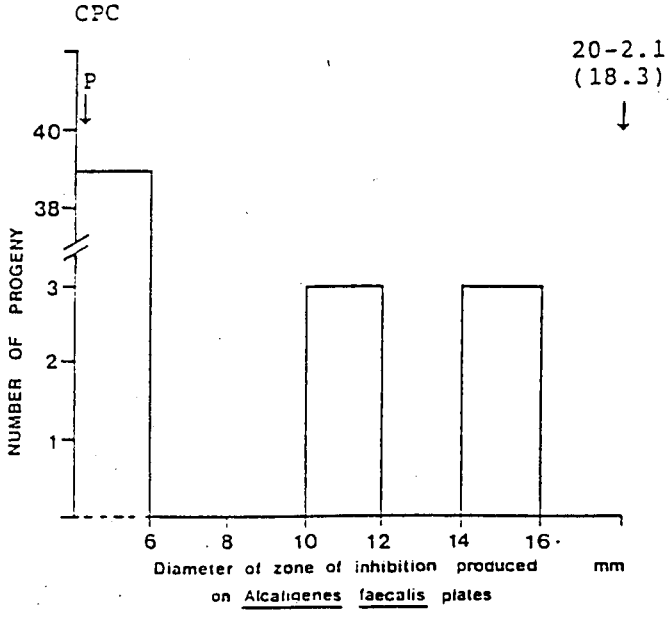
Overall CPC+/- ratio = 6:39

given by the results for group II progeny selected from MM plus histidine. However, this is not substantiated amongst the other progeny groups. Examination of the variation in Pen N and CPC titres amongst the progeny reveals that the titre characteristics of both parental strains were recovered amongst the progeny (Fig. 4.14 and Appendix III). A group of recombinants which on assay produced less inhibition of Sarcina lutea ATCC 9341 than the 20-3.11 parent were also recovered, showing similarity with a group of progeny from Cross 9. CPC producing recombinants were found showing the complementation of cnp-6 and cnp-7. The numbers of CPC producers from both selective media were low, falling below the level expected if two unlinked genes were segregating freely. However, there is no strong case for linkage shown in the data. The low recovery of CPC producers could then be a reflection of differences in the selective advantage or disadvantage of certain phenotypes under the conditions of this fusion. An understanding of factors which could result in bias amongst the progeny recovered could only be gained as further crosses involving the parental strains were examined to reveal any trends in the types of progeny recovered.

Fig. 4.14 Distribution of antibiotic titre amongst progeny recovered from cross 10

A. Progeny recovered on MM + histidine

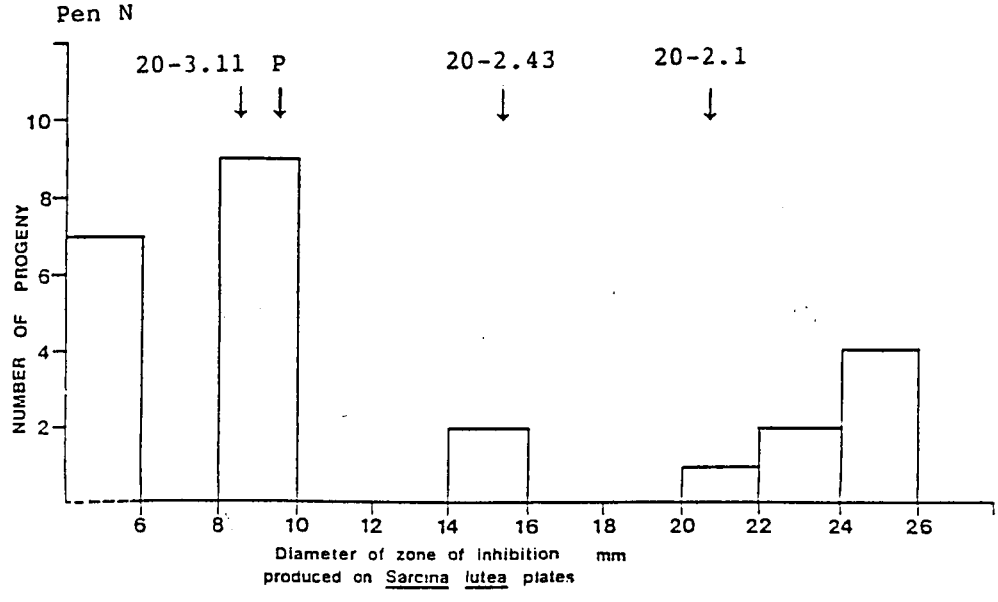


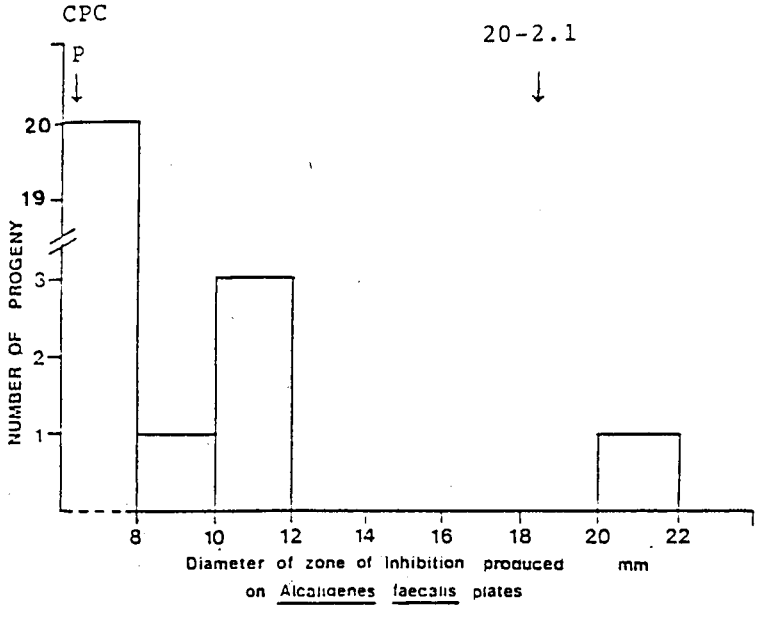


P= Progeny mean.

Fig. 4.14 Continued

B. Progeny recovered on MM + leucine



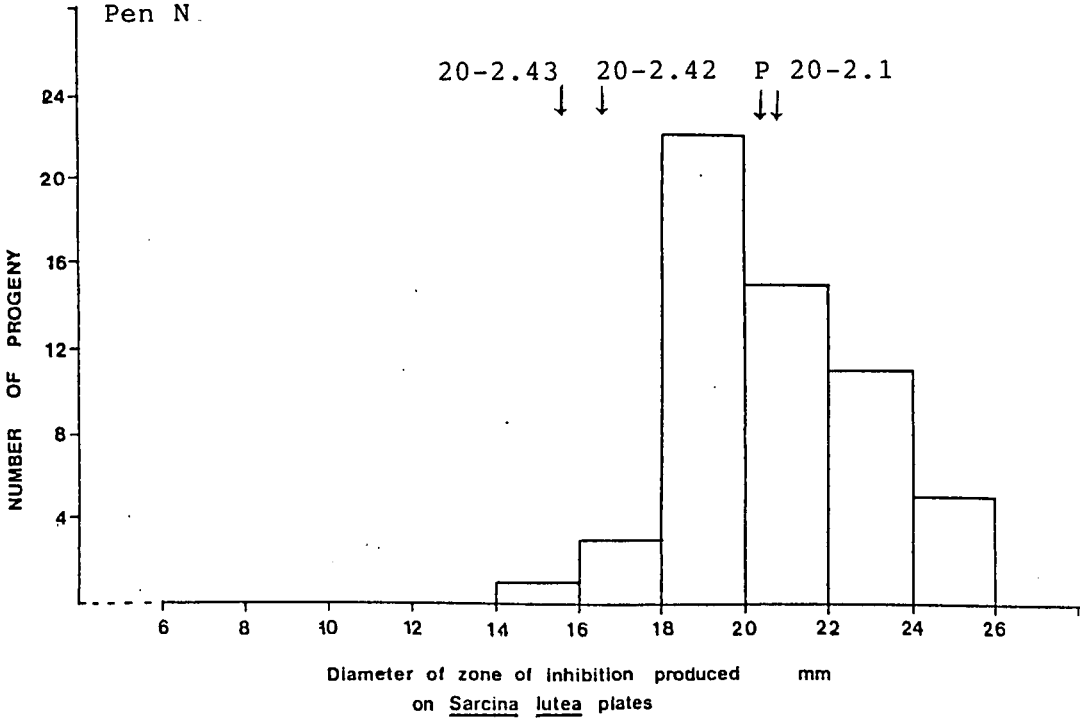


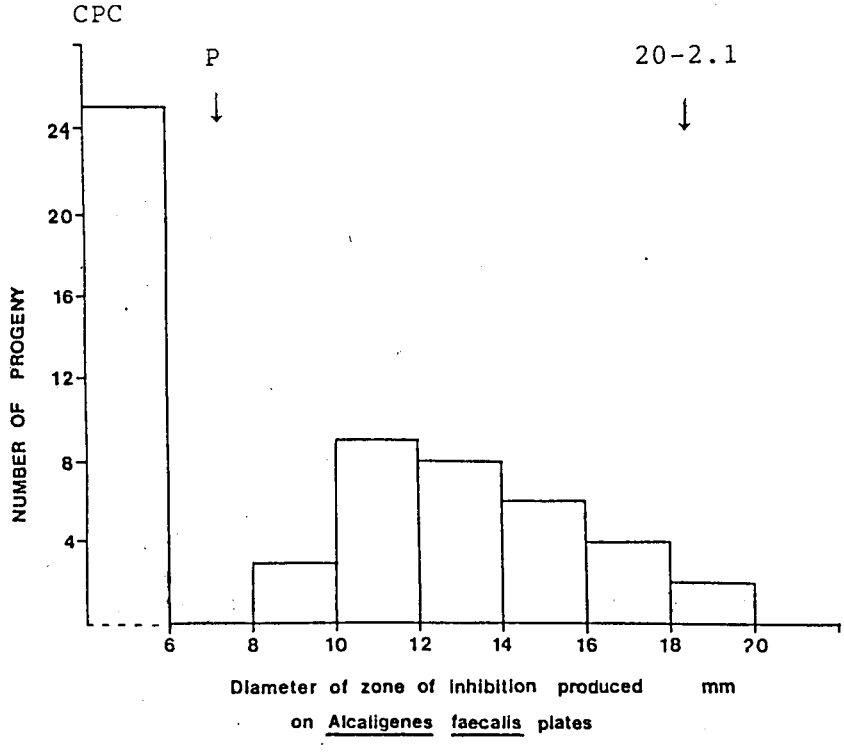
p = Progeny mean.

4.3.6 Cross 11 20-2.42 (*cnp-5* *orn-1*) x 20-2.43 (*cnp-6* *met-13*).

Putative haploid progeny were recovered on MM following this fusion. On purification a tendency to produce heterogeneous populations of colonies was seen but the problem was not as severe as amongst the progeny of crosses 9 and 10. Of the 57 prototrophic progeny finally obtained, 37 appeared stable following the first purification on MM and the remaining 20 were isolated after a second round of purification on MM supplemented with 15-20 μM thiabendazole. These were assessed for B-lactam production by plug assay giving the results presented in Fig. 4.15. Over half of the progeny (32 out of 57) tested were CPC producers, showing the *cnp-5* and *cnp-6* mutations to be complementary. Neither of the wild-type alleles of the *cnp-5* and *cnp-6* markers had been lost as a result of the selection against the linkage groups carrying *met-13* and *orn-1*. The higher number of CPC producing progeny obtained would suggest that *cnp-5* and *cnp-6* are not linked and could also be indicative of linkage between either *cnp-5* and *orn-1* or *cnp-6* and *met-13*. Further investigations of these possibilities would require the introduction of further selectable markers into the parental strains to allow the recovery and study of progeny carrying the *orn-1* or *met-13* markers.

Fig. 4.15 Distribution of antibiotic titres amongst progeny recovered from cross 11.





P = Progeny mean.

4.3.7 Cross 12 20-2.44 (*cnp-1 ino-2*) x 20-2.49 (*cnp-6 met-13 tbz-9*)

The markers present in the parental strains used in this fusion allowed the selection of possible haploid recombinants on either MM or MM supplemented with inositol plus 30μM thiabendazole. Selectants were obtained on both media. This proved to be a more typical cross and the problems due to heterogeneity of fusion products described previously did not arise. Progeny were analysed with respect to the relevant non-selective marker for the selection system used and for β-lactam production. Table 4.7 gives the distribution of phenotypes recovered and Fig. 4.16 illustrates the distribution of titres found amongst the progeny. There was no indication of linkage between the *met-13*, *tbz-9* and *ino-2* markers. The low recovery of *ino-2*$^+$ *tbz-9*$^+$ colonies on MM could be indicative of linkage but it is not consistent with the recovery of considerable numbers of progeny carrying the *ino-2* marker on MM supplemented with inositol and thiabendazole. For progeny obtained from both selection systems the range of Pen N titres found spans the titres of both the parental strains. In both cases a number of CPC producers were isolated although the level of restored production was decreased compared to the production of CPC by the 20-

Table 4.7 Phenotype analysis for cross 12

A. Phenotype analysis of progeny recovered on MM

Phenotype				Number
met	ino	TBZ*	CPC*	
+	+	+	-	4
+	+	+	+	2
+	+	-	-	71
+	+	-	+	5
				82

Ratio CPC +/- = 7:75

B. Phenotype analysis of progeny recovered on MM + inositol + thiabendazole

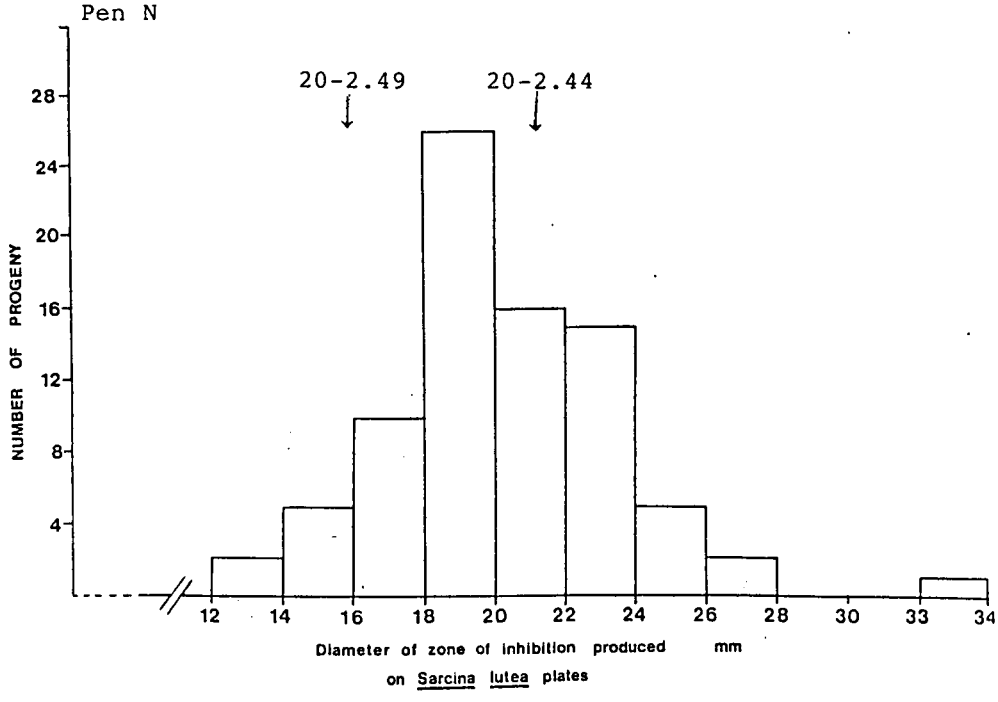
Phenotype				No.
met	ino	TBZ*	CPC*	
+	+	-	-	61
+	-	-	-	17
+	+	-	+	4
+	-	-	+	23
				105

Ratio CPC +/- + 27:78

* + = wild type i.e. sensitive to thiabendazole or CPC producing

Fig. 4.16 Distribution of antibiotic titres amongst progeny recovered from Cross 12

A. Progeny recovered on MM



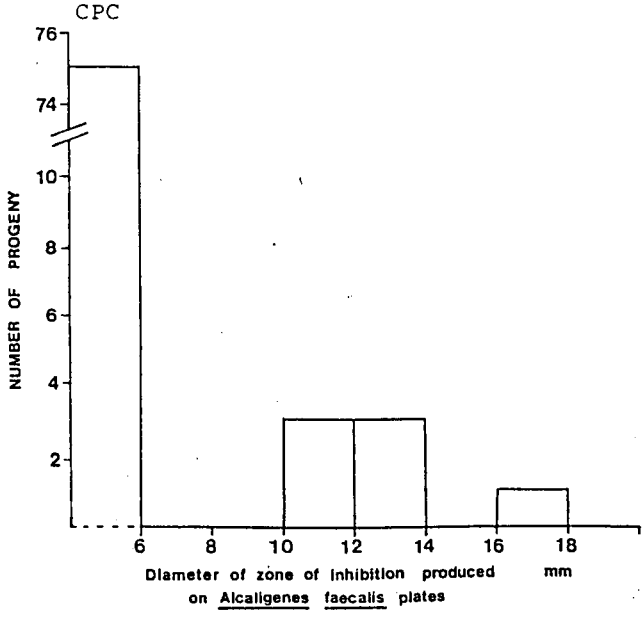
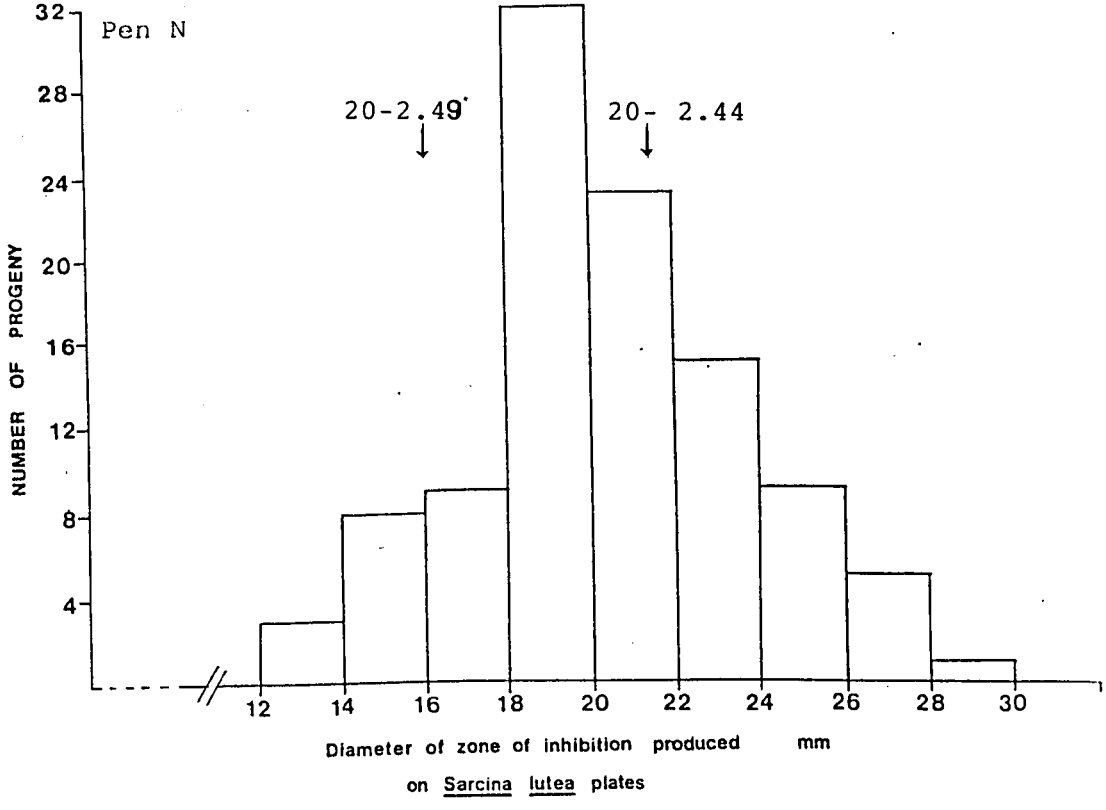
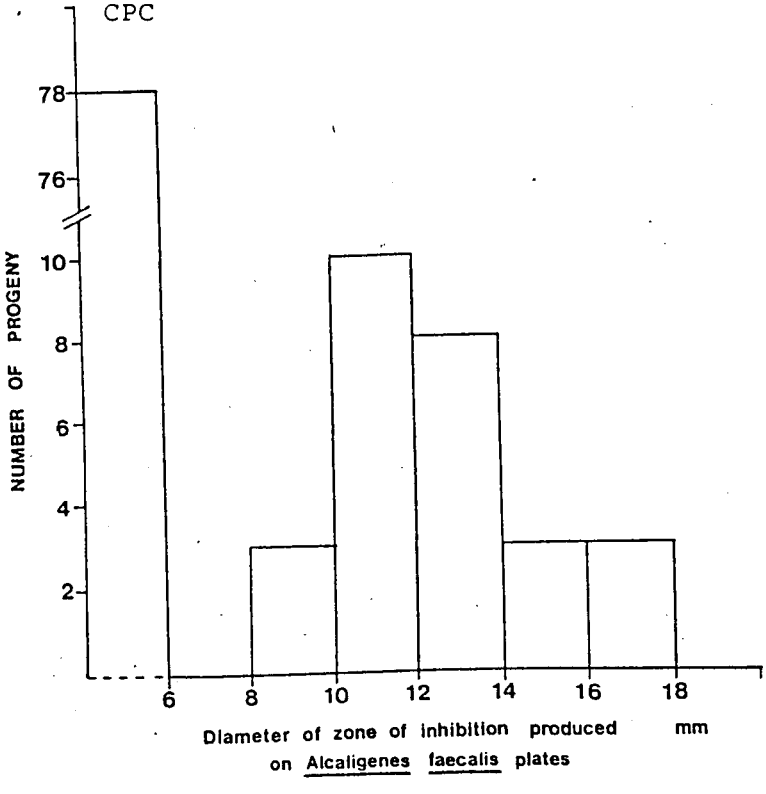


Fig. 4.16 Continued

(B) Progeny recovered on MM + inositol + TBZ





2.1 controls. That CPC production at any level should be restored is of interest since both the parental strains were deficient in the conversion of Pen N into DAOC. The ratio of CPC producers to non-producers isolated from MM supplemented with inositol plus thiabendazole suggests that cnp-1 and cnp-6 are non-allelic and unlinked either to each other or to tbz-9 or met-13.

In contrast a lower ratio of producers to non-producers was found amongst the progeny obtained by selection on MM. Linkage of cnp-6 to the wild-type allele of ino-2 could be one explanation for this, with the number of inositol independent progeny competent to produce CPC being low (8.5% of the progeny selected on MM and 3.8% of those selected on MM plus inositol and thiabendazole) and with similar numbers of inositol requiring CPC producers and non-producers being recovered. However, it is possible that the difference in numbers of CPC producing progeny recovered could be the result of the differential survival of recombinant types under the two selection systems used.

4.4 Discussion

A number of C. acremonium strains carrying mutations having major effects on CPC biosynthesis

were used in protoplast fusion crosses designed to test the complementary nature of the mutations involved. Not all possible pairs of mutations were examined because of the time scale involved, however, a representative group of tests were carried out.

Progeny were examined primarily to determine their ability or lack of ability to synthesise CPC with the quantitative aspects of the analysis being of secondary concern. In a number of cases, this led to the use of a plug assay system as a means of evaluation of β-lactam titre. The plug assay method facilitated the screening of larger numbers of progeny per given period of time than would have been possible had shake flask fermentations been used. Results of crosses described in Chapter 3 suggested the system to be reasonably reliable in giving a profile of the spectrum of β-lactam titres found amongst the progeny of a cross. The system is not as satisfactory though for providing data for quantitative analysis in that no measure of growth is taken into account and absolute potency values cannot be calculated for the progeny. Neither is the detailed analysis of fermentation broths by HPLC which was of use in crosses 5, 6 and 7 possible. Ideally shake flask fermentations would have been carried out on the progeny from all of the crosses to allow the data regarding antibiotic titre obtained to be processed to

examine the statistical evidence for or against the linkage of factors affecting titre and other markers present.

Complementation to give CPC progeny was observed in the majority of crosses (Table 4.8). The results of three crosses (5, 6 and 7) were more difficult to interpret. Because of the leaky nature of the nic-1 marker, only a very small number of progeny was obtained in crosses 6 and 7. The failure to observe recombination in cross 5 to yield CPC producing progeny, despite the nature of the biochemical differences of the parental strains suggesting that this should have been possible, was also a reflection of the number of progeny obtained. This was not sufficient to either confirm or eliminate the possibility of linkage between the two mutated CPC genes. In the case of linkage the recovery of CPC producing progeny would be the consequence of mitotic recombination. The results of cross 9 showed that recombination between strain carrying cnp-5 and cnp-7 gave progeny with a restored capacity to synthesise CPC. Earlier biochemical evidence had suggested that mutations cnp-5 and cnp-7 both resulted in impaired IPNS activity although the phenotype conferred by cnp-7 appeared stable whilst the strain carrying cnp-5 showed a tendency to possess IPNS activity on subculturing. Genetic results indicate that cnp-5

Table 4.8 **Complementation between *C. acremonium* strains carrying mutations resulting in impaired CPC biosynthesis.**

Cross 1.	Parental strains	*cnp* mutations present	Presence/Absence (+/-) of complementation ? denotes results inconclusive
5	20-3.4	*cnp*-7	?
	20-5.2	*cnp*-8	
6	20-3.4	*cnp*-7	?
	20-3.5	*cnp*-9	
7	20-3.5	*cnp*-9	?
	20-5.2	*cnp*-8	
8	20-3.10	*cnp*-7	?
	20-2.32	*cnp*-5	
9	20-3.11	*cnp*-7	+
	20-2.42	*cnp*-5	
10	20-3.11	*cnp*-7	+
	20-2.43	*cnp*-6	
11	20-2.43	*cnp*-6	+
	20-2.42	*cnp*-5	
12	20-2.49	*cnp*-6	+
	20-2.44	*cnp*-1	

and cnp-7 are not allelic and the frequency of CPC producing recombinants recovered from MM supplemented with histidine also suggests that they may not be located on the same linkage group. Ramos et al (1986) proposed that the mutation resulting in the loss of CPC production carried by strain N-2 (20-3.1) the progenitor of 20-3.11 is regulatory in nature. They suggested that the expression of the genes coding for IPNS, isopenicillin N epimerase and deacetoxycephalosporin C synthetase was eliminated by the mutation carried in N-2 (20-3.1).

A comparison of DNA sequence data for the cloned C. acremonium IPNS gene with that for the same gene isolated from strain N-2 (20-3.1) though does not support this view. On the contrary strain N-2 (20-3.1) would appear to produce an inactive IPNS enzyme as the result of a single base pair change in the coding region of the gene (Harford pers. com.). Therefore cnp-7 would appear to correspond to a mutation in the structural gene for IPNS. This coupled with the complementation shown between cnp-7 and cnp-5 would suggest that cnp-5 may effect a gene other than that coding for the IPNS enzyme itself. The nature and role of the gene affected in the cnp-5 strain is not known, although speculations could be made concerning either a role connected with regulation or co-factor availability.

Cross 12 also involved two strains which showed phenotypic similarity in both being deficient in the ring expansion of Pen N to yield DAOC. The results of this cross suggest that the mutations involved, cnp-1 and cnp-6 are not linked, with a considerable number of CPC producing progeny being recovered on MM supplemented with inositol plus thiabendazole. Linkage between cnp-6 and the wild-type allele of ino-2 being a possible explanation for the low recovery of the CPC producing phenotype on MM. The mutations impairing CPC biosynthesis in the parental strains would, therefore, appear to be affecting different genes whilst resulting in similar phenotypes. From the information available it was not possible to draw any conclusions as to the identity of the biochemical functions affected in each of the two strains. It would be of interest to determine whether or not deacetoxycephalosporin C synthetase activity is present in the strains carrying cnp-1 or cnp-6. Felix et al (1981) reported the isolation of a mutant C. acremonium strain which overproduced Pen N apparently as a result of the mislocation of this enzyme in the cell such that when preparations of permeabilized cells were assayed active enzyme was present and able to convert exogenously supplied Pen N into DAOC. This would provide one possibility as to the types of mutations other than those affecting the

structural gene which could result in the phenotype shown by the strains carrying cnp-1 and cnp-6. Other possibilities could involve a regulatory function or a role concerned with co-factor availability for one or both of the genes affected. Scheidegger et al (1984) showed the close association of deacetoxycephalosporin C synthetase and deacetoxycephalosporin C hydroxylase activity during purification. The two enzyme activities have recently been found to remain associated in a ratio of 7 to 1 deacetoxycephalosporin C synthetase to hydroxylase on purification with a single monomeric protein of molecular weight 41,000 +/- 2,000 most probably appearing to be responsible for both activities (Dotzlaf and Yeh 1987) giving support to Scheidegger et al (1984) who suggested that a single bifunctional enzyme existed. Dotzlaf and Yeh (1987) comment that the stoichiometry of the conversion of Pen N into DAOC and DAC does not eliminate the possibility that these stages may involve the existence of enzyme bound intermediates. Were this so it is possible that an explanation for the role of the genes mutated in the strains carrying cnp-1 and cnp-6 might be related to the formation of such intermediates.

Several of the crosses carried out yielded progeny which were more difficult to purify than the colonies normally isolated from fusion plates. Colonies

resembling putative haploid recombinants showed considerable heterogeneity which in many cases persisted throughout several rounds of purification. This was particularly noticeable in the divergent crosses 9 and 10 between strains from the 20-3.1 (N-2) and 20-2.1 (CO728) lineages. The inclusion of thiabendazole at low levels in the growth medium did not appear to significantly increase the degree of haploidisation occurring. Hamlyn (1982) reported the occurrence of abnormal heterozygous colonies following a fusion between two strains derived from the M8650 progenitor strain. These colonies were presumed to be aneuploids and on transfer from a minimal to a complete medium they produced sectors carrying one particular auxotrophic requirement from one of the parental strains. The presence of a translocation which would result in the only viable haploid segregants able to be produced from the aneuploid being auxotrophic was suggested as an explanation for the stability of the aneuploids whilst on minimal medium. The instability of progeny obtained from A. nidulans crosses in which one of the parental strains carried a non-recipricol translocation has also been described. This mitotic non-conformity was observed amongst progeny carrying the translocated chromosomal segment as a duplication. These strains showed slower growth compared to the parents and had a

modified morphology. The loss of all or part of one of the duplicated chromosomal segments resulted in the production of sectors of growth approaching wild-type with respect to growth rate and morphology (Azevedo and Roper 1970). Speculations could be made as to the existence of differences in chromosomal arrangement between strains derived from 20-3.1 (N-2) and those derived from the 20-2.1 (CO728) lineage which might act in a similar manner to produce the heterozygous type of fusion products recovered from crosses 9 and 10. The data available does not clearly implicate the involvement of any particular linkage group in such an effect. For an understanding of the reasons behind the occurrence of the heterozygous colonies the inclusion of additional genetic markers into the parental strains would be necessary to allow a more detailed analysis of the segregation of linkage groups in future crosses.

A number of progeny purified from crosses 9 and 10 were found to produce no detectable Pen N despite the fact that the lowest titre parental strain always gave a small though detectable activity against Sarcina lutea ATCC 9341. Many of the progeny falling into this group were isolated in group III i.e. those purified from YEA/Casamino acids medium. This phenomenon is also noticeable in other divergent crosses, 5 and 6 for example, but not in the sister

strain crosses 11 and 12. A skewness in B-lactam titre distribution amongst progeny from crosses with a tendency for the progeny mean titre to fall below the parental mean titre was observed in a number of crosses by Perez-Martinez (1984) and attributed to epistatic interactions.

The data from the crosses described give little consistent evidence for linkage between the genetic markers segregating. Cases where two markers appeared linked, for example leu-4 and his-2 in fusion 9 are seen but the linkages are only apparent amongst progeny selected under certain circumstances, and are not supported when the data from all the crosses are considered as a whole. These inconsistencies are reminescent of some data obtained by Hamlyn (1982) which showed aberrant linkage relationships amongst the progeny of some crosses compared to the established C. acremonium linkage map. The possible operation of undefined selective pressures when progeny are recovered on selective media directly following fusion could explain the apparent bias towards the recovery of certain phenotypes. A selective advantage of progeny carrying the leu^+ allele over those carrying leu^- has been described previously (Perez-Martinez, 1984). Evidence for linkage involving the cephalosporin C non-producing markers was suggested in cases where unexpectedly high

numbers of CPC producing progeny were recovered. Cross 11 showed possible linkages of either cnp-5 with orn-1 or cnp-6 with met-13 but these are not totally consistent with the results of fusions 9 and 10. The strongest case for linkage is perhaps that for the linkage of cnp-6 to the wild-type allele of the ino-2 marker already discussed for cross 12. However, in all cases the future establishment of linkage relationships between these markers would depend upon determining a consistent pattern of behaviour for them over a number of crosses.

CHAPTER 5

General Discussion

Studies of the genetics of the imperfect fungus C. acremonium (Hamlyn and Ball 1979; Hamlyn 1982), began with attempts to apply the parasexual methods which had proved successful in A. nidulans (Pontecorvo and Roper 1952; Roper 1952; Pontecorvo et al 1953; Roper and Pritchard 1955; Pontecorvo 1952; Forbes 1959) and P. chrysogenum (Pontecorvo and Sermonti 1953, 1954; Sermonti 1954, 1956, 1957, 1961). The initial difficulty in achieving the formation of a heterokaryon stage was overcome by the use of protoplast fusion and a method for parasexual genetic analysis in C. acremonium was subsequently described (Hamlyn and Ball 1979; Hamlyn 1982). In approximately 1% of cases, protoplast fusion is followed by nuclear fusion giving rise to a transient diploid stage. Two types of progeny may then be recovered for analysis; haploid recombinants arising after spontaneous haploidisation which are isolated by plating fusion mixtures on a range of selective media; and heterozygous colonies, believed to be aneuploid for one or more chromosomes or even diploid in some cases which sector to yield haploid recombinants. The use of this system for parasexual analysis enabled the development of a linkage map for C. acremonium (Hamlyn et al 1985). Quantitative studies demonstrated the polygenic nature

of CPC biosynthesis and allowed the location, with respect to other markers, of effective factors influencing antibiotic titre (Perez-Martinez 1984).

The work described here was aimed towards developing the study of the genetic basis of CPC production towards a point at which information concerning genes encoding key enzymes in the biosynthetic pathway could be obtained.

In the course of these studies it was observed that the usual expectation that the product of an C. acremonium protoplast fusion cross is a transient diploid which rapidly gives rise to haploid segregants was not always fulfilled. The persistent heterogeneity of many of the fusion products obtained in two of the crosses (9 and 10) would question whether the diploid or heterozygous phase is always as transient as previously assumed. There have been previous reports of rare 'stable heterozygotes' which have been accounted for by proposing that a chromosomal abnormality present in one of the parental strains concerned, resulted in the unusual stability of an aneuploid stage under the particular selective conditions employed (Hamlyn 1982, Perez-Martinez 1984). As the pool of information concerning the parasexual genetics of C. acremonium is expanded it may be anticipated that the genetic basis underlying both the transient nature of the diploid stage and the

observed exceptions to this will be revealed. The possibility of constructing stable strains aneuploid for linkage groups carrying genes either encoding rate limiting enzymes in CPC biosynthesis or responsible for regulatory effects along with possible implications for strain improvement might also be envisaged but would require the mapping of genes implicated in the biosynthesis pathway to linkage groups.

A number of strains of C. acremonium carrying mutations resulting in blocks of CPC biosynthesis isolated in different laboratories were available for use in mapping studies. Complementation and the restoration of CPC production has been demonstrated between two such strains (Perez-Martinez 1984). The strains available had been derived from divergent lineages of C. acremonium raised titre strains. Therefore the use of these in mapping studies was considered to be limited as in any cross the diversity of the genetic backgrounds of the two parents might be anticipated to mask to some degree the segregation of the genes of particular interest, by either pleiotropic or more direct regulatory effects. The existence of barriers to recombination between strains as a result of the introduction of changes in chromosome structure during the different programmes of strain improvement was also a potential problem.

A closely related group of C. acremonium strains carrying mutations affecting CPC biosynthesis were therefore isolated and characterised following the U.V. mutagenesis of a raised titre strain. After the induction of selectable markers to facilitate protoplast fusion, these strains were used alongside those already discussed in genetic studies. Complementation tests were performed between pairs of blocked strains. It is of interest that complementation was shown by two strains which both carried mutations (cnp-1 and cnp-6), resulting in apparently identical phenotypes i.e. in a loss of the ability to produce DAOC from Pen N. This would suggest that more than one gene function may be required for this particular step in CPC biosynthesis.

Complementation was also shown between the cnp-5 and cnp-7 mutations which both appeared to result in an inability to convert the ACV tripeptide precursor of CPC into isopenicillin N.

As the linkage map for C. acremonium was established using marked strains of the M8650 low titre lineage, information regarding the linkage group location of the mutations present in the blocked strains could not be derived from the crosses designed to assess complementation. However, linkage relationships between these mutations and the markers introduced to allow the selection of fusion products

were studied and evidence for the linkage of cnp-6 and ino-2 was obtained.

The future mapping of the mutations carried by the blocked strains would require the use of a CPC producing strain, closely related to and preferably derived from the same progenitor strain as the blocked mutant strains. This strain would ideally carry a sufficient number of markers to allow the segregation of each C. acremonium linkage group to be followed and would, therefore, act as a master strain for the genetic analysis of antibiotic production in this organism. Data obtained from mapping studies would be expected not only to yield information concerning structural genes involved in the CPC biosynthetic pathway, but also to allow the location of genes playing regulatory roles. A knowledge of the location of such genes with respect to other genetic markers, could be envisaged to be of use as the techniques of molecular biology are applied to C. acremonium with a view to the cloning and modification of genes underlying CPC biosynthesis for which a readily applicable selection system might not necessarily be available.

Crosses aimed at relating markers present in a C. acremonium raised titre master strain to those present in a number of M8560 strains would be of use in relating information concerning the location of genes

implicated in CPC biosynthesis to the C. acremonium linkage map and could reveal differences in chromosomal arrangement induced in the raised titre lineage during strain improvement.

The contribution of classical genetic studies to the industrial exploitation of organisms such as C. acremonium would appear to be somewhat limited. The induction of the necessary markers and the construction of a sufficiently detailed linkage map to allow a truly planned breeding strategy to be used in strain improvement would be extremely time consuming in comparison to the increase in antibiotic titre already achieved through programmes of mutation and selection. However, the recovery, following protoplast fusion in C. acremonium, of progeny combining the improved antibiotic titre of one parent strain with the good sporulation characteristics of the other parent has been reported (Hamlyn and Ball 1979) and Van der Beek et al (1986) described the use of protoplast fusion in P. chrysogenum to improve the characteristics of strains used in penicillin production. In the latter case, the genetic markers introduced in order to allow the selection of fusion products did not, in themselves, affect the antibiotic titre of the parental production strains. The development of selection systems for C. acremonium which would avoid the introduction of possibly deleterious mutations

whilst allowing strains of industrial use to be crossed, would therefore be desirable.

The recent development of transformation systems and cloning vectors for use with C. acremonium (Queener et al 1984), Penalva et al 1986), the isolation and sequencing of the gene encoding IPNS and the recent isolation of a gene encoding expendase and hydroxylase activities from this organism (Samson et al 1985, Harford et al 1986, Ignolia et al 1987) has opened the way for a more direct study of the genetic basis of CPC biosynthesis than has hitherto been possible. Such studies may be expected to have considerable implications for the development of strains with improved CPC production characteristics, in allowing the investigation of the control of the expression of key genes in CPC biosynthesis and the effect on CPC production of adjusting the copy number of such genes. The future possibilities for the genetic manipulation of CPC biosynthesis would therefore appear to be promising.

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APPENDIX I

APPENDIX I

Mapping Crosses with Strains From the M8650 lineage.

A number of protoplast fusion crosses were carried out with the aim of providing additional information on the linkage relationships between certain markers present in the M8650 derived strains which had been used in the construction of the C. acremonium linkage map.

Crosses A1 and A2 (Table 1) were repeats of crosses originally carried out by Hamlyn (1982), the results of which had in part conflicted with those expected given the linkage relationships between the markers involved established from other crosses. Hamlyn's data for cross A1 showed a lack of recovery of progeny carrying the ben-1$^-$ allele from selective plates supplemented with leucine thus questioning the established linkage between ane-5 and ben-1. For cross A2 the data showed the apparent linkage of the ben-1, ino-1, red-1 and pyt-1 markers. When the crosses were repeated the data (Table 2) did not display the same trends. Progeny carrying the ben-1 allele were recovered from cross A1, although there was a lack of progeny carrying the ane-5$^-$ allele. The leaky nature of the ane-5 allele, however, did pose problems in the identification of progeny

TABLE 1 Crosses between strains of the M8650 lineage.

CROSS	PARENTAL STRAINS
A1	20-1.4 azu-1 arg-1 mor-1 leu-1 red-1 pyt-1 ben-1
	20-1.8 ane-5 ino-1
A2	20-1.16 red-1 arg-2 pyt-1
	20-1.20 leu-1 ino-1 ben-1 mor-1
A3	20-1.8 ane-5 ino-1
	20-1.13 lys-1 phe-1
A4	20-1.4 azu-1 arg-1 mor-1 leu-1 red-1 pyt-1 ben-1
	20-1.13 lys-1 phe-1

Table 2 Pairwise analysis tables for crosses A1 and A2

Cross A1

From MM supplemented with arginine plus aneurin

		ane		azu		ben		red	
		+	-	+	-	+	-	+	-
arg	+	(12)	5	10	(7)	8	(9)	4	(13)
	-	14	(1)	(8)	7	(3)	12	(2)	13
ane	+			(14)	12	(10)	16	(3)	23
	-			4	(2)	1	(5)	3	(3)
azu	+					7	(11)	3	(15)
	-					(4)	10	(3)	11
ben	+							1	(10)
	-							(5)	(6)

From MM supplemented with leucine plus inositol

		ino		azu		ben		red	
		+	-	+	-	+	-	+	-
leu	+	(0)	2	1	(1)	2	(0)	2	(0)
	-	0	(0)	(0)	0	(0)	0	(0)	0
ino	+			(0)	0	(0)	0	(0)	0
	-			1	(1)	2	(0)	2	(0)
azu	+					1	(0)	1	(0)
	-					(1)	0	(1)	0
ben	+							2	(0)
	-							(0)	0

Table 2 Continued

From MM supplemented with leucine plus aneurin

		ane		azu		ben		red		pyt	
		+	-	+	-	+	-	+	-	+	-
leu	+	(7)	0	5	(2)	4	(3)	2	(5)	6	(1)
	-	10	(0)	(5)	5	(2)	8	(1)	9	(3)	7
ane	+			(10)	7	(6)	11	(3)	14	(9)	8
	-			0	(0)	(0)	0	0	(0)	0	(0)
azu	+					4	(6)	3	(7)	6	(4)
	-					(2)	5	(0)	7	(3)	4
ben	+							1	(5)	4	(2)
	-							(2)	9	(5)	6

From MM supplemented with arginine plus inositol

		ino		azu		ben		red	
		+	-	+	-	+	-	+	-
arg	+	(1)	4	4	(1)	0	(5)	5	(0)
	-	2	(2)	(1)	3	(1)	3	(2)	2
ino	+			(1)	2	(1)	2	(1)	2
	-			4	(2)	0	(6)	6	(0)
azu	+					0	(5)	5	(0)
	-					(1)	3	(2)	2
ben	+							0	(1)
	-							(7)	1

Table 2 Continued

Cross A2

From MM supplemented with inositol

		pyt		ben		mor		red	
		+	-	+	-	+	-	+	-
ino	+	(2)	15	12	(5)	15	(2)	(1)	16
	-	16	(6)	(6)	16	(7)	15	21	(1)
pyt	+			(2)	15	(1)	17	16	(2)
	-			15	(6)	21	(0)	(6)	15
ben	+					16	(2)	(6)	12
	-					(6)	15	16	(5)
mor	+							(7)	15
	-							15	(2)

From MM supplemented with leucine

		pyt		ben		mor		red	
		+	-	+	-	+	-	+	-
leu	+	(1)	18	16	(3)	17	(2)	(1)	18
	-	5	(1)	(1)	5	(2)	4	0	(6)
pyt	+			(1)	5	(1)	5	0	(6)
	-			16	(3)	18	(1)	(1)	18
ben	+					15	(2)	(0)	17
	-					(4)	4	1	(7)
mor	+							(1)	18
	-							0	(6)

carrying it and so could be an explanation for this observation. The results for cross A2 (Table 2) showed the ben-1 and ino-1 markers to recombine freely. Recombination between ane-5 and ino-1 appeared limited, again this was possibly due to a bias against the identification of progeny carrying the ane-5⁻ allele. The frequency of recombination between ino-1 and red-1 on linkage group III calculated from the data shown here is 15.8% which is in the order of the figure of 12.9% determined by Perez-Martinez (1984). No other consistent evidence of linkage was apparent.

Two crosses, A3 and A4 in Table 1 were carried out with a view to positioning the phe-1 marker on the C. acremonium linkage map. Previous work (Perez-Martinez 1984) had shown some indication of the linkage of phe-1 to lys-1 on linkage group VI. However, the evidence was not conclusive and a cross between strain 20-1.4 (azu-1 arg-1 met-1 leu-1 ben-1 pyt-1 ben-1) and 20-1.13 (lys-1 phe-1) had failed to demonstrate linkage between the azu-1 marker, previously mapped to linkage group VI and phe-1. The results obtained here (Table 3) again demonstrated that phe-1 does not map onto linkage groups I, II, III, V, VII or VIII. The linkage of phe-1 and lys-1 on group VI was again also a possibility but recombination between phe-1 and azu-1

Table 3 Pairwise analysis tables for crosses A3 and A4.

Cross A3

From MM Supplemented with aneurine plus lysine

		lys +	lys -
ane	+	9	8
	-	5	0

From MM Supplemented with aneurine

		phe +	phe -
ane	+	16	2
	-	17	2

No progeny were recovered from MM supplemented with inositol plus lysine or inositol plus phenylalanine

Table 3 Continued

Cross A4

From MM supplemented with arginine plus lysine

		lys		azu		pyt		ben		red		moi	
		+	-	+	-	+	-	+	-	+	-	+	-
arg	+	(7)	1	1	(7)	4	(4)	2	(6)	5	(3)	7	(1)
	-	7	(0)	(0)	7	(4)	3	(2)	5	(4)	3	(3)	4
lys	+			(0)	14	(7)	7	(3)	11	(8)	6	(9)	5
	-			1	(0)	1	(0)	1	(0)	1	(0)	1	(0)
azu	+					1	(0)	1	(0)	1	(0)	1	(0)
	-					(7)	7	(3)	11	(8)	6	(9)	5
pyt	+							4	(4)	6	(2)	6	(2)
	-							(0)	7	(3)	4	(4)	3
ben	+									4	(0)	3	(1)
	-									(5)	6	(7)	4
red	+											7	(2)
	-											(3)	3

Cross A4

From MM supplemented with arginine plus phenylalanine

		phe		azu		pyt		ben		red		mor	
		+	-	+	-	+	-	+	-	+	-	+	-
arg	+	(9)	3	0	(12)	6	(6)	7	(5)	8	(4)	12	(0)
	-	2	(1)	(0)	3	(2)	1	(2)	1	(2)	1	(1)	2
phe	+			(0)	11	(5)	6	(6)	5	(6)	5	(9)	2
	-			0	(4)	3	(1)	3	(1)	4	(0)	4	(0)
azu	+					0	(0)	0	(0)	0	(0)	0	(0)
	-					(8)	7	(9)	6	(10)	5	(13)	2
pyt	+							5	(3)	7	(1)	7	(1)
	-							(4)	3	(3)	4	(6)	1
ben	+									6	(3)	8	(1)
	-									(4)	2	(5)	1
red	+											9	(1)
	—											4	1

Table 3 Continued

From MM supplemented with leucine plus phenylalanine

		phe		azu		ben		red	
		+	-	+	-	+	-	+	-
leu	+	(6)	2	0	(8)	3	(5)	5	(3)
	-	4	(2)	(0)	6	(3)	3	(5)	1
phe	+			(0)	10	(3)	7	(6)	4
	-			0	(4)	3	(1)	4	(0)
azu	+					0	(0)	0	(0)
	-					(6)	8	(10)	4
ben	+							5	(1)
	-							(5)	3

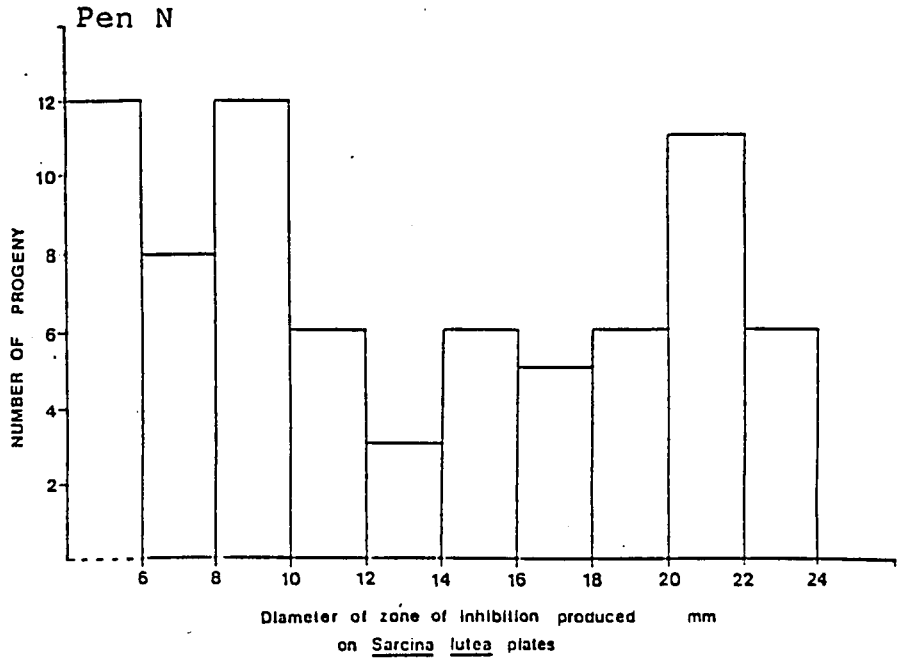
was detected amongst the progeny selected on media supplemented with either arginine plus phenylalanine or leucine plus phenylalanine. The analysis of the segregation of the phe-1 marker in further crosses would then be necessary in order to position this marker on the C. acremonium M8650 linkage map.

APPENDIX II

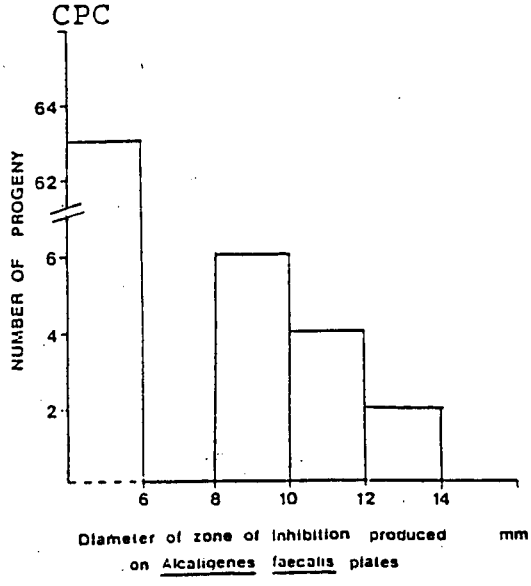
APPENDIX II

Antibiotics titres of progeny recovered from Cross 9. The distribution of titres amongst the separate groups (I, II and III) of progeny recovered.

A. Progeny recovered on MM + leucine

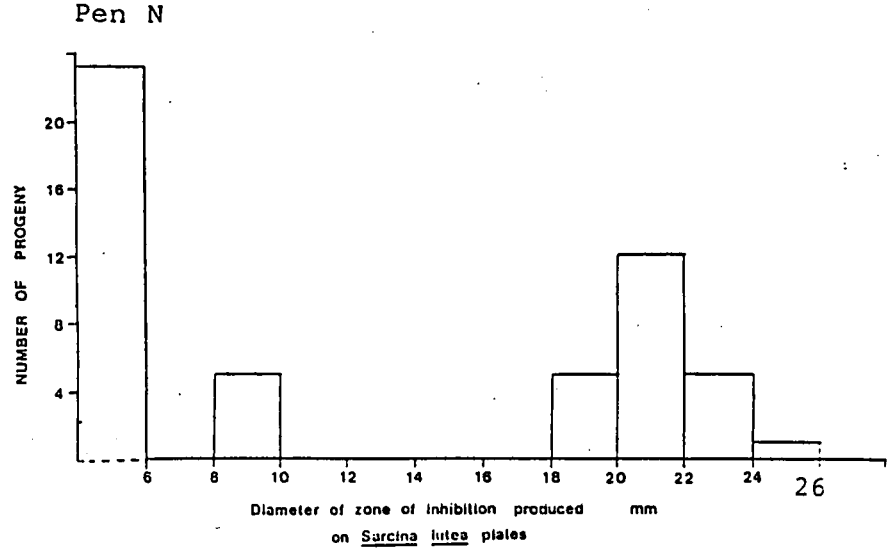


Progeny group										
I	1	2	4	2	2	3	3	2	7	4
II	1	2	3	3		2			2	1
III	10	4	5	1	1	1	2	4	2	1

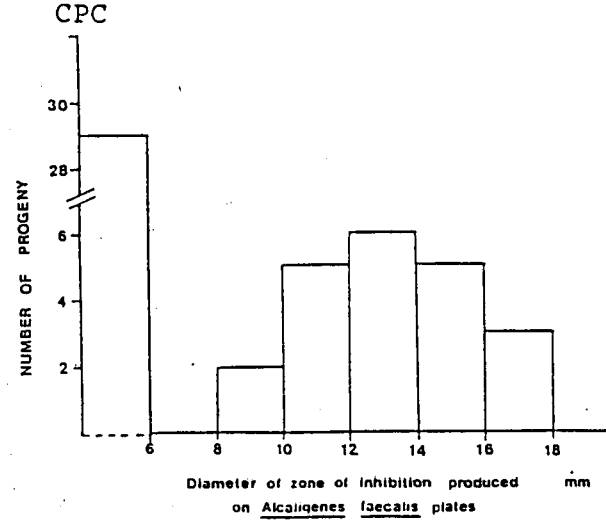


Progeny group					
I	26		1	3	
II	11		2		1
III	26		3	1	1

B. Progeny recovered on MM + histidine



Progeny group									
	I	3		1			4	12	4	1
	II			3						
	III	20		1			1		1	



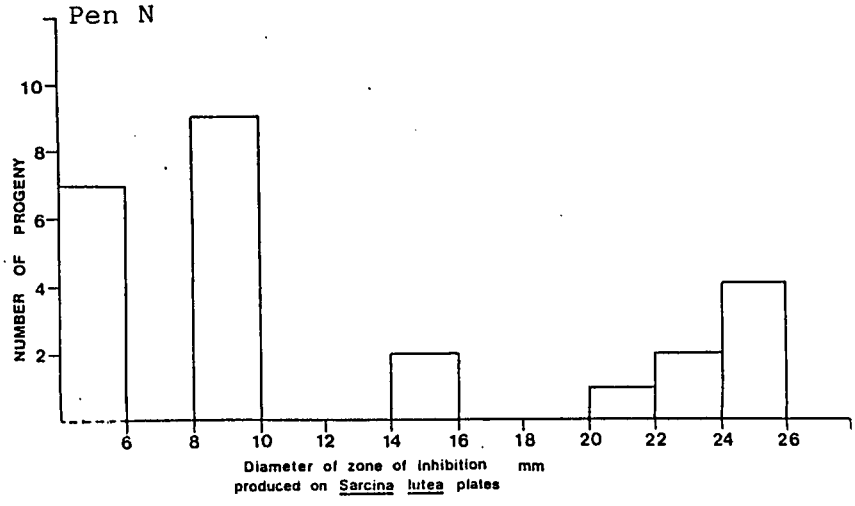
Progeny group								
	I	5	2	5	6	5	2	
	II	3						
	III	21			1		1	

APPENDIX III

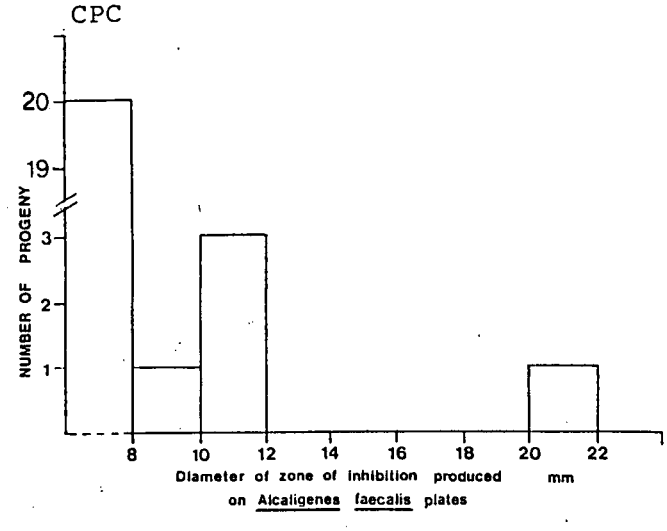
APPENDIX III

Antibiotic titres of progeny recovered from Cross 10. The distribution of titres amongst the separate groups (I, II and III) of progeny recovered.

A. Progeny recovered on MM + leucine

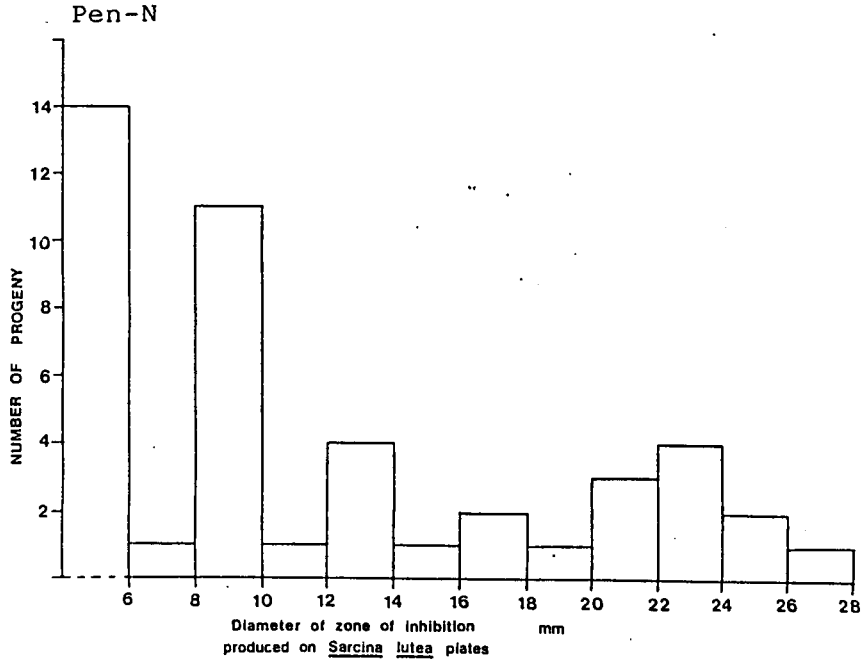


Progeny group										
	I									
	II			4		1				1
	III	7		5		1		1	2	3

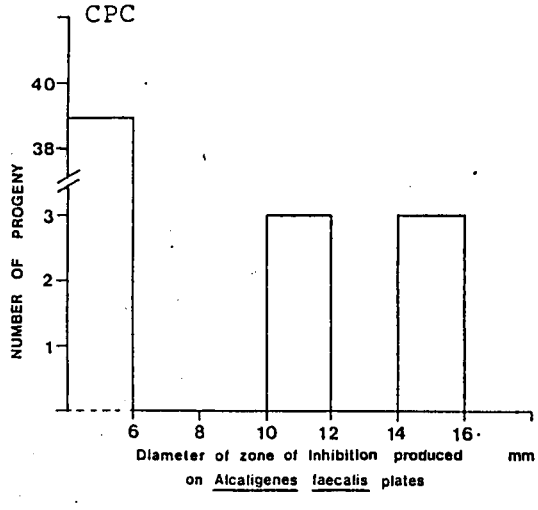


Progeny group										
	I									
	II	5		1						
	III	15	1	2				1		

B. Progeny recovered on MM + histidine



Progeny group														
	I													
	II	2		1	6		4	1	2	1	2	2	1	1
	III	12			5	1					1	2	1	



Progeny group					
	I				
	II	21		2	
	III	18		1	3

APPENDIX IV

Appendix IV

Allele Symbols

leu$^-$	requirement for leucine
arg$^-$	" arginine
met$^-$	" methionine
ade$^-$	" adenine
cys$^-$	" cysteine
ino$^-$	" inositol
his$^-$	" histidine
orn$^-$	" ornithine
S$^-$	" reduced/organic sulphur
nic$^-$	" nicotinic acid
ane$^-$	" aneurin
phe$^-$	" phenylalanine
red$^-$	produces red pigmentation
mor$^-$	morphological marker
azu$^-$	resistance to 6-azauracil
tbz$^-$	resistance to thiabendazole
pyt$^-$	resistance to pyrithiamine
ben$^-$	resistance to benomyl
cnp$^-$	impaired cephalosporin C biosynthesis