MOLECULAR AND CLASSICAL GENETIC ANALYSIS OF PENICILLIN BIOSYNTHESIS IN 'ASPERGILLUS NIDULANS'

Maureen B. R. Riach

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at the
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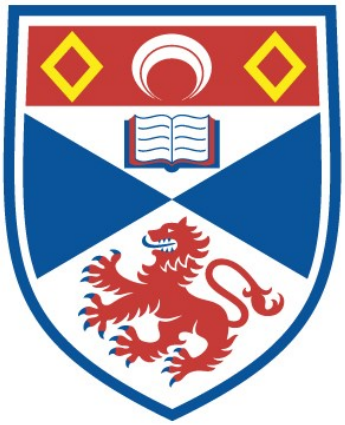

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MOLECULAR AND CLASSICAL GENETIC ANALYSIS OF PENICILLIN BIOSYNTHESIS IN *ASPERGILLUS NIDULANS*

MAUREEN B.R. RIACH B.Sc.(Hons.)

Plant Sciences Laboratory,
Sir Harold Mitchell Building,
University of St. Andrews,
Greenside Place,
St. Andrews,
Fife, KY16 9TH,
Scotland, U.K.

DECLARATION

I, Maureen Birrell Rankin Riach, hereby certify that this thesis has been composed by myself, that it is a record of my own work and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

Signed Date 30th July 1993

I was admitted to the Faculty of Science of the University of St. Andrews under Ordinance No. 12 on 1st October 1987 and as a candidate for the degree of Ph.D. on 1st October 1988.

Signed Date 30th July 1993

CERTIFICATE

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the degree of Ph.D.

Signature of Supervisor Date

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I would first like to thank my supervisor Dr. Jim Kinghorn for his help and guidance throughout the period of this research and acknowledge funding by the Science and Engineering Research Council (Biotechnology Directorate). I am also indebted to Drs. Andrew MacCabe and Shiela Unkles who acted as bench supervisors and gave patient advice. The heterologous hybridisation of the *P. chrysogenum* IPNS gene to *A. nidulans npeA*$^{+}$ and *npeA*0049/1 genomic DNA; the isolation and characterisation of the *A. nidulans* genomic clones pSTA200, pSTA201 and pSTA207 and subclones pSTA203, pSTA204 and pSTA807; the construction of *A. nidulans* strain SAA101; the physical analysis of *npeA* mutants and *npeA*0022 complementing transformants; Southern blot analysis of sequences 5′ and 3′ of IPNS; and identification of the ACVS coding region; were carried out by Dr. MacCabe, whilst the isolation and characterisation of pSTA18 and the initial identification of the penicillin biosynthetic gene transcripts were performed by Dr. Unkles.

I am grateful to many friends and former colleagues for the various contributions they made to the preparation of this thesis and for making my stay in St. Andrews a memorable one.

Finally, I dedicate this thesis to my parents, in gratitude for all their help and encouragement over the years.

CONTENTS

	Page
DECLARATION	i
CERTIFICATE	ii
COPYRIGHT	iii
ACKNOWLEDGEMENTS	iv
CONTENTS	v
ABBREVIATIONS	1
SUMMARY	4
CHAPTER 1: INTRODUCTION	6
1.1 HISTORICAL ASPECTS	6
1.2 BIOCHEMICAL ASPECTS	19
1.3 GENETIC ASPECTS	21
1.4 REGULATION ASPECTS	26
1.5 MOLECULAR ASPECTS	27
1.6 ISOLATION OF THE PENICILLIN BIOSYNTHETIC GENES	30
1.7 RESEARCH OBJECTIVES	33
CHAPTER 2: MATERIALS AND METHODS	34
2.1 CHEMICALS	34
2.2 STRAINS	34
2.2.1 Bacterial Strains	34
2.2.2 Fungal Strains	35

2.3 MAINTENANCE OF CULTURES 37
2.3.1 Bacterial Strains 37
2.3.2 Fungal Strains 38
2.4 MEDIA 38
2.4.1 Bacterial Culture Media 38
2.4.2 Fungal Media 39
2.5 ISOLATION OF SPONTANEOUS NITRATE-ASSIMILATION DEFECTIVE *A. nidulans* MUTANTS 42
2.6 CLASSICAL GENETIC TECHNIQUES 43
2.7 QUALITATIVE PROTEASE ASSAY 44
2.8 PROTOPLAST PREPARATION AND TRANSFORMATION 45
2.8.1 *A. nidulans* 45
2.8.2 *A. niger and A. oryzae* 46
2.9 PENICILLIN BIOASSAY 48
2.10 DETERMINATION OF β-GALACTOSIDASE ACTIVITY 49
2.10.1 Qualitative assay 49
2.10.2 Quantitative assay 50
2.11 DETERMINATION OF PROTEIN CONCENTRATION 51
2.12 PROTEIN PURIFICATION AND SEQUENCING 52
2.13 PLASMIDS AND COSMIDS 52
2.14 PREPARATION OF COMPETENT *E. coli* CELLS 54
2.15 TRANSFORMATION OF COMPETENT *E. coli* CELLS 54
2.16 PHENOL/CHLOROFORM EXTRACTION AND ETHANOL PRECIPITATION OF NUCLEIC ACIDS 56
2.17 PLASMID DNA PREPARATIONS 57
2.17.1 Small Scale Plasmid DNA Preparations 57
2.17.2 Large Scale Plasmid DNA Preparation 57
2.18 PREPARATION OF FUNGAL CHROMOSOMAL DNA 60

2.19 QUANTITATION OF DNA 60

2.20 DNA ELECTROPHORESIS 61

2.21 RESTRICTION ENZYME DIGESTION OF DNA 61

2.22 RECOVERY OF DNA FRAGMENTS FROM AGAROSE GELS 62

2.23 CONSTRUCTION OF HYBRID PLASMIDS 63

2.24 ^{32}P LABELLING OF DNA 65

2.25 ISOLATION OF *A. nidulans* TOTAL RNA 66

2.26 QUANTITATION OF RNA 68

2.27 ELECTROPHORESIS OF RNA, NORTHERN TRANSFER AND HYBRIDISATION 68

2.28 SOUTHERN TRANSFER AND HYBRIDISATION 72

2.29 DNA SEQUENCE ANALYSIS 75

CHAPTER 3: CHARACTERISATION OF THE *A. nidulans npeA* LOCUS 78

3.1 INTRODUCTION 78

3.2 HETEROLOGOUS HYBRIDISATION OF *P. chrysogenum* IPNS TO *A. nidulans npeA*$^{+}$ AND *npeA*0049/1 GENOMIC DNA 79

3.3 ISOLATION AND CHARACTERISATION OF *A. nidulans* GENOMIC CLONES 79

3.4 PHYSICAL ANALYSIS OF *npeA* MUTANTS 80

3.5 CONSTRUCTION OF DOUBLE MUTANT STRAINS WITH THE *argB2* ALLELE 81

3.6 COMPLEMENTATION OF *A. nidulans npeA* MUTANTS 82

3.7 TIME COURSE OF PENICILLIN PRODUCTION BY *A. nidulans* AND *A. oryzae* 84

3.8 PHYSICAL ANALYSES OF *npeA*0022 TRANSFORMANTS 85

3.9 CLASSICAL GENETIC ANALYSIS OF *npeA*0022 TRANSFORMANTS 86

3.10 CLASSICAL GENETIC ANALYSIS OF THE *npeA*0049/1 MUTATION 87

3.11 HYBRIDISATION STUDIES OF SEQUENCES 5′ OF IPNS 88

3.12 CONSTRUCTION OF *lacZ* FUSION RECOMBINANTS 88

3.13 IDENTIFICATION OF BIDIRECTIONAL PROMOTER ACTIVITIES 5′ OF IPNS 91

3.14 GENE DISRUPTION STUDIES USING A FRAGMENT 5′ OF IPNS 93

3.15 IDENTIFICATION AND CHARACTERISATION OF THE ACVS CODING REGION 95

3.16 HYBRIDISATION STUDIES OF SEQUENCES 3′ OF IPNS 95

3.17 IDENTIFICATION OF PENICILLIN BIOSYNTHETIC GENE TRANSCRIPTS 96

3.18 BIOASSAY OF PENICILLIN PRODUCTION IN STRAINS GROWN UNDER DEREPRESSED AND REPRESSED CONDITIONS 98

3.19 HYBRIDISATION STUDIES OF SEQUENCES 5′ OF *acvA* AND 3′ OF *acyA* 99

CHAPTER 4: REGULATION OF EXPRESSION OF THE *A. nidulans* PENICILLIN BIOSYNTHETIC GENES 101

4.1 INTRODUCTION 101

4.2 PRELIMINARY ANALYSIS OF *A. nidulans acvA* AND *ipnA* EXPRESSION SIGNALS USING *lacZ* FUSION ANALYSIS 101

4.3 RNA TRANSCRIPT ANALYSIS TO DETERMINE THE REGULATION OF EXPRESSION OF PENICILLIN BIOSYNTHETIC GENE EXPRESSION IN *A. nidulans* 104

4.3.1 Temporal Regulation 104

4.3.2 Transcription of Penicillin Biosynthetic Genes in Mutants with Impaired or Enhanced Penicillin Biosynthetic Capabilities 105

4.3.3 Effect of Various Regulatory Mutations on the Expression of the Penicillin Biosynthetic Genes 107

4.3.3.1 Carbon catabolism mutations 107

4.3.3.2 Nitrogen metabolism regulatory mutations 109

4.3.3.3 Sulphur metabolism regulatory mutations 110

4.3.3.4 pH regulatory mutations 111

4.3.4 Effect of Various Carbon and Nitrogen Sources on the Expression of the Penicillin Biosynthetic Genes 112

4.3.4.1 Effect of 1 % glucose or 1 % lactose with 10 mM proline or 5 mM ammonium tartrate as carbon and nitrogen sources 113

4.3.4.2 Effect of 1 % glucose and 10 mM ammonium tartrate; or 1 % glucose and 10 mM ammonium tartrate switched to 1 % glucose and 10 mM proline; as carbon and nitrogen sources 115

4.3.4.3 Effect of 1 % glucose and 5 mM ammonium tartrate; 3.5 % lactose and 10 mM proline; or 3.5 % lactose and 0.01 % casamino acids as carbon and nitrogen sources 117

4.3.4.4 Effect of 1 % sodium acetate and 5 mM ammonium tartrate as carbon and nitrogen sources 118

4.3.5 Effect of Various Additions to Minimal Medium on the Expression of the Penicillin Biosynthetic Genes 119

4.3.5.1 Effect of the amino acid precursors of penicillin 120

4.3.5.2 Effect of the side-chains of penicillin G and penicillin V 121

4.3.5.3 Effect of acetate 122

4.3.5.4 Effect of methionine and lysine 122

CHAPTER 5: CONCLUSIONS 125

REFERENCES 147

APPENDIX 164

ABBREVIATIONS

A_{420nm}	absorbance at 420 nm
A_{595nm}	absorbance at 595 nm
bp	base pair(s)
BSA	bovine serum albumin
C-	carbon
cm	centimetre(s)
CNBr	cyanogen bromide
conc	concentration
dATP	2′-deoxy-adenosine-5′-triphosphate
dCTP	2′-deoxy-cytidine-5′-triphosphate
dGTP	2′-deoxy-guanosine-5′-triphosphate
dH2O	distilled water
dTTP	2′-deoxy-thymidine-5′-triphosphate
Da	Dalton
DTT	dithiothreitol
DEPC	diethly pyrocarbonate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylenediaminetetraacetic acid
e.g.	*exempli gratia* (for example)
et al.	*et alia* (and others)
Fig.	Figure
g	gram(s)
h	hour(s)
i.e.	*id est* (that is)

IPTG	isopropyl-ß-D-thiogalactopyranoside
kb	kilobase pair(s)
kDa	kiloDalton(s)
kV	kiloVolt(s)
l	litre(s)
lb	pounds
µCi	microCuries
µg	microgram(s)
µl	microlitre(s)
µM	micromolar
M	molar
mg	milligram(s)
min	minute(s)
ml	millilitre(s)
mm	millimetre(s)
mM	millimolar
MOPS	morpholinopropanesulfonic acid
N-	nitrogen
No.	number
Nos.	numbers
ng	nanogram(s)
nm	nanometre(s)
^{o}C	degrees Centigrade
OD	optical density
OD600	optical density at 600 nm (wavelength)
o-NP	o-nitrophenol
o-NPG	o-nitrophenyl-ß-D-galactopyranoside
PABA	para-amino benzoic acid

PAGE	polyacrylamide-gel electrophoresis
PEG	polyethylene glycol
PMSF	phenylmethylsulfonyl fluoride
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
sec	second(s)
TE	10 mM Tris-HCl pH 8.0, 1 mM EDTA
Tris	Tris(hydroxymethyl)methylamine
UV	ultra violet
x	times
x g	times force of gravity (centrifugation)
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside
≡	equivalent to
>	greater than

SUMMARY

Clones of *A. nidulans* genomic DNA (pSTA200, pSTA201 and pSTA207) spanning 20 kb have been isolated and demonstrated by a combination of classical and molecular genetic means to represent the *npeA* locus, involved in the synthesis of penicillin, located on linkage group VI of *A. nidulans*. As well as containing the gene encoding the second enzyme for penicillin biosynthesis, namely isopenicillin N synthetase (IPNS) (designated *ipnA*), results presented here show that these clones contain two additional genes to form a cluster of three contiguous penicillin biosynthetic genes. Our evidence suggests that these genes encode σ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACVS) and acyltransferase (ACYT) (designated *acvA* and *acyA* respectively), the first and third enzymes required for penicillin biosynthesis, with the genetic order being *acvA-ipnA-acyA*. Transcripts have been identified for the three genes and their approximate sizes determined: *acvA* >9.5 kb, *ipnA* 1.4 kb and *acyA* 1.6 kb. Results indicate that the *acvA* and *ipnA* genes are divergently transcribed, whilst *acyA* is transcribed in the same orientation as *ipnA*. Northern blot and hybridisation analyses of the regulation of expression of the structural genes for penicillin biosynthesis indicated that all three genes are subject to some form of regulation, the mechanism of which is not clear from our studies. Hybridisation data appeared to suggest that

if any regulatory genes exist for penicillin biosynthesis, they are not located immediately 5′ of *acvA* or 3′ of *acyA* at the *A. nidulans npeA* locus.

CHAPTER 1

INTRODUCTION

1.1 HISTORICAL ASPECTS

For many centuries fungi, especially moulds, have been used empirically in folk medicine for the treatment of infections, and during the latter half of the nineteenth century, several scientists recorded the antagonism of *Penicillium* species to bacterial growth. However, it is not known whether any of the antibacterial activity observed was due to the presence of penicillin. It was not until 1928 that penicillin was discovered by Alexander Fleming at St. Mary's Hospital, London, through a chance observation, and the history of ß-lactam antibiotics began.

While examining plates which had been seeded with staphylococci and left on a laboratory bench during a vacation, Fleming noticed that on one plate, in the vicinity of a large contaminating colony of mould, the bacteria were undergoing lysis. Realising that this fungus was secreting a bacteriolytic substance into the medium, he subcultured the mould and carried out experiments to ascertain something of the properties of the antibacterial substance. Fleming grew the mould, initially identified wrongly as *Penicillium rubrum* but later correctly as *P. notatum*, in broth and demonstrated that the resulting culture filtrate had potent

antibacterial activity against a number of Gram-positive pathogens and Gram-negative cocci, but not Gram-negative bacilli. Fleming named this "mould broth filtrate", penicillin and found that it was non-toxic to rabbits or mice even in large doses, non-toxic and non-irritant to man and furthermore did not interfere with leucocyte function to a greater extent than ordinary broth. Fleming's initial interest was in the use of penicillin as an agent for selecting penicillin resistant microbes in differential cultures, in order to separate different types of bacteria. However, although he did not carry out any animal experiments to establish the clinical usefulness of penicillin, he was not unaware of the broader possibilities of penicillin and suggested in his original paper (Fleming, 1929) that "it may be an efficient antiseptic for application to, or injection into, areas infected with penicillin-sensitive microbes". A recent study of Fleming's original notebooks (Wainwright, 1991) has indicated that soon after he discovered penicillin, he began a search for new antibiotic producing moulds and bacteria. Throughout the 1930s, he continued to work with penicillin, studying its effects on anaerobes and whether or not staphylococci develop resistance to it. In addition, he attempted to improve its production methods.

Recently unearthed casenotes (Wainwright and Swan, 1987), have shown that C.G Paine, a hospital bacteriologist in Sheffield and an ex-student of Fleming,

was achieving cures with crude culture filtrates of *P. notatum*, subcultured from Fleming's original strain, as early as 1930. Although Fleming used crude penicillin topically on infections soon after its discovery in 1928, no clinical records have been found to indicate that he used penicillin as a curative agent prior to 1932 when he successfully used it to treat a pneumococcal eye infection. Paine continued his work with penicillin and in 1932 he informed H.W. Florey, the then newly appointed professor of pathology at the University of Sheffield, about his promising results, but at that time Florey showed no interest in the work (Selwyn, 1980; Wainwright and Swan, 1987). This work on the therapeutic potential of crude penicillin was discontinued because of the variability of the antibiotic producing strain and the belief that pure penicillin would have to be produced before it could be effectively used clinically. In 1929, Fleming's junior colleagues F. Ridley and S.R. Craddock prepared crude extracts of penicillin from mould culture filtrates but, daunted by its instability and the difficulty in purifying it, they stopped the work prematurely. Comparable work was carried out in 1932 by P.W. Clutterbuck, R. Lovell and H. Raistrick at the London School of Hygiene, but they found that the antibacterial activity of penicillin could readily be lost and abandoned further work on it.

In 1938, Florey and E.B. Chain at Oxford decided to make a detailed study of certain microbially-produced

antibacterial substances, including penicillin - a decision which both Florey and Chain have stated was motivated by purely academic interest, not by the thought that it would lead to the isolation of valuable therapeutic agents. By the summer of 1939, Chain had made little progress in his attempts to purify penicillin but towards the end of that year, N.G. Heatley joined the group and he suggested the use of a process which led to the preparation of material that was approaching a purity of 1 %. Florey showed conclusively in 1940 that this crude penicillin was non-toxic and when given sub-cutaneously to mice, it could protect them from otherwise fatal staphylococcal and streptococcal infections. E.P. Abraham, A.D. Gardner and A.G. Sanders, who were also by this time working on the penicillin project at Oxford, became engaged in a major effort to further purify penicillin and produce sufficient material for a small clinical trial. In January 1941, the first patient ever to be given crude penicillin intravenously developed a sharp temperature rise and a rigor, but this pyrogenic activity was due to an impurity in the preparation, not the penicillin itself, and was removed chromatographically by Abraham. Early in 1941, Florey and C.M. Fletcher treated five patients in Oxford who were gravely ill with staphylococcal or streptococcal infections. The penicillin used was only 0.3 % to 0.7 % pure and the supply was inadequate despite the major improvements in concentration brought about by Chain,

Abraham and Heatley, and the savings made by recovering the antibiotic from patients' urine during treatment. Nevertheless, the results indicated that penicillin could control very severe infections and was non-toxic to man.

During World War II, the industrial production of penicillin was developed in a joint Anglo-American co-operation. In June 1941, Florey and Heatley took Fleming's *P. notatum* strain from Britain to the United States of America in the hope that penicillin could be produced there in larger quantities. At the Northern Regional Research Laboratory (NRRL), Peioria, the use of deep fermentation, rather than stationary surface culture normally carried out in Roux flasks, was developed to improve large scale penicillin production. In addition, a suitable culture medium was sought on which to grow the *Penicillium*. Fleming had used digested bull heart which was the standard culture medium of the day, whilst Raistrick and colleagues used the synthetic medium Czapek-Dox. However, *Penicillium* grew slowly and produced very low levels of penicillin on both and alterations made by Heatley to the Czapek-Dox recipe did not improve the culture medium. Heatley working with A.J. Moyer at NRRL, in 1941, greatly improved the yield of penicillin by introducing into the fermentation, corn steep liquor which was both abundant and cheap. Corn steep liquor not only increased the penicillin yield but it also favoured the production of benzyl penicillin (penicillin G) rather than 2,pentenyl penicillin (penicillin F) and other

natural penicillins, because it contains phenylacetic acid, the side-chain precursor of penicillin G. Consequently phenylacetic acid, which was cheaply chemically synthesised, was used as an additional ingredient in the fermentation medium, and this also increased penicillin yields and stimulated the formation of penicillin G. A search was then conducted at NRRL by R.G. Coghill, K.B. Raper and colleagues for fungal strains which produced higher yields of penicillin than the original *P. notatum* and samples from a wide variety of sources were screened for the production of penicillin. One of the better isolates was *Penicillium chrysogenum* (NRRL 1951) which also grew easily in submerged culture. It is from this strain that most of today's industrial strains have been derived by mutagenesis, commonly by irradiation.

In 1941, the United States government decided to spend money on the penicillin project and Merck, Squibb and Pfizer were the first major companies to become involved. This led to a vast output of penicillin and the foundation of the modern pharmaceutical industry. By 1942, ICI and Kemball Bishop in the U.K., were producing penicillin on a small scale, and production was also continued by Florey's group in Oxford. This British effort provided sufficient crude penicillin for Florey to perform a second clinical trial from which further information was obtained about methods of administration and dosage of the antibiotic. Meantime, in the U.S.A.,

larger amounts of penicillin were becoming available and larger scale clinical trials were conducted which confirmed earlier conclusions that penicillin was valuable for the treatment of staphylococcal, streptococcal, pneumococcal and gonococcal infections and also confirmed and extended previous observations on its pharmacokinetics. Wartime efforts to produce penicillin in large amounts by fermentation exceeded all expectations. Sufficient penicillin was available by 1943 to be used in successful studies of its use in war surgery and in the treatment of war wounds, and in 1944 it was used to treat all British and American battle casualties. During this time, penicillin was independently isolated in limited amounts from *P. baculatum* by researchers at Gist-brocades in the Netherlands who were isolated from the scientific world because of the war situation. This Dutch penicillin was subsequently found to be identical to that produced in America (Veenstra *et al.*, 1989).

As it was unforeseen that fermentation could achieve a significant increase in yield, in parallel with efforts to produce penicillin by this method, a major project was conducted with the objective to manufacture penicillin in quantity by total chemical synthesis. In order for this to be achieved, it was first necessary to determine the structure of the penicillin molecule. To this end, Abraham and Chain began a collaboration in 1942 with R.

Robinson, W. Baker and J.W. Cornforth of another Oxford laboratory, to begin chemical investigations.

In 1943, Chain and Abraham succeeded in purifying the penicillin molecule as a barium salt. The American company Squibb had also independently purified and crystallised penicillin but as a sodium salt and on hearing this information, Abraham converted the British penicillin to a sodium salt which led to its spontaneous crystallisation. However, British and American penicillins were not identical, the former contained as its side-chain a 2,pentenyl group (designated penicillin F), whilst the latter contained a benzyl group (designated penicillin G).

Abraham in 1943 proposed a ß-lactam structure (Fig. 1) for penicillin which was favoured by Chain and Baker but this proposed structure was not universally accepted. By the end of 1944, R.B. Woodward had provided further evidence for the ß-lactam structure of penicillin and he proposed a rational explanation for the reactivity of its ß-lactam ring. However, it was not until 1945 that the ß-lactam structure was shown conclusively to be correct, following x-ray crystallographic analysis by D.C. Hodgkin and B.W. Low at Oxford. Attempts to chemically synthesise this antibiotic in a rational manner were unsuccessful until 1957 when J.C. Sheehan and colleagues at the Massachusetts Institute of Technology (MIT) produced phenoxymethyl penicillin (penicillin V) which has as its side-chain phenoxyacetic acid, and 6-aminopenicillanic

acid (6-APA), the nucleus of the penicillin molecule. Total chemical synthesis of penicillin was, however, too expensive to be commercially viable as major improvements in fermentation technology meant that the production of penicillin by fermentation on a large scale had become very efficient and economical.

Following the determination of the difference between penicillin F and penicillin G, other natural penicillins were found which differed in their related side-chains. O.K. Behrens and co-workers at the Lilly Research Laboratories, Indianapolis showed that in *P. notatum* a variety of amino acids could function as side-chain precursors, and thus a variety of biosynthetic penicillins could be obtained by simply adding synthetic precursors to the *Penicillium* fermentation. In 1948, this group produced penicillin V, the first useful chemical modification of the penicillin molecule, which was readily formed by the addition of phenoxyacetic acid to the medium during fermentation. Penicillin V came into general clinical use in 1954 for oral administration as it is relatively stable in dilute acid and is well absorbed when given orally, unlike penicillin G which is unstable in acid. However, this method had restricted potential since only a limited number of compounds are accepted as substrates by the enzymatic system for penicillin biosynthesis.

The next major step forward was dependent upon finding an easy method of obtaining 6-APA, the penicillin

nucleus, to which side-chains could be chemically added, as required, to produce alternatives to the natural penicillins. In 1953, K. Kato in Japan found small amounts of 6-APA in fermentations of *P. chrysogenum*, whilst three years earlier, K. Sakaguchi and S. Murao also in Japan reported that the penicillin nucleus could be obtained by removing the N-acyl side-chain from penicillin using an amidase produced by *P. chrysogenum*. G.N. Rolinson, F.R. Batchelor, F.P. Doyle and J.H.C. Nayler of the Beecham Research Laboratories (U.K.) in 1958 isolated 6-APA in crystalline form from fermentation broths of *P. chrysogenum* to which no side-chain precursor had been added. The availability of 6-APA in reasonably large amounts opened up the way to the semisynthesis of a series of new penicillins by simple acylation which could not be obtained by fermentation. Subsequently, the production of 6-APA in quantity was performed by removing the side-chain from penicillin G using bacterial amidases. As previously mentioned, Sheehan in 1957 chemically synthesised 6-APA - however it is too expensive to be used on an industrial scale and is produced more economically by fermentation and enzymic or chemical modification of penicillin G or penicillin V. The only penicillins obtained by the simple addition of side-chain precursors to *Penicillium* fermentation which are in clinical use today are penicillin G and penicillin V. In addition, a number of clinically useful semisynthetic derivatives are now available which have

extended the range of activity of penicillins. Some semisynthetic derivatives e.g. methicillin are resistant to the activity of penicillinases which are a major cause of bacterial resistance to penicillins, whilst other "broad spectrum" penicillins e.g. ampicillin have activity against a wider variety of Gram-negative organisms than penicillin G (although they are more active against Gram-positive than against Gram-negative bacteria). In contrast the aminido-penicillins possess more activity against Gram-negative than against Gram-positive microorganisms.

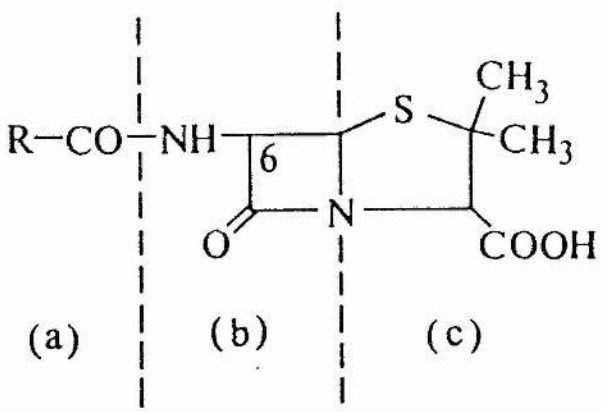
Exploitation of cephalosporins, the second major class of ß-lactams produced by fungi, began in 1945 when G. Brotzu of the Institute of Hygiene, Cagliari (Italy), isolated a strain of *Cephalosporium acremonium* from the sea near a sewage outfall. Culture filtrates of this organism were shown to have broad antibacterial activity against both Gram-negative and Gram-positive bacteria. Abraham and colleagues in the 1950's ascertained that *C. acremonium* produced three types of antibiotic: acidic steroids, collectively known as cephalosporin P, and active only against Gram-positive microorganisms (Burton *et al.*, 1956); cephalosporin N, which was a new penicillin with a broad spectrum of activity, later renamed penicillin N (Newton and Abraham, 1954); and cephalosporin C which was isolated as a contaminant in a crude preparation of penicillin N (Newton and Abraham, 1956). Cephalosporins have a structure similar to

penicillins, except that cephalosporins have the ß-lactam ring fused to a 6-membered dihydrothiazine ring rather than a 5-membered thiazolidine ring (Fig. 1). Although cephalosporin C has good resistance to various ß-lactamases and is active against Gram-negative and Gram-positive organisms, it has only weak antibacterial activity and is not clinically used.

However, chemical or chemical and enzymatic removal of the cephalosporin C side-chain gives 7-amino-cephalosporanic acid (7-ACA), the cephalosporin nucleus, which can be chemically acylated to produce semisynthetic derivatives. 7-ACA has also been demonstrated to be synthesised by a strain of *C. acremonium* transformed with a novel hybrid 7-ACA biosynthetic operon (Isogai *et al.*, 1991). Unlike penicillins, cephalosporins can be modified not only at the side-chains but also by chemical modification of the acetoxy group at position 3 of the nucleus of cephalosporin C. A large number of semisynthetic cephalosporins are now available, several of which are currently in use against a broad spectrum of bacteria.

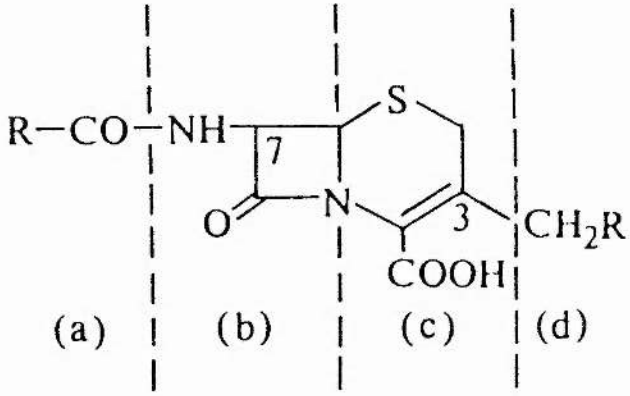
The classical ß-lactam antibiotics, i.e. penicillins and cephalosporins, are produced by a variety of lower eukaryotes (filamentous fungi) and filamentous prokaryotic microorganisms (*Streptomyces* species). In addition, a number of new ß-lactams have been discovered in prokaryotic microorganisms, including species of the actinomycetes and a number of genera of Gram-negative

Fig. 1 Basic chemical structure of penicillins and cephalosporins (from Lancini and Parenti, 1982).



Penicillins

(a) = Side chain
(b) = β-lactam ring
(c) = Thiazolidine ring



Cephalosporins

(a) = Side chain
(b) = β-lactam ring
(c) = Dihydrothiazine ring
(d) = Group at position 3

bacteria (reviewed by Elander, 1983; Martin and Liras, 1985).

Of these new antibiotics, cephamycins, produced by *Streptomyces* species, closely resemble cephalosporins and are highly resistant to a number of ß-lactamases. Semisynthetic derivatives have been derived by chemical modification of the naturally occurring cephalosporin C, and those in clinical use are recommended mainly for treating Gram-negative organisms.

Industrial screening programmes, in which *Streptomyces* strains were tested for ß-lactam antibiotics and ß-lactamase inhibitors, led to the discovery of other novel, non-classical ß-lactams. These include the ß-lactamase inhibitors, the carbapenems, and the clavam, clavulanic acid. Carbapenems, in addition to being good ß-lactamase inhibitors, have significant antibacterial activity e.g. thienamycin is highly active against a broad range of Gram-positive and Gram-negative microorganisms. Clavulanic acid has only weak antibacterial activity but significantly inhibits ß-lactamases and has synergistic activity when combined with vulnerable ß-lactams against a wide range of ß-lactamase - producing Gram-positive and Gram-negative bacteria.

A third group of non-classical antibiotics, monocyclic ß-lactams, have been isolated from fermentation broths of various bacteria. The nocardicins are produced by *Nocardia* species, whilst the monobactams

have been shown to be produced by a number of Gram-negative bacteria including species of *Pseudomonas*, *Chromobacterium*, *Gluconobacter*, *Acetobacter* and *Agrobacterium* (Elander, 1983). These antibiotics have activity against Gram-negative bacteria and good resistance to ß-lactamases.

Finally, artificial ß-lactams which do not occur in nature are available and include the 1-oxacephems and 1-carbacephems which are nuclear analogues of the cephalosporins and are produced by total chemical synthesis. 1-oxacephems are also produced by chemical conversion from the 6-APA molecule. Penems are another man-made ß-lactam which are chemically synthesised from penicillin V, and share features of the penicillin (penam) and cephalosporin (cephem) ring systems. (For a fuller historical perspective see reviews by Abraham, 1983, 1990; Elander, 1983; Lancini and Parenti, 1982; Martin and Liras, 1985; Miller, 1983; Selwyn, 1980, 1983; Sheehan, 1982; Sykes and Wells, 1985; Wolfe *et al.*, 1984).

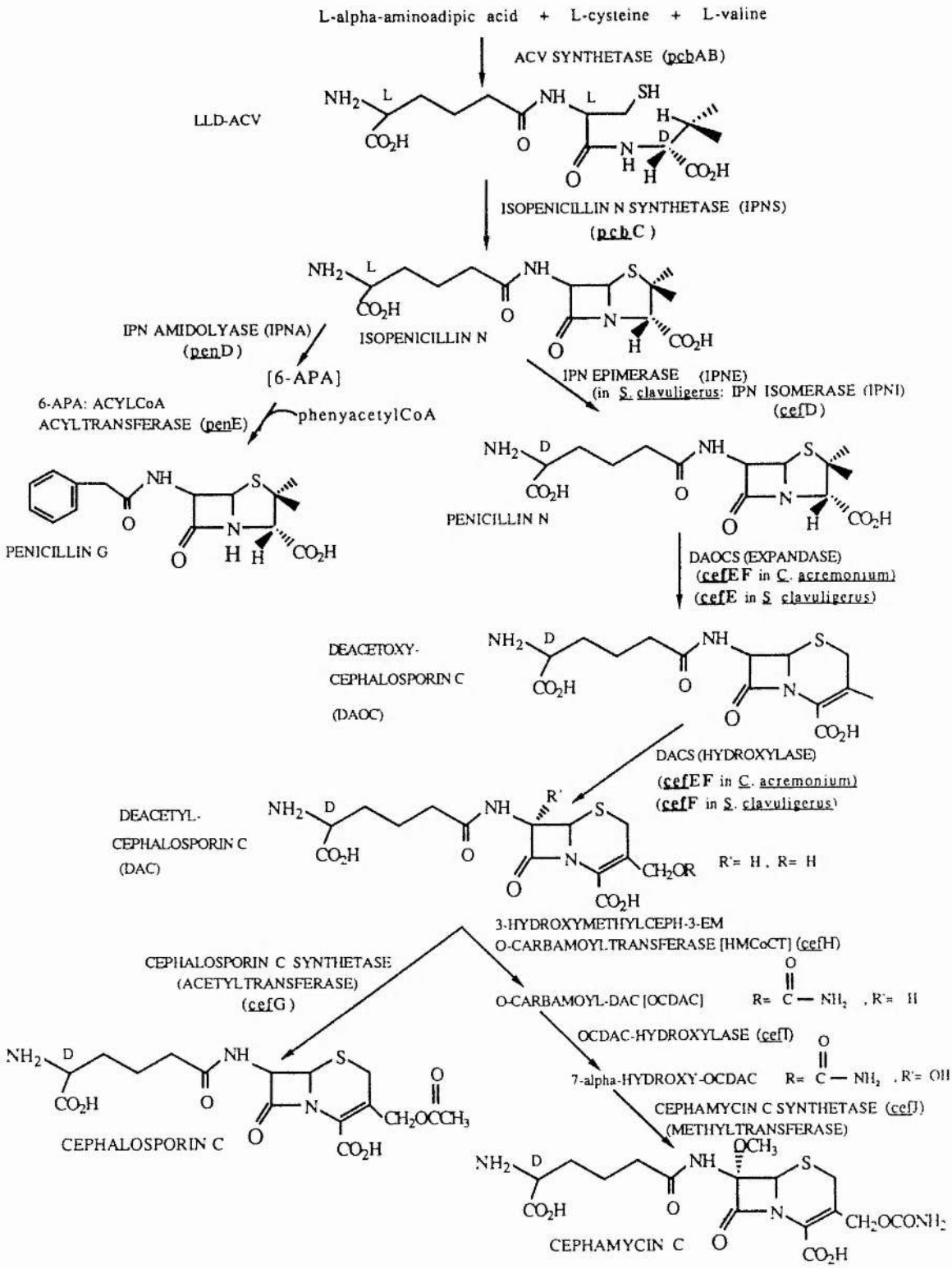
1.2 BIOCHEMICAL ASPECTS

Progress in understanding the biosynthesis and regulation of ß-lactam antibiotics has been slow, despite their medical and industrial importance, due, in part, to the instability of the enzymes involved (Martin, 1987). However, with the development of cell-free systems from ß-lactam producing organisms, in

which cells are disrupted by various means and the enzyme activity of the resulting cell-free extract is studied, the biochemical properties of some ß-lactam biosynthetic enzymes have been described (Demain, 1983; Jensen *et al.*, 1985, 1988; Martin and Liras, 1989; Miller and Ingolia, 1989a; Nuesch *et al.*, 1987).

The initial steps in the formation of hydrophobic penicillins and hydrophilic cephalosporins and cephamycins are identical (Fig. 2). In each of these pathways, the amino acid precursors L-α-aminoadipic acid, L-cysteine and L-valine are condensed into the noncyclic tripeptide σ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV), by the enzyme ACV synthetase (ACVS). ACV is then cyclised by the enzyme isopenicillin N synthetase (IPNS) to form isopenicillin N (IPN), the first ß-lactam in the pathway, which is an intermediate having an L-α-aminoadipyl side-chain attached to the ß-lactam-thiazolidine ring of penicillin. It is at this point that the pathways diverge. In cephalosporin- and cephamycin- producing microorganisms, IPN is converted to penicillin N by isomerisation of the α-aminoadipyl side-chain. This is followed by expansion of the five-membered thiazolidine ring of penicillins into the six-membered dihydrothiazine ring common to cephalosporins and cephamycins which is in turn converted through a variety of intermediates into cephalosporins and cephamycins. During penicillin biosynthesis, however, a transacetylation reaction, catalysed by the enzyme

Fig. 2 Biosynthetic pathways to the sulphur-containing ß-lactam antibiotics penicillin G, cephalosporin C and cephamycin C (from Miller and Ingolia, 1989a).


L-alpha-aminoadipic acid + L-cysteine + L-valine
ACV SYNTHETASE (pcbAB)
LLD-ACV
ISOPENICILLIN N SYNTHETASE (IPNS)
(pcbC)
IPN AMIDOLYASE (IPNA)
(penD)
ISOPENICILLIN N
[6-APA]
IPN EPIMERASE (IPNE)
(in S. clavuligerus: IPN ISOMERASE (IPNI)
(cefD)
6-APA: ACYLCoA
ACYLTRANSFERASE (penE)
phenyacetylCoA
PENICILLIN G
PENICILLIN N
DAOCS (EXPANDASE)
(cefEF in C. acremonium)
(cefE in S. clavuligerus)
DEACETOXY-
CEPHALOSPORIN C
(DAOC)
DACS (HYDROXYLASE)
(cefEF in C. acremonium)
(cefF in S. clavuligerus)
DEACETYL-
CEPHALOSPORIN C
(DAC)
R'= H , R= H
3-HYDROXYMETHYLCEPH-3-EM
O-CARBAMOYLTRANSFERASE [HMCoCT] (cefH)
CEPHALOSPORIN C SYNTHETASE
(ACETYLTRANSFERASE)
(cefG)
O-CARBAMOYL-DAC [OCDAC]
R= C — NH2 , R'= H
OCDAC-HYDROXYLASE (cefI)
7-alpha-HYDROXY-OCDAC
R= C — NH2 , R'= OH
CEPHAMYCIN C SYNTHETASE (cefJ)
(METHYLTRANSFERASE)
CEPHALOSPORIN C
CEPHAMYCIN C

acyltransferase (ACYT), exchanges the α-aminoadipyl side-chain for phenylacetic acid or phenoxyacetic acid, forming penicillin G or V respectively.

1.3 GENETIC ASPECTS

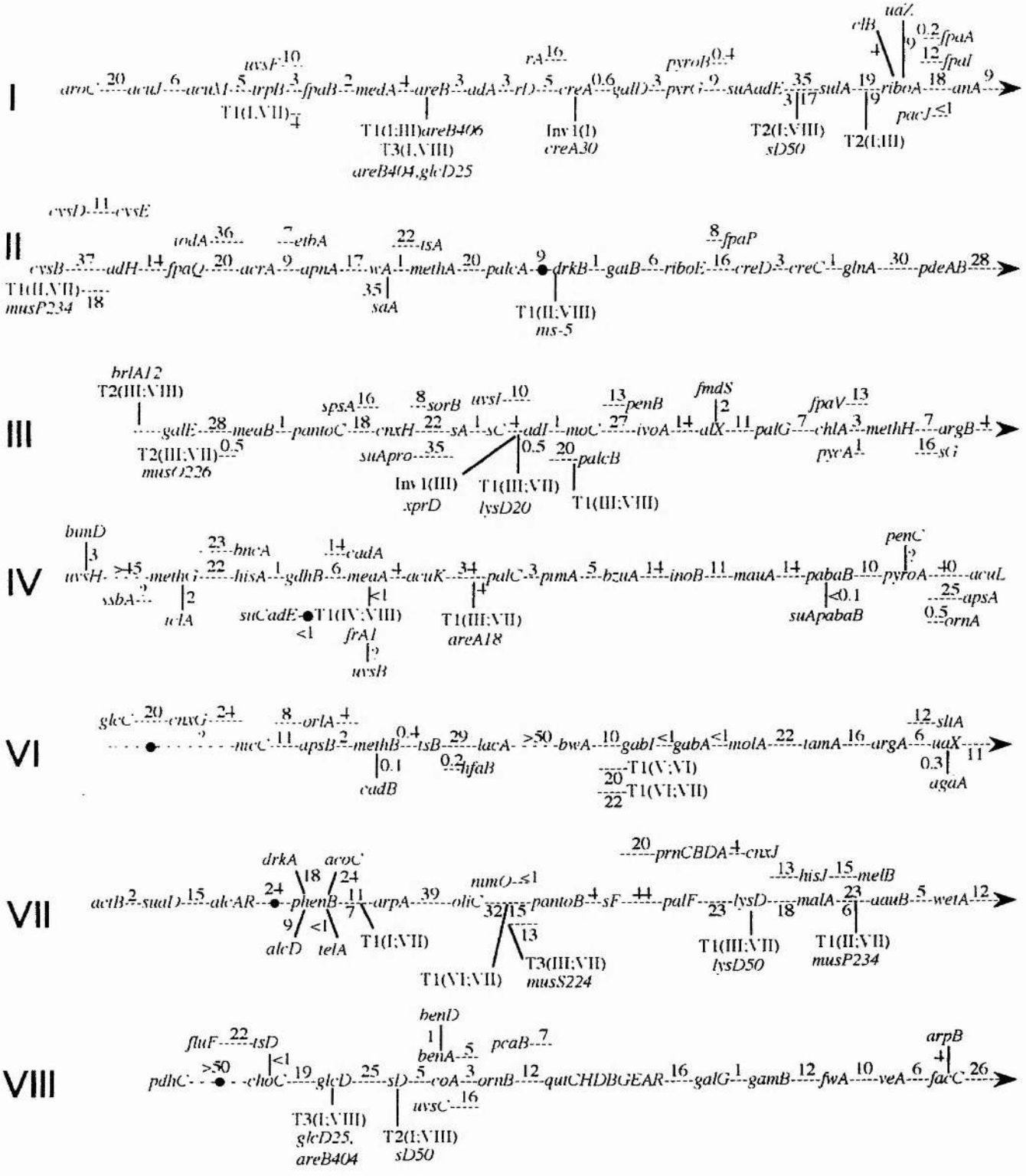
Although there exists considerable empirical knowledge concerning the industrial production of ß-lactam antibiotics by fermentation, until recently relatively little was known regarding the molecular biology of their biosynthesis. This was partly due to the sparsity of information concerning the genetics of the filamentous fungi *Penicillium chrysogenum* and *Cephalosporium acremonium*, the two most important commercial sources of ß-lactams. Alteration of antibiotic titre by genetic means has been based largely on the laborious empirical method of screening large numbers of mutagenised cells for ß-lactam over-producers and non-producers. Recently, however, molecular techniques have been applied to analyse antibiotic production and regulation (reviewed in Elander, 1983; Ingolia and Queener, 1989; Martin and Demain, 1980; Martin and Liras, 1985; Miller and Ingolia, 1989a; Simpson and Caten, 1980; Veenstra *et al.*, 1989).

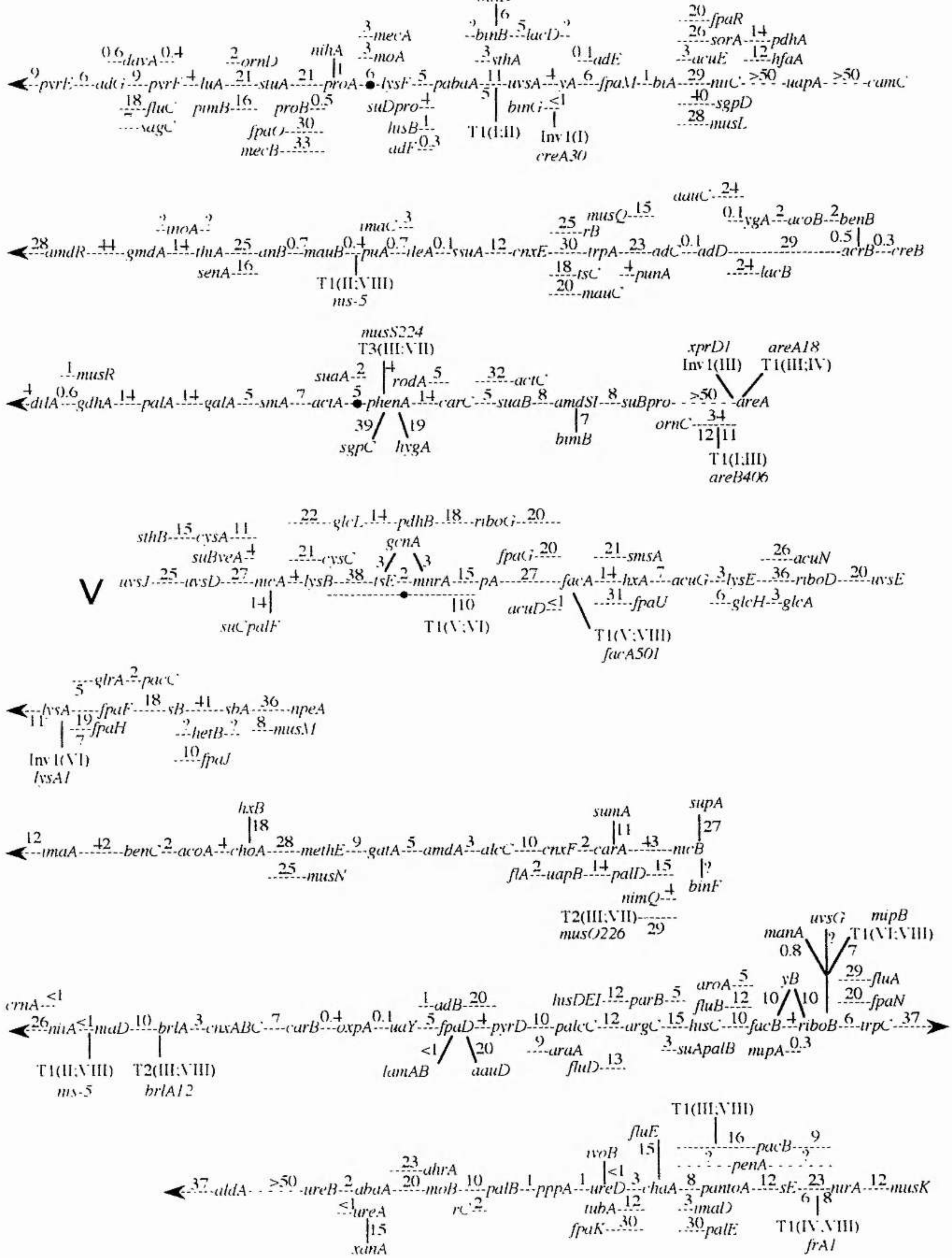
The generally high penicillin titre produced by the imperfect fungus *P. chrysogenum* renders it the preferred organism for industry and for the biochemical investigation of penicillin production. However,

several filamentous fungi synthesise this ß-lactam including the ascomycete *Aspergillus nidulans*, which has been employed in this study as a model organism for the investigation of penicillin biosynthesis. Although *A. nidulans* produces comparatively low levels of penicillin, it has the advantage of being amenable to rigorous formal genetic analysis by means of both its sexual and parasexual cycles, and is thus genetically well characterised, having a detailed chromosome map (Fig. 3, Clutterbuck, 1993; and reviewed by Ditchburn *et al.*, 1976). Furthermore, efficient transformation systems are available for *A. nidulans*, and a number of *A. nidulans* mutants defective in penicillin production (designated *npe*) have been described.

The existence of at least four unlinked genetic loci which impair penicillin production in *A. nidulans* has been revealed (Ditchburn *et al.*, 1976; Edwards *et al.*, 1974; Holt and Macdonald, 1968; Holt *et al.*, 1976; Makins *et al.*, 1981, 1983) using chemically or radiologically induced mutants defective in penicillin production (designated *npe*), which exhibit penicillin titres of 10 % or less than that of the wild type. Their corresponding mutations were designated *npeA*, *npeB*, *npeC* and *npeD*, and are located on linkage groups VI, III, IV and II, respectively, as determined by parasexual haploidisation and meiotic recombination analysis. In addition, a set of over-producing *A. nidulans* mutants with raised penicillin yields have

Fig. 3 Linkage map of *A. nidulans* (from Clutterbuck, 1993).





binD
0.6 davA 0.4
2 ornD
nihA
3 mecA
3 moA
binB 5 lacD
3 sthA
0.1 adE
20 fpaR
26 sorA 14 pdhA
3 acuE 12 hfaA
9 pyrE 6 adG 9 pyrF 4 luA 21 stuA 21 proA 6 lysF 5 pabaA 11 uvsA 4 yA 6 fpaM 1 biA 29 nicC >50 uapA >50 camC
18 fluC
sagC
pimB 16
proB 0.5
fpaO 30
mecB 33
suDpro 4
lysB 1
adF 0.3
binG ≤1
T1(I;II)
Inv1(I)
creA30
40 sgpD
28 musL
2 moA 2
imaC 3
musQ 15
aauC 24
0.1 ygA 2 acoB 2 benB
28 amdR 44 gmdA 14 thiA 25 anB 0.7 mauB 0.4 puA 0.7 ileA 0.1 ssuA 12 cnxE 30 trpA 23 adC 0.1 adD 29 0.5 acrB 0.3 creB
25 rB
senA 16
T1(II;VIII)
ms-5
18 tsC
20 mauC
4 punA
24 lacB
musS224
T3(III;VII)
1 musR
suaA 2
rodA 5
32 actC
xprD1
Inv1(III)
areA18
T1(III;IV)
4 dilA 0.6 gdhA 14 palA 14 galA 5 smA 7 actA 5 phenA 14 carC 5 suaB 8 amdSI 8 suBpro >50 areA
39 sgpC
19 lysA
7 bimB
ornC 34
12 11
T1(I;III)
areB406
22 glcL 14 pdhB 18 riboG 20
gcnA
sthB 15 cysA 11
suBveA 4
21 cysC
fpaG 20
21 smsA
26 acuN
V
uvsJ 25 uvsD 27 nicA 4 lysB 38 tsE 2 mnrA 15 pA 27 facA 14 hxA 7 acuG 3 lysE 36 riboD 20 uvsE
14
suCpalF
10
T1(V;VI)
acuD ≤1
31 fpaU
6 glcH 3 glcA
T1(V;VIII)
facA501
5 glrA 2 pacC
11 lysA fpaF 18 sB 41 sbA 36 npeA
19 fpaH
2 hetB 2
8 musM
Inv1(VI)
lysA1
10 fpaJ
hxB
18
sumA
11
supA
27
12 imaA 42 benC 2 acoA 4 choA 28 methE 9 gatA 5 amdA 3 alcC 10 cnxF 2 carA 43 nicB
25 musN
flA 2 uapB 14 palD 15
nimQ 4
binF
T2(III;VII)
musO226 29
manA
uvsG
mipB
T1(VI;VIII)
0.8
7
29 fluA
20 fpaN
cnxA ≤1
1 adB 20
hisDEI 12 parB 5
aroA 5
fluB 12
yB
26 niiA ≤1 maD 10 brlA 3 cnxABC 7 carB 0.4 oxpA 0.1 uaY 5 fpaD 4 pyrD 10 palcC 12 argC 15 hisC 10 facB 4 riboB 6 trpC 37
T1(II;VIII)
ms-5
T2(III;VIII)
brlA12
<1
20
lamAB
aauD
9 araA
fluD 13
3 suApalB
mipA 0.3
T1(III;VIII)
fluE
16
pacB 9
penA
23 ahrA
woB
37 aldA >50 ureB 2 abaA 20 moB 10 palB 1 pppA 1 ureD 3 chaA 8 pantoA 12 sE 23 nirA 12 musK
≤1 ureA
15
xanA
rC 2
tubA 12
fpaK 30
3 malD
30 palE
6 8
T1(IV;VIII)
frA1

been isolated and the corresponding mutations designated *penA*, *penB* and *penC* have been assigned to linkage groups VIII, III and IV, respectively. Investigations of non-producer mutations have revealed the existence of a somewhat analogous situation in *P. chrysogenum*, which has five complementation groups designated *npeV*, *npeW*, *npeX*, *npeY* and *npeZ* (Normansell *et al.*, 1979).

The preponderance of both mutagenised strains (Edwards *et al.*, 1974) and natural isolates (Cole *et al.*, 1976) of *A. nidulans* penicillin non-producers carry mutations within the *npeA* locus. Most of the *P. chrysogenum npe* mutants belong to the *npeY* complementation group (Normansell *et al.*, 1979). An explanation for the high frequency of mutations occurring within the *A. nidulans npeA* and *P. chrysogenum npeY* loci may be that these loci (i) encompass large DNA regions and (ii) have an exceedingly high mutation rate. The *npeA* and *npeY* loci thus appear to play a crucial role in penicillin biosynthesis.

The biochemical functions of the products of the *npe* loci are not well defined. However, *A. nidulans* strains carrying the *npeA* mutation are able to produce penicillin when supplied with ACV, and *npeY* mutants of *P. chrysogenum* are unable to produce the ACV tripeptide. Thus the *npeA* and *npeY* loci may be equivalents, associated with ACVS (Makins *et al.*,

1981), the first enzyme of the penicillin biosynthetic pathway. Investigations into the effect of pH on penicillin production in *A. nidulans* have found that wild type strains produce more penicillin at alkaline than at acid pH. Furthermore, mutations affecting the pH regulatory system have profound effects on penicillin production. When grown at neutral pH, mutants in five genes *palA*, *palB*, *palC*, *palE* and *palF* mimic the effects of growth at acid pH, considerably reducing penicillin production, whereas mutants in *pacC* mimic the effects of growth at alkaline pH thus considerably elevating penicillin production (Caddick *et al.*, 1986). Classical genetic analysis has revealed that *npeD* is allelic with *palF* and that *npeC* has a phenotype consistent with it being a *pal* mutant and is either allelic with *palA* or defines a new *pal* gene. The phenotype of *npeB* is difficult to study as such mutants produce significant levels of penicillin. This may be due to the *cnxA*5 mutation, also carried by these mutants which confers some resistance to the inhibitory effects of low pH on penicillin biosynthesis (H.N. Arst Jr., personal communication). Therefore, it would appear that of the four *npe* loci in *A. nidulans*, only *npeA* is concerned with ß-lactam biosynthesis *per se*.

Transformation of penicillin-producing fungi, initially hampered by the lack of efficient vectors and recipient strains, was first developed in *A. nidulans*. Much information on transformation methods has

accumulated in the related fungi *A. nidulans* and *A. niger* (for a selection of such methods see: Ballance and Turner, 1985; Buxton *et al.*, 1985; Johnstone *et al.*, 1985; Kelly and Hynes, 1985; Tilburn *et al.*, 1984; van Hartingsvelt *et al*, 1987; Ward *et al.*, 1988; Yelton *et al.*, 1984; reviewed in Johnstone, 1985; Saunders *et al.*, 1986) and has since been applied to *P. chrysogenum* (for a selection of methods see: Beri and Turner, 1987; Cantoral *et al.*, 1987; Diez *et al.*, 1987; Sanchez *et al.*, 1987; Whitehead *et al.*, 1989; reviewed in Martin, 1987). Development of efficient gene mediated transformation systems, including the *argB* system (Johnstone *et al.*, 1985) utilised in this work, in which genetic material is introduced into and expressed in an organism such as *A. nidulans*, whose classical genetics has been well explored, has greatly facilitated progress in both the understanding and exploration of its molecular genetics. Transformation has provided a method for the isolation of specific genes by complementation of relevant, previously well-studied, mutants using e.g. gene banks cloned in shuttle vectors. In addition, it permits the *in vitro* manipulation of cloned genes and reintroduction of such genes into the organism by transformation, thereby providing a powerful means for studying the regulation of gene expression.

1.4 REGULATION ASPECTS

Penicillin is elaborated by penicillin-producing filamentous fungi only during certain stages in their life cycle, typically when a trophophase (growth phase) is followed by a much longer idiophase (production phase) (Holt *et al.*, 1976; Martin and Demain, 1980). With the availability of cell-free systems, regulatory mechanisms important for the initiation of ß-lactam biosynthesis and for the precursor flow involved have been studied in a number of ß-lactam-producing organisms. The regulation of penicillin biosynthesis is complex and is tightly controlled, both at the level of precursor availability and at the level of the biosynthetic enzymes themselves. In addition to temporal repression, ß-lactam biosynthesis is subject to strong carbon catabolite and nitrogen metabolite repression, the molecular basis for which appears to be at the transcriptional and/or translational level, resulting in repressed formation of crucial enzymes (reviewed by Martin and Aharonowitz, 1983; Martin and Demain, 1980; Nuesch *et al.*, 1987). ß-lactam biosynthesis is poorly sensitive to phosphate regulation (reviewed by Liras *et al.*, 1990; Martin, 1977) but has been shown to be subject to regulation at the level of sulphur metabolism (reviewed by Martin and Aharonowitz, 1983; Nuesch *et al.*, 1987), pH regulation (Shah *et al.*, 1991) and to end product regulation (reviewed by Martin and Aharonowitz, 1983; Martin and

Demain, 1980). Greater understanding of the regulatory mechanisms controlling ß-lactam biosynthesis would be extremely valuable with respect to increasing antibiotic yields.

1.5 MOLECULAR ASPECTS

The isolation and analysis of penicillin biosynthetic genes and their products should greatly enhance progress in determining the control mechanisms involved in, and the number and relative importance of the various signals that affect, penicillin biosynthesis. Such studies should identify rate-limiting or feedback inhibited steps, and show whether there are any specific regulatory genes controlling ß-lactam biosynthesis or whether control is exerted via overall metabolism of the cell. A greater understanding of the way in which these control mechanisms work at the molecular level is invaluable if ß-lactam over-producing strains of filamentous fungi manipulated by recombinant DNA technology are to be obtained e.g one of the direct ways to obtain high-producing strains is by the selective elimination of bottlenecks in antibiotic pathways.

It is possible to amplify the copy number of ß-lactam biosynthetic genes by cloning and re-introducing such genes to the host organism. If a cloned gene corresponds to a rate-limiting step, increased production of the enzyme it encodes could conceivably

result in a concomitant increase in penicillin production. Another goal is to increase expression of such structural genes by fusing them to strong fungal promoters, while an understanding of the upstream control regions of the genes encoding ACVS, IPNS, and ACYT may permit the generation of defined, deregulated upstream mutants, with consequent over-production of the end product of the pathway. The isolation, manipulation and transformation into the host organism of regulatory genes associated with ß-lactam synthesis, if they exist, may however be even more important for strain improvement than the introduction of e.g. multiple copies of the genes that encode the biosynthetic enzymes. Amplification or elimination of such genes may be possible to stimulate precursor flow in the penicillin pathway. Genes controlling the regulation of antibiotic biosynthesis are often located near the genes encoding antibiotic biosynthetic enzymes, and therefore DNA fragments containing the ß-lactam biosynthetic genes may be useful in the study of such regulatory elements. Conversion of some intermediates e.g. ACV into ß-lactam antibiotics may be feasible *in vitro* by immobilised enzyme technology, and the cloning of ß-lactam antibiotic biosynthetic genes and the subsequent overproduction of the individual enzymes in *E. coli* should facilitate this and also the bioconversion of substrate analogues to novel penicillins and other ß-lactam antibiotics (reviewed in

Ingolia and Queener, 1989). A significant number of novel ß-lactams have even been prepared with IPNS from natural sources (reviewed by Jensen, 1985). With the enhanced availability of proteins synthesised by the cloned genes, information on the biochemical and biophysical properties of the ß-lactam biosynthetic enzymes will be more readily available and any data so obtained could be used for rational protein engineering. To this end, *in vitro* mutagenesis of cloned genes could be used to produce a new generation of more active and stable biosynthetic enzymes with enlarged or modified substrate specificity, resulting in new biosynthetic derivatives of already existing enzymes e.g. determination of the primary sequence of a protein produced may allow the engineering of its sequence to specific requirements (Floss, 1987).

Studying the regulation of the penicillin biosynthetic genes is a useful means of determining how penicillin biosynthesis is regulated. The expression signals of a number of *A. nidulans* genes have been analysed with the aid of *lacZ* fusion vectors (van Gorcom *et al.*, 1985, 1986). Using this system, the presence of promoter sequences of DNA is reported by the expression of an active ß-galactosidase (ß-gal) fusion protein comprising the 5' upstream expression signals of the gene under analysis and the ß-gal activity of a partially deleted *E.coli lacZ* gene. This, therefore, is a useful system with which to study the

regulation of genes involved in penicillin biosynthesis, whose products are not easily assayed directly, because it is the hybrid protein ß-gal activity which is detected rather than the actual product of the gene of interest.

1.6 ISOLATION OF THE PENICILLIN BIOSYNTHETIC GENES

Several approaches have been used in attempts to clone antibiotic biosynthetic genes:

(i.) Phenotypic complementation, where DNA cloned in vectors is transformed in to, and tested for, complementation of non-producing mutants. This phenotypic rescue of mutants requires the availability of the relevant mutants and a gene transfer capability of the host organism;

(ii.) Gene disruption (mutational cloning) techniques, which do not require the prior isolation of mutants, since the required DNA clones are recognised by their ability to disrupt relevant genes giving the appropriate non-producing mutant phenotype;

(iii.) Cloning of antibiotic resistance genes that might be linked to biosynthetic genes. This allows the isolation of co-cloned antibiotic production genes by the initial selection of suitable resistant clones and is only possible with pathways for compounds to which the potential recipient strain is sensitive;

(iv.) Reverse genetics, in which the amino acid sequence of the relevant purified enzyme is determined,

then DNA probes based on this sequence are synthesised and used to screen for hybridising clones in a DNA library; and

(v.) Gene homology, where cross-hybridisation allows the isolation of clones containing homologous genes from different organisms.

At the commencement of this research, such studies had resulted in the identification and isolation of the gene encoding IPNS, the second enzyme in the penicillin biosynthetic pathway, from *C. acremonium*, *P. chrysogenum* and *A. nidulans* (Carr *et al.*, 1986; Ramon *et al.*, 1987; Samson *et al.*, 1985). In addition, the *cefEF* gene encoding the enzymes hydroxylase and expandase, important in cephalosporin biosynthesis (Fig. 2), had been cloned from *C. acremonium* (Samson *et al.*, 1987).

The IPNS gene was first cloned from *C. acremonium* using the reverse genetics approach (Samson *et al.*, 1985). A partial amino acid sequence (23 amino acid residues) of the purified IPNS protein (Pang *et al.*, 1984) was determined and a region selected that had a relatively low level of degeneracy in the codon choices. Sets of DNA oligonucleotides (two pools of 17-mer oligonucleotides with 32 different oligonucleotides in each pool) from this region were synthesised and the mixed oligonucleotides were used to screen a cosmid library of *C. acremonium* DNA. A cosmid hybridising with the probe was isolated and the IPNS gene was localised

by restriction enzyme digestion and Southern blot analysis before being sequenced. This determined the open reading frame with the predicted amino acid sequence which matched the sequence of the purified IPNS protein. Further proof that the cloned gene was that of IPNS was obtained by expressing the gene in *E. coli* (which has no endogenous IPNS) and demonstrating that the resultant protein, which co-migrated with authentic IPNS on SDS-polyacrylamide gels, had IPNS activity as judged by bioassay.

Using the *C. acremonium* IPNS gene as a heterologous hybridisation probe, IPNS genes were subsequently isolated from genomic libraries of *P. chrysogenum* (Carr *et al.*, 1986) and *A. nidulans* (Ramon *et al.*, 1987), and their DNA sequences determined. No authentic purified IPNS protein was available from these organisms. Proof that each of the hybridising clones contained the IPNS gene was obtained by expression of the cloned gene in *E. coli*. Also, the *P. chrysogenum* putative IPNS protein from *E. coli*, which migrated slightly slower than the *C. acremonium* IPNS protein on SDS-polyacrylamide gels, strongly bound antibodies to *C. acremonium* IPNS.

The *C. acremonium cefEF* gene was cloned by the "reverse genetic" approach in a similar fashion to the *C. acremonium* IPNS gene. Proof of identity of the *cefEF* gene was also obtained by DNA sequencing and expression of the cloned genes in *E. coli* (Samson *et al.*, 1987).

1.7 RESEARCH OBJECTIVES

The initial aim of the project was to analyse the control of expression of the *A. nidulans* IPNS gene (*ipnA*) by *lacZ* fusion reporter analysis. This was undertaken in order to help understand more about the regulation of the penicillin biosynthetic pathway. Preliminary data suggested the existence of a second promoter region located adjacent to, and reading away from, the IPNS gene. Evidence available from work carried out, by Dr. A.P. MacCabe, in our laboratory suggested that the *A. nidulans* ACVS gene (*acvA*) was located close to and 5′ of *ipnA*. The possibility therefore existed that this second promoter activity, revealed by *lacZ* fusion analysis of a stretch of DNA which contained *ipnA*, was actually that of *acvA*. Consequently, a major goal became the determination of whether or not the ACVS gene, which had not previously been isolated from any organism, had indeed been cloned and corresponded to the previously uncharacterised *npeA* locus. Experiments were subsequently performed in order to characterise further the *A. nidulans npeA* locus. Finally, northern blot and hybridisation analyses were carried out to study the regulation of expression of the penicillin biosynthetic genes in an attempt to gain more of an insight into how penicillin biosynthesis is regulated.

CHAPTER 2

MATERIALS AND METHODS

2.1 CHEMICALS

Analytical grade chemicals were used wherever possible and were obtained from Sigma, BDH, Aldrich, Boehringer Mannheim, Oxoid, May and Baker, Kodak, Lab M and Fisons. DNA modifying enzymes, restriction enzymes and their dilution buffers were purchased from Pharmacia or NBL. Novozym 234 was obtained from Novobiolabs, and corn steep liquor was provided by Glaxochem. Water was distilled prior to use. α-^{32}P-dCTP and α-^{35}S-dATP were obtained from Amersham.

2.2 STRAINS

2.2.1 Bacterial Strains

Escherichia coli strain DH5 (F^-, *endA*1, *hsdR*17 (r_k^-, m_k^-) *supE*44, *thi*-1, lambda, *gyrA*96, *recA*1) or DH5α (F^-, *endA*1, *hsdR*17, *supE*44, *thi*-1, *recA*1, *gyrA*96, *relA*1, $\phi 80^R$, *lacZ*ΔM15 was used for the propagation of all plasmids except the *lacZ* fusion vectors pAN923-41B, -42B and -43B, which were harboured in *E. coli* strain JA221 (*hsdM*$^+$, *hsdR*$^-$, *lacY*, *leuB*6, *trpE*5, *recA*1). Cosmid clone No. 35 was propagated in *E. coli* K12 strain DH1 (F^-, *recA*1, *endA*1, *gyrA*1, *thi*-1, *hsdR*17, *supE*44).

Bacillus subtilis C107 was used as the indicator organism for the penicillin bioassay.

2.2.2 Fungal Strains

Nomenclature of gene loci in *A. nidulans* was according to Clutterbuck (1984).

Dr. A.J. Clutterbuck (University of Glasgow) provided the *A. oryzae argB*$^-$ and *A. niger argB*$^-$ strains, and the following *A. nidulans* strains: *biA*1, used as the "wild type" strain; G0049/1, the natural penicillin non-producer (*yA*2, *npeA*0049/1); G0156 (*biA*1, *pacC*5), G415 (*biA*1, *cnxE*14, *palC*4), G0215 (*biA*1, *methH*2, *su*-6 *methH*2), G0216 (*biA*1, *methH*2, *su*-7 *methH*2) and G0217 (*biA*1, *methH*2, *su*-8 *methH*2) which are regulatory mutant strains used in northern blotting experiments; and the *argB*2 mutant strains G034 (*biA*1, *argB*2), G34 (*yA*2, *methH*2, *argB*2) and G324 (*yA*2, *wA*3, *methH*2, *argB*2, *galA*1, *ivoA*1, *sC*12), which were used in e.g. the construction of various double mutants for use in transformation experiments. The "Glasgow" strains were all derived from a single wild isolate of *A. nidulans*, NRRL194 (Pontecorvo *et al.*, 1953).

Dr. J.H. Croft (University of Birmingham) supplied the following *A. nidulans* natural penicillin non-producing strains: Birmingham isolate Nos. 49, 108, 112, 123, 132 and 136, belonging to heterokaryon-compatibility group F; and Birmingham isolate Nos. 51 and 143, belonging to heterokaryon-compatibility group G (Cole *et*

al., 1976; J.H. Croft, personal communication). Birmingham isolate Nos. 51 and 143 were however found to produce penicillin and consequently their study was not further pursued.

Professor G. Holt (Polytechnic of Central London) provided G69 (*cha*, *penA*1), an *A. nidulans* penicillin over-producing strain used for the isolation of ACVS protein; and also the following induced penicillin non-producing strains of *A. nidulans*: GH79 (*yA*2, *pyroA*4, *cnxA*5, *npeA*0022), GH44 (*yA*2, *pyroA*4, *cnxA*5, *npeA*005) and GH36 (*galA*1, *pyroA*4, *facA*303, *s*3, *nic*8, *ribo*2, *npeA*002), which were crossed with the *A. nidulans argB*2 allele to construct double mutants for use in transformation and complementation experiments.

The *A. nidulans* double mutant *npeA*0049/1, *pyrG*89 strain M1.4 (*pabaA*1, *niaD*, *pyrG*89, *npeA*0049/1), was constructed by Dr. S. Assinder (University of St. Andrews) by fusing protoplasts from the naturally occuring penicillin non-producing strain G0049/1 and G191 (*pabaA*1, *pyrG*89, *fwA*1, *uaY*9), an *A. nidulans* uridine auxotrophic strain obtained from Professor G. Turner (University of Sheffield).

A. nidulans strains MH837 (*biA*1, *xprD1*), MH8 (*biA*1, *niiA*4, *areA*102) and MH205 (*biA*1, *niiA*4, *areA*19); were obtained from Professor M.J. Hynes (University of Melbourne). All three strains were used in northern blotting experiments, whilst MH837 and MH205 were used to

construct double mutants with the *argB2* marker for use in transformation and complementation experiments.

Dr. J. Kelly (Flindus University) provided *A. nidulans* C46 (*pabaA1*, *niiA4*, *argB2*, JA1, *creC27*) and 481 (*pabaA1*, *creA1*).

The following sulphur regulatory mutant strains: (*yA1*, *pyroA4*, *mapA25*); (*yA1*, *pyroA4*, *mapB1*); (*yA1*, *pabaA2*, *mapB2*); (*yA1*, *pyroA4*, *mapC3*); and (*yA1*, *pyroA4*, *mapD6*); used in northern blotting experiments, were obtained from Professor P. Weglenski (University of Warsaw).

Dr. B. Tomsett (University of Liverpool) provided *A. nidulans* strain B418 (*biA1*, *puA2*, *niaD52*), which was used in the classical genetic analysis of transformants.

P. chrysogenum V992 and *C. acremonium* M8650, used in this study, were provided by Dr. M. Ramsden (Glaxochem).

The *N. crassa nit*-1 mutant was obtained from Dr. R. Mendel (Akademie der Wissenschaften).

2.3 MAINTENANCE OF CULTURES

2.3.1 Bacterial Strains

Strains cultured on Luria agar plates for short term storage were kept at 4^{o}C. For long term storage, cultures were held at -70^{o}C in Luria broth containing 20 % glycerol (final concentration).

2.3.2 Fungal Strains

Strains were cultured on complete medium plates for medium term storage, and held on silica gel (Roberts, 1969) for long term storage. Stocks were kept at 4°C.

Unless otherwise indicated, *A. nidulans* was incubated at 37°C, whilst *A. oryzae* and *A. niger* were incubated at 30°C.

2.4 MEDIA

Unless otherwise indicated, the preparation of solid media was by the addition of 1.2 % agar to the appropriate liquid media, and the sterilisation of all media and solutions was carried out by autoclaving at 15 lb/inch^2 for 15 min.

2.4.1 Bacterial Culture Media

Luria broth was prepared according to Sambrook *et al.* (1989).

Where appropriate, selective Luria medium was prepared by the addition of 100 µg/ml ampicillin (final concentration) to medium which had been autoclaved and precooled to 55°C.

SOC medium was prepared thus:

2.0 %	tryptone
0.5 %	yeast extract
10.0 mM	NaCl
2.5 mM	KCl

made to pH 7.4 with NaOH and autoclaved. To this was then added sterile 5 mM $MgCl_2$, 5 mM $MgSO_4$ plus 20 mM glucose, and the whole filter sterilised just prior to use.

Bioassay medium, based on that of Ditchburn *et al.* (1974), was prepared as follows:

0.5 % peptone
0.3 % Lab-Lemco
0.1 % sodium citrate
1.2 % agar

2 ml of a 5 day (sporulating) culture of *B. subtilis* C107 and 1 ml of 0.5 % 2,3,5-triphenyl tetrazolium chloride (filter sterilised), were added to 200 ml of bioassay medium precooled to 50°C. Petri dishes (22 cm x 22 cm) were used for the penicillin bioassay.

2.4.2 Fungal Media

Complete and minimal medium for *Aspergillus* were based on the recipes described by Cove (1966) and Pontecorvo *et al.* (1953).

Complete medium:

10 g glucose
50 ml salts solution (see below)
1 ml trace elements solution (see below)
1 ml vitamin solution (see below)
2 g peptone
1 g yeast extract
1 g casein hydrolysate

made up to 1 l with dH_2O and pH 6.5 with 5 M NaOH.

Minimal medium: (nitrogen-less)

10 g glucose
50 ml salts solution (see below)
1 ml trace elements solution (see below)

made up to 1 l with dH_2O and pH 6.5 (unless otherwise indicated) with NaOH.

Unless otherwise stated, 10 mM ammonium tartrate was routinely added to *Aspergillus* minimal medium, however 5 mM or 10 mM ammonium tartrate; 10 mM $NaNO_3$; 10 mM $NaNO_2$; 1 mM hypoxanthine; 10 mM proline; 5 mM or 10 mM glutamate; were used as alternative nitrogen sources, as required.

Nitrogen sources: The various nitrogen sources were either incorporated directly into the medium prior to autoclaving or were kept as sterile 1 M stock solutions and added to nitrogen-less minimal medium precooled to 55°C.

Trace elements solution:

1.1 g $(NH_4)_6MO_7O_{24}.4H_2O$
11.1 g H_3BO_4
1.6 g $CoCl.6H_2O$
1.6 g $CuSO_4.5H_2O$
50.0 g EDTA (disodium salt)
5.0 g $FeSO_4.7H_2O$
5.0 g $MnCl_2.7H_2O$
22.0 g $ZnSO_4.7H_2O$

made up to 1 l with dH_2O and boiled with stirring. The solution was then cooled to 60°C, adjusted to pH 6.5-6.8 with KOH and stored in the dark at 4°C.

Vitamin solution:

25.0 mg	biotin
2.5 g	nicotinic acid
0.8 g	para-amino benzoic acid
1.0 g	pyridoxine HCl
2.0 g	pantothenic acid
2.5 g	riboflavin
1.5 g	aneuric acid
20.0 g	choline chloride

made up to 1 l with dH_2O.

Supplements: The following supplements were sterilised by filtration and stored as concentrated aqueous solutions at 4°C. The appropriate amount of supplements were added to media which had previously been cooled to 55°C, as required.

Supplement	Concentration of stock solution	Amount added to 100 ml medium
arginine-HCl	4.2 g/100 ml	1 ml
biotin	10.0 mg/100 ml	1 ml
choline-HCl	2.0 g/100 ml	1 ml
1 M glutamate	18.7 g/100 ml	1 ml
methionine	0.5 g/100 ml	1 ml
nicotinic acid	10.0 mg/100 ml	1 ml
PABA	0.14 g/100 ml	0.5 ml
1 M proline	11.15 g/100 ml	1 ml
putrescine	0.2 g/100 ml	1 ml
pyridoxine-HCl	0.5 g/100 ml	1 ml
riboflavin	0.8 g/100 ml	1 ml
1 M uridine	24.42 g/100 ml	1 ml

Salts solution:

10.4 g KCl
10.4 g $MgSO_4.7H_2O$
30.4 g KH_2PO_4

made up to 1 l with dH_2O.

Saline tween solution:

0.01 % Tween 80
0.9 % NaCl

Fermentation medium, based on the recipe of Ditchburn *et al.* (1974), was prepared as follows:

3.5 % lactose
0.45 % $MgSO_4$
1.0 % $CaCO_3$
0.21 % nitrogen as corn steep liquor

made to pH 5.2 with 5 M KOH. Solid medium for fermentation plugs was prepared by the addition of agar to a final concentration of 4 %.

2.5 ISOLATION OF SPONTANEOUS NITRATE-ASSIMILATION DEFECTIVE *A. nidulans* MUTANTS

Spontaneous mutants of *A. nidulans*, defective in the genes required for nitrate assimilation, were isolated by positive selection for resistance to 470 mM chlorate on minimal medium containing 10 mM glutamate as the sole source of nitrogen (reviewed by Cove, 1979). Such mutants were assigned by growth tests (Table I) to one of the gene loci of the nitrogen assimilation pathway (Fig. 4; Unkles *et al.*, 1989).

Table I

A. nidulans mutants defective in nitrate assimilation.

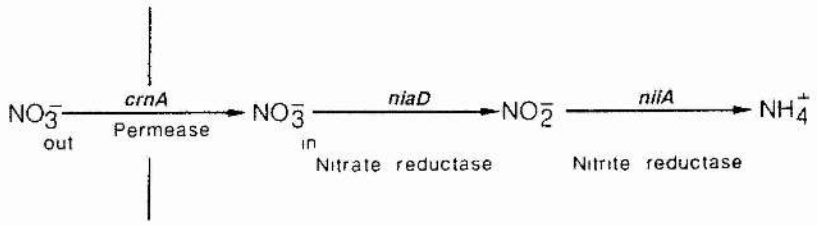
Gene mutation[a]	Chlorate resistance[b]	Utilisation of sole nitrogen source[c]			
		NO_3	NO_2	NH_4	hypoxanthine
niaD$^-$	R	-	+	+	+
crnA$^-$	R	+	+	+	+
cnxA-J$^-$	R	-	-	+	-
nirA$^-$	R	-	-	+	+
wt	S	+	+	+	+

[a] The minus (-) superscripts denote loss-of-function mutations. wt denotes the wild type (*biA*1) strain.

[b] R denotes resistance to chlorate, such mutants having the ability to grow on minimal medium containing 470 mM chlorate and 10 mM glutamate as the sole nitrogen source. S denotes sensitivity to chlorate.

[c] Symbol (+) denotes wild type levels of growth; symbol (-) denotes poor growth.

Fig. 4 The catalytic conversion of nitrate to ammonium ions. Extracellular nitrate is transported to the cell by a permease encoded by the *crnA* gene, converted to nitrite by the action of nitrate reductase encoded by *niaD*, and finally converted to ammonium ions by nitrite reductase, the *niiA* gene product (from Unkles *et al.*, 1989).


NO3−
out
crnA
Permease
NO3−
in
niaD
Nitrate reductase
NO2−
niiA
Nitrite reductase
NH4+

2.6 CLASSICAL GENETIC TECHNIQUES

Sexual crosses and the characterisation of the resulting progeny were performed according to the procedures of Pontecorvo *et al.* (1953) and Clutterbuck (1974).

Crosses were initiated by mixing conidia of the two parent strains, which differed both in auxotrophic markers and in spore colour, with two loopfuls of complete medium and a loopful of arginine or uridine (if required) in a 1-2 cm diameter area in the centre of a plate of thick minimal medium containing 10 mM ammonium tartrate or 10 mM $NaNO_3$ as the nitrogen source, as appropriate. The mixture was then streaked out towards the edge of the petri dish which was then sealed to restrict air exchange, and incubated at 37°C for 7-10 days. Cleistothecia were rolled on 2 % agar to remove any adhering conidia and Hulle cells, then crushed in e.g. 10 ml saline tween to yield a suspension of ascospores. Trial platings were then made by spreading e.g. 100 µl ascospore suspension on complete medium (one plate per cleistothecium), followed by incubation at 37°C. The ascospore suspensions of hybrid cleistothecia were then plated out onto complete medium at an appropriate dilution to give 20-30 colonies per dish.

Progeny resulting from a cross were inoculated onto a complete medium "master plate" such that their position corresponded to that of needles on a multipin replicator, which consisted of a perspex sheet carrying 25 pins in a

5 x 5 array and one eccentric pin as an orientation marker. Colonies were transferred to various test media by stabbing the replicator or inoculating wire (as appropriate) upwards into the medium in an inverted plate, to prevent scatter of conidia. To test for the presence of auxotrophic markers, colonies were inoculated onto minimal medium containing all the supplements except that required to allow the growth of progeny containing the mutant allele under examination. The presence of *xprD1* and *npeA* alleles in progeny was detected by assaying for protease and penicillin production, respectively. Control colonies of both parents were classified along with the progeny of each cross.

Wild type colonies excrete biotin therefore, when testing for this auxotrophic marker, plates should be incubated for no longer than 1-2 days to prevent cross-feeding of biotin-requiring colonies by prototrophic ones (Pontecorvo *et al.*, 1953).

2.7 QUALITATIVE PROTEASE ASSAY

Qualitative assays of protease production, to test for the presence of the *xprD1* allele in the progeny of certain sexual crosses of *A. nidulans*, were based on the method of Cohen (1972). Colonies were inoculated onto plates of appropriately supplemented minimal medium containing 10 mM ammonium tartrate as a nitrogen source, and a final concentration of 2 % dried skimmed milk solution (10 % stock concentration in dH_2O, sterilised by

autoclaving at 15 lb/inch^2 for 15 min), incubated at 37^{o}C for 2 days then placed at 4^{o}C overnight. Extracellular protease activity was detected as clear halos around the organism from which the protease had diffused, solubilising the casein component of the skimmed milk which gives the milk its opacity. The transfer of plates to 4^{o}C arrests the growth of the colonies but not the production of protease thus accentuating the periphery of any halo produced. As controls, *A. nidulans* strains wild type (*biA*1) and MH837 (*xprD1*) were inoculated on each assay plate. Extracellular protease is produced by strains containing the *xprD1* mutant allele, but not in the wild type strain, when grown on medium containing ammonium tartrate as the nitrogen source (Fig. 5).

2.8 PROTOPLAST PREPARATION AND TRANSFORMATION

2.8.1 *A. nidulans*

The preparation of protoplasts, and the transformation of *A. nidulans* were based on the method of Tilburn *et al.* (1983). *A. nidulans* conidia were seeded onto appropriately supplemented minimal medium, and grown for 14 h at 37^{o}C with orbital shaking at 200 rpm. The mycelium was filtered through sterile muslin, rinsed with 0.8 M $MgSO_4$, 10 mM sodium phosphate pH 5.8; and resuspended in 100 ml of the same buffer. 0.1 g Novozym 234 was added to the suspension and the whole incubated at 30^{o}C with gentle orbital shaking. The mixture was

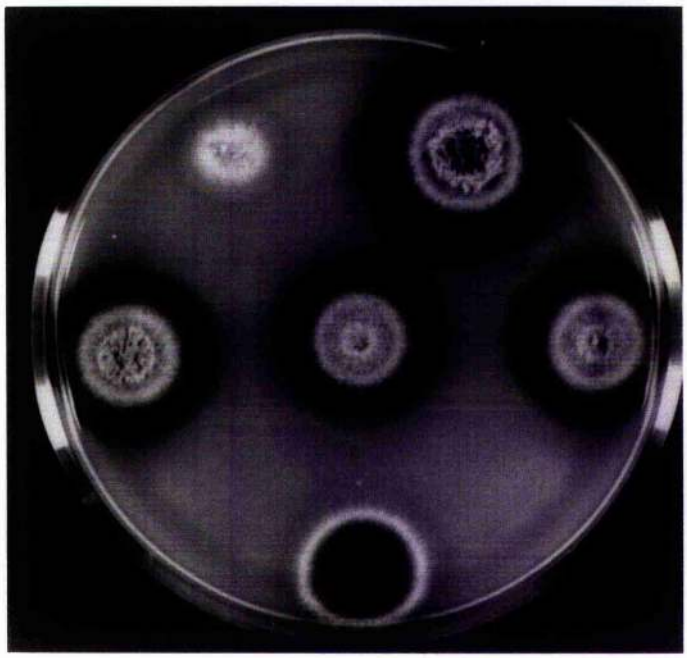
Fig. 5 Qualitative protease assay plate. Extracellular protease production is detected as clear halos around *xprD1* mutant strains but not wild type strains when ammonium is present as the sole nitrogen source.

A. nidulans wild type (*biA*1), G34 and *xprD*1 mutant strains MH837 and SAA244; grown on appropriately supplemented minimal medium containing 10 mM ammonium tartrate as a nitrogen source, and a final concentration of 2 % dried skimmed milk solution.

G34 MH837

SAA244 SAA244 SAA244

(*biA*1)



decanted into sterile 20 ml plastic containers and centrifuged at room temperature, 2400 x g for 20 min. The protoplast pellicle was removed and washed four times in 1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris-HCl pH 7.5; at room temperature by centrifugation. The final pellet was resuspended in 1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris-HCl pH 7.5; to yield 3 x 10^8 protoplasts/ml.

For transformation, 200 μl of the above protoplast suspension was incubated with 5-10 μg of plasmid DNA at room temperature for 20 min. 10 volumes (2 ml) of 50 % PEG 4000, 10 mM $CaCl_2$, 10 mM Tris-HCl pH 7.5; were added and the mixture incubated at room temperature for 10 min. The preparation was diluted with 5 volumes (10 ml) of 1.2 M sorbitol, 10 mM $CaCl_2$, 10 mM Tris-HCl pH 7.5; and added to 300 ml transformation selection medium (molten minimal agar solution containing 1.2 M sorbitol and appropriate supplements except for that required for growth of untransformed organisms) maintained at 50°C. The mixture was plated out and incubated at 37°C for up to 4 days. Control protoplasts were treated as above but without the addition of DNA. Protoplast viability was tested by plating "untransformed" protoplasts on non-selective medium.

2.8.2 *A. niger* and *A.oryzae*

The preparation of protoplasts, and the transformation of *A. niger* or *A. oryzae* were based on the method of Unkles *et al.* (1989). *A.niger* or *A. oryzae*

conidia were seeded into 250 ml of appropriately supplemented minimal medium, and grown at 30°C, with orbital shaking at 250 rpm, for 12 h. The mycelium was harvested by filtration through sterile muslin, rinsed with 0.8 M $MgSO_4$, 10 mM sodium phosphate pH 5.8; and resuspended in 25 ml of the same buffer. 0.1 g Novozym 234 was then added, and the mixture incubated at 30°C, with orbital shaking at 250 rpm, for 45 min, then the protoplasts were pelleted by centrifugation at 1500 x g for 10 min. Protoplasts were washed three times in 1.2 M sorbitol, 50 mM $CaCl_2$, 10 mM Tris-HCl pH 7.5; and finally resuspended in the same buffer at a concentration of 2 x 10^8 protoplasts/ml.

For transformation, 100 µl of the above protoplast suspension was mixed with 5-10 µg of plasmid DNA and incubated on ice for 20 min. To this, 1 ml of 50 % PEG 4000, 50 mM $CaCl_2$, 10 mM Tris-HCl pH 7.5; was added to 100 ml transformation selection medium (molten minimal agar solution containing 1.2 M sorbitol and appropriate supplements except that required for growth by untransformed organisms) maintained at 50°C. This was then overlayed onto transformation selection medium and incubated at 30°C for up to 5 days. Control protoplasts were treated as above but without the addition of DNA, and protoplast viability was tested by plating control protoplasts on non-selective medium.

2.9 PENICILLIN BIOASSAY

Penicillin yields of various fungal species were assessed using a method based on that adopted by Ditchburn *et al.* (1974). Using a sterile steel cork borer (9 mm internal diameter), plugs were punched in plates of solidified fermentation agar then transferred to the surface of a sterile petri dish, with not more than 12 plugs placed on each fresh dish. Individual fermentation plugs were inoculated with conidia and incubated at 25°C for 5 days, in a humid environment to prevent dessication of the agar. Plugs were then placed on thin bioassay medium seeded with *B. subtilis* C107 spores and incubated at 37°C overnight. As controls, plugs inoculated with known penicillin-producing strains were treated as above, and 0.5 µl of penicillin G was spotted onto bioassay medium prior to incubation at 37°C. Alternatively, to determine whether or not organisms in liquid culture were producing penicillin, 5 µl and 10 µl aliquots of culture supernatants were spotted directly onto the bioassay medium prior to incubation at 37°C. Antibiotic production was detected as clear halos due to the inhibition of the indicator bacterium *B. subtilis*. The inclusion of the tetrazolium salt in the bioassay medium accentuated the peripheries of the zones of inhibition, thus facilitating measurements: where the bacterium grew, the tetrazolium salt was reduced to red formazan but the zone of inhibition remained colourless.

2.10 DETERMINATION OF ß-GALACTOSIDASE ACTIVITY

2.10.1 Qualitative assay

Qualitative assays of *lacZ* fusion product activity in transformants containing *lacZ* fusion recombinants were made on minimal agar which was based on M9 minimal medium (Sambrook *et al.*, 1989) but contained either 0.2 % glucose or maltose as the carbon source and 1 % NH_4Cl or 10 mM glutamate as the nitrogen source, and to which agar was added at 1 % and X-gal (stock concentration 5 mg/ml in methanol) was added to yield a final concentration of 40 µg/ml. As controls to distinguish between endogenous *A. nidulans* and hybrid protein ß-gal activity, transformants containing pAN923-21B, the *trpC* :: *lacZ* fusion plasmid of van Gorcom *et al.* (1986), were also inoculated onto the above media. Activity of the hybrid protein was detected in ß-gal non-inducing media by the hydrolysis of X-gal to yield a blue mycelium, initially at the centre of the colony, after approximately 24-48 h incubation at 37°C. Endogenous *A. nidulans* ß-gal activity was detected by the hydrolysis of X-gal to yield a blue mycelium, initially at the periphery of the colony after approximately 48 h in non-inducing medium, and approximately 24 h in medium containing lactose (0.2 %), which is an inducer of the endogenous *lacZ* gene (Fantes and Roberts, 1973).

2.10.2 Quantitative assay

Solutions required:

Reaction buffer:	20 μM PMSF (20 mM stock in DMSO)
	50 mM ß-mercaptoethanol
	100 mM $NaPO_4$ pH 7.4
Substrate solution:	10 mg/ml o-NPG in reaction buffer
Stop mix:	1 M Na_2CO_3

Quantitative assays of *lacZ* fusion product activity in transformants were based on the method of van Gorcom *et al.* (1985). *A. nidulans* wild type (*biA1*) strain was used as a control to determine whether or not endogenous ß-gal activity was being detected under the growth conditions used. Conidia were seeded into 250 ml minimal medium containing various carbon and nitrogen sources, at final concentrations of 1 % and 10 mM respectively, and incubated at 37°C, with orbital shaking at 200 rpm, for 18 h. The mycelium was harvested by filtration through sterile muslin, washed with sterile dH_2O, blotted dry and frozen in liquid N_2. 2 g pressed wet weight of mycelium was ground in liquid N_2, suspended in 5 ml reaction buffer and incubated on ice for 15 min. The suspension was then centrifuged at 32570 x g, 4°C for 15 min, and the supernatant (cell free extract) decanted and placed on ice. To 100 μl of substrate solution was added 100 μl enzyme extract plus 300 μl reaction buffer, and the whole was incubated at 37°C for 8 min. This gave the optimum reaction rate and linear reactions with respect to the time of incubation and volume of extract used. After this

time, 500 µl of stop mix was added to terminate enzyme activity. A time zero blank was prepared for each reaction by adding 500 µl of stop mix to the cell free extract/reaction buffer mixture before the addition of 100 µl of substrate solution. The absorbance of the reaction was measured against the time zero blank at 420nm. Enzyme activities were determined by estimating the amount of O-NP produced, from a standard curve of o-NP (stock concentration 10 mg/ml, dissolved in reaction buffer) against A_{420nm} (Table II, Fig. 6), then normalised with respect to protein concentrations using the method of protein determination developed by Bradford (1976). All reactions were carried out in triplicate and the A_{420nm} of all standards was measured in triplicate.

It may be necessary to determine protein concentrations of enzyme extracts on the day of their preparation, as problems have been experienced with precipitation of protein in enzyme extracts stored at -70°C.

2.11 DETERMINATION OF PROTEIN CONCENTRATION

The protein concentration of enzyme extracts was determined according to the method of Bradford (1976). For this, Bradford's reagent, a protein binding dye, was prepared as follows: 100 mg Coomassie brilliant blue G was dissolved in 55 ml 96 % ethanol with stirring, to which was added 100 ml orthophosphoric acid. This was then made up to 1 l with dH_2O, filtered twice through

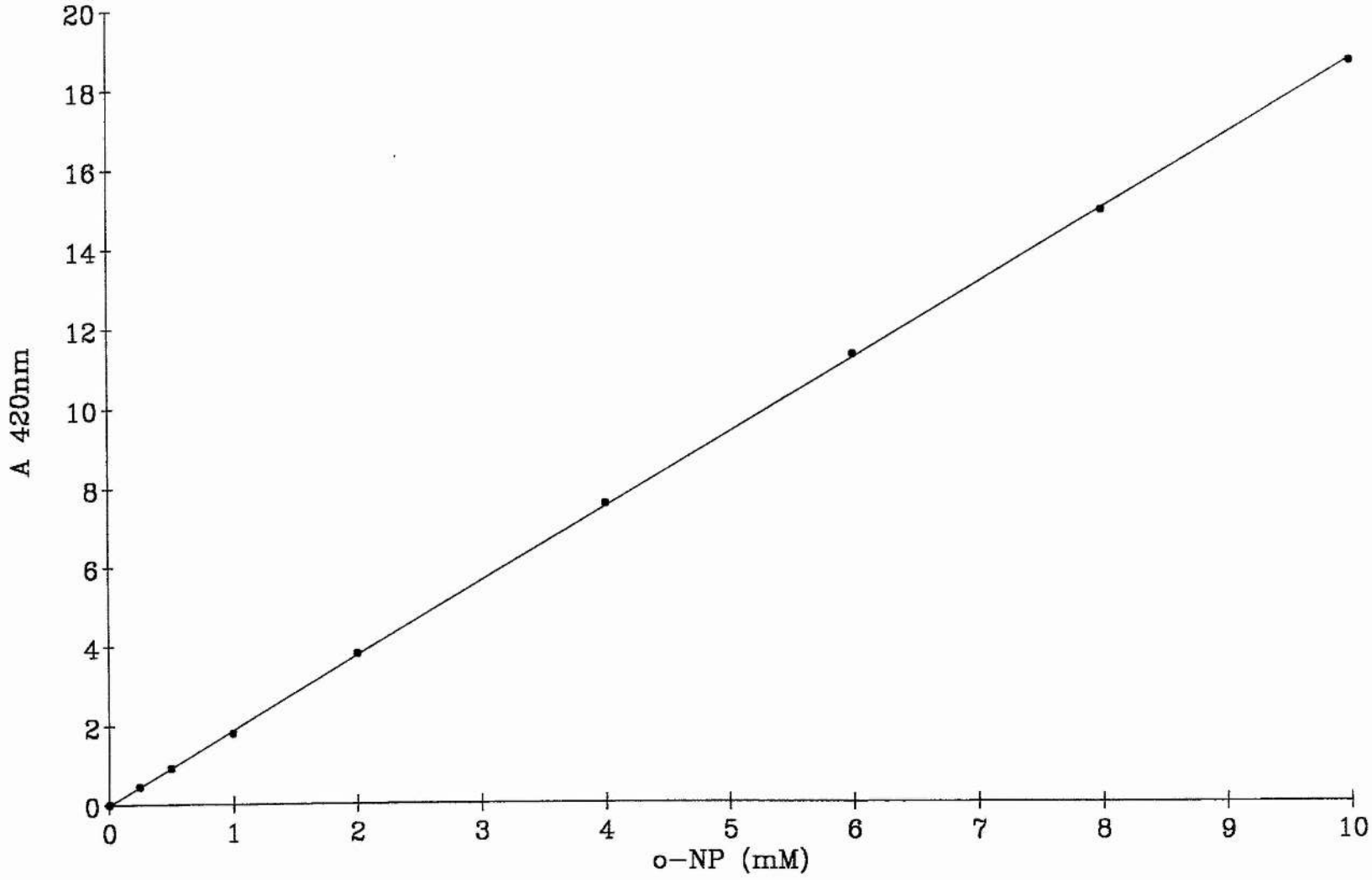
Table II

Spectrophotometric measurement of o-NP concentration.

o-NP conc (mM)	A_{420nm}[a]			Average A_{420nm}
0	0	0	0	0
0.25	0.466	0.456	0.475	0.466
0.5	0.904	0.902	0.968	0.925
1	1.787	1.797	1.837	1.807
2	3.820	3.580	3.983	3.790
4	7.347	7.313	8.100	7.590
6	11.240	10.820	11.900	11.320
8	15.180	14.075	15.600	14.950
10	19.120	17.747	19.330	18.730

[a] A_{420nm} values derived from three separate standard curves of o-NP concentration, each standard having been measured in triplicate.

Fig. 6 Typical standard curve of o-NP. A_{420nm} values shown are averaged from three separate standard curves.


20
18
16
14
12
10
8
6
4
2
0
A 420nm
0
1
2
3
4
5
6
7
8
9
10
o-NP (mM)

Whatman No.1 filter paper and stored in the dark at room temperature. For the assay itself, 20 μl of enzyme extract was made up to 100 μl with dH_2O, mixed with 5 ml Bradford's reagent, and the reaction allowed to proceed for 5-10 min. The absorbance of the reaction was measured at 595nm against a suitable blank (100 μl dH_2O plus 5 ml Bradford's reagent). Protein concentrations were estimated from a standard curve of BSA (stock concentration 1 mg/ml in dH_2O) concentrations against absorbance at 595nm (Table III, Fig. 7). The A_{595nm} of all standards and the protein concentration of the enzyme extracts were determined in triplicate.

2.12 PROTEIN PURIFICATION AND SEQUENCING

Purified ACVS protein was prepared by the method of van Liempt *et al.* (1989), from 6 x 10 l fermentation cultures of *A. nidulans* (strain G69) grown for 32 h at 28^{o}C, conditions which result in penicillin production, and amino acid sequencing was performed as detailed in MacCabe *et al.* (1990, 1991).

2.13 PLASMIDS AND COSMIDS

All standard techniques employed in the cloning and generation of recombinant plasmids were as detailed by Sambrook *et al.* (1989).

Cloning vectors pUC13, pUC18 and pUC19 were obtained from Pharmacia, whilst pILJ16 was provided by Dr. I.L. Johnstone (University of Glasgow).

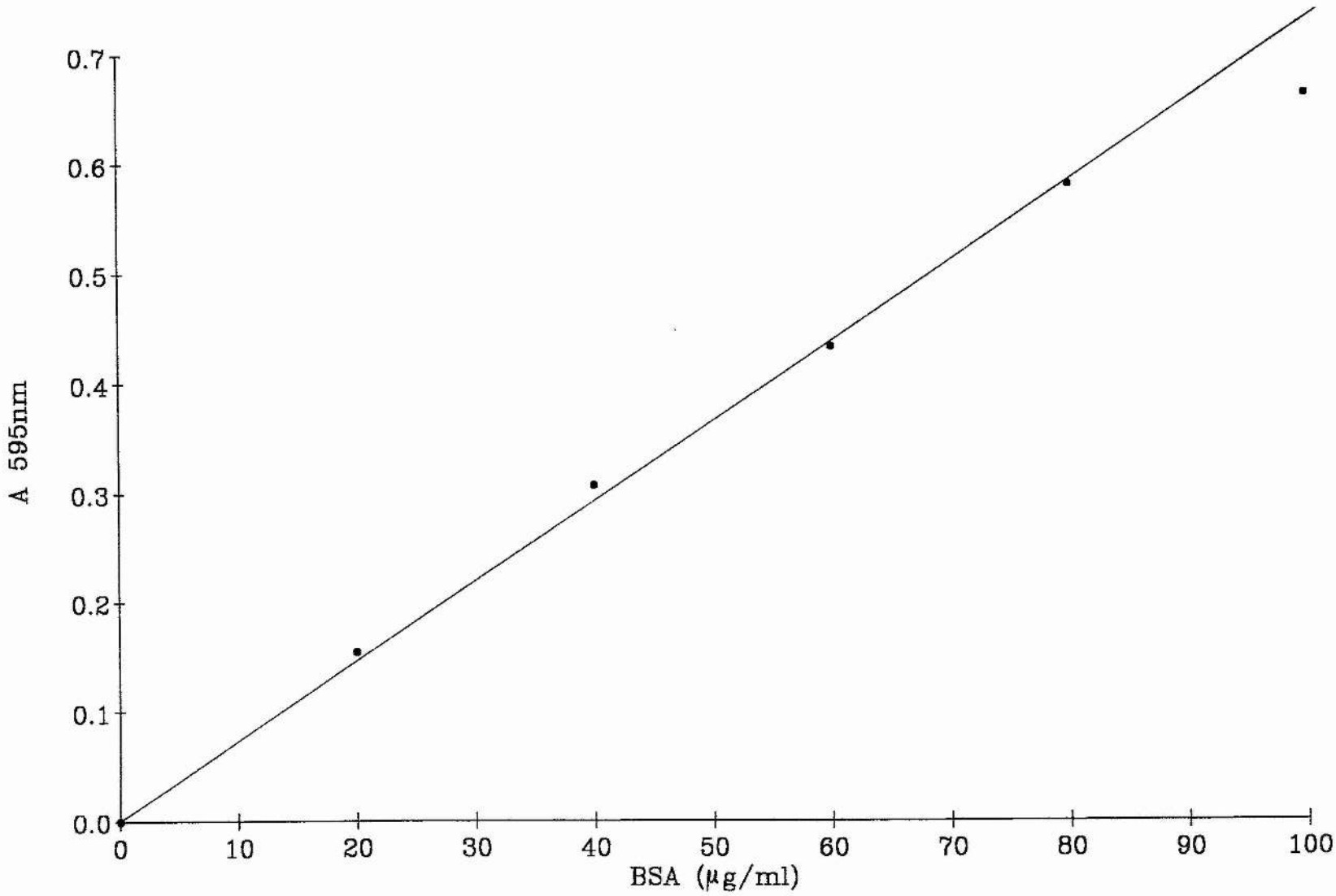
Table III

Spectrophotometric measurement of BSA concentration.

BSA conc (μg/ml)	A_{595nm}[a]			Average A_{595nm}
0	0	0	0	0
20	0.124	0.196	0.143	0.154
40	0.282	0.361	0.278	0.307
60	0.396	0.479	0.425	0.433
80	0.554	0.647	0.538	0.580
100	0.606	0.728	0.653	0.662

[a] A_{595nm} values derived from three separate standard curves of BSA concentration, each standard having been measured in triplicate.

Fig. 7 Typical standard curve of BSA. A_{595nm} values shown are averaged from three separate standard curves.


A 595nm
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
10
20
30
40
50
60
70
80
90
100
BSA (µg/ml)

Plasmids pSTA200, pSTA201 and pSTA207 were isolated (Dr. A.P. MacCabe, University of St. Andrews) from an *A. nidulans argB* based genomic library (Johnstone *et al.*, 1985). pSTA200 and pSTA201 were isolated using an *Xba*I-*Bam*HI fragment prepared from pIPNSp18B, a recombinant plasmid containing the *P. chrysogenum* IPNS coding region (provided by Dr. M. Ramsden, Glaxochem). pSTA207 was isolated using a 600 bp *Kpn*I-*Sal*I fragment isolated from pSTA201 (Fig. 8.II). Plasmids pSTA807, pSTA203 and pSTA204 are deletion subclones derived from plasmid pSTA201 (Fig. 8.II; Dr. A.P. MacCabe, University of St. Andrews).

Using the *Xba*I-*Bam*HI fragment of pIPNSp18B, pSTA18 was isolated (Dr. S.E. Unkles, University of St. Andrews) from an *Eco*RI *P. chrysogenum* genomic library generated in pUC13. pSTA18 contains the *P. chrysogenum* IPNS gene as well as sequences downstream which have been shown to encode the gene for ACYT (Veenstra *et al.*, 1989; J.F. Martin, personal communication).

Plasmid pDJB2, which contains the *N. crassa pyr*4 gene, was obtained from Professor G. Turner (University of Sheffield), as was the cosmid clone No. 35 which contains *A. nidulans* genomic DNA sequences including *acvA*, *ipnA*, *acyA* and sequences 5′ of *acvA*. Plasmid pSTA230 was generated by cloning the 5.5 kb *Hin*dIII-*Hin*dIII fragment (containing sequences of *A. nidulans* DNA 5′ to *acvA*) from cosmid clone No. 35 into pUC18 (Dr A.P. MacCabe, University of St. Andrews).

Fig. 8 The *A. nidulans* IPNS region.

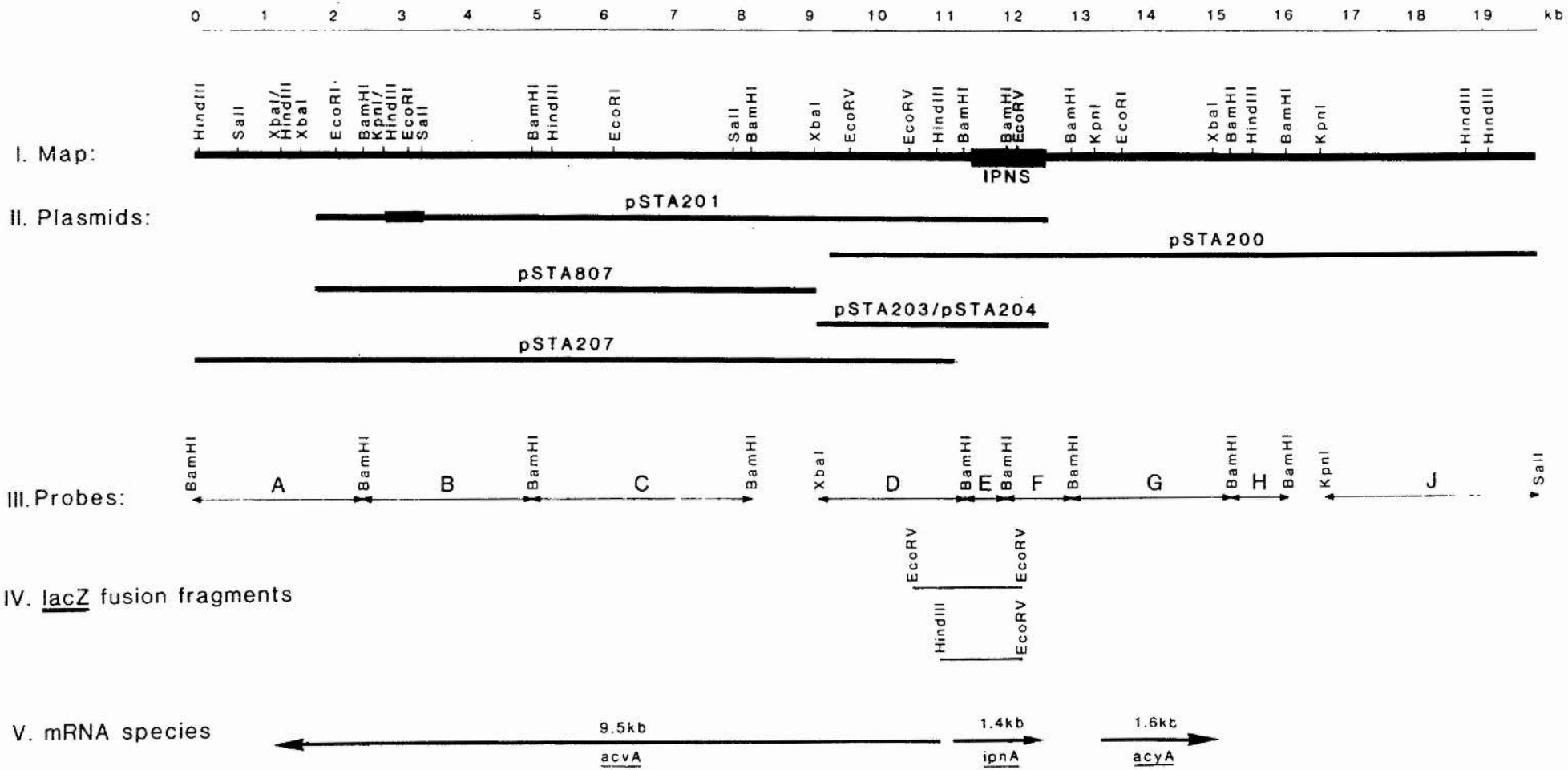
I. Restriction endonuclease map. The heavy bar represents the gene encoding IPNS (Ramon *et al.*, 1987). Only *Eco*RV sites relevant to the *lacZ* fusions are shown.

II. pSTA200, pSTA201 and pSTA207 were isolated from an *A. nidulans* genomic library in pILJ16 (Johnstone *et al.*, 1985). Plasmids pSTA203, pSTA204 and pSTA807 are subclones of pSTA201.

III. DNA fragments used to generate ^{32}P-labelled probes for hybridisation experiments. Fragment A was isolated from pSTA207; fragments B, C and E were isolated from pSTA201; whilst fragments D, F, G, H and J were isolated from pSTA200. The 0.6 kb *Kpn*I-*Sal*I fragment from pSTA201, used as a probe to isolate pSTA807, is represented by a heavy bar on pSTA201.

IV. Fragments inserted into *lacZ* fusion vectors in both orientations.

V. mRNA species revealed by northern blotting analyses. The approximate position of the genes was deduced from northern blotting, complementation and cross-hybridisation studies. The direction of transcription is indicated by arrows (determined by Ramon *et al.*, 1987, for *ipnA*). Not all *Sal*I sites in fragments B and C are located on the map.


0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 kb
I. Map:
HindIII SalI XbaI/HindIII XbaI EcoRI BamHI KpnI/HindIII EcoRI SalI BamHI HindIII EcoRI SalI BamHI XbaI EcoRV EcoRV HindIII BamHI BamHI EcoRV BamHI KpnI EcoRI XbaI BamHI HindIII BamHI KpnI HindIII HindIII
IPNS
II. Plasmids:
pSTA201
pSTA200
pSTA807
pSTA203/pSTA204
pSTA207
III. Probes:
BamHI A BamHI B BamHI C BamHI XbaI D BamHI E BamHI F BamHI G BamHI H BamHI KpnI J SalI
IV. lacZ fusion fragments
EcoRV EcoRV
HindIII EcoRV
V. mRNA species
9.5kb acvA
1.4kb ipnA
1.6kb acyA

The series of *argB* based *lacZ* fusion vectors, designated pAN923-41B, -42B and -43B; and the *trpC* :: *lacZ* fusion plasmid pAN923-21B was provided by Dr. C.A.M.J.J. van den Hondel (TNO, The Netherlands). The strategy used for subcloning the relevant fragments is described by van Gorcom *et al.* (1985).

2.14 PREPARATION OF COMPETENT *E. coli* CELLS

The preparation of competent *E. coli* was based on methods detailed by Sambrook *et al.* (1989).

200 ml of Luria broth was inoculated with 200 µl of an overnight culture of *E. coli* cells in Luria broth and incubated at 37°C with vigorous orbital shaking, until OD_{600} was 0.15-0.2. Cells were pelleted at 4225 x g for 10 min, resuspended in 50 ml ice-cold 100 mM $MgCl_2$ and incubated on ice for 5 min. Following centrifugation at 4225 x g, 4°C for 10 min, cells were resuspended in 50 ml ice-cold 100 mM $CaCl_2$ and incubated on ice for 30 min. The now competent cells were pelleted as above, resuspended in 2 ml 14 % glycerol, 100 mM $CaCl_2$ and stored at -70°C in 50 µl or 100 µl aliquots.

2.15 TRANSFORMATION OF COMPETENT *E. coli* CELLS

Stored aliquots of competent *E. coli* cells were thawed on ice for up to 1 h then transformation carried out using either of the following procedures:

i) DNA (in TE, sterile dH_2O or ligation mix) was added to 50 µl aliquots of competent cells and the whole

incubated on ice for 30 min. Cells were heat shocked at 42°C for 2.5 min then 950 µl Luria broth, prewarmed to 37°C, was added to each aliquot and the mixture incubated at 37°C for 1 h. 200 µl of each transformation mix was spread onto dry Luria agar plates and incubated at 37°C overnight.

ii) DNA (in TE, sterile dH_2O or ligation mix) was added to 50 µl aliquots of competent cells and incubated on ice for 30 min. Cells were heat shocked at 42°C for 90 sec then transferred onto ice for 2 min before 950 µl SOC was added and the mixture incubated 37°C, with orbital shaking at 225 rpm, for 1 h. 200 µl of each transformation mix was spread onto dry Luria agar selection plates and incubated at 37°C overnight.

Luria agar selection plates contained the appropriate concentration of antibiotic required for the selection of transformants e.g. 100 µg/ml ampicillin (final concentration).

If an *E. coli* strain containing a mutated *lacZ* gene e.g. *E. coli* DH5α, was the transformation recipient of a plasmid whose polylinker/cloning sites were contained within a *lacZ* gene, then X-gal (final concentration 60 ng/ml; dissolved in dimethylformamide) and IPTG (final concentration 60 ng/ml; dissolved in sterile dH_2O) were added to the transformation selection medium. Expression of the *lacZ* gene in transformants results in the formation of blue colonies due to cleavage of the X-gal to galactose and 5-bromo,4-chloro,2-indole indicative

that the *lacZ* gene is intact in the plasmid DNA. White colonies are indicative that either the plasmid is a recombinant containing a fragment cloned into the *lacZ* gene of the vector, or that the *lacZ* gene has become mutated in some way. This facilitates screening for transformants containing recombinant plasmids in cloning experiments.

2.16 PHENOL/CHLOROFORM EXTRACTION AND ETHANOL PRECIPITATION OF NUCLEIC ACIDS

Proteins were removed from nucleic acid solutions by the addition of phenol/chloroform reagent (1:1 phenol:chloroform where phenol is equilibrated with 100 mM Tris-HCl pH 8.0 then 10 mM Tris-HCl pH 8.0 until the pH of the aqueous layer is greater than 7.6, and contains 0.1 % hydroxyquinilone, and "chloroform" is a 24:1 mixture of chloroform:isoamyl alcohol), mixed to form an emulsion then centrifuged at 15000 x g for 2 min at room temperature to separate the aqueous and organic phases. The aqueous phase was removed and the process repeated 1-2 times or until the interphase was clear. Samples were chloroform extracted, to remove any traces of phenol from the sample, by the addition of an equal volume of 24:1 chloroform:isoamyl alcohol, mixed and spun at 15000 x g for 2 min to separate the aqueous and organic phases. The aqueous phase was removed and the process repeated 1-2 times or until the interphase was clear.

Nucleic acids were concentrated by precipitation with 2.5 volumes of chilled (-20°C) 96 % ethanol after the addition of 0.1 volume of 3 M NaAc pH 5.5. The solution was mixed and incubated at 4°C overnight; -70°C for 30 min or -20°C for 1 h or until required. Precipitates were pelleted by centrifugation at 15000 x g for 15 min, washed with 70 % ethanol at room temperature, vacuum dried and the nucleic acids dissolved in an appropriate volume of sterile dH_2O or TE.

2.17 PLASMID DNA PREPARATIONS

2.17.1 Small Scale Plasmid DNA Preparations

The small scale preparation of plasmid DNA was by the alkaline lysis method detailed in Sambrook *et al.* (1989).

2.17.2 Large Scale Plasmid DNA Preparation

The preparation of plasmid DNA on a large scale was carried out using either of the following modifications of the alkaline lysis method detailed in Sambrook *et al.* (1989).

Solutions required:

Solution I: 50 mM glucose
25 mM Tris-HCl pH 8.0
10 mM EDTA

Solution II: 1.0 % SDS
0.2 M NaOH

Solution III: 3 M KoAc pH 4.8

TE: 10 mM Tris-HCl pH 8.0
1 mM EDTA

i) 400 ml of Luria broth containing the appropriate antibiotic was inoculated with a 10 ml starter culture of the *E. coli* strain harbouring the required plasmid and incubated overnight at 37°C, with orbital shaking at 200 rpm. The cells were pelleted at 8281 x g, 4°C for 10 min, washed with 250 ml 50 mM Tris-HCl pH 8.0, repelleted at 8281 x g, 4°C for 10 min and the supernatant discarded. Following storage at -70°C for 20-30 min, the pellet was resuspended in 50 ml ice-cold solution I and held on ice for 30 min. Lysis of the cells was completed by the addition of 80 ml freshly prepared solution II which was thoroughly mixed and incubated on ice for 10 min. To precipitate chromosomal DNA, 40 ml ice-cold solution III was then added, thoroughly mixed and incubated on ice for 10-15 min. The precipitate was removed by centrifugation at 8281 x g, 4°C for 10 min and the supernatant filtered through two layers of muslin to separate the solution from remaining cell debris and the precipitate. Nucleic acids were precipitated by the addition of 100 ml chilled (-20°C) isopropanol, followed by centrifugation at 10810 x g, 4°C for 10 min. The supernatant was discarded and the pellet dried, dissolved in 8 ml TE containing 50 µg/ml final concentration DNase-free RNase A and incubated at 37°C for 1 h. The sample was phenol/chloroform extracted twice, chloroform extracted twice and the DNA precipitated by the addition of 2.5 volumes of 96 % ethanol and 0.1 volumes of 3 M NaAc pH

5.5, incubated at -20°C for 1-2 h and centrifuged at 11950 x g, 4°C for 10 min. The pellet was washed with 70 % ethanol at room temperature, dried under vacuum and the plasmid DNA purified by centrifugation to equilibrium in a caesium chloride- ethidium bromide density gradient. The pellet was dissolved in 9.05 ml sterile dH_2O to which was added 9.25 g caesium chloride and 400 µl of a 10 mg/ml ethidium bromide solution. The mixture was poured into a Sorvall 11.5 ml "ultracrimp" tube (Dupont), the air was expelled by the addition of liquid paraffin and the sample centrifuged at 253100 x g, 20°C for at least 16 h. The plasmid band was recovered and the ethidium bromide removed by repeated extraction with sterile dH_2O saturated butan-1-ol until the sample appeared colourless, then the aqueous phase was dialysed against two changes of 5 l of TE at 4°C overnight. Plasmid DNA was precipitated using 2.5 volumes of ethanol and 0.1 volume of 3 M NaAc pH 5.5, kept at -20°C for 1-2 h, pelleted by centrifugation at 11950 x g, 4°C for 10 min and washed with 70 % ethanol at room temperature. Vacuum dried DNA was dissolved in TE and stored at -20°C.

ii) 200 ml Luria broth containing the appropriate antibiotic was inoculated with a 10 ml starter culture of the *E. coli* strain carrying the required plasmid and incubated overnight at 37°C, with orbital shaking at 200 rpm. Cells were pelleted at 8281 x g, 4°C for 10 min, resuspended in 4 ml solution I and incubated for 5 min at room temperature. To this, 8 ml freshly prepared solution

II was added, thoroughly mixed and incubated on ice for 5 min, followed by the addition of 6 ml ice-cold solution III and incubation on ice for a further 10 min. The precipitate was removed by centrifugation at 17210 x g, 4°C for 20 min, the supernatant was immediately decanted into a fresh tube then extracted with an equal volume of phenol/chloroform. An equal volume of isopropanol was added and incubated at room temperature for 30 min before centrifugation at 17210 x g, 18°C for 20 min. The resulting pellet was washed with 70 % ethanol, vacuum dried, dissolved in 4 ml TE containing 100 μg/ml final concentration boiled RNase A and incubated at 37°C for 1-2 h. Plasmid DNA was purified from this solution using a Qiagen-pack 500 column (Diagen) according to the manufacturer's instructions, and stored in TE at -20°C.

2.18 PREPARATION OF FUNGAL CHROMOSOMAL DNA

All large scale preparation of fungal genomic DNA was as detailed by Tilburn *et al.* (1983). Small scale preparation of DNA for rapid analysis e.g. of transformants was by the technique of Leach *et al.* (1986).

2.19 QUANTITATION OF DNA

The concentration and quality of plasmid and genomic DNA preparations were determined spectrophotometrically by measuring the optical density at 260_{nm} and 280_{nm}. An OD of 1 corresponds to approximately 50 μg/ml double

stranded DNA. Purity of DNA preparations was estimated from the ratio of OD_{260}/OD_{280} measurements. If the preparation is pure, this ratio should be approximately 1.8 (Sambrook *et al.*, 1989).

2.20 DNA ELECTROPHORESIS

Agarose gel electrophoresis of DNA was carried out essentially as described by Sambrook *et al.* (1989).

Horizontal 1 % agarose gels containing 0.5 μg/ml ethidium bromide were prepared and run in TEA electrophoresis buffer (40 mM Tris-HCl, 20 mM NaAc, 1.8 mM EDTA, pH 7.4 with glacial acetic acid).

*Hin*dIII digested lambda phage DNA and/or 123 bp ladder (Pharmacia) were used as DNA molecular weight markers.

DNA samples were mixed with 0.1 volume of gel loading buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol FF, 25 % Ficoll 400 in sterile dH_2O) and electrophoresis continued until the bromophenol blue dye had migrated to within the last third of the gel.

DNA binding ethidium bromide in agarose gels was visualised using a short wavelength UV transilluminator (Ultra-Violet Products) and photographed using a Polaroid MP-4 land camera and Polaroid Type 667 film (Polaroid).

2.21 RESTRICTION ENZYME DIGESTION OF DNA

DNA samples were routinely incubated at 37°C with 2-5 fold excess of restriction enzymes, according to the

manufacturers recommendations, in appropriate commercially prepared restriction enzyme dilution buffers to ensure total digestion of DNA. The reaction was stopped by incubation for 10-15 min at 68°C. When DNA was to be cleaved by one or more restriction enzymes, the digestions were carried out simultaneously if both enzymes worked in the same buffer. However, when enzymes required different buffers, the enzyme that worked in the buffer of lower ionic strength was used first, the reaction stopped, then the buffer conditions changed to higher ionic strength before the second enzyme was added and incubation continued. When further manipulation of the DNA was required, the sample was phenol/chloroform and chloroform extracted then the DNA sodium acetate/ethanol precipitated as detailed in section 2.16.

2.22 RECOVERY OF DNA FRAGMENTS FROM AGAROSE GELS

DNA restriction fragments were recovered by direct extraction from low melting point agarose gels, or by electroelution from low melting point (LMP) or ordinary agarose gels, as described below.

i) Direct Extraction From LMP Agarose Gels

DNA digests were run out on ordinary agarose gels to ensure sufficient separation of the desired band which was located under UV light, excised and then run out on an LMP agarose gel. The desired fragment was then excised from the LMP agarose gel and placed into an microfuge tube with a small hole pierced in the bottom which was

plugged with siliconised glass wool, and the whole placed into a second microfuge tube. This was then centrifuged at 3180 x g for 10 min and the DNA purified and concentrated from the resulting solution by phenol/chloroform and chloroform extraction and sodium acetate/ethanol precipitation as detailed in section 2.16.

ii) Electroelution into dialysis tubing

DNA digests were electrophoresed in an agarose gel until the desired fragment was resolved and could be excised from the gel. DNA fragments were recovered by electroeluting the DNA from the gel slice into a small volume of sterile TEA buffer in a sealed dialysis bag. Purification and concentration of the DNA was by phenol/chloroform and chloroform extraction and sodium acetate/ethanol precipitation as detailed in section 2.16.

2.23 CONSTRUCTION OF HYBRID PLASMIDS

DNA fragments were cloned into plasmid vectors and amplified in *E. coli* strains. Linearised plasmid vectors were prepared for ligation by dephosphorylation of 5′ ends using Calf Intestinal Phosphatase (CIP). 5 μg of DNA in 5 μl of 1 x CIP buffer (50 mM Tris-HCl pH 9.0, 1 mM $MgCl_2$, 0.1 mM ZnCl. 1 mM spermidine) was incubated with 1 μl CIP (@ 1.5 units/μl) for 30 min at 37^{o}C before a second 1 μl aliquot of CIP (@ 1.5 units/μl) was added and incubation continued for 30 min at 45^{o}C. The reaction was

heat killed at 68°C for 15 min then the DNA purified by two phenol/chloroform extractions followed by ether extraction (Sambrook *et al.*, 1989). The DNA was concentrated by sodium acetate/ethanol precipitation as detailed in section 2.16.

Where required, 3′ recessed termini of fragments and plasmids were blunt ended using the enzyme Klenow. 10 μg vacuum dried DNA was taken up in 36.5 μl sterile dH_2O to which was added 5 μl of 10 x Klenow buffer (0.5 M Tris-HCl pH 7.2, 0.1 M $MgSO_4$, 1 mM DTT, 500 μg/ml BSA), 2 μl dATP (@ 2 mM), 2 μl dTTP (@ 2 mM), 2 μl dGTP (@ 2 mM), 2 μl dCTP (@ 2 mM) plus 0.5 μl Klenow (@ 7.5 units/μl). The reaction was carried out at room temperature for 20 min then heat killed at 68°C for 10 min. DNA was purified by phenol/chloroform and chloroform extraction and concentrated by sodium acetate/ethanol precipitation.

Ligation of blunt-ended DNA molecules was carried out using a 10 x ligation buffer giving a final concentration of 50 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 1 mM DTT, 1 mM ATP, 100 μg/ml BSA; 250 ng of prepared vector, purified DNA fragment such that vector:insert molar ratio was 10:1, 1:1 and 1:10, and 1.5 units T_4 DNA ligase; in a final volume of 10 μl. Control ligations of CIP-treated and non-CIP-treated linearised vector were also performed. Ligation reactions and controls were incubated at room temperature for 6 h or at 14°C overnight, then reactions terminated by incubation at 68°C for 10-15 min. Suitable aliquots e.g. 2.5 μl of ligation mixtures and

controls were used to transform *E. coli* strain DH5 or DH5α.

Ligation of DNA molecules with cohesive ends was performed using a 10 x ligation buffer giving a final concentration of 66 mM Tris-HCl pH 7.6, 6.6 mM $MgCl_2$, 1 mM DTT, 0.66 mM ATP; 100 ng of prepared vector DNA, purified DNA fragments such that the vector:insert molar ratio was 10:1, 1:1 and 1:10, and 0.1 units T_4 DNA ligase in a final volume of 10 μl. Control ligations of CIP-treated and non-CIP-treated linearised vector were also carried out. Ligation reactions and controls were incubated at room temperature for 1-3 h then heat killed at 68°C for 10-15 min. Suitable aliquots e.g. 2.5 μl of ligation mixtures and controls were transformed into *E. coli* DH5 or DH5α.

2.24 ^{32}P LABELLING OF DNA

The 23-base oligomer was end-labelled with α-^{32}P-dCTP according to Sambrook *et al.* (1989).

All other probes were radiolabelled by the method of Feinberg and Vogelstein (1983). Typically, 30-50 ng of DNA were labelled with 50μCi of α-^{32}P-dCTP. Incorporated and unincorporated nucleotides were separated by passing the solution down a Nick column (Pharmacia) and eluting with TE. Fractions containing incorporated radionucleotides were pooled, stored at -20°C until required, then boiled for 5 min, quenched on ice for 10 min and hybridised to the appropriate filters.

2.25 ISOLATION OF *A. nidulans* TOTAL RNA

A. nidulans strains were grown at 26°C, with orbital shaking at 250 rpm, in the various media and for the lengths of time indicated in section 3.17 and chapter 4 In each case the pH of the medium was 5.2. Mycelium was harvested by filtration through sterile muslin and rinsed with DEPC treated sterile dH_2O, frozen in liquid N_2 and stored at -70°C until required. Total RNA was isolated from the mycelium essentially according to the method of Cathala *et al.* (1983).

1 g pressed wet weight of mycelium was ground in liquid N_2, 3 ml lysis buffer (5 M guanidine thiocyanate, 50 mM Tris-HCl pH 7.5, 10 mM EDTA) and 0.3 ml ß-mercaptoethanol was added with stirring then the mycelium was homogenised by passage through a 21 gauge then a 23 gauge hypodermic needle. To precipitate the RNA, 15 ml 4 M LiCl was added with stirring and the solution homogenised by passage through a 21 gauge and a 23 gauge hypodermic needle several times. The sample was incubated at room temperature for 20 min followed by 4°C overnight, then mixed by inversion, spun at 1020 x g, 4°C for 5 min, the supernatant collected and spun at 10440 x g, 4°C for 90 min. The pellet was resuspended in 10 ml 3 M LiCl by passage through a 21 gauge and a 23 gauge hypodermic needle several times and the RNA pelleted by centrifugation at 10440 x g, 4°C for 1 h. The pellet was resuspended in solubilisation buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.1 % SDS) by passage through a 21 gauge

and a 23 gauge hypodermic needle several times then phenol/chloroform extracted and the aqueous phase collected. The organic phase and interphase were back extracted by addition of an equal volume of solubilisation buffer which was mixed well, the phases separated by centrifugation at 15000 x g for 2 min and the aqueous phase collected and pooled with the first aqueous phase. Phenol/chloroform extraction was continued until no interphase was obtained, the sample was chloroform extracted three times, divided into four aliquots, and the RNA was concentrated and stored in 2.5 volumes of 96 % ethanol plus 0.1 volume of 3 M NaAc pH 5.4, at -20^{o}C until required. RNA was pelleted by centrifugation at 15000 x g for 20 min, washed with 70 % ethanol, vacuum dried and dissolved in an appropriate volume of DEPC treated sterile dH_2O.

Extreme care was taken throughout the isolation, analysis and manipulation of RNA to ensure that RNase contamination did not occur or was minimised. Glassware was baked at 260^{o}C for 6 h or treated with 0.1 % DEPC solution and autoclaved at 15 lb/inch^2 for 15 min. Magnetic fleas were treated with the latter. Plasticware and mortars and pestles were autoclaved twice. Solutions were treated with 0.1 % DEPC prior to autoclaving, except for those containing Tris-HCl which were made up using 0.1 % DEPC treated sterile dH_2O and, as far as possible, RNase-free equipment, and autoclaved in RNase-free bottles. Gloves were worn at all times during the

preparation of materials and solutions used for the isolation and analysis of RNA and during all manipulations involving RNA.

2.26 QUANTITATION OF RNA

The concentration and quality of RNA preparations was determined spectrophotometrically by measuring the optical density at 260nm and 280nm. An OD of 1 corresponds to approximately 40 µg/ml for single stranded RNA. Purity of RNA preparations was estimated from the ratio of OD_{260}/OD_{280} measurements. If the preparation is pure, this ratio should be approximately 2.0 (Sambrook *et al.*, 1989).

2.27 ELECTROPHORESIS OF RNA, NORTHERN TRANSFER AND HYBRIDISATION

a) Stock Solutions

10 x MOPS buffer: 0.2 M MOPS
0.05 M NaAc
0.01 M EDTA

20 x SSC: 3.0 M NaCl
0.3 M sodium citrate, pH 7.0

20 x SSPE: 3.0 M NaCl
0.2 M NaH_2PO_4
25.0 mM EDTA
pH 7.4

100 x Denhardt's solution: 2 % Ficoll 400
2 % polyvinylpyrrolidone
2 % BSA (Pentax Fraction V)
Stored in aliquots at -20°C

100 mg/ml heat denatured herring sperm DNA:

Herring sperm DNA (Sigma) was dissolved in sterile dH_2O at a final concentration of 10 mg/ml, sheared by ultrasonication and passage through a 23 gauge hypodermic needle several times, and stored at -20°C until required. The appropriate volume of herring sperm DNA stock solution was heat denatured in a boiling water bath for 10 min then quenched on ice for 5 min before addition to prehybridisation solutions.

b) Electrophoresis and Transfer of RNA

Electrophoresis and northern blotting of RNA was performed essentially as described by Davis *et al.* (1986).

Horizontal 1 % agarose gels containing 0.66 M formaldehyde and 0.2 µg/ml ethidium bromide, were prepared and run in 1 x MOPS buffer. Gel rigs, gel formers and combs were washed with dilute detergent (Decon) and rinsed well with dH_2O before use to help reduce RNase contamination. 10 µg samples (unless otherwise stated) of total RNA in 5 µl DEPC treated sterile dH_2O were mixed with 15 µl of loading buffer (Davis *et al.*, 1986), heated to 95°C for 5 min, quenched on ice and loaded onto a formaldehyde gel. Electrophoresis was continued until the bromophenol blue dye had migrated to within the last third of the gel. The 0.24-9.5 kb RNA ladder (BRL) was used as molecular weight markers.

RNA binding ethidium bromide in gels was visualised using a short wavelength UV transilluminator and photographed using a Polaroid MP-4 land camera and Polaroid Type 667 film.

Unused areas of gels were removed then gels were washed twice for 20 min each wash in 500 ml of 10 x SSC to remove formaldehyde from the gel, then blotted overnight onto Amersham Hybond-N filters using 10 x SSC, essentially as detailed by Davis *et al.* (1986). The orientation of the gel and position of the gel slots were marked on the nylon membrane with a pencil. The membrane was allowed to air dry on a piece of Whatman 3MM paper at 37^{o}C, wrapped between two layers of cling film, placed RNA-side down on a UV transilluminator and exposed to short wavelength UV light for 5 min to covalently link the RNA to the membrane. The filter was either probed immediately or stored at room temperature in cling film until required. To check that RNA had been transferred to the filter, the gel was stained with 0.5 µg/ml ethidium bromide in 200 ml dH_2O for 15 min and visualised under UV light.

c) Hybridisation

Filters were incubated for 4 h to overnight at 42^{o}C in prehybridisation solution (5 x SSPE, 5 x Denhardt's solution, 40 % deionised formamide, 500 µg/ml denatured herring sperm DNA), and hybridised overnight in the same buffer with the ^{32}P-labelled probe. Unhybridised probe

was removed by washing the filter sequentially in 5 x, 3 x, 1 x, 0.5 x and 0.2 x SSC containing 0.1 % SDS and 0.1 % sodium pyrophosphate, as required, for 20 min periods at 42^{o}C, until background radiation on the membrane was insignificant.

d) Autoradiography

Following hybridisation and washing, membranes were sealed in plastic bagging, placed against Fuji RX film or the more sensitive Kodak XAR5 film in an autoradiography cassette fitted with intensifying screens, and stored at -70^{o}C. The film was removed and processed in a Fuji RG II X-ray film processor. Exposure times varied according to the strength of the signal expected.

When rehybridisation of the membrane was required with a different probe, filters were stripped of the previous probe by successively boiling the membrane in large volumes of 0.1 % SDS until the radiation on the membrane was insignificant.

e) Verification of Uniformity of *A. nidulans* RNA Transfer

In order to verify uniformity of RNA transfer of different samples and hence concentrations of RNA found in each lane, northern blots were stripped of the previous probe then rehybridised with a fragment specific for the constitutively expressed *A. nidulans actA* gene (Fidel *et al.*, 1988), as detailed above.

2.28 SOUTHERN TRANSFER AND HYBRIDISATION

a) Stock Solutions

20 x SSC: 3.0 M NaCl
0.3 M trisodium citrate, pH 7.0

20 x SET: 3.0 M NaCl
0.4 M Tris-HCl pH 7.8
20.0 mM EDTA

20 x SSPE: 3.0 M NaCl
0.2 M NaH_2PO_4
25.0 mM EDTA

pH 7.4

100 x Denhardt's solution: 2 % Ficoll 400
2 % polyvinylpyrrolidone
2 % BSA (Pentax Fraction V)
Stored in aliquots at -20°C

10 % dried skimmed milk: Boil for 5 min, cool on ice then store at 4°C for up to 2 weeks.

b) DNA Transfer

DNA fragments were separated by electrophoresis. Unused areas of the gel were then removed and the gel was rinsed in dH_2O and sequentially depurinated, denatured and neutralised then blotted overnight onto Amersham Hybond-N nylon membrane or Hybond-C nitrocellulose membrane using 10 x SSC, essentially as described by Sambrook *et al.* (1989). The orientation of the gel, and position of the gel slots were marked on the membranes with a pencil and the DNA was fixed to the filters as follows:

i) Hybond-N nylon membrane was briefly washed in 3 x SSC, air dried on a piece of Whatman 3MM paper at 37°C, wrapped between two layers of cling film, placed DNA-side down on a UV transilluminator and exposed to short wavelength UV light for 3-5 min to covalently link the DNA to the membrane. The filter was either probed immediately or stored at room temperature in the cling film until required.

ii) Hybond-C nitrocellulose membrane was washed in 4 x SET for 5 min then air died on a piece of Whatman 3MM paper at 37°C for 30 min. The filter was placed between two fresh pieces of Whatman 3MM paper in foil and baked in a vacuum oven at 80°C for 2 h. Blots were either probed immediately or stored overnight at room temperature or for up to several months dry at 4°C.

c) Hybridisation

In general, the temperature of prehybridisation, hybridisation and washing of filters was 56°C when a heterologous fragment was used as the probe, or 65°C when homologous probes were used.

i) Hybond-N membrane was wetted with dH_2O, drained and incubated for 4 h to overnight in prehybridisation solution (5 x SSPE, 30 % PEG 6000, 0.5 % dried skimmed milk, 1 % SDS, 0.1 % $Na_2P_4O_7.10H_2O$, 250 μg/ml denatured herring sperm DNA), with shaking, at a suitable temperature. The ^{32}P-labelled probe was added to the same buffer and incubation continued overnight. Unhybridised

probe was removed by washing the filter sequentially in 5 x, 3 x, 1 x, 0.5 x, 0.2 x and 0.1 x SSC containing 0.1 % SDS and 0.1 % $Na_2P_4O_7.10H_2O$, as appropriate, for 30 min periods at the same temperature as used for hybridisation, until background radiation on the membrane was insignificant.

ii) Hybond-C membrane was wetted in 4 x SET, 0.1 % SDS for 20 min, drained then prehybridised for 12 h, at a suitable temperature with shaking, in 4 x SET, 0.1 % $Na_2P_4O_7.10H_2O$, 5 x Denhardt's solution, 0.1 % SDS, 100 μg/ml denatured herring sperm DNA. The ^{32}P-labelled DNA probe was added to the same buffer and incubation continued overnight. Unhybridised probe was removed by washing the filter sequentially in 4 x, 1 x, 0.5 x and 0.2 x SET containing 0.1 % SDS and 0.1 % $Na_2P_4O_7.10H_2O$, as appropriate, for 30 min periods at the same temperature as used for hybridisation, until background radiation on the membrane was insignificant.

When rehybridisation of a membrane was required with a different probe, the previous probe was stripped off by successively boiling the membrane in large volumes of 0.1 % SDS until the radiation on the membrane was insignificant.

d) Autoradiography

Following hybridisation and washing, membranes were sealed in plastics bagging, placed against Fuji RX film or Kodak XAR5 film in an autoradiography cassette fitted

with intensifying screens, and stored at -70°C. The film was removed and processed in a Fuji RG II X-ray film processor. Exposure times varied according to the strength of signal expected.

2.29 DNA SEQUENCE ANALYSIS

DNA was subcloned into pUC18 and/or pUC19 and the insert sequenced by means of the dideoxy chain termination method using a Sequenase version 2.0 kit (Unites States Biochemical Corporation). DNA was radiolabelled using α-^{35}S-dATP. The "universal" primer used was supplied with the kit, however the "reverse" primer was synthesised in the Department of Biochemistry, University of St. Andrews.

a) Solutions

10 x TBE: 0.89 M Tris-borate
0.89 M boric acid
0.02 M EDTA pH 8.0

Sequencing gel solution: 63.0 g urea
0.3 g bisacrylamide
5.7 g acrylamide
15.0 ml 10 x TBE

Made up to 1 l in dH_2O. The solution was prepared, filtered through Whatman No.1 filter paper and degassed, immediately prior to use.

b) Preparation of sequencing gels

Electrophoresis was performed using a Seqi-Gen sequencing gel rig (Bio-rad) which was prepared and assembled according to the manufacturer's instructions. The bottom of the gel was sealed using 30 ml sequencing gel solution plus 120 µl 25 % ammonium persulphate and 120 µl TEMED (N, N, N', N'-tetramethylethylenediamine) which was allowed to set for approximately 30 min. The gel was poured using 120 ml sequencing gel solution plus 120 µl 25 % ammonium persulphate and 120 µl TEMED. Two 0.4 mm thick "sharks tooth" combs were inserted at the top of the gel space to a depth of 5 mm, clamped in place, and the gel allowed to set for at least 3 h. The sharks tooth combs were then removed, the top of the gel irrigated with 1 x TBE and the sharks tooth combs replaced, with the points of the teeth just touching the gel. The gel was prerun at 2 kV with 400 ml 1 x TBE in the bottom reservoir and approximately 1.4 l in the back buffer tank, until the gel temperature reached 45-50^{o}C.

c) Electrophoresis and autoradiography

Immediately prior to loading, samples were heated to 80^{o}C for 2 min and quenched on ice. The top of the gel was irrigated with 1 x TBE to flush out urea from the wells, 2 µl samples were loaded into appropriate wells and electrophoresis continued for 3 h with the voltage adjusted to maintain the gel temperature at 45-50^{o}C. The gel was soaked in 10 % acetic acid, 12 % methanol for 15

min to remove the urea, then transferred onto Whatman 3MM paper and dried using a Bio-rad gel drier at 80^{o}C for 45 min. The dried gel was exposed to Fuji RX or Kodak XAR5 film at room temperature in an autoradiography cassette without intensifying screens and the film processed using a Fuji RG II X-ray film processor. Exposure times varied according to the intensity of the signal expected.

CHAPTER 3

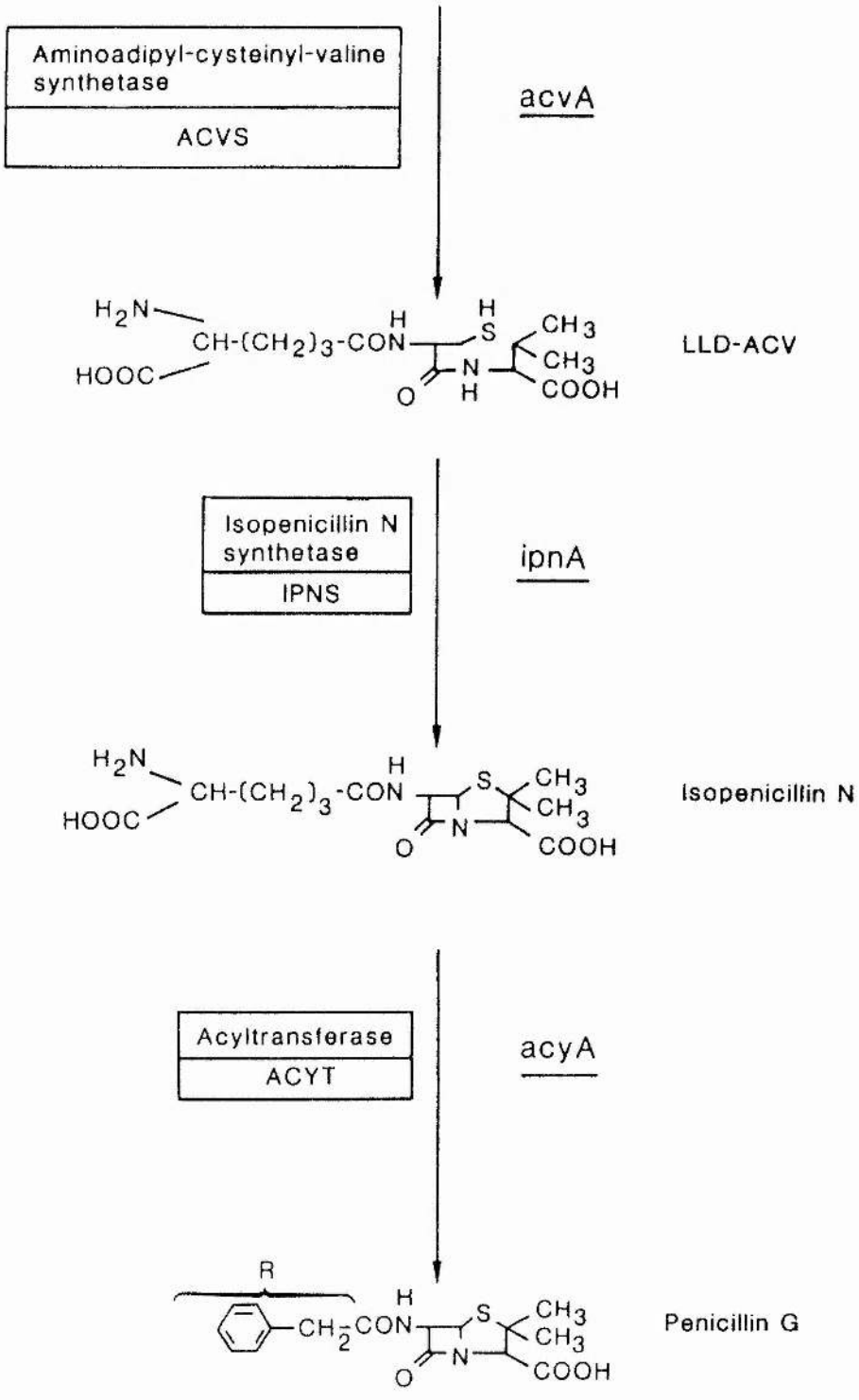
CHARACTERISATION OF THE *A. nidulans npeA* LOCUS

3.1 INTRODUCTION

This chapter concerns the isolation and analysis of *A. nidulans* genomic DNA clones which we have found to be counterparts of the previously uncharacterised *npeA* locus. The evidence available indicates that, in addition to containing the gene encoding IPNS, such clones contain two further penicillin biosynthetic genes, one encoding ACYT and the other encoding ACVS. Ingolia and Queener (1989) suggested that, following precedents accepted for other "branched" pathways, genes involved in penicillin and cephalosporin biosynthesis should be designated as follows: *pcb* for genes involved in the conversion of primary metabolites to IPN; *cef* for genes involved in the conversion of IPN to cephalosporins; and *pen* for genes involved in the conversion of IPN to penicillin G or V (see Fig. 2). In *Aspergillus* genetics, however, genes are commonly named after the enzymes they encode, thus we propose that in *A. nidulans* the genes encoding ACVS, IPNS and ACYT be designated *acvA*, *ipnA* and *acyA*, respectively (Fig. 9).

Fig. 9 The biochemical and genetic components of the penicillin biosynthetic pathway in *A. nidulans* (from MacCabe *et al.*, 1990, 1991).

L-α-Aminoadipic Acid+L-Cysteine+L-Valine



3.2 HETEROLOGOUS HYBRIDISATION OF *P. chrysogenum* IPNS TO *A. nidulans npeA*$^{+}$ AND *npeA*0049/1 GENOMIC DNA

The *P. chrysogenum* IPNS gene probe was cross-hybridised to *A. nidulans* and *C. acremonium* DNA (Fig. 10). Differences in hybridisation pattern were observed between a naturally occurring *A. nidulans* penicillin non-producing mutant (*npeA*0049/1) and the wild type strain (*npeA*$^{+}$). No hybridisation was observed in *npeA*0049/1 implying a deletion of sequences homologous to the IPNS gene probe. This suggested that the *npeA* locus in *A. nidulans* may contain the IPNS gene. To investigate this further, several *A. nidulans* IPNS-containing genomic clones were isolated.

3.3 ISOLATION AND CHARACTERISATION OF *A. nidulans* GENOMIC CLONES

An *A. nidulans* genomic library (Johnstone *et al.*, 1985) was screened at low stringency using the *P. chrysogenum* IPNS gene probe. Of 17 strongly hybridising clones, two were found to overlap by 3 kb but extend for 10 kb in opposite directions, namely pSTA200 and pSTA201. A map of the genomic *A. nidulans* IPNS region was generated (Fig. 8.I) and the IPNS gene located by hybridisation to the *P. chrysogenum* IPNS probe. Comparison of this map to that of Ramon *et al.* (1987), confirmed the location and orientation of the *A. nidulans* IPNS gene on pSTA200 and pSTA201.

Fig. 10 Hybridisation of the *P. chrysogenum* IPNS gene to *A. nidulans* and *C. acremonium* DNA. 2 μg genomic DNA digests were electrophoresed on a 1 % agarose gel at 70 V for 6 h and transferred onto Amersham Hybond-C. The blot was prehybridised at 56°C for 12 h with 4 x SET, 0.1 % $Na_4P_2O_7.10H_2O$, 0.1 % SDS, 100 μg/ml denatured herring sperm DNA, 0.1 % polyvinylpyrrolidone, 0.1 % Ficoll 400, 0.1 % BSA; and hybridised overnight to the ^{32}P-labelled *P. chrysogenum* IPNS gene probe in the same buffer at 56°C. The filter was washed sequentially with 4 x, 1 x and 0.5 x SET in 0.1 % $Na_4P_2O_7.10H_2O$, 0.1 % SDS; at 56°C for 30 min periods, then autoradiographed with Fuji RX film for 12 h. Molecular size markers (kb) are shown on the right margin. *npeA*0049/1 and *npeA*$^+$ are the relevant markers of the *A. nidulans* strains.

HindIII
PstI
EcoRI
npeA0049/1
npe+
C. acremonium
P. chrysogenum
23.1
9.4
6.6
4.4
2.3
2.0
0.6

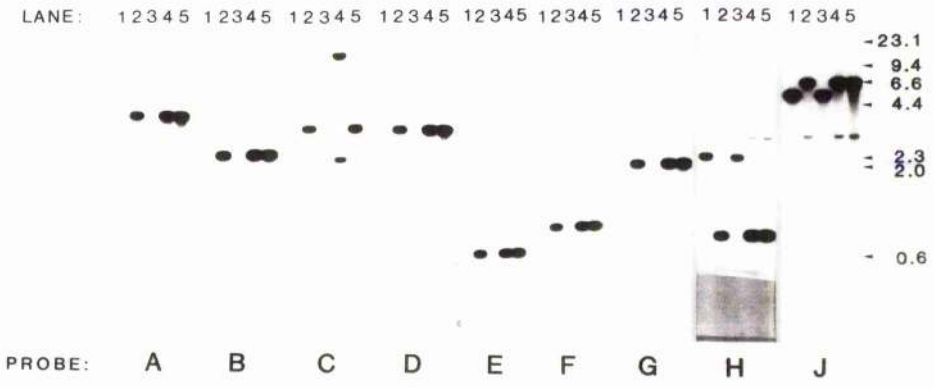
The *A. nidulans* library was re-screened using a *Kpn*I-*Sal*I fragment isolated from the end of the pSTA201 genomic insert distal to IPNS (Fig. 8.II), with a view to isolating a clone similar to pSTA201 but excluding the IPNS region. Such a clone was isolated and designated pSTA207 (Fig. 8.II).

3.4 PHYSICAL ANALYSIS OF *npeA* MUTANTS

Restriction enzyme digests of genomic DNA isolated from various *npeA* mutants and the wild type *A. nidulans* were blotted and probed with DNA fragments spanning pSTA200, pSTA201 and pSTA207 (Fig. 11).

The mutation *npeA*0049/1 appears to be an extensive deletion of genomic DNA extending from the furthest cloned sequences 5′ of IPNS to the region of probe H (Fig. 8.III), 3′ of IPNS. This suggests that the cloned DNA fragments encompassing the IPNS gene correspond to the *npeA* region. Sequences in the vicinity of the right-most extremity of the deletion are probably rearranged, as probe J (Fig. 8.III) hybridised to fragments of different sizes in *npeA*0049/1 and the wild type. A second mutation analysed, namely *npeA*0022 appears to have resulted in the perturbation of genomic sequences homologous to probe C (Fig. 8.III). The data can be interpreted as representing an insertion of approximately 14 kb of DNA into the region cross-hybridising to *Bam*HI fragment C (Fig. 8.III; A.P. MacCabe, personal communication). A third mutation,

Fig. 11 Southern blot analysis of *A. nidulans npeA* mutants. 5 μg *Bam*HI genomic DNA digests were electrophoresed on 1 % agarose gels at 50 V overnight and blotted onto Amersham Hybond-N. Blots were prehybridised at 56°C for 12 h in 5 x SSPE, 6 % PEG 6000, 0.5 % skimmed milk, 1 % SDS, 0.1 % $Na_4P_2O_7.10H_2O$, 250 μg/ml denatured herring sperm DNA; and hybridised overnight in the same buffer at 56°C with the specified ^{32}P-labelled probes (see Fig. 8.III). The blots were washed sequentially with 4 x, 1 x and 0.2 x SSC in 0.1 % $Na_4P_2O_7.10H_2O$, 0.1 % SDS; at 56°C for 30 min periods, before autoradiography with Fuji RX film for 12 h. Molecular size markers in kb are shown on the right margin. Relevant genotypes of DNA in each track are as follows: lane 1 - *npeA*0049/1, *argB*$^+$; lane 2 - *npeA*$^+$, *argB*$^+$; lane 3 - *npeA*0049/1, *argB*$^+$; lane 4 - *npeA*0022, *argB*2; and lane 5 - *npeA*005, *argB*$^+$. Molecular size markers (kb) are shown on the right margin.


LANE: 12345 12345 12345 12345 12345 12345 12345 12345 12345
23.1
9.4
6.6
4.4
2.3
2.0
0.6
PROBE: A B C D E F G H J

*npeA*005 showed no obvious difference in genomic organisation within the region probed, in comparison to wild type. This may reflect a point mutation, or a small insertion or deletion of DNA.

Southern blot analysis was also carried out on genomic DNA digests from six independently isolated naturally occurring *npeA* strains (Birmingham isolate Nos. 49, 108, 112, 123, 132 and 136), all belonging to heterokaryon-compatibility group F (Cole *et al.*, 1976). The banding patterns observed were identical to those seen for the naturally occurring *npeA*0049/1 mutation, indicating a similar deletion of this 20 kb region in all seven of the wild isolate *npeA* mutants analysed. Since the left-most extremity of the deletion in *npeA*0049/1 and each of the Birmingham isolate deletion mutants has not been established, they may not be identical.

3.5 CONSTRUCTION OF DOUBLE MUTANT STRAINS WITH THE *argB2* ALLELE

The ***A. nidulans argB***2 mutant strain G34 was crossed with ***areA***19 and *xprD*1 alleles to construct the double mutants *areA*19, *argB*2 (strain SAA236) and *xprD*1, *argB*2 (strain SAA244; see Fig. 5), respectively, for use in transformation experiments (see section 4.2).

Various *A. nidulans argB*2 mutant strains were crossed with *npeA*0022, *npeA*005, *npeA*002 and *npeA*0049/1 alleles, to construct double mutant strains for use in

transformation and complementation experiments, as follows: G324 was crossed with *npeA*0022 and *npeA*005 to give *npeA*0022, *argB*2 (strain SAA101) and *npeA*005, *argB*2 (strain SAA243), respectively; G324 was crossed with *npeA*002 to yield *npeA*002, *argB*2 (strain SAA260); and G034 was crossed with *npeA*0049/1 to yield *npeA*0049/1, *argB*2 (strain SAA246).

Prior to crossing, a forcing marker had to be built into strain G0049/1 which carries the *npeA*0049/1 allele. This was achieved by isolating spontaneous mutants of *A. nidulans* defective in the genes required for nitrate assimilation. One such chlorate resistant mutant (strain SAA235) failed to grow on appropriately supplemented minimal medium containing 10 mM $NaNO_3$ or 10 mM $NaNO_2$ as the sole nitrogen source, but grew when 10 mM ammonium tartrate or 1 mM hypoxanthine was used as the sole nitrogen source. This was used in a sexual cross with G034 to yield SAA246 (see above).

3.6 COMPLEMENTATION OF *A. nidulans npeA* MUTANTS

Double mutant *npeA*, *argB*2 strains were used as recipients for transformation by recombinant plasmids pSTA200, pSTA201 and pSTA207; and their derivatives pSTA203, pSTA204 and pSTA807 (Fig. 8.II). Transformants were selected on the basis of arginine prototrophy and screened for penicillin production (Table IV).

Of the three genomic clones, only pSTA201 and pSTA207 efficiently complemented the phenotype of

Table IV

Transformation and complementation of *npeA*, *argB2* mutants.

Allele	Plasmid	No. of *argB*$^+$ transformants analysed	No. of *npeA*$^+$ phenotypes
*npeA*0022	pSTA200	103	0
	pSTA201	259	59
	pSTA207	100	39
	pSTA203	224	0
	pSTA204	194	0
	pSTA807	977	1
*npeA*005	pSTA200	417	0
	pSTA201	99	32
	pSTA207	510	0
	pSTA204	218	0
	pSTA807	322	0
	pSTA200+pSTA207[a]	203	2
*npeA*002	pSTA200	23	0
	pSTA201	60	27
	pSTA207	57	26
	pSTA204	4	0
	pSTA807	41	0
*npeA*0049/1	pSTA200+pSTA201[a]	399	0
	pSTA200+pSTA207[a]	418	0

[a] Cotransformation experiments with equimolar plasmid concentrations (5 μg of each).

mutation *npeA*0022. Subclones pSTA203 and pSTA204, both of which contain the intact IPNS gene, failed to complement this mutation although pSTA807, which lacks the IPNS gene, yielded a very low frequency of complementation. These results indicate that a penicillin biosynthetic gene lies 5′ to IPNS and may be intact on pSTA201 and pSTA207 (but see section 3.14 and chapter 5), as judged by the frequency of complementation, but incomplete on pSTA807. Evidence that the fragment responsible for the complementation of *npeA*0022 is not IPNS is provided by the fact that pSTA207 (which does not include IPNS) complemented *npeA*0022, whilst pSTA203 and pSTA204 (which include IPNS) failed to do so.

Only pSTA201 reversed the phenotype of mutant *npeA*005. pSTA200 and pSTA207 complemented at a low frequency but only when transformed together into this mutant.

Preliminary analysis indicated that the general pattern of complementation of the *npeA*002 allele was similar to that of *npeA*0022. Some confusion previously existed as to the exact *npeA* allele number and genotype of the original strain (GH36) carrying the *npeA*002 mutation (M.W. Adlard, Polytechnic of Central London, personal communication), therefore complementation studies of *npeA*002 were not further pursued for fear that this allele was actually equivalent to *npeA*0022.

The deletion mutant *npeA*0049/1 was not complemented by pSTA200, pSTA201 or pSTA207 in cotransformation experiments.

3.7 TIME COURSE OF PENICILLIN PRODUCTION BY *A. nidulans* AND *A. oryzae*

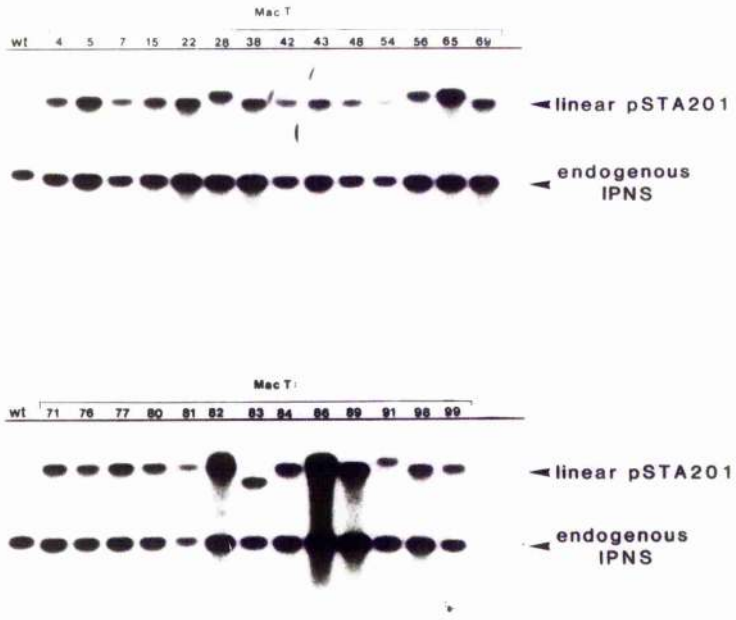
A. oryzae strain *argB*$^-$, which was judged by bioassay to naturally be a ß-lactam non-producing strain, was cotransformed with pSTA200 and pSTA201 or pSTA207, to determine whether or not these plasmids could confer on this strain/species the ability to produce penicillin. A selection of such transformants was bioassayed and found to produce penicillin, however, in two out of three subsequent bioassays, the *A. oryzae argB*$^-$ transformation recipient strain was found to produce penicillin *per se*. Consequently, a time course experiment was set up in which the *A. oryzae argB*$^-$ and *A. nidulans* wild type strains were bioassayed at specific regular intervals over a 10 day period. *A. oryzae* was found to be capable of producing penicillin, but it only began to do so after 95 h incubation at 25^{o}C before plugs were transferred to bioassay plates (M.B.R. Riach, unpublished). *A. nidulans*, however, began to produce penicillin after only 30 h incubation at 25^{o}C with halos of approximately the same size produced after 3-10 days incubation at 25^{o}C, prior to the transfer of plugs to bioassay plates (M.B.R. Riach, unpublished).

Originally, fermentation plugs were transferred to the bioassay medium after 3-5 days incubation at 25°C hence penicillin production was faithfully detected in *A. nidulans*, but not in *A. oryzae*, penicillin-producing strains. Thereafter, to accommodate this finding, fermentation plugs were bioassayed after 5 days incubation at 25°C.

3.8 PHYSICAL ANALYSES OF *npeA*0022 TRANSFORMANTS

A random sample of 27 arginine prototrophs (designated MacT) arising from pSTA201 complementation of the *npeA*0022, *argB*2 double mutant (strain SAA101), which were concomitantly found to produce penicillin (i.e. $npeA^{+}$), were examined by Southern blotting. *Xba*I digests of genomic DNA were blotted and hybridised to the *A. nidulans* IPNS gene, the results of which are shown in Fig. 12. Whilst the wild type strain and all transformants analysed exhibited the endogenous IPNS fragment, an additional larger band was present in all transformants. For the majority (over 70 %), this signal corresponded to the size of linearised pSTA201 (e.g. MacT5, MacT48 and MacT69). This observation is consistent with homologous integration of the transforming plasmid at the genomic IPNS site. Of the remaining transformants, only one (MacT83) was shown, by re-probing with *argB*, to have integrated at the *argB* locus (data not shown). The sites of integration in

Fig. 12 Southern blot analysis of transformants. DNA minipreps were made from 10 ml cultures of MacT transformants and the *A. nidulans* wild type (*biA*1) strain (designated wt in figure) and digested with *Xba*I. Samples were electrophoresed for 12 h at 50 V on a 1 % agarose gel and transferred to Amersham Hybond-N. The blot was prehybridised and hybridised at 68^{o}C as described in the legend to Fig. 10 using probe E (Fig. 8.III) prior to autoradiography with Fuji RX film for 20 h. The blot was stripped and rehybridised as above using the *argB* probe (data not shown).


Mac T
wt 4 5 7 15 22 28 38 42 43 48 54 56 65 69
linear pSTA201
endogenous IPNS
Mac T
wt 71 76 77 80 81 82 83 84 86 89 91 98 99
linear pSTA201
endogenous IPNS

MacT28, MacT56, MacT65, MacT82, MacT86 and MacT91 are unknown.

3.9 CLASSICAL GENETIC ANALYSIS OF *npeA*0022 TRANSFORMANTS

Five transformants which were analysed by Southern blotting were also subjected to classical genetic analysis to determine the genomic location of the integrated sequences, by crossing to an *A. nidulans* *npeA*$^{+}$, *argB*$^{+}$ strain (B418) (Table V). Integration of pSTA201 at the *npeA* locus would mean that recombination between the respective *npeA*0022, (*npeA*$^{+}$) and *npeA*$^{+}$ alleles of the MacT transformants and the *npeA*$^{+}$, *argB*$^{+}$ strain, during meiosis, would be unlikely and therefore result in the absence of *npeA*$^{-}$ progeny. This, however, would allow the possibility of recombination between the *argB*2 allele of the MacT transformants and the *argB*$^{+}$ allele of the *npeA*$^{+}$, *argB*$^{+}$ strain during meiosis, and ideally 25 % of the progeny would be *argB*2 mutants. Conversely, if pSTA201 integrated at the *argB* locus of the transformants, all progeny would be arginine independent (i.e. *argB*$^{+}$) and ideally 25 % would be *npeA*$^{-}$. Integration of pSTA201 at an ectopic site would allow recombination between the *npeA*$^{-}$ and *npeA*$^{+}$ alleles of the respective transformant and *npeA*$^{+}$, *argB*$^{+}$ parent strains, thus both *npeA*$^{-}$ and *argB*$^{-}$ progeny would be recovered from such a cross.

Table V

Genetic linkage observed in transformant strains.

Transformant[a]	No. of randomly isolated progeny screened	Phenotype of progeny			
		argB$^+$	*argB*$^-$	*npeA*$^+$	*npeA*$^-$
MacT5	60	56	4	60	0
MacT48	60	54	6	60	0
MacT69	120	99	21	120	0
MacT83	60	60	0	36	24
MacT91	60	54	6	30	30

[a] Transformants of the putative genotype *argB*2, (*argB*$^+$), *npeA*0022, (*npeA*$^+$) where the genes in brackets denote incoming DNA, were crossed to strain B418 of genotype *argB*$^+$, *npeA*$^+$.

The genetic data show that MacT5, MacT48 and MacT69 carry integrates at or near the *npeA* locus (no *npeA*$^-$ phenotypic progeny were recovered from such crosses). The integration of pSTA201 at the IPNS site in transformants MacT5, MacT48 and MacT69 as judged by Southern analyses, combined with formal genetic mapping of such integration events to the *npeA* locus, support the suggestion that the IPNS region and the *npeA* locus are counterparts. Furthermore, classical genetic analyses confirm that pSTA201 has integrated in MacT83 and MacT91 at sites other than *npeA*: in MacT83, pSTA201 mapped at the *argB* locus, whilst in MacT91, integration occurred at an ectopic site.

A summary of the combined physical and genetic transformation data is given in Table VI.

3.10 CLASSICAL GENETIC ANALYSIS OF THE *npeA*0049/1 MUTATION

A cross was set up between the *npeA*0049/1 mutant (strain M1.4) and the better genetically characterised *npeA*0022 mutant (strain GH79), to ensure that the *npeA*0022 mutation does, in fact, map at the *npeA* locus. No *npeA*$^+$ strains were found in 100 progeny screened for penicillin production, suggesting that the *npeA*0049/1 and *npeA*0022 mutations are closely linked.

Table VI

Summary of plasmid integration from DNA hybridisation and classical genetic analyses.

Transformant	DNA hybridisation	Classical genetics
MacT5	IPNS	*npeA*
MacT48	IPNS	*npeA*
MacT69	IPNS	*npeA*
MacT83	*argB*	*argB*
MacT91	elsewhere	elsewhere

3.11 HYBRIDISATION STUDIES OF SEQUENCES 5′ OF IPNS

Complementation studies of the *npeA*0022 mutation indicate the existence of a gene 5′ of IPNS which has a role in the biosynthetic pathway of ß-lactams. Heterologous hybridisation was carried out using probe C (Fig. 8.III) to *Eco*RI digests of genomic DNA isolated from different fungal species. Specific cross-hybridisation did occur to DNA from *P. chrysogenum*, *C. acremonium* and *A. oryzae*, all of which produce ß-lactams. Significantly, a specific signal was not detected for *N. crassa*, an organism which has not been found to produce antibiotic activity on bioassay plates (M.B.R. Riach, unpublished).

3.12 CONSTRUCTION OF *lacZ* FUSION RECOMBINANTS

In order to investigate the control of expression of the *A. nidulans* IPNS gene, fragments of DNA in the region 5′ of IPNS were cloned into the *lacZ* fusion vectors pAN923-41B, -42B and -43B (van Gorcom *et al.*, 1986). Each of these three integration vectors has a unique *Bam*HI site in one of three different translational reading frames in front of the *E. coli* *′lacZ* gene, which is comprised of the protein coding region of the *E. coli lacZ* gene lacking the 5′ upstream control region and the first eight codons thus is not transcribed *per se*. The unique restriction site in front of the *′lacZ* gene facilitates in-phase translational fusion of an *A. nidulans* gene together

with its 5′ upstream expression signals to *E. coli* *′lacZ*. This system, therefore, reports the presence of promoter sequences on cloned stretches of DNA by the production of a hybrid protein product comprising the amino terminal region of the gene under analysis and the ß-gal activity of the partially deleted *lacZ* gene.

Two DNA fragments in the IPNS region: a 1.2 kb *Hin*dIII-*Eco*RV fragment, and a 1.6 kb *Eco*RV-*Eco*RV fragment, were cloned into these vectors in all possible reading frames and orientations with respect to the *lacZ* reporter gene, as detailed below.

The 1.2 kb *Hin*dIII-*Eco*RV insert fragment had one blunt end created by *Eco*RV digestion, and one 5′ sticky end created by *Hin*dIII digestion of pSTA200, neither of which were compatible with the 5′ sticky ends created during linearisation of the vector molecules using *Bam*HI. The enzyme Klenow was used to fill in the 3′ recessed termini of the insert and vector to yield flush ended molecules. Following dephosphorylation of pAN923-41B, -42B and -43B; using calf-intestinal phosphatase (CIP) to prevent intramolecular ligation of vector molecules, the vector and insert fragments were ligated using T_4 DNA ligase, thus creating recombinant plasmids.

Problems were, however, encountered because pSTA200 contains two non-resolving 1.2 kb *Hin*dIII-*Eco*RV fragments which were thus isolated together, and cloned into the *lacZ* fusion vectors. Recombinant plasmids,

containing either of the inserts, were transformed into *E. coli* DH5 for amplification of the DNA. Transformants were then screened using a α-^{32}P-labelled 660 bp *Bam*HI-*Bam*HI fragment of pSTA200, which contains part of the IPNS structural gene, as a homologous probe to determine which recombinants contained the desired *Hin*dIII-*Eco*RV insert. Plasmid DNA was then prepared from any colonies which hybridised to this probe.

The 1.6 kb *Eco*RV-*Eco*RV fragment, cloned from pSTA201, is similar to the 1.2 kb *Hin*dIII-*Eco*RV fragment from pSTA200, but has an additional 400 bp upstream from the 5' region of the *A. nidulans* IPNS structural gene (Fig. 8.IV). Restriction of pSTA201 with *Eco*RV yielded a flush-ended insert, therefore only the vector molecules, linearised with *Bam*HI, required Klenow treatment. Vectors were then dephosphorylated, ligated to the insert, and transformed into *E. coli* DH5. Problems were again encountered because the 1.6 kb *Eco*RV-*Eco*RV fragment also ran as a non-resolving doublet and both fragments were isolated together and cloned into the *lacZ* fusion vectors. In this case, plasmid DNA was prepared from a selection of the transformants obtained and screened for the presence of the required fragment, integrated singly into the vector, by digestion with restriction enzymes (see below). Only plasmids exhibiting the appropriate restriction profile were studied further.

Plasmids from both sets of clonings were screened for the presence of an insert fragment by *Bam*HI digestion of the DNA. This differentiated between recombinants and recircularised vectors because a 660 bp *Bam*HI-*Bam*HI fragment was obtained only if plasmids contained the appropriate insert fragment. *Bam*HI/*Xho*I digests were subsequently used to orientate the insert fragment within the recombinant plasmids. In total, twelve caesium chloride-purified plasmids were prepared: six containing the 1.2 kb *Hin*dIII-*Eco*RV insert, designated pSTA800, pSTA801, pSTA804, pSTA806, pSTA811 and pSTA812; and six containing the 1.6 kb *Eco*RV-*Eco*RV insert, designated pSTA813, pSTA814, pSTA815, pSTA816, pSTA817 and pSTA818 (Table VII).

3.13 IDENTIFICATION OF BIDIRECTIONAL PROMOTER ACTIVITIES 5′ OF IPNS

For preliminary analysis, the twelve transformants generated above were transformed into the *npeA*0022, *argB*2 double mutant (strain SAA101), and a randomly chosen representative sample of *argB*$^+$ transformants containing each of the these plasmids (Table VII) was qualitatively analysed for the expression of the hybrid protein ß-gal activity. Colonies were inoculated onto appropriately supplemented M9-based minimal agar medium containing various carbon and nitrogen sources and X-gal, a gratuitous inducer of *lacZ* which yields a blue colour when cleaved by ß-gal. The endogenous *A.*

Table VII

Qualitative analysis of ß-gal activity in a randomly chosen, representative sample of *A. nidulans npeA*0022 transformants containing the *lacZ* fusion recombinant plasmids indicated.

Plasmid	Vector backbone	5′fragment	Orientation of fragment[a]	Hybrid ß-gal expressed[b]
pSTA800	pAN923-41B	*Hin*dIII-*Eco*RV	5′-3′	-
pSTA801	"	(1.2 kb) from	3′-5′	-
pSTA804	pAN923-43B	pSTA200	5′-3′	-
pSTA806	"	"	3′-5′	-
pSTA811	pAN923-42B	"	3′-5′	-
pSTA812	"	"	5′-3′	+
pSTA813	pAN923-41B	*Eco*RV-*Eco*RV	3′-5′	-
pSTA814	"	(1.6 kb) from	5′-3′	-
pSTA815	pAN923-42B	pSTA201	3′-5′	-
pSTA816	"	"	5′-3′	+
pSTA817	pAN923-43B	"	3′-5′	+
pSTA818	"	"	5′-3′	-
pAN923-21B[c]				+
- (wild type strain)[c]				-

[a] Orientation of fragment in relation to the direction of transcription of the IPNS gene.

[b] Symbol (+) denotes expression of hybrid protein ß-gal activity; symbol (-) denotes lack of ß-gal activity.

[c] As controls: i. *A. nidulans npeA*022 transformed with pAN923-21B i.e. *trpC* :: *lacZ* fusion plasmid (van Gorcom *et al.*, 1986). ii. *A. nidulans* wild type (*biA*1) assayed for hybrid protein ß-gal activity.

nidulans lacZ gene is induced by lactose and repressed by glucose (Fantes and Roberts, 1973), however, glucose has been found to repress the enzymes ACVS and IPNS, and hence penicillin biosynthesis in *P. chrysogenum*, although it does not affect ACYT (Revilla *et al.*, 1986). Maltose was therefore used as an alternative carbon source to glucose in an attempt to overcome any problems this might have caused, although fusion protein ß-gal activity was detected but to a lesser extent when glucose was used as a carbon source. Hybrid protein ß-gal activity in transformants containing pSTA812, pSTA816 and pSTA817 was easily distinguishable from endogenous ß-gal activity when glucose or maltose and ammonium or glutamate were used as carbon and nitrogen sources.

Predictably, one of the six plasmids containing the 1.2 kb *Hin*dIII-*Eco*RV fragment (pSTA812) showed the presence of promoter activity, reading only in the direction of the IPNS gene (Table VII). Contrary to expectations, however, two of the six plasmids containing the larger 1.6 kb *Eco*RV-*Eco*RV fragment (pSTA816, pSTA817) (Table VII) exhibited promoter activities: one apparently identical to that in the 1.2 kb *Hin*dIII-*Eco*RV fragment (i.e. same direction and reading frame; pSTA816), and a second in the opposite orientation (pSTA817). It is possible that the IPNS gene promoter permits transcription of IPNS from two different directions, however, the *'lacZ* gene lacks

both its promoter and the first eight codons, thus requires both a promoter and ATG start codon for it to be faithfully transcribed and translated. This would suggest that the 0.4 kb *Eco*RV-*Hin*dIII stretch of the 1.6 kb *Eco*RV-*Eco*RV fragment contains the transcriptional start site of a gene 5′ to, and reading away from, the IPNS gene. Surprisingly, on pSTA817, the ATG start site allowing expression of *lacZ* from promoter sequences in the opposite orientation to IPNS was in fact fortuitous, resulting from ligation of the blunt ended 1.6 kb *Eco*RV-*Eco*RV fragment into the *BamH*I cloning site of the *lacZ* fusion vector pAN923-43B which had been blunt-ended using Klenow. However, the remainder of the 1.6 kb *Eco*RV-*Eco*RV fragment reading away from this ATG site into the *lacZ* gene must indeed contain authentic promoter sequences in order for the *lacZ* gene to be correctly transcribed and translated.

3.14 GENE DISRUPTION STUDIES USING A FRAGMENT 5′ OF IPNS

pSTA807 is a subclone of pSTA201 which contains a 7.5 kb region 5′ of IPNS (Fig. 8.II), but probably does not contain an intact penicillin biosynthetic gene as judged by the very low frequency at which pSTA807 complements the *npeA*0022 mutation (Table IV). pSTA807 was introduced by transformation into an *A. nidulans* *argB*2, *npeA*$^{+}$ strain (G34), and it was observed that 59 % (59/100) of arginine prototrophic transformants

exhibited penicillin non-producing phenotypes. This high frequency of penicillin non-producers is indicative of homologous integration of an incomplete gene and, by extension, the existence of a penicillin biosynthetic gene 5′ of IPNS. As a control, pILJ16, the vector on which pSTA807 is based, was transformed into the *A. nidulans argB2*, *npeA*$^{+}$ strain (G34) and 2 % (1/50) of arginine prototrophic transformants were observed to exhibit a penicillin non-producing phenotype. This may have been due to non-homologous integration of the plasmid into the *A. nidulans* penicillin biosynthetic genes, however the copy number and site of integration of pILJ16 into the genomic DNA remains to be elucidated.

That gene disruption by pSTA807 occurs at such a high frequency, suggests that this construct lacks both 5′ and 3′ termini. pSTA201 therefore must also lack the 3′ terminus (see Fig. 8.II) in view of the *lacZ* fusion data detailed in section 3.13. Consequently, complementation of *npeA*0022 by pSTA807 must be a rare transformation event, involving a double cross-over at the *npeA* site as well as a second integration at *argB*. The molecular basis of the apparent contradiction in the fact that pSTA201 complements *npeA*0022 following integration at non-homologous sites yet apparently lacks at least the 3′ terminus, is as yet unclear.

3.15 IDENTIFICATION AND CHARACTERISATION OF THE ACVS CODING REGION

A. nidulans ACVS protein was purified in the laboratory and used to generate partial amino acid sequence data. An internal CNBr fragment of the purified amino-terminally blocked protein was used to obtain a 15 amino acid segment of sequence, from which a set of mixed 23-mer oligonucleotides was synthesised and subsequently used to probe Southern filters of *Bam*HI digested pSTA200 and pSTA201 (Fig. 13). Only a 3.0 kb *Bam*HI fragment of pSTA201 (Fig. 8.III) was detected using this probe. No hybridisation was observed to any fragment of pSTA200, suggesting that the 1 kb *Bam*HI-*Xba*I nucleotide stretch between fragments C and D (Fig. 8.III) contained the region homologous to the probe. DNA sequence data of this region (MacCabe *et al.*, 1991) has confirmed this conclusion, as the 15 amino acid segment of sequence obtained from the purified ACVS protein matched that predicted from the DNA sequence, and shown that the ACVS gene is transcribed in the opposite orientation relative to IPNS (Fig. 8.V).

3.16 HYBRIDISATION STUDIES OF SEQUENCES 3′ OF IPNS

A 1.9 kb *Hin*dIII-*Eco*RI *P. chrysogenum* probe isolated from pSTA18, which contains part of the gene coding for ACYT (Barredo *et al.*, 1989b; Diez *et al.*, 1989), was hybridised to *Bam*HI digests of the *A. nidulans* genomic clones pSTA200 and pSTA201 (Fig. 14).

Fig. 13 Identification of the ACVS coding region. Panel A shows the *Bam*HI digestion pattern of plasmids pSTA200 (lane 2) and pSTA201 (lane 3) after electrophoresis on a 1 % agarose gel. Lanes 1 and 4 contain lambda/*Hin*dIII and BRL 123 bp markers, respectively. DNA was transferred onto Amersham Hybond-N and the blot was prehybridised at 30°C with 5 x SSPE, 6 % PEG 6000, 0.5 % skimmed milk, 1 % SDS, 0.1 % $Na_4P_2O_7.10H_2O$, 250 µg/ml denatured herring sperm DNA; and hybridised overnight in the same buffer at 30°C with 100 ng end-labelled mixed 23-mer probe. Washing was performed in 5 x SSC, 0.1 % SDS, 0.1 % $Na_4P_2O_7.10H_2O$; at 30°C for 40 min, and repeated in the same buffer at 40°C. Panel B shows the result of autoradiography with Fuji RX film for 6 h.

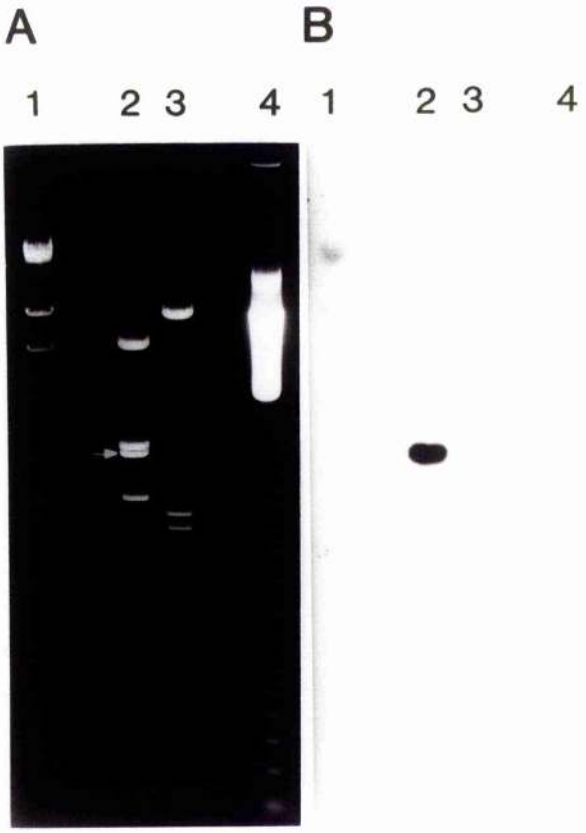
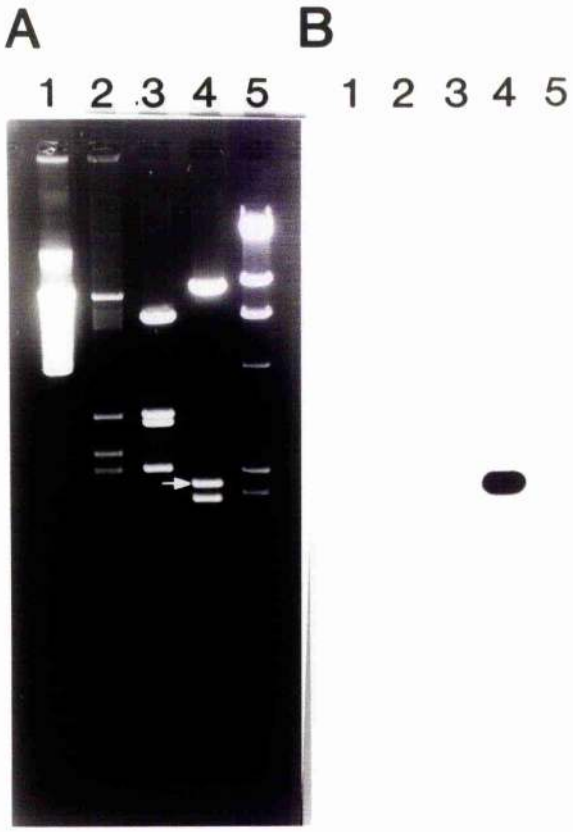

A
B
1
2
3
4
1
2
3
4

Fig. 14 Identification of the *A. nidulans* acyltransferase gene. Panel A shows the *Bam*HI digestion pattern obtained from plasmids pSTA207 (lane 2), pSTA201 (lane 3) and plasmid pSTA200 (lane 4). Lanes 1 and 5 contain the BRL 123 bp ladder and lambda/*Hin*dIII markers respectively. Panel B shows the result of hybridisation of the *P. chrysogenum* ACYT probe to the plasmid digests under the same conditions as described in the legend to Fig. 11, after washing down to 1 x SSC at 56^{o}C.


A
B
1 2 3 4 5
1 2 3 4 5

This probe hybridised specifically to the 2.4 kb pSTA200 *Bam*HI fragment designated G (Fig. 8.III).

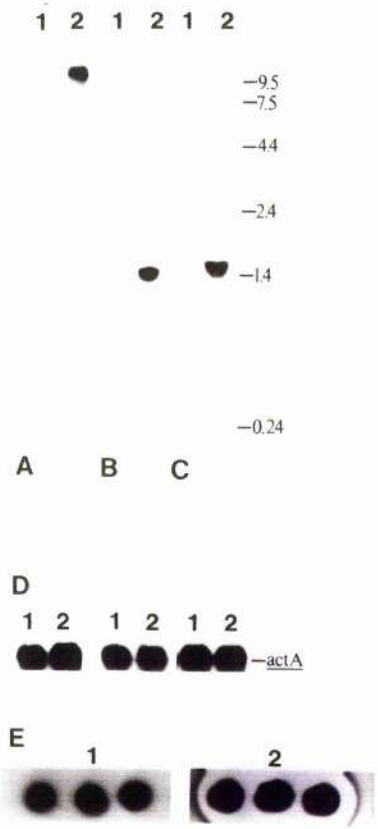
To determine the orientation of transcription of ACYT, two probes: a 0.7 kb *Xho*I-*Hpa*I fragment containing the 5′ end of the *P. chrysogenum* ACYT gene, and a 0.9 kb *Eco*RI-*Sal*I fragment containing the 3′ end of the *P. chrysogenum* ACYT gene (Barredo *et al.*, 1989b; Diez *et al.*, 1989; J.F. Martin, personal communication), isolated from pSTA18 ; were hybridised to various restriction endonuclease digests of pSTA200. Such experiments (data not shown) suggested that the direction of transcription of the ACYT gene is the same as that of IPNS (i.e. from left to right in Fig. 8.V).

An attempt was made to sequence the *A. nidulans* genomic DNA region 3′ to IPNS encompassing the ACYT gene. DNA fragments from the area spanned by probes G and H (Fig. 8.III) were subcloned from pSTA200 into pUC18, and preliminary sequence analysis performed. However, this work was aborted as problems were encountered with obtaining oligonucleotide primers, and during this time it was learned that the *A. nidulans* ACYT gene had been sequenced at the nucleotide level by Tobin *et al.* (1990).

3.17 IDENTIFICATION OF PENICILLIN BIOSYNTHETIC GENE TRANSCRIPTS

Three major transcripts were detected by northern blot analysis (Fig. 15, Panel A, B and C) of total RNA

Fig. 15 Northern blots of transcripts. Northern blots of total RNA prepared from *A. nidulans* wild type (*biA*1) mycelium grown for 26 h at 26°C, with orbital shaking at 250 rpm, under (1) repressed conditions in minimal medium pH 5.2 containing 1 % glucose and 10 mM ammonium tartrate; and (2) derepressed conditions in fermentation medium pH 5.2. Blots were hybridised to the following probes: Panel A, the putative ACVS fragment C (Fig. 8.III); Panel B, the IPNS specific fragment E (Fig. 8.III); Panel C, the putative ACYT fragment G (Fig. 8.III). The molecular sizes (kb) of the BRL ladder are indicated. Panel D. In order to verify the uniformity of RNA transfer, blots were stripped and reprobed with a fragment specific for *A. nidulans actA* (Fidel *et al.*, 1988). Prehybridisation, hybridisation, washing of filters and autoradiography was as described in Materials and Methods. Panel E. Bioassay of wild type (*biA*1) mycelium grown on (1) minimal medium pH 5.2 containing 1 % glucose and 10 mM ammonium tartrate; and (2) fermentation medium pH 5.2. Areas of clearing around plugs indicate ß-lactam antibiotic activity.


1 2 1 2 1 2
−9.5
−7.5
−4.4
−2.4
−1.4
−0.24
A B C
D
1 2 1 2 1 2
−actA
E
1
2

prepared from wild type mycelium grown for 26 h, at 26°C with orbital shaking at 250 rpm, in fermentation medium pH 5.2 (derepressed conditions). No transcripts were detected in RNA isolated from mycelium grown for 26 h, at 26°C with orbital shaking at 250 rpm, in minimal medium pH 5.2 containing 1 % glucose as the carbon source and 10 mM ammonium tartrate as the nitrogen source (repressed conditions).

A probe specific for the IPNS gene (Fig. 8.III; fragment E) hybridised to an RNA species of 1.4 kb. This species was of the size expected from the *A. nidulans* inferred IPNS polypeptide length of 331 amino acids (Ramon *et al.*, 1987) and protein size of 39 kDa (Ramos *et al.*, 1985). A probe specific for the ACVS gene (Fig. 8.III; fragment C) hybridised to a species of greater than 9.5 kb. This is suggested to be the ACVS message although this mRNA size is rather larger than that predicted from the protein size of 230 kDa (van Liempt *et al.*, 1989). This discrepancy was as a result of the protein size being underestimated, the revised size being 422 kDa (MacCabe *et al.*, 1991). The ACVS gene has been sequenced at the nucleotide level and found to encode a polypeptide of predicted length 3,770 amino acids, molecular mass 422,486 Da (MacCabe *et al.*, 1991). Total RNA probed with a nucleotide stretch 3′ of IPNS (Fig. 8.III; fragment G) revealed a transcript of 1.6 kb. The size of this mRNA is in reasonable accord with the inferred polypeptide length

of 357 amino acids reported for the *A. nidulans* and *P. chrysogenum* ACYT genes (Barredo *et al.*, 1989b; Tobin *et al.*, 1990), and this 1.6 kb transcript is suggested to be the ACYT message.

The appearance of all three transcripts is in accord with the regulation of the genes being, at least in part, at the level of mRNA accumulation.

3.18 BIOASSAY OF PENICILLIN PRODUCTION IN STRAINS GROWN UNDER DEREPRESSED AND REPRESSED CONDITIONS

P. chrysogenum V992 and the following *A. nidulans* strains: *npeA*$^{+}$ (*biA*1), *npeA*0022, *npeA*$^{+}$ (MacT5, MacT48, MacT69 and MacT156), *penA*1 (G69), *npeA*0022 (SAA101) and *npeA*0049/1 (G0049/1); were inoculated onto fermentation agar plugs, and also plugs of appropriately supplemented *Aspergillus* minimal medium containing 10 mM ammonium tartrate as the nitrogen source and 4 % agar. Plugs were incubated at 25^{o}C in a humid environment for 5 days then transferred to bioassay medium and incubated at 37^{o}C overnight. Halos, indicative of ß-lactam antibiotic production, were observed around *npeA*$^{+}$ cells grown on fermentation medium (derepressed conditions) but not those grown on minimal medium (repressed conditions) (Fig. 15; Panel E). This confirmed that the production of penicillin, as judged by bioassay, follows a pattern similar to that of the presence of the ACVS, IPNS and ACYT gene transcripts in RNA isolated from *A. nidulans* wild type

cells grown under derepressed and repressed conditions (see section 3.17). No penicillin was produced by control *npeA*$^-$ strains grown on plugs of either minimal or fermentation medium.

3.19 HYBRIDISATION STUDIES OF SEQUENCES 5′ OF *acvA* AND 3′ OF *acyA*

The *npeA* locus of *A. nidulans* has been shown to contain the three structural genes encoding the enzymes required for penicillin biosynthesis (Fig. 9), however, it is not known whether or not there exists any regulatory genes to control this process. An attempt was thus made to determine if such a regulatory gene is clustered with the structural genes for penicillin biosynthesis at the *npeA* locus.

Total RNA was isolated from *A. nidulans* wild type (*biA*1) grown at 26^{o}C, with orbital shaking at 250 rpm, for 24 h in fermentation medium pH 5.2 and 24 h or 48 h in the various minimal media pH 5.2 described in section 4.3.4.1. This RNA was then subjected to northern blot analysis using as probes, the 5.5 kb *Hin*dIII-*Hin*dIII fragment isolated from pSTA230, a subclone of cosmid clone No. 35 which contains *A. nidulans* genomic DNA sequences 5′ of *acvA*; and the 3 kb *Hin*dIII-*Hin*dIII fragment isolated from pSTA200 (Fig. 8.II) containing *A.nidulans* genomic DNA sequences 3′ of *acyA*. Each RNA sample was loaded onto the gel at three times the usual concentration (i.e. 30 μg loaded instead of 10 μg) in an

attempt to circumvent the possibility of a transcript not being detected simply due to the gene being poorly transcribed and hence the mRNA being present in low abundance. No mRNA species was found to hybridise to either of the probes detailed above, although detection of the *A. nidulans actA* gene indicated that RNA had indeed been transferred to the filter from the gel during blotting (Fig. 20.II, panel A.2). It therefore appears that if any regulatory genes exist for penicillin biosynthesis, they are not located immediately 5′ of *acvA* or 3′ of *acyA* at the *A. nidulans npeA* locus.

CHAPTER 4

REGULATION OF EXPRESSION OF THE *A. nidulans* PENICILLIN BIOSYNTHETIC GENES

4.1 INTRODUCTION

This chapter concerns the analysis of the regulation of *A. nidulans* penicillin biosynthetic gene expression, aimed at gaining more of an insight into how penicillin biosynthesis is regulated. To this end, preliminary analysis of the *A. nidulans acvA* and *ipnA* expression signals was carried out by *lacZ* fusion studies, and northern blot and hybridisation analyses of the regulation of *A. nidulans acvA*, *ipnA* and *acyA* expression were also performed.

4.2 PRELIMINARY ANALYSIS OF *A. nidulans acvA* AND *ipnA* EXPRESSION SIGNALS USING *lacZ* FUSION ANALYSIS

Preliminary experiments were carried out which were aimed at determining the regulation of *A. nidulans acvA* and *ipnA* gene expression. Hybrid protein ß-gal activity was quantitatively assayed in transformants of *A. nidulans* SAA101, the *npeA*0022, *argB*2 double mutant strain, containing pSTA816 and pSTA817, the *ipnA* :: *lacZ* and *acvA* :: *lacZ* fusion plasmids, respectively (see section 3.12). Such transformants were grown in appropriately supplemented minimal medium containing 1 % glucose, 1 % maltose or 1 % lactose as the carbon source;

and 10 mM ammonium tartrate (≡ 20 mM ammonium ion concentration) or 10 mM proline (≡ 10 mM ammonium ion concentration) as the nitrogen source; and ß-gal activity was detected by spectrophotometric measurement of the cleavage of o-NPG (colourless) to o-NP (yellow). Endogenous *A. nidulans* ß-gal activity was detected in the wild type (*biA*1) strain and transformants analysed when lactose, but not glucose or maltose, was used as the carbon source. Although Southern blot and classical genetic analysis of these transformants is required to determine the integration site(s) and the number of integrated copies of the transforming plasmids, preliminary evidence (Table VIII) suggests that the ACVS and IPNS genes are not highly regulated.

It had been the intention that the regulation of expression of *acvA* and *ipnA* would be further studied by transforming pSTA816 and pSTA817 into a number of *A. nidulans* strains with different genetic backgrounds and subsequently quantitatively assaying ß-gal fusion protein activity in such transformants grown under a variety of physiological conditions. To this end, *A. nidulans* *areA*19, *argB*2 and *xprD*1 (an allele at the *areA* locus; Arst and Cove, 1973), *argB*2 double mutant strains (SAA236 and SAA244, respectively), suitable for transformation with these plasmids and which were not available from external sources, were constructed by classical genetic crossing experiments (see section 3.5). Expression of endogenous *A. nidulans* ß-gal activity can lead to

Table VIII

ß-galactosidase activities of *lacZ* fusion recombinant transformant and wild type strains, grown in minimal media with various carbon and nitrogen sources (as indicated), normalised with respect to protein concentrations of enzyme extracts.

Strains grown in minimal medium		μM o-NP produced/mg protein/min by				
C- source	N- source	*biA1*[a]	SAA215[b]	SAA217[b]	SAA221[c]	SAA229[c]
glucose	proline	0	0.769	0.175	0.205	0.278
glucose	ammonium	0	0.535	0.376	0.106	0.693
maltose	proline	0	0.844	0.506	0.083	0.312
maltose	ammonium	0	1.607	0.200	0.065	0.194
lactose	proline	0.083	2.476	0.638	0.139	0.172
lactose	ammonium	0.304	3.398	2.360	0.677	0.625

[a] *A. nidulans* wild type strain, untransformed.

[b] *A. nidulans npeA*0022 transformed with pSTA816 (see Table VII).

[c] *A. nidulans npeA*0022 transformed with pSTA817 (see Table VII).

problems when using *lacZ* as the reporter system, but this is typically overcome by using glucose to repress the endogenous *lacZ gene*. However, this is not an ideal solution when the system being studied, such as penicillin biosynthesis, is thought to be subject to carbon catabolite repression, thus it was decided that these experiments should be curtailed.

An alternative system would be to clone the penicillin biosynthetic gene expression signals into GUS fusion vectors in which the *E. coli uidA* gene, coding for ß-glucuronidase (GUS), acts as a reporter gene (Roberts *et al.*, 1989). No *uidA* gene homologue has been reported in *A. nidulans* and thus GUS activity in transformants containing GUS fusion recombinants can be attributed solely to expression of the fusion protein. Active GUS fusion protein comprising the 5′ upstream expression signals of the *A. nidulans* glyceraldehyde-phosphate dehydrogenase gene (*gpdA*) and the *uidA* coding sequence has been shown to be expressed in the filamentous fungi *A. nidulans*, *A. oryzae* and *Fulvia fulva* (Roberts *et al.*, 1989). However, the GUS fusion vectors were not available at the time the *lacZ* fusion work was being undertaken, and when they subsequently became available, due to time constraints, the work was not carried out.

4.3 RNA TRANSCRIPT ANALYSIS TO DETERMINE THE REGULATION OF EXPRESSION OF PENICILLIN BIOSYNTHETIC GENE EXPRESSION IN *A. nidulans*

In each of the following northern blot and hybridisation experiments described, *A. nidulans* total RNA was probed, unless otherwise indicated, with *A. nidulans* genomic DNA fragments C, E and G (Fig. 8.III) specific for the ACVS, IPNS and ACYT genes, respectively. Filters were then stripped of their radioactivity and back-hybridised with a probe specific for the *A. nidulans* actin gene (*actA*) (Fidel *et al.*, 1988) in order to determine the extent of uniformity of RNA concentration between each lane.

Total RNA was extracted from a number of *A. nidulans* strains with different genetic backgrounds, grown for varying lengths of time under a variety of physiological conditions, as indicated in the text. In each case the pH of the medium was 5.2 and cultures were incubated at 26^{o}C with orbital shaking at 250 rpm.

4.3.1 Temporal Regulation

A time course was set up to determine the times at which the transcripts of the *A. nidulans* penicillin biosynthetic genes were detectable in fermentation medium, i.e. under conditions derepressed for penicillin biosynthesis. *A. nidulans* wild type (*biA*1) mycelium was harvested at 8 h intervals after 16 h to 56 h incubation in fermentation medium. Northern blot analysis of total

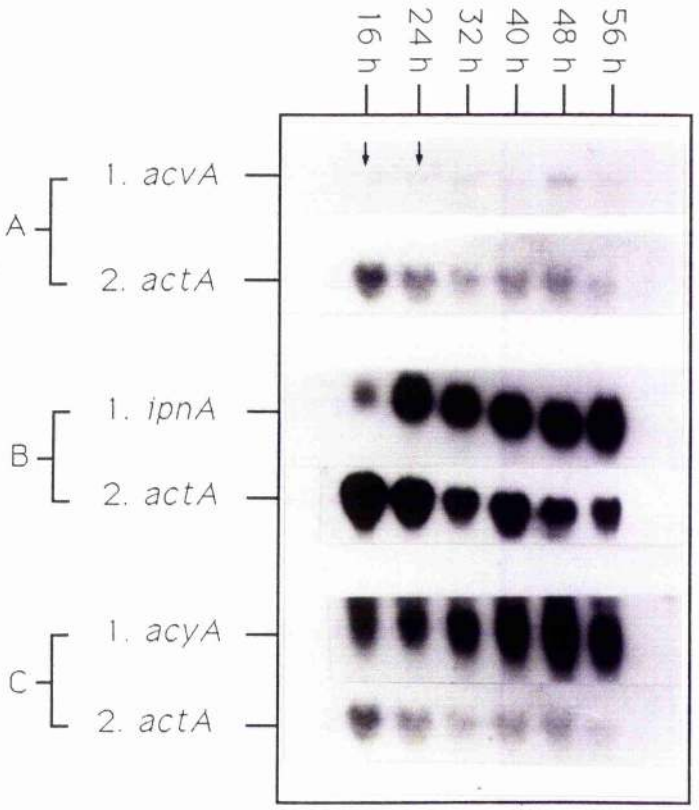
RNA from such mycelia revealed that *acvA*, *ipnA*, and *acyA* mRNAs were detectable at all time points analysed. Transcript levels appeared to perhaps increase slightly with time up to 56 h incubation (Fig. 16), with the most marked increase in the amount of transcript produced occurring between the 16 h and 24 h samples. 24 h was therefore chosen as a convenient time point at which to study transcription of the penicillin biosynthetic genes in different *A. nidulans* strains grown in fermentation medium.

Supernatants of each culture were bioassayed for penicillin production. No penicillin was detected before 24 h incubation in fermentation medium, and the zones of inhibition, indicative of ß-lactam activity, increased with time up to 56 h incubation. It would appear therefore that penicillin biosynthesis is subject to temporal regulation and that a lag occurs between the time of production of the penicillin biosynthetic gene transcripts and the detection of ß-lactam activity.

4.3.2 Transcription of Penicillin Biosynthetic Genes in Mutants with Impaired or Enhanced Penicillin Biosynthetic Capabilities

Northern blot analysis was carried out on total RNA prepared from mycelia of the *A. nidulans* *npeA*0022 and *npeA*005 non-penicillin producing mutant strains GH79 and GH44 respectively, and the *penA*1 penicillin over-producing strain G69, grown in fermentation medium for 24

Fig. 16 Northern blots of total RNA extracted from *A. nidulans* wild type (*biA*1) grown in fermentation medium pH 5.2 at 26°C, with orbital shaking at 250 rpm, for a variety of time periods (indicated above lanes), and hybridised to the following homologous gene-specific probes: Panel A.1, *acvA*; Panel B.1, *ipnA*; and Panel C.1, *acyA*. In addition, each blot was stripped and back-hybridised to the *A. nidulans actA* gene-specific probe (Panels A.2, B.2 and C.2) to determine the extent of uniformity of RNA concentration in each lane. Prehybridisation, hybridisation, washing of filters and autoradiography was as described in Materials and Methods. Bands present on the original autoradiograph but undetectable in the photograph are indicated by arrows.


16 h
24 h
32 h
40 h
48 h
56 h
A
1. acvA
2. actA
B
1. ipnA
2. actA
C
1. acyA
2. actA

h. The *penA*1 mutant strain synthesised *acvA*, *ipnA* and *acyA* transcripts at similar or perhaps slightly reduced levels compared to the wild type strain (Fig. 17) and the *npeA*005 mutant strain produced all three penicillin biosynthetic gene transcripts at comparable levels to the wild type (Fig. 18). In the *npeA*0022 mutant strain, *ipnA* and *acyA* mRNA species were present at similar levels to those found in the wild type (Fig. 18) and an *acvA* transcript which was of a size greater than that expected for the ACVS gene hybridised to the *acvA* specific probe (for a possible explanation, refer to the discussion).

Southern blot and hybridisation analysis (see section 3.4) revealed that with the *npeA*005 mutation there was no obvious difference in genomic organisation within the regions probed compared to the wild type. Thus it was expected that all three penicillin biosynthetic gene transcripts would be present in strains carrying this mutation. On the other hand, Southern blot analysis (see section 3.4) indicated that the *npeA*0022 mutation has resulted in an alteration of genomic sequences homologous to the DNA fragment used as the *acvA* specific probe in northern blot and hybridisation analysis (probe C; Fig. 8.III), which may have resulted from an insertion of approximately 14 kb of DNA into this region (A.P. MacCabe, personal communication). This could possibly explain why a transcript which was larger than that expected for *acvA* was detected in the *npeA*0022 mutant strain using this probe. In addition, ACVS activity has

Fig. 17 Northern blots of total RNA extracted from a number of *A. nidulans* mutant strains (relevant alleles indicated above lanes) and wild type strain (*biA1*; designated wt in figure) grown in fermentation medium pH 5.2 at 26°C, with orbital shaking at 250 rpm, for 24 h. Blots were hybridised to the following homologous gene-specific probes: Panel A.1, *acvA*; Panel B.1, *ipnA*; and Panel C.1, *acyA*. In addition, each blot was stripped and back-hybridised to the *A. nidulans actA* gene-specific probe (Panels A.2, B.2 and C.2) to determine the extent of uniformity of RNA concentration in each lane. Prehybridisation, hybridisation, washing of filters and autoradiography was as described in Materials and Methods.

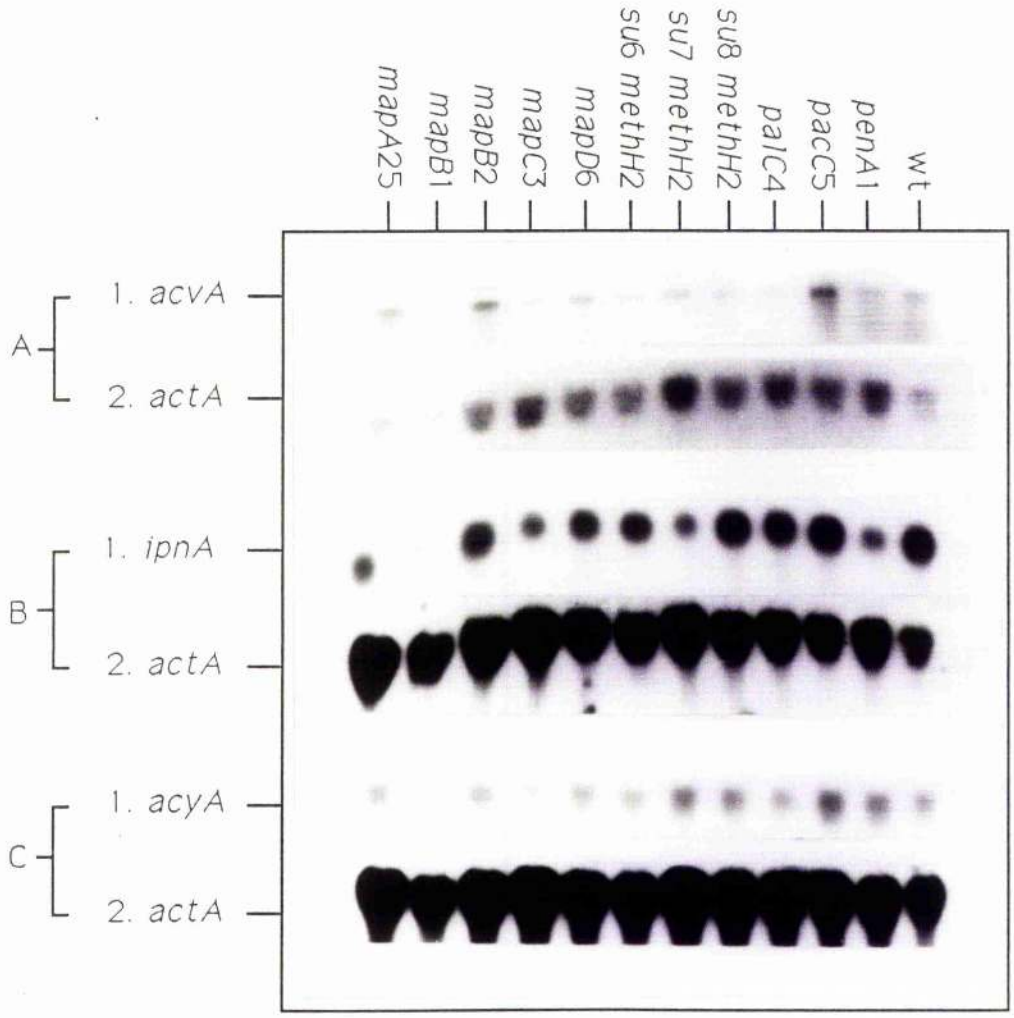
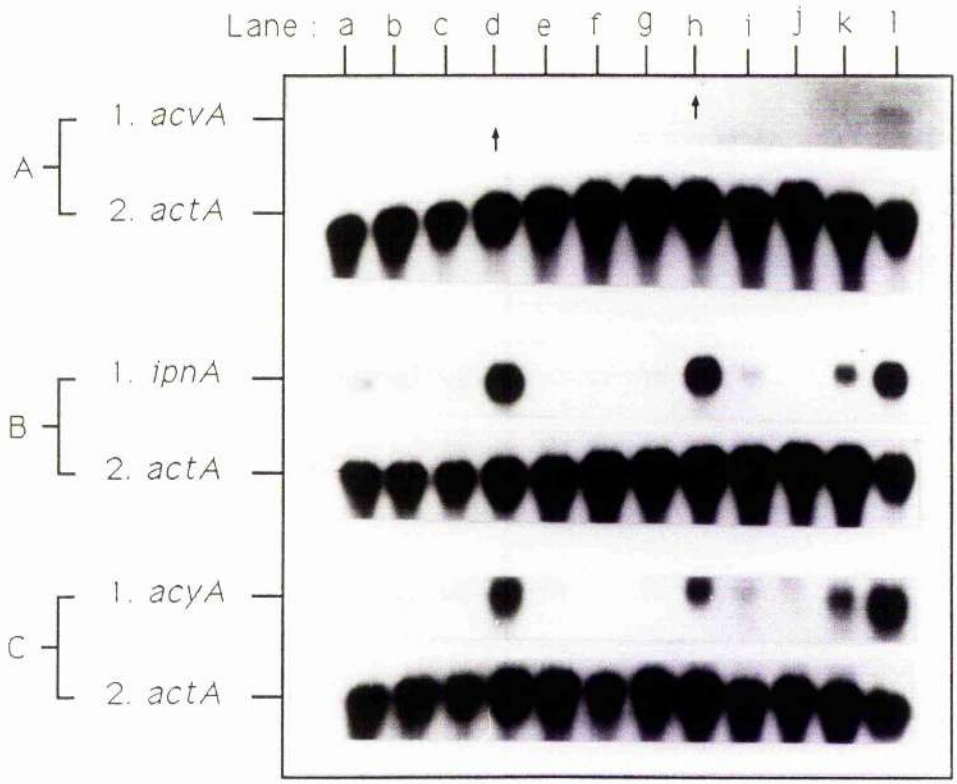

mapA25
mapB1
mapB2
mapC3
mapD6
su6 methH2
su7 methH2
su8 methH2
palC4
pacC5
penA1
wt
A
1. acvA
2. actA
B
1. ipnA
2. actA
C
1. acyA
2. actA

Fig. 18 Northern blots of total RNA extracted from *A. nidulans* wild type (*biA*1) grown at 26°C, with orbital shaking at 250 rpm, for 48 h in fermentation medium pH 5.2 (lane 1) and the following minimal media pH 5.2. Lanes a to c - 3.5 % lactose and 0.01 % casamino acids: unsupplemented (lane a), supplemented with 1 mM L-α-aminoadipic acid, 1 mM L-cysteine and 1 mM L-valine (lane b), or 2 mM phenylacetic acid (PAA) and 2 mM phenoxyacetic acid (PXA) (lane c); lanes e to g - 3.5 % lactose and 10 mM proline: unsupplemented (lane e), supplemented with 1 mM L-α-aminoadipic acid, 1 mM L-cysteine and 1 mM L-valine (lane f), or 2 mM PAA and 2 mM PXA (lane g); lanes i to k - 1 % glucose and 5 mM ammonium tartrate: unsupplemented (lane i), supplemented with 1 mM L-α-aminoadipic acid, 1 mM L-cysteine and 1 mM L-valine (lane j), or 2 mM PAA and 2 mM PXA (lane k).

Total RNA extracted from *A. nidulans* mutant strains *npeA*005 (lane d) and *npeA*0022 (lane h), grown for 24 h in fermentation medium pH 5.2 at 26°C, with orbital shaking at 250 rpm for 24 h, was also included on the blot.

Blots were hybridised to the following homologous gene-specific probes: Panel A.1, *acvA*; Panel B.1, *ipnA*; and Panel C.1, *acyA*. In addition, each blot was stripped and back-hybridised to the *A. nidulans actA* gene-specific probe (Panels A.2, B.2 and C.2) to determine the extent of uniformity of RNA concentration in each lane. Prehybridisation, hybridisation, washing of filters and autoradiography was as described in Materials and Methods. Bands present on the original autoradiograph but undetectable in the photograph are indicated by arrows.


Lane : a b c d e f g h i j k l
A
1. acvA
2. actA
B
1. ipnA
2. actA
C
1. acyA
2. actA

been found to be lacking in this strain (H. van Liempt, unpublished) which may be thus due to the insertion disrupting the *acvA* sequence, causing a non-functional gene product to be synthesised.

There is no obvious explanation for the fact that the *penA*1 mutant strain produces comparable or reduced levels of *acvA*, *ipnA* and *acyA* transcripts yet produces elevated levels of penicillin. Perhaps the *penA*1 mutation has pleiotropic effects which result in the over-production of penicillin in such strains.

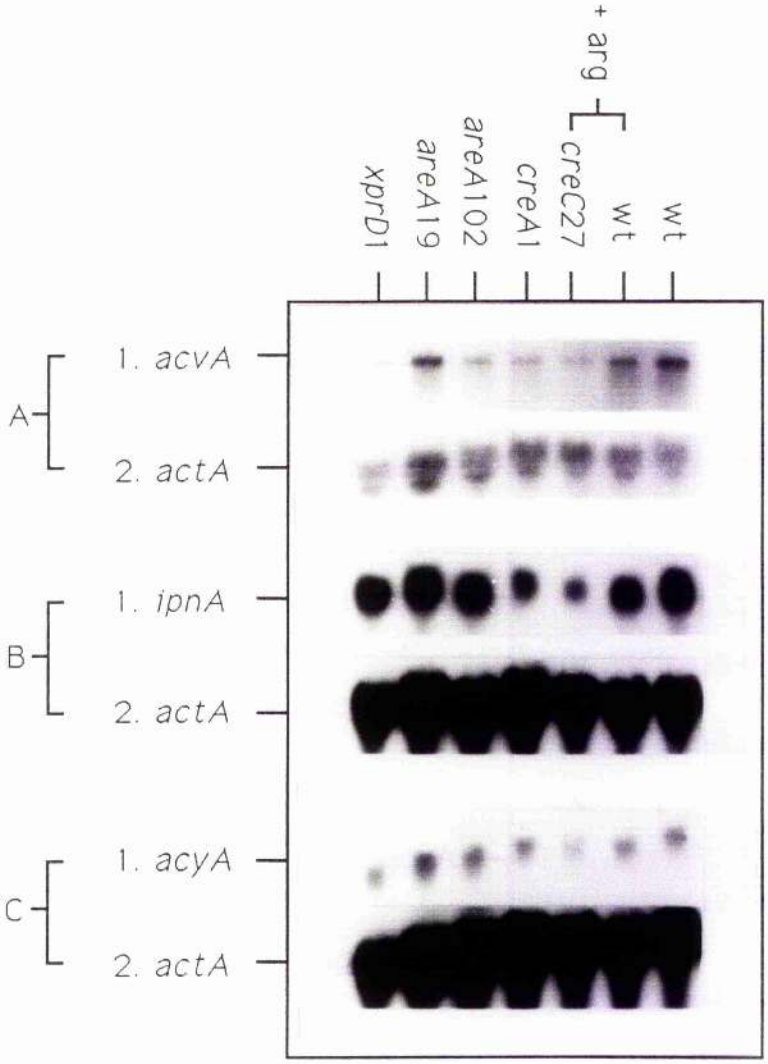
4.3.3 Effect of Various Regulatory Mutations on the Expression of the Penicillin Biosynthetic Genes

In order to determine the effects of carbon catabolism, nitrogen metabolism, sulphur metabolism and pH regulation on transcription of the penicillin biosynthetic genes, northern blot analysis was carried out on total RNA prepared from *A. nidulans* strains with a variety of genetic backgrounds which were grown in fermentation medium for 24 h.

4.3.3.1 Carbon catabolism mutations

A. nidulans strains C46 and 481 altered in carbon catabolite repression carrying the *creC*27 and *creA*1 mutations respectively, both produced *acvA*, *ipnA* and *acyA* transcripts, but at apparently lower levels compared to those produced by the wild type strain (Fig. 19).

Fig. 19 Northern blots of total RNA extracted from a number of *A. nidulans* mutant strains (relevant alleles indicated above lanes) and wild type strain (*biA1*; designated wt in figure) grown in fermentation medium pH 5.2 at 26°C, with orbital shaking at 250 rpm, for 24 h. Fermentation medium was supplemented with 100 mM arginine (arg) for the arginine-requiring *creC27* mutant strain and a control wild type (*biA1*) strain as indicated in the figure. Blots were hybridised to the following homologous gene-specific probes: Panel A.1, *acvA*; Panel B.1, *ipnA*; and Panel C.1, *acyA*. In addition, each blot was stripped and back-hybridised to the *A. nidulans actA* gene-specific probe (Panels A.2, B.2 and C.2) to determine the extent of uniformity of RNA concentration in each lane. Prehybridisation, hybridisation, washing of filters and autoradiography was as described in Materials and Methods.


xprD1
areA19
areA102
creA1
creC27
wt
wt
+ arg
A
1. acvA
2. actA
B
1. ipnA
2. actA
C
1. acyA
2. actA

This may be because the *creA* and *creC* strains grow more slowly than the wild type strains. Therefore, as penicillin biosynthesis is subject to temporal regulation (see section 4.3.1) which is related to the stage in the producing organism's life cycle (Holt *et al.*, 1976; Martin and Demain, 1980), it may be that the penicillin biosynthetic genes have been switched on later than in the wild type strain, with the result that at the time point analysed, the *creA* and *creC* strains produce less transcript than the wild type. In order to ascertain whether or not this is the case, a time course of the *creA* and *creC* mutant strains in fermentation medium should be carried out. Time constraints however, prevented this experiment from being performed.

The *creA* and *creC* mutant strains are carbon catabolite derepressed and in these strains, systems which are normally switched off by glucose are no longer subject to such repression. Consequently, it might be expected that if penicillin biosynthesis was subject to carbon catabolite repression, then *acvA*, *ipnA* and *acyA* transcripts would be present in elevated amounts in *cre* strains in comparison to the wild type when grown under the same conditions. This did not appear to be the case with *cre* mutants grown in fermentation broth, which may indicate that penicillin biosynthesis is not carbon catabolite repressed. However, in this medium, lactose is the main carbon source and should in itself be expected to alleviate carbon catabolite repression. To investigate

this further, a comparison could be made between *cre* and wild type strains grown in minimal medium containing glucose as the carbon source.

As *A. nidulans* C46 is an *argB*2 strain, the fermentation medium required supplementation with arginine to assist the growth of this strain. Northern blot analysis was also carried out on total RNA prepared from wild type (*biA*1) mycelium grown in fermentation medium supplemented with arginine to determine whether or not arginine affects transcription of the penicillin biosynthetic genes. No significant difference was apparent between levels of *acvA*, *ipnA* and *acyA* mRNA produced by the wild type in arginine supplemented or unsupplemented fermentation medium (Fig. 19), and it appears that arginine, at the concentration tested (100 mM), does not significantly affect penicillin biosynthesis.

4.3.3.2 Nitrogen metabolism regulatory mutations

A. nidulans mutant strains MH205 and MH8 harbouring the *areA*19 and *areA*102 mutations respectively, and strain MH837 carrying the mutation *xprD*1, all produced similar levels of *ipnA* and *acyA* transcripts to the wild type strain. The mutants *areA*102 and *xprD*1 both produced apparently reduced levels of *acvA* mRNA, whilst the *areA*19 strain produced similar amounts of this message compared to the wild type (Fig. 19). The reasons for this are unclear.

The positive-acting regulatory gene *areA* mediates nitrogen metabolite repression and its protein product is thought to act at the level of transcription (Arst, 1984; Kudla *et al.*, 1990). Loss-of-function mutations *areA*19 and *areA*102 are nitrogen metabolite super-repressed whereas the mutation *xprD*1 (=*areA*^{d}) leads to extreme nitrogen metabolite derepression. If penicillin biosynthesis is subject to nitrogen metabolite repression then *xprD*1 strains would be expected to produce elevated levels of the penicillin biosynthetic gene transcripts, whilst *areA*19 and *areA*102 would exhibit reduced levels of such mRNA. This was not demonstrated here, perhaps because in fermentation medium *A. nidulans* is apparently derepressed for penicillin biosynthesis. However, transcription of *acvA*, *ipnA* and *acyA* does appear to be affected by the concentration of ammonium in the growth medium (see section 4.3.4.2). Therefore, the predicted effects of the *areA* and *xprD* mutations might be apparent in minimal medium containing ammonium as the sole nitrogen source.

4.3.3.3 Sulphur metabolism regulatory mutations

A. nidulans sulphur metabolism mutant strains G0215, G0216 and G0217 harbouring the mutations *su*-6 *methH*2, *su*-7 *methH*2 and *su*-8 *methH*2 respectively; and *mapA*25, *mapB*1, *mapB*2, *mapC*3 and *mapD*6 mutants all produced *acvA*, *ipnA* and *acyA* mRNA at similar levels to those transcribed in the wild type (Fig. 17).

The *su*-6 *methH2*, *su*-7 *methH2* and *su*-8 *methH2* mutants are derepressed for methionine biosynthesis, as in these strains, several enzymes of methionine synthesis are constitutive (Lukaszkiewicz and Paszewski, 1976), whilst *map* mutations release sulphur metabolism from the control of methionine-mediated regulatory systems (Nadolska-Lutyk *et al.*, 1989). It would be expected that if penicillin biosynthesis in *A. nidulans* was subject to regulation at the level of sulphur metabolism, then these strains would produce altered quantities of penicillin biosynthetic gene transcripts in comparison to the wild type. However, the sulphur regulatory mutations studied here appear to have little effect on the production of penicillin, but this may be because the strains were grown in fermentation medium, i.e. under conditions derepressed for penicillin biosynthesis.

4.3.3.4 pH regulatory mutations

A. nidulans pH regulatory mutant strains G0156 and G415 carrying the mutations *pacC*5 and *palC*4 respectively, both produced *ipnA* and *acyA* transcripts at similar or slightly reduced levels compared to the wild type strain. The *palC*4 mutant gave a lower level of *acvA* mRNA, whilst the *pacC*5 strain produced an increased amount of this transcript compared to the wild type strain (Fig. 17).

pacC and *palC* mutations appear to affect primarily the pH regulatory mechanism. Mutation in the *pacC* gene results in an abnormally acidic internal pH and mimics

the effects of growth of the organism at alkaline pH. In contrast, mutation in the *palC* gene results in an abnormally alkaline internal pH, thus mimicking acidic growth conditions (Caddick *et al.*, 1986). It has been shown that production of penicillin G by *A. nidulans* is subject to regulation by the pH of the growth medium, and wild type strains synthesise more penicillin at alkaline than at acid pH, with the optimum pH being 8 (Shah *et al.*, 1991; M.W. Adlard, personal communication). This form of regulation could occur at the level of transcription of the ACVS gene because in the *pacC*5 mutant, *acvA* mRNA levels are increased whereas in the *palC*4 mutant the amount of *acvA* transcript is decreased in comparison to those of the wild type. Similar quantities of *ipnA* and *acyA* transcripts were present in both the *pacC*5 and *palC*4 mutant strains compared with wild type levels (Fig. 17).

4.3.4 Effect of Various Carbon and Nitrogen Sources on the Expression of the Penicillin Biosynthetic Genes

In order to determine the effects of carbon catabolism and nitrogen metabolism on the regulation of penicillin biosynthesis, northern blot analysis was carried out on total RNA prepared from *A. nidulans* strains grown for 48 h (unless otherwise stated) in fermentation medium or minimal medium containing various carbon and nitrogen sources. The fermentation medium sample (i.e. derepressed conditions) was used as a

standard to check that hybridisations were reliable and to give an indication of the effect of the various carbon and nitrogen sources on the quantity of message produced.

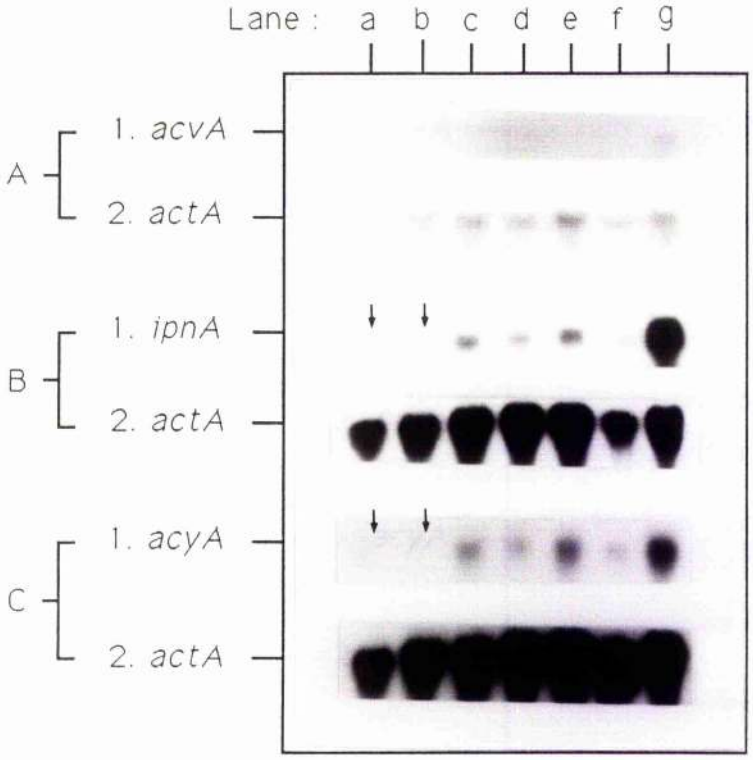
4.3.4.1 Effect of 1 % glucose or 1 % lactose with 10 mM proline or 5 mM ammonium tartrate as carbon and nitrogen sources

As a preliminary experiment, *A. nidulans* wild type (*biA*1) was grown for 24 h and 48 h in minimal medium containing 1 % glucose as the carbon source and 5 mM ammonium tartrate (≡ 10 mM ammonium ion concentration) or 10 mM proline (≡ 10 mM ammonium ion concentration) as the nitrogen source; and 48 h in minimal medium containing 1 % lactose as the carbon source with 5 mM ammonium tartrate or 10 mM proline as the nitrogen source. The *acvA* mRNA was detected in the fermentation medium sample but not in any of the minimal media tested, although detection of the *actA* gene indicated that RNA was indeed transferred from the gel to the filter during blotting. *ipnA* and *acyA* messages were detected in all the media tested (Fig. 20.I). When 1 % glucose was used as the carbon source, approximately equal quantities of transcripts were observed after 24 h and 48 h incubation when both 5 mM ammonium tartrate and 10 mM proline were used as the nitrogen source. On the other hand, when lactose was used as the carbon source, insufficient mycelium had grown within 24 h to be able to prepare RNA, therefore 48 h was chosen as a suitable time point at

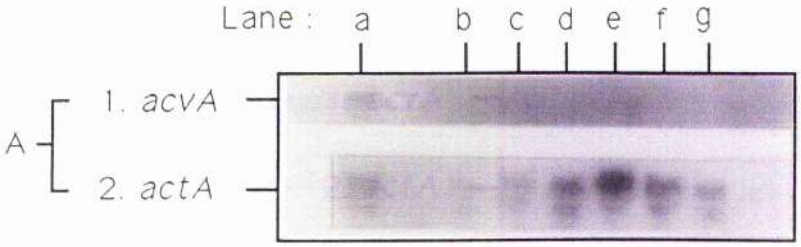
Fig. 20.I Northern blots of total RNA extracted from *A. nidulans* wild type (*biA*1) grown in a variety of media at 26^{o}C, with orbital shaking at 250 rpm, for varying lengths of time. Lanes a to f - minimal medium pH 5.2 containing 1 % lactose and 5 mM ammonium tartrate for 48 h (lane a), 1 % lactose and 10 mM proline for 48 h (lane b), 1 % glucose and 5 mM ammonium tartrate for 24 h (lane c) and 48 h (lane d), 1 % glucose and 10 mM proline for 24 h (lane e) and 48 h (lane f); lane g - fermentation medium pH 5.2. Blots were hybridised to the following homologous gene-specific probes: Panel A.1, *acvA*; Panel B.1, *ipnA*; and Panel C.1, *acyA*. In addition, each blot was stripped and back-hybridised to the *A. nidulans actA* gene-specific probe (Panels A.2, B.2 and C.2) to determine the extent of uniformity of RNA concentration in each lane. Prehybridisation, hybridisation, washing of filters and autoradiography was as described in Materials and Methods. Bands present on the original autoradiograph but undetectable in the photograph are indicated by arrows.

Fig. 20.II As Fig. 20.I, Panel A, but with three times the concentration of RNA loaded onto each lane, and with an altered lane order as follows: lane a - fermentation medium pH 5.2 for 24 h; lanes b to g - minimal medium pH 5.2 containing 1 % lactose and 5 mM ammonium tartrate for 48 h (lane b), 1 % lactose and 10 mM proline for 48 h (lane c), 1 % glucose and 5 mM ammonium tartrate for 24 h (lane d) and 48 h (lane e), 1 % glucose and 10 mM proline for 24 h (lane f) and 48 h (lane g). Blots were hybridised to the following homologous gene-specific probes: Panel A.1, *acvA*; Panel B.1, *ipnA*; and Panel C.1, *acyA*. In addition, each blot was stripped and back-hybridised to the *A. nidulans actA* gene-specific probe (Panels A.2, B.2 and C.2) to determine the extent of uniformity of RNA concentration in each lane. Prehybridisation, hybridisation, washing of filters and autoradiography was as described in Materials and Methods.

I.


Lane : a b c d e f g
A
1. acvA
2. actA
B
1. ipnA
2. actA
C
1. acyA
2. actA

II.


Lane : a b c d e f g
A
1. acvA
2. actA

which to study the effect of various factors on the expression of the penicillin biosynthetic genes.

Glucose and ammonium were classically thought to repress penicillin biosynthesis (reviewed by Martin and Aharonowitz, 1983) whereas lactose and proline were thought to lead to derepression of penicillin biosynthesis. However, greater quantities of *ipnA* and *acyA* mRNA were seen in each case where 1 % glucose was used as the carbon source compared to 1 % lactose, and when 5 mM ammonium tartrate compared to 10 mM proline was used as the nitrogen source in 1 % lactose minimal medium. In no case though, was the quantity of mRNA produced in minimal medium as great as that produced in fermentation medium (Fig. 20.I). In an attempt to circumvent any possibility that *acvA* mRNA was not detected simply due to its low abundance, an additional gel was run which was identical to that in Fig. 20.I except that each RNA sample was loaded at three times the usual concentration (i.e. 30 μg loaded instead of 10 μg). The gel was then northern blotted and hybridised with the *acvA*-specific probe, however again no *acvA* transcript was detected (Fig. 20.II). This may be because 1 % lactose grown cultures are slower growing than 1 % glucose grown cultures, and ammonium tartrate is a richer nitrogen source and sustains better growth than proline, when a poor carbon source is used. It could be therefore that the comparatively slower growing cultures are at an earlier stage in their life cycle and hence produce less

mRNA at the time point sampled, than in cultures at a later stage in their life cycle. As a preliminary experiment to clarify this point, northern blot analysis of a time course of the wild type strain in minimal medium containing 1 % glucose and 5 mM ammonium tartrate (≡ 10 mM ammonium ion concentration) was attempted. However, the batch cultures in shake flasks quickly became exhausted of nutrients and strains began to lyse after approximately 2 days incubation. Thus a time course could not be carried out without replenishing the exhausted nutrients to prevent lysis of the strains. However, this would not give a representative picture of the pattern of mRNA synthesis upon exhaustion of carbon and nitrogen sources which may regulate transcription of the genes being studied.

4.3.4.2 Effect of 1 % glucose and 10 mM ammonium tartrate; or 1 % glucose and 10 mM ammonium tartrate switched to 1 % glucose and 10 mM proline; as carbon and nitrogen sources

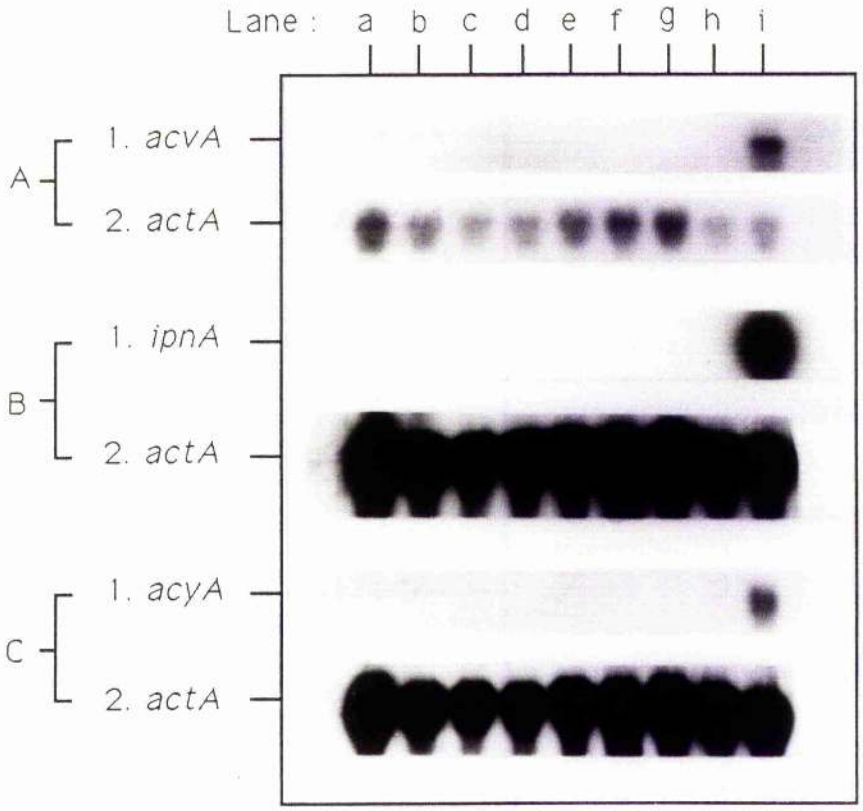
In an attempt to determine whether or not penicillin biosynthesis is subject to carbon catabolite and nitrogen metabolite regulation, northern blot analysis was carried out on *A. nidulans* wild type (*biA*1), *areA*19, *xprD*1, *creA*1 and *creC*27 strains grown in minimal medium containing 1 % glucose and 10 mM ammonium tartrate (≡ 20 mM ammonium ion concentration), and in addition, wild type, *areA*19 and *xprD*1 strains switched from this medium to minimal medium

containing 1 % glucose and 10 mM proline for 5 h. All three penicillin biosynthetic gene transcripts were detected in the control wild type strain grown in fermentation medium. No *acvA*, *ipnA* or *acyA* transcripts were detected, however, for any of these strains in any of the minimal media tested, yet the presence of *actA* bands for each sample indicated that RNA was present in each lane blotted (Fig. 21).

If penicillin biosynthesis is subject to nitrogen metabolite and carbon catabolite repression, it would be expected that *xprD1*, *creA1* and *creC27* strains would show derepressed expression and hence produce elevated levels of *acvA*, *ipnA* and *acyA* transcripts in comparison to that of wild type strains minimal medium and in fermentation medium. Furthermore, the *areA19* mutant strain would be repressed for penicillin biosynthesis in fermentation medium and in minimal medium containing 1 % glucose and 10 mM ammonium tartrate. Conversely, when grown in minimal medium with a rich nitrogen source such as 10 mM ammonium tartrate, then switched to that with a poor nitrogen source such as 10 mM proline, the *areA19* strain would be expected to show derepressed expression of the penicillin biosynthetic gene transcripts (see sections 4.3.3.1 and 4.3.3.2 for further explanation).

The pattern of expression of the penicillin biosynthetic gene transcripts obtained was somewhat unexpected, especially as *ipnA* and *acyA* transcripts were detected in minimal medium containing 1 % glucose and 5

Fig. 21 Northern blots of total RNA extracted from a number of *A. nidulans* mutant strains and wild type (*biA*1) grown in a variety of media at 26°C, with orbital shaking at 250 rpm, for varying lengths of time. Lanes a and b - wild type (*biA*1) (lane a) and mutant strain *areA*19 (lane b) grown in minimal medium pH 5.2 containing 1 % glucose and 10 mM ammonium tartrate for 48 h, switched to minimal medium pH 5.2 containing 1 % glucose and 10 mM proline for 5 h; lanes c to f - wild type (*biA*1) (lane c) and mutant strains *areA*19 (lane d), *xprD*1 (lane e) and *creA*1 (lane f) grown in minimal medium pH 5.2 containing 1 % glucose and 10 mM ammonium tartrate for 48 h; lanes g and h - mutant strain *creC*27 (lane g) and wild type (*biA*1) (lane h) grown in minimal medium pH 5.2 containing 1 % glucose and 10 mM ammonium tartrate supplemented with 100 mM arginine for 48 h; lane i - wild type (*biA*1) grown in fermentation medium pH 5.2 for 24 h. Blots were hybridised to the following homologous gene-specific probes: Panel A.1, *acvA*; Panel B.1, *ipnA*; and Panel C.1, *acyA*. In addition, each blot was stripped and back-hybridised to the *A. nidulans actA* gene-specific probe (Panels A.2, B.2 and C.2) to determine the extent of uniformity of RNA concentration in each lane. Prehybridisation, hybridisation, washing of filters and autoradiography was as described in Materials and Methods.


Lane : a b c d e f g h i
A
1. acvA
2. actA
B
1. ipnA
2. actA
C
1. acyA
2. actA

mM ammonium tartrate (see section 4.3.4.1). However, a similar lack of such transcripts was observed in minimal medium containing 1 % glucose and 10 mM ammonium tartrate in section 3.17. It therefore appears that increasing the concentration of ammonium tartrate from 5 mM to 10 mM (equivalent to an increase in ammonium ions from 10 mM to 20 mM) completely inhibits transcription of the penicillin biosynthetic genes in each of the strains tested, and that penicillin biosynthesis is, in fact, subject to nitrogen metabolite regulation. This high concentration of ammonium appears to be over-riding any derepression of nitrogen metabolite regulation occurring in the *xprD*1 nitrogen metabolism derepressed strain, or in the *areA*19 strain when switched to the nitrogen metabolite derepressed medium.

4.3.4.3 Effect of 1 % glucose and 5 mM ammonium tartrate; 3.5 % lactose and 10 mM proline; or 3.5 % lactose and 0.01 % casamino acids as carbon and nitrogen sources

Northern blot analysis was carried out on total RNA prepared from *A. nidulans* wild type (*biA*1) grown for 48 h in minimal medium containing 1 % glucose and 5 mM ammonium tartrate as the carbon and nitrogen sources respectively; or 3.5 % lactose as the carbon source and 10 mM proline or 0.01 % casamino acids as the nitrogen source; in order to ascertain whether 3.5 % lactose, proline or casamino acids would lead to derepressed

expression of the penicillin biosynthetic genes. In this case, 3.5 % lactose was used as the carbon source compared to 1 % lactose used in section 4.3.4.1 because 3.5 % is the concentration of lactose added to fermentation medium and it may be that this quantity is required to give sufficient growth of the mycelium and relieve any carbon catabolite repression. Proline and casamino acids were each used as the nitrogen source since it was thought that they might relieve any nitrogen metabolite repression which may be operating. *acvA* mRNA was detected only in the fermentation medium sample although probing for actin confirmed that RNA had in fact been transferred on to the filter probed. Transcripts were detected for *ipnA* and *acyA* in very low amounts in each of the samples tested, though at reduced quantities compared to that found in fermentation medium (Fig. 18). Expression of these genes did not appear to be significantly affected when lactose, proline or casamino acids were used compared to glucose or ammonium tartrate, although the lactose grown cultures again grew very slowly compared to the glucose grown cultures and it could be that the effects of temporal regulation on gene expression are being observed.

4.3.4.4 Effect of 1 % sodium acetate and 5 mM ammonium tartrate as carbon and nitrogen sources

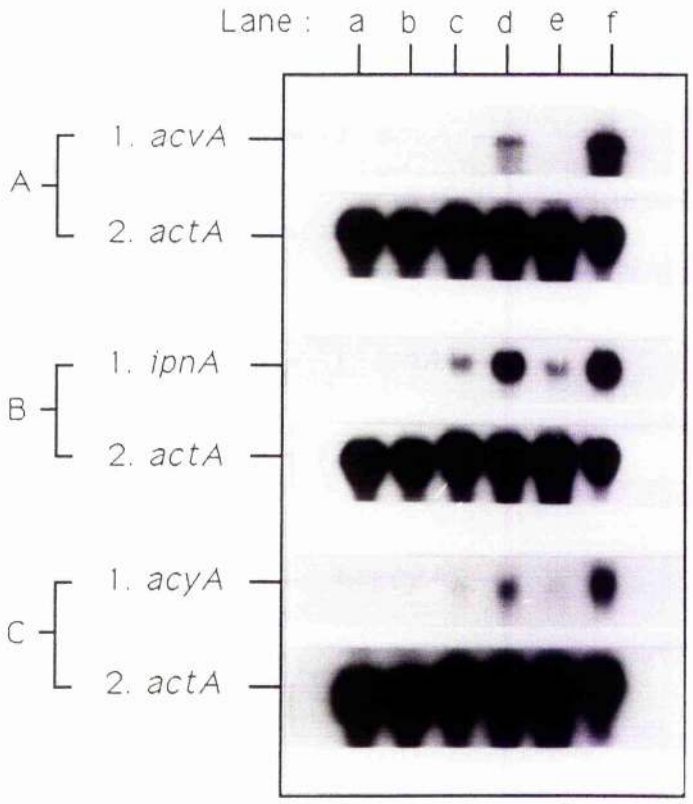
Northern blot analysis was carried out on total RNA prepared from *A. nidulans* wild type (*biA*1) grown for 48 h

in minimal medium containing 1 % sodium acetate and 5 mM ammonium tartrate as the carbon and nitrogen sources respectively. All three penicillin biosynthetic gene transcripts were detected in this medium, although in very small amounts in comparison to the sample in fermentation medium (Fig. 22). This was the only unsupplemented minimal medium tested where a band was detected corresponding to the *acvA* signal. It therefore appears that acetate stimulates the production of penicillin, possibly by allowing expression of the ACVS gene. This is confirmed by the fact that of all the various minimal media described in section 4.3.4 used to grow cultures from which RNA was prepared, this was the only one in which ß-lactam activity was detected during bioassay of the resulting culture supernatant. Conversely, ß-lactam activity was detected in the culture supernatant of the *A. nidulans* wild type (*biA*1) strain, when grown in fermentation medium.

4.3.5 Effect of Various Additions to Minimal Medium on the Expression of the Penicillin Biosynthetic Genes

In order to determine whether or not expression of the penicillin biosynthetic genes is induced by the presence of the amino acid precursors of penicillin; the side-chains of penicillin G and V; methionine; lysine; and acetate; northern blot analysis was carried out on *A. nidulans* wild type (*biA*1) grown for 48 h in various minimal media to which had been added L-α-aminoadipic

Fig. 22 Northern blots of total RNA extracted from *A. nidulans* wild type (*biA*1) grown in a variety of media at 26°C, with orbital shaking at 250 rpm, for 48 h. Lanes a, b, d, and e - minimal medium pH 5.2 containing 1 % glucose and 5 mM ammonium tartrate, supplemented with 1 mM lysine (lane a), 1 mM methionine (lane b), 1 % sodium acetate (lane d) and unsupplemented (lane e); lane c - minimal medium pH 5.2 containing 1 % sodium acetate and 5 mM ammonium tartrate; and lane f - fermentation medium pH 5.2. Blots were hybridised to the following homologous gene-specific probes: Panel A.1, *acvA*; Panel B.1, *ipnA*; and Panel C.1, *acyA*. In addition, each blot was stripped and back-hybridised to the *A. nidulans actA* gene-specific probe (Panels A.2, B.2 and C.2) to determine the extent of uniformity of RNA concentration in each lane. Prehybridisation, hybridisation, washing of filters and autoradiography was as described in Materials and Methods.


Lane : a b c d e f
A
1. acvA
2. actA
B
1. ipnA
2. actA
C
1. acyA
2. actA

acid, L-cysteine and L-valine; phenylacetic acid (PAA) and phenoxyacetic acid (PXA); methionine; lysine; and sodium acetate; respectively.

4.3.5.1 Effect of the amino acid precursors of penicillin

A. nidulans wild type (*biA*1) was grown in minimal medium containing 1 % glucose and 5 mM ammonium tartrate; 3.5 % lactose and 10 mM proline; and 3.5 % lactose and 0.01 % casamino acids; as the carbon and nitrogen sources, respectively, which had been supplemented with 1 mM L-α-aminoadipic acid, 1 mM L-cysteine and 1 mM L-valine. No *acvA* mRNA was detected in cells grown in any of the media tested here, however, a very faint *acyA* message was detected in each case (see Fig. 18). No *ipnA* message was seen when 1 % glucose and 5 mM ammonium tartrate were used as the carbon and nitrogen sources respectively, although an *actA* band was detected for this sample. A very faint *ipnA* mRNA signal was observed in the other media tested. Apart from the absence of the *ipnA* band in 1 % glucose and 5 mM ammonium tartrate minimal medium supplemented with precursors, the reason for which is as yet unclear, no significant difference was observed between the levels of transcripts found in minimal media supplemented with such precursors from those found in the equivalent unsupplemented minimal media (Fig. 18). The use of various carbon and nitrogen sources was in an attempt to circumvent any interference in induction of

the penicillin biosynthetic genes by carbon catabolite and nitrogen metabolite repression.

4.3.5.2 Effect of the side-chains of penicillin G and penicillin V

A. nidulans wild type (*biA*1) was grown in minimal medium containing 1 % glucose and 5 mM ammonium tartrate; 3.5 % lactose and 10 mM proline; and 3.5 % lactose and 0.01 % casamino acids, respectively, which had been supplemented with 2 mM phenylacetic acid (PAA) and 2 mM phenoxyacetic acid (PXA), the side-chains of penicillin G and V respectively. *acvA* mRNA was detected only in the minimal medium containing 3.5 % lactose, 0.01 % casamino acids and side-chains, but at a much reduced level compared to the fermentation medium sample (see Fig. 18). No *acvA* transcript was detected in the corresponding unsupplemented minimal medium. Both *ipnA* and *acyA* transcripts were detected in all the media tested, with apparently no significant difference observed between the amounts of message found in the corresponding unsupplemented minimal media (Fig. 18). ß-lactam activity was observed very weakly in the culture supernatant of the sample grown in minimal medium containing 3.5 % lactose and 0.01 % casamino acids plus side-chains, but was undetected in any of the other media tested here. The penicillin G and V side chain precursors PAA and PXA respectively, appear to stimulate penicillin production possibly by inducing expression of *acvA*, but only in one

of the minimal media tested here, that containing 3.5 % lactose and 0.01 % casamino acids.

4.3.5.3 Effect of acetate

A. nidulans wild type (*biA*1) was grown in minimal medium containing 1 % glucose and 5 mM ammonium tartrate as the carbon and nitrogen sources respectively, supplemented with 1 % sodium acetate. Transcripts were observed for each of the penicillin biosynthetic genes in this medium. The *acvA* mRNA species was present in a significant amount, as were the *ipnA* and *acyA* transcripts which were present in much greater quantities than were observed in the corresponding unsupplemented minimal medium (Fig. 22). ß-lactam activity was also observed in the supernatant of the sodium acetate supplemented culture during bioassay, but not in the unsupplemented culture. It therefore appears that the addition of sodium acetate to minimal medium stimulates penicillin production, inducing expression of the ACVS gene and increasing the expression of the IPNS and ACYT genes.

4.3.5.4 Effect of methionine and lysine

A. nidulans wild type (*biA*1) was grown in minimal medium containing 1 % glucose and 5 mM ammonium tartrate as the carbon and nitrogen sources respectively, supplemented with 1 mM methionine or 1 mM lysine. No *acvA*, *ipnA* or *acyA* mRNA was observed in any of the media tested, although *actA* transcripts were detected for each

sample indicating that the RNA had indeed been blotted onto the filter (Fig. 22). *ipnA* and *acyA* transcripts were detected in the corresponding unsupplemented minimal media samples which could suggest that methionine and lysine are repressing the expression of these genes (Fig. 22).

It should be noted that the northern blot and hybridisation experiments described above were preliminary attempts to determine the effect on transcription of the penicillin biosynthetic genes, of growing *A. nidulans* strains with different genetic backgrounds under different physiological conditions.

Due to time limitations, and the laborious and time-consuming nature of these experiments, each was carried out only once, but would have to be repeated before any meaningful conclusions could be drawn from the results. The above experiments were only semi-quantitative, and for a more quantitative analysis of the data, densitometry could be carried out on the autoradiographs obtained. Alternatively, as gene-specific probes are available, more accurate dot blot and hybridisation analysis of the RNA samples could be performed, followed by densitometry of the resulting autoradiographs.

Additionally, in northern blot and hybridisation experiments carried out using mutants with various genetic backgrounds, where only one allele of a given

locus was studied, the results obtained could be specific to that particular allele and not characteristic of that mutation. Therefore, such experiments would have to be repeated using a number of mutant strains harbouring different alleles of the same locus in order to determine whether the results were allele-specific or mutation-specific.

CHAPTER 5

CONCLUSIONS

Clones of *A. nidulans* genomic DNA (pSTA200, pSTA201 and pSTA207) spanning 20 kb have been isolated and determined by a combination of classical and physical genetic means to represent the *npeA* locus, which has been identified, by the data presented here, to contain at least one gene, designated *ipnA*, which encodes IPNS, the second enzyme of the penicillin biosynthetic pathway. pSTA201, which harbours *ipnA*, was found to integrate at the resident IPNS genomic site in the majority of transformants analysed by Southern hybridisation. Significantly, transformants, which on the basis of Southern blotting experiments appeared to have integrated at the IPNS site, were found by formal genetic analysis to carry integrates at the *npeA* locus. Mutant strain *npeA*0049/1 is deleted in the IPNS region which further supports the notion that the IPNS region and the *npeA* locus are counterparts. The *ipnA* mRNA was found to be 1.4 kb which is in accord with the size of *A. nidulans* transcript expected from the *A. nidulans* IPNS protein data of Ramos *et al.* (1985) and inferred polypeptide length of 331 amino acids (Ramon *et al.*, 1987).

An additional gene required for penicillin biosynthesis is located 5′ of *ipnA*. Mutant strain *npeA*0022, which is defective in ACVS activity (Makins *et*

al., 1981; H. van Liempt, personal communication), was found to have an alteration in its DNA sequences between 3 kb and 6 kb upstream of IPNS coding sequences. pSTA207 does not include *ipnA* yet it complements *npeA*0022 at a relatively high frequency (39 %), which could imply that it harbours an intact penicillin biosynthetic gene. Furthermore, *lacZ* fusion studies suggest that sequences leading into this putative gene give transcriptional activity and that the gene is transcribed 3'-5' with respect to the direction of *ipnA* transcription. Transformation of a penicillin-producing strain by a plasmid carrying an incomplete penicillin biosynthetic gene lacking both termini would lead to the loss of penicillin production in a high frequency of transformants, due to disruption of the functional gene as a consequence of homologous integration. This was indeed observed following transformation of an *npeA*$^{+}$ strain with pSTA807. Sequence conservation between ß-lactam producing fungi detected in the region designated C (Fig. 8.III) may be indicative of some evolutionary conservation of DNA sequences, and hence the presence of another gene. The sequences 5' of IPNS hybridised to mRNA species of greater than 9.5 kb from cells grown under conditions which lead to the derepression of penicillin biosynthesis. This transcript was of sufficient length to encode the *A. nidulans* ACVS protein, estimated to be a monomer of 422 kDa (MacCabe *et al.*, 1991), suggesting that this region contains the gene, designated *acvA*,

which encodes ACVS, the first enzyme of the penicillin biosynthetic pathway. Hybridisation of a 23-base mixed oligomer probe, derived from ACVS amino acid sequence data,to a fragment of pSTA201, gave definitive proof of the identification and location of *acvA*. In addition, subsequent DNA sequencing of this hybridising region showed the presence of coding potential for the amino terminal amino acid sequence of the 20 kDa CNBr polypeptide fragment from which the oligomer probe was derived, thus confirming that the *acvA* gene indeed encodes ACVS and is transcribed in the opposite orientation to *ipnA*. The entire *acvA* gene of *A. nidulans* has been sequenced (MacCabe *et al.*, 1991) and further positive identification of these sequences as the ACVS structural gene was achieved by matching amino acid sequences determined from polypeptides generated from the purified ACVS protein with those predicted from the nucleotide sequence. This also provides additional verification of the reading frame deduced from the *acvA* DNA sequence.

Evidence indicates that a third gene required for penicillin biosynthesis is also located at the *npeA* locus. The *P. chrysogenum* gene for ACYT strongly cross-hybridises to a single fragment located 3′ to *A. nidulans ipnA*, clearly indicating that the gene, designated *acyA*, which encodes ACYT, the third enzyme of the penicillin biosynthetic pathway, is found at the *npeA* locus in *A. nidulans*. A 1.6 kb mRNA is observed in derepressed cells

but not in repressed cells, and it is likely that this transcript represents the *acyA* message. The transcript size is in reasonable accord with the inferred polypeptide length of 357 amino acids reported for the *A. nidulans* ACYT gene (Tobin *et al.*, 1990), and this 1.6 kb transcript is suggested to represent the *acyA* message.

The IPNS gene has been cloned from several microorganisms by various groups. Three are derived from fungal strains: *C. acremonium* (Samson *et al.*, 1985), *P. chrysogenum* (Barredo *et al.*, 1989a; Carr *et al.*, 1986; Diez *et al.*, 1989, 1990; Smith *et al.*, 1990a) and *A. nidulans* (MacCabe *et al.*, 1990; Ramon *et al.*, 1987; Smith *et al.*, 1990a; Veenstra *et al.*, 1989; Weigel *et al.*, 1988); six from Gram-positive microorganisms: *S. clavuligerus* (Leskiw *et al.*, 1988; Smith *et al.*, 1990a), *S. jumojinensis* (Shiffman *et al.*, 1988), *S. lipmanii* (Shiffman *et al.*, 1988; Weigel *et al.*, 1988), *S. griseus* (Garcia-Dominguez *et al.*, 1991) and *N. lactamdurans* (Coque *et al.*, 1991); and one from a Gram-negative unicellular prokaryote *Flavobacterium* sp SC 12,154 (Shiffman *et al.*, 1990; Smith *et al.*, 1990a).

Our estimate of the size of the *A. nidulans ipnA* transcript size of 1.4 kb is in reasonable accord with the size of transcripts obtained from *P. chrysogenum* (Diez *et al.*, 1989, 1990) and *C. acremonium* (Gutierrez *et al.*, 1991) and good accord with the size of *P. chrysogenum* mRNA obtained by Veenstra *et al.* (1989) and Smith *et al.* (1990a); and also the transcript size

expected from the IPNS protein data and inferred polypeptide sizes from the various organisms described above.

After our work on the cloning of the *acvA* gene and characterisation *npeA* locus of *A. nidulans* was completed and published, papers were published which described the cloning of the ACVS genes of *A. nidulans* (Smith *et al.*, 1990a), *P. chrysogenum* (Diez *et al.*, 1990; Smith *et al.*, 1990a), *C. acremonium* (Gutierrez *et al.*, 1991), *S. clavuligerus* (Smith *et al.*, 1990a), *N. lactamdurans* (Coque *et al.*, 1991) and *Flavobacterium* sp SC 12,154 (Smith *et al.*, 1990a). In each case, our estimate of the size of the *A. nidulans acvA* message of greater than 9.5 kb, was in good accord with the size of the ACVS gene mRNA obtained from *P. chrysogenum* (Diez *et al.*, 1990; Smith *et al.*, 1990a) and *C. acremonium* (Gutierrez *et al.*, 1991); and the transcript size expected from the ACVS protein data and inferred polypeptide sizes from *A. nidulans* (MacCabe *et al.*, 1991), *P. chrysogenum* (Diez *et al.*, 1990; Smith *et al.*, 1990a), *C. acremonium* (Gutierrez *et al.*, 1991) and *N. lactamdurans* (Coque *et al.*, 1991).

The ACYT gene has been cloned from two penicillin producing fungal strains: *A. nidulans* (MacCabe *et al.*, 1990; Tobin *et al.*, 1990) and *P. chrysogenum* (Barredo *et al.*, 1989b; Diez *et al.*, 1990; Smith *et al.*, 1990a; Veenstra *et al.*, 1989). Our estimate of the size of *A. nidulans acyA* mRNA of 1.6 kb was in reasonable accord with the size of ACYT gene transcript in *C. acremonium*

(Smith *et al.*, 1990c) and in *P. chrysogenum* as estimated by Smith *et al.* (1990a), Tobin *et al.* (1990) and Veenstra *et al.* (1989); and but a larger discrepancy exists between our size estimate and that for the *P. chrysogenum acyA* message estimated by Barredo *et al.* (1989b) and Diez *et al.* (1989, 1990). Our estimated *A. nidulans acyA* mRNA size of 1.6 kb is also in reasonable agreement with that expected from the protein data and inferred polypeptide length reported for *A. nidulans* (Tobin *et al.*, 1990) and *P. chrysogenum* (Barredo *et al.*, 1989b; Tobin *et al.*, 1990) ACYT.

We have no explanation for the discrepancies between the difference in the estimates of transcript sizes of *acvA, ipnA* and *acyA* obtained by the different groups.

The *A. nidulans npeA* locus, therefore, represents a complex gene cluster which contains the ACVS, IPNS and ACYT genes, respectively, in the genetic order *acvA-ipnA-acyA*. The biochemical and genetic relationships of the pathway are represented in Fig. 9. This situation is rather unusual, but not unique, for lower eukaryotes (for reviews: Arst, 1984; Gurr *et al.*, 1987; Rambosek and Leach, 1987) e.g. the ß-lactam biosynthetic genes are closely linked in *S. clavuligerus* (Coque *et al.*, 1991; Ingolia and Queener, 1989; Miller and Ingolia, 1989a, 1989b; Smith *et al.*, 1990a), *N. lactamdurans* (Coque *et al.*, 1991) and *Flavobacterium* sp SC 12,154 (Smith *et al.*, 1990a). In filamentous fungi, structural genes of closely related fungi are often not closely linked but are

instead distributed, almost at random, throughout the fungal genome. Several exceptions to this general pattern exist in *A. nidulans* where gene clusters are found which are comprised of several tightly linked genes such as the seven *qut* genes for quinic acid breakdown, the four *prn* genes for protein breakdown, and the smaller *alcC-alcR* alcohol cluster for ethanol utilisation (reviewed by Gurr *et al.*, 1987). In addition, *A. nidulans* contains a nitrate cluster (*crnA-niiA-niaD*) for nitrate assimilation in which the nitrate reductase (*niaD*) and the nitrite reductase (*niiA*) genes are transcribed in opposite orientations from a common promoter (Johnstone *et al.*, 1990). The *A. nidulans acvA* and *ipnA* genes are divergently transcribed from an intergenic region of 872 nucleotides (MacCabe *et al.*, 1991). The direction of *ipnA* transcription has been genetically determined (Ramon *et al.*, 1987), whilst evidence that *acvA* is transcribed in the opposite orientation comes from the *lacZ* fusion data and comparison of *acvA* nucleotide and ACVS amino acid sequence data. The *A. nidulans acyA* is transcribed in the same direction i.e. 5′-3′ with respect to the direction of transcription of *ipnA*, and this has been confirmed by Tobin *et al.* (1990). The penicillin biosynthetic genes have also been shown to be clustered in *P. chrysogenum* in the same genetic order and orientation as in *A. nidulans* (Burnham *et al.*, 1989; Diez *et al.*, 1989; Smith *et al.*, 1990a, 1990b; Veenstra *et al.*, 1989). In addition, the ACVS and IPNS genes for early cephalosporin biosynthesis

have been found to be clustered in *C. acremonium*, in the same orientation as in *A. nidulans* and *P. chrysogenum* (Gutierrez *et al.*, 1991). The ACVS and IPNS genes of the prokaryotes *N. lactamdurans* and *S. clavuligerus* are also clustered together but are transcribed in the same orientation relative to each other (Coque *et al.*, 1991) i.e. the opposite orientation relative to those of the fungal genes, and in *N. lactamdurans, acvA* and *ipnA* appear to be expressed as a single transcript (J.R.R. Coque, P. Liras and J.F. Martin, unpublished).

Extensive amino acid and DNA sequence similarity between IPNS-encoding genes from the prokaryotes *S. clavuligerus* (Leskiw *et al.*, 1988), *S. lipmanii* (Weigel *et al.*, 1988), *S. jumojinensis* (Shiffman *et al.*, 1991), *S. griseus* (Garcia-Dominguez *et al.*, 1991), *N. lactamdurans* (Coque *et al.*, 1991) and *Flavobacterium* sp SC 12,154 (Shiffman *et al.*, 1991); and those from the eukaryotes *C. acremonium* (Samson *et al.*, 1985), *P. chrysogenum* (Carr *et al.*, 1986) and *A. nidulans* (Ramon *et al.*, 1987; Weigel *et al.*, 1988), suggests a close evolutionary relationship between these genes. It has been proposed from DNA sequence comparisons, that this gene first arose in the prokaryotes, possibly in *Streptomyces* species, and was horizontally transferred to a eukaryotic progenitor of those filamentous fungi which synthesise ß-lactam antibiotics after the eukaryotes and prokaryotes diverged (Carr *et al.*, 1986; Leskiw *et al.*, 1988; Ramon *et al.*, 1987; Weigel *et al.*, 1988). Further

evidence exists to corroborate that the IPNS gene first arose in *Streptomyces* and was transferred to the filamentous fungi. The *C. acremonium* and *P. chrysogenum* *ipnA* genes have an unusually high G/C content with respect to other fungal genes, and *Streptomyces* species are known to have a high G/C content in their genome (Carr *et al.*, 1986). Additionally, *Streptomyces* species contain the most extensive ß-lactam biosynthetic pathways, whereas the fungal pathways are truncated versions of the bacterial pathways and ß-lactam biosynthetic genes are dispersed in the *C. acremonium* genome (Skatrud and Queener, 1989) but physically linked in the *S. clavuligerus* genome (Smith *et al.*, 1990a) thus it is more likely that initially linked genes were scattered after transfer than dispersed genes being brought together and transferred as a single unit. Also, to avoid autotoxicity, *Streptomyces* species have specific resistance genes clustered together with genes of the antibiotic biosynthetic pathway which are absent from fungi which are not sensitive to ß-lactam antibiotics. It is therefore unlikely that the pathway was transferred from fungi to bacteria without simultaneous transfer of the resistance gene, and the likelihood is that this gene arose with the biosynthetic genes in *Streptomyces*.

The fungi perhaps acquired part of the ß-lactam biosynthetic pathway from *Streptomyces*: *C. acremonium* expresses most of the pathway, whereas *P. chrysogenum* and *A. nidulans* express only the first few steps. Results

presented here, showing that *acvA*, *ipnA* and *acyA* are tightly linked, might suggest that the genes for the whole penicillin biosynthetic pathway were transferred to the filamentous fungi as a single unit. ß-lactam producing *Streptomyces* species however, do not appear to produce penicillin G or V, which may suggest that they lack ACYT activity and hence the *acyA* gene. It is therefore possible that *acyA* subsequently evolved, or was sequestered from another cell function. in penicillin-producing fungi, or that it was lost from *Streptomyces*, after the transfer of *acvA* and *ipnA*. Cloning and sequence analysis of the ACYT genes from *A. nidulans* (Tobin *et al.*, 1990) and *P. chrysogenum* (Barredo et al., 1989b; Tobin *et al.*, 1990) has revealed extensive homology between the genes at the DNA and predicted amino acid sequence level and, unlike all other penicillin biosynthetic genes, *acyA* contains three introns, found at identical positions in both ACYT genes, which suggests that the ACYT gene may have evolved from a eukaryotic progenitor (Tobin *et al.*, 1990). This would require that the close linkage of the ACVS and IPNS genes to that of ACYT was selected after the transfer of *acvA* and *ipnA* to penicillin-producing fungi which is difficult to visualise, but might imply that tight linkage of the three penicillin biosynthetic genes gives a selective advantage to the organism. Alternatively, it has been suggested that *acyA* may be of prokaryotic origin (Barredo *et al.*, 1989b) or part prokaryotic and part eukaryotic

origin (P.L. Skatrud, personal communication). A somewhat analogous situation exists in *C. acremonium* in which the *cefEF* and *cefG* genes are closely linked forming a cluster of "late" cephalosporin biosynthetic genes encoding the enzymes that carry out the final three steps of cephalosporin biosynthesis (Fig. 2). It has been proposed that the intron-less *cefEF* gene originated from the *Streptomyces* but the *cefG* gene, which contains two introns, is of eukaryotic origin (reviewed by Martin and Gutierrez, 1992).

If the horizontal transfer hypothesis is correct and if all of the ß-lactam biosynthetic genes were transferred simultaneously, the same high degree of sequence identity would be expected to be displayed in other ß-lactam biosynthetic genes in widely different microorganisms as is found for the IPNS genes. Indeed, the genes encoding deacetoxy-cephalosporin C synthetase (DAOCS) activity from *C. acremonium* (*cefEF*) and *S. clavuligerus* (*cefE*) are similar and have been shown to possess almost the same percentage similarity at the DNA and predicted amino acid level as the corresponding IPNS genes and proteins from these species (Kovacevik *et al.*, 1989; Miller and Ingolia, 1989a, 1989b). Similarly, cloning and DNA sequence analysis of the ACVS genes from the filamentous fungi *A. nidulans* (MacCabe *et al.*, 1991), *P. chrysogenum* (Diez *et al.*, 1990; Smith *et al.*, 1990d) and *C. acremonium* (Gutierrez *et al.*, 1991); and the prokaryote *N. lactamdurans* (Coque *et al.*, 1991) have

indicated that the *acvA* genes in each of these organisms contain a single open reading frame of similar length encoding proteins which, by extension, are of similar deduced amino acid length. The deduced molecular weights of the proteins in *P. chrysogenum*, *C. acremonium* and *N. lactamdurans* are also in good agreement with the experimentally derived molecular mass of the *A. nidulans* ACVS (MacCabe *et al.*, 1991). Furthermore, the ACVS genes of *A. nidulans*, *P. chrysogenum*, *C. acremonium* and *N. lactamdurans* exhibit almost the same percentage sequence identity at the predicted amino acid level as do the IPNS genes from these organisms (Coque *et al.*, 1991; Martin and Gutierrez, 1992). Also, in each of these species, the derived amino acid sequence of ACVS contains three repeated domains which have high homology with one another and with two multienzyme peptide synthetases, gramicidin S synthetase I and tyrocidine synthetase I, from *Bacillus brevis* (Coque *et al.*, 1991; Diez *et al.*, 1990; Gutierrez *et al.*, 1991; MacCabe *et al.*, 1991; Smith *et al.*, 1990d). In addition, these repetitive domains in *A. nidulans* (MacCabe *et al.*, 1991) and *P. chrysogenum* (Smith *et al.*, 1990d) share similarity with the adenylate-forming enzymes parsley 4-coumarate-CoA ligase and firefly luciferase. The precise function of these homologous regions in ACVS is unclear but it has been speculated that each corresponds to a functional domain that recognises one of the three substrate amino acids

(Coque *et al.*, 1991; Diez *et al.*, 1990; Gutierrez *et al.*, 1991; MacCabe *et al.*, 1991; Smith *et al.*, 1990d).

The similarities between the different ACVS genes would thus appear to support the horizontal transfer hypothesis. However, the finding that the *acvA-ipnA* gene cluster is organised differently in *N. lactamdurans* and *S. clavuligerus* relative to the fungal genes is intriguing in relation to the hypothesis of a single event in which these genes were horizontally transferred from actinomycetes to filamentous fungi. Perhaps a rearrangement of the genes occurred soon after the transfer to an ancestral fungus. Further controversy exists regarding the proposed horizontal transfer event. Originally, it was thought that a single transfer event occurred about 370 million years ago (Carr *et al.*, 1986; Leskiw *et al.*, 1988; Ramon *et al.*, 1987; Weigel *et al.*, 1988), long after the eukaryotes and prokaryotes diverged about 2 billion years ago. However, Cohen *et al.* (1990) have proposed a modified version of this hypothesis in which a single transfer of the ß-lactam genes from prokaryotes to the ß-lactam producing filamentous fungi took place close to the divergence between the Gram-positive and Gram-negative bacteria estimated to be approximately 1-1.5 billion years ago. Alternatively, they postulate that several transfer events occurred between Gram-positive, Gram-negative and eukaryotic microorganisms, unless the rate of IPNS gene evolution is not constant in all lineages. On the other hand, Penalva

et al. (1990) corroborate earlier suggestions of horizontal transfer about 370 million years ago but propose that two different events of horizontal transfer of the IPNS gene from *Streptomyces* to filamentous fungi occurred at this time, one to the progenitor of *A. nidulans* and *P. chrysogenum* and the second to an ancestor of *C. acremonium*. More extensive studies of the ß-lactam biosynthetic genes from a variety of organisms are required to resolve the question of whether a transfer mechanism, which in itself is a controversial issue in evolution, best accounts for the spread of ß-lactam biosynthetic genes in nature, and if so when exactly it occurred.

Regarding the *npeA* mutational events, pSTA201 alone is able to achieve complementation of *npeA*005. Co-transformation of *npeA*005 by pSTA200 and pSTA207 also gives complementation, albeit at a reduced frequency. According to Southern analyses, no obvious changes have taken place at the DNA level in *npeA*005 and its primary physiological defect has not been detected. Northern blot and hybridisation analysis was performed on total RNA extracted from *A. nidulans npeA*005 and *npeA*0022 mutant strains grown in fermentation medium (derepressed conditions). The *npeA*005 mutant strain produced all three penicillin biosynthetic gene transcripts in comparable amounts to the wild type, therefore under derepressed conditions the effect of the *npeA*005 mutation does not appear to be at the level of transcription. As expected

from Southern blot and hybridisation analysis, *ipnA* and *acyA* mRNA species were present in the *npeA*0022 mutant strain at similar levels to those found in the wild type and the *acvA* transcript was undetected. However, the *acvA* specific probe detected a transcript in *npeA*0022 which was of a size greater than that expected for the ACVS gene and may be due to the insertion of DNA into the *acvA* region which, from Southern analysis, is thought to account for the mutation in *npeA*0022 (A.P. MacCabe, personal communication). The lack of ACVS activity in this strain (H. van Liempt, personal communication) may thus be due to the insertion disrupting the *acvA* sequence resulting in the synthesis of a non-functional protein. The deletion mutant *npeA*0049/1 has not been complemented by transformation or cotransformation with pSTA200, pSTA201 or pSTA207. This may be due to several factors, not the least of which is the absence of a homologous *npeA* genomic location, which has been demonstrated to be the preferred site of integration of pSTA201, and the fact that the *A. nidulans* ACVS gene sequence was found to be incomplete on pSTA201 and pSTA207 (MacCabe *et al.*, 1991) with these plasmids lacking the *acvA* 3′ terminus. However, the possibility cannot be excluded that there may also be a further genetic determinant required for penicillin biosynthesis, such as a regulatory gene, which is deleted in *npeA*0049/1 and which is not represented on these clones. Hybridisation studies suggested that if any such regulatory genes exist for penicillin biosynthesis,

they are not located immediately 5′ of *acvA* or 3′ of *acyA* at the *A. nidulans npeA* locus.

In order to gain more of an insight into how penicillin biosynthesis is regulated, preliminary analysis of the regulation of expression of the *A. nidulans* penicillin biosynthetic genes was carried out by *lacZ* fusion studies and by northern blot and hybridisation analyses.

Preliminary *lacZ* fusion analysis of the *A. nidulans acvA* and *ipnA* expression signals suggests that the ACVS and IPNS genes are not highly regulated.

Northern blot and hybridisation analyses of the regulation of *A. nidulans acvA*, *ipnA* and *acyA* expression were performed by probing total RNA extracted from a number of *A. nidulans* strains with different genetic backgrounds, grown for varying lengths of time under a variety of physiological conditions, with *A. nidulans* genomic DNA fragments specific for the ACVS, IPNS and ACYT genes. Identification and characterisation of specific mRNAs involved indicates that only monocistronic transcripts are produced, a common feature of fungal gene clusters (Gurr *et al.*, 1986). All three messenger species were observed in wild type (*biA*1) cells grown under derepressed conditions in fermentation medium but not in repressed conditions in minimal medium, and the presence of transcripts followed a pattern similar to the production of penicillin as judged by bioassay. These results suggest that the expression of the *acvA*, *ipnA* and

acyA genes is at least partly mediated by the mRNA accumulated in response to growth conditions. This could be due either to increased transcription or to slower turnover of these mRNAs in cells grown in fermentation medium. Penicillin biosynthesis additionally appears to be subject to temporal regulation: the amounts of penicillin biosynthetic gene transcripts appear to increase with time and a lag occurs between the time of production of these transcripts and detection of ß-lactam activity by bioassay.

In order to determine the effects of carbon catabolism, nitrogen metabolism, sulphur metabolism and pH regulation on penicillin biosynthesis, the production of penicillin biosynthetic gene transcripts was monitored in a number of such *A. nidulans* regulatory mutant strains grown in fermentation medium. It has previously been shown that production of penicillin G by *A. nidulans* is subject to regulation by the pH of the growth medium (Shah *et al.*, 1991), and it appears that this regulation may occur at the level of transcription of the ACVS gene. pH regulatory mutant strains produce altered levels of *acvA* mRNA in comparison to those of the wild type when grown in fermentation medium: in the *pacC*5 and *palC*4 mutant strains which respectively mimic the effects of growth of the organism at alkaline and acid pH, the levels of *acvA* transcript produced are increased and decreased respectively; whilst the quantity of *ipnA* and *acyA* transcript produced by each strain is similar to

that of the wild type. If, as was classically thought (reviewed by Martin and Aharonowitz, 1983; Martin and Demain, 1980; Nuesch *et al.*, 1987), penicillin biosynthesis is subject to carbon catabolite and nitrogen metabolite repression, then strains which are carbon catabolite or nitrogen metabolite derepressed, or nitrogen metabolite super-repressed respectively, would be expected to produce elevated or reduced levels of penicillin biosynthetic gene transcripts in comparison to the wild type strain grown under the same conditions. However, this was not found to be the case but this may have been because the strains were grown in fermentation medium i.e. under conditions derepressed for penicillin biosynthesis. In addition, results obtained with the carbon catabolite repressed mutants may have been influenced by the effects of temporal repression of penicillin biosynthesis on these slow-growing strains. Similarly, if penicillin biosynthesis is subject to regulation at the level of sulphur metabolism as has been previously shown (reviewed by Martin and Aharonowitz, 1983; Nuesch *et al.*, 1987), then mutant strains altered in sulphur metabolism would be expected to produce altered levels of penicillin biosynthetic gene transcripts in comparison to the wild type strain grown under the same conditions. Again, this was not found to be the case but may have been because the strains were grown under derepressed conditions.

The effects of carbon and nitrogen metabolism on the regulation of penicillin biosynthesis were studied by northern blot and hybridisation analyses of *A. nidulans* strains grown in minimal media containing various carbon and nitrogen sources. Glucose and ammonium were classically thought to repress penicillin biosynthesis (reviewed by Martin and Aharonowitz, 1983) whereas poorer carbon and nitrogen sources such as lactose and proline or casamino acids, respectively, were thought to lead to derepression of penicillin biosynthesis. Generally, this was not reflected in the results obtained here, but the effects of temporal repression on penicillin biosynthetic gene expression were possibly being observed. However, it was found that an increase in ammonium ions from 10 mM to 20 mM completely inhibits transcription of the penicillin biosynthetic genes in each of the strains tested and that penicillin biosynthesis is, in fact, subject to nitrogen metabolite regulation. In each of the unsupplemented minimal media tested when glucose or lactose was used as the carbon source, *ipnA* and *acyA* were the only penicillin biosynthetic gene transcripts detected and no ß-lactam activity was detectable in the culture supernatants. However, when 1 % sodium acetate was used as the carbon source, all three penicillin biosynthetic gene transcripts were detected as was ß-lactam activity in the culture medium. It therefore appears that acetate, at the concentration tested, stimulates the production of

penicillin, possibly by allowing the expression of the ACVS gene.

Experiments were carried out to determine whether or not the expression of the penicillin biosynthetic genes is induced by the presence of the amino acid precursors of penicillin; the side-chains of penicillin G or V; methionine; lysine; and acetate. Under the conditions tested and at the concentrations used, the precursors of penicillin, L-α-aminoadipic acid, L-cysteine and L-valine, did not appear to induce expression of the *acvA*, *ipnA* and *acyA* transcripts. The penicillin G and V side chain precursors phenylacetic acid and phenoxyacetic acid respectively, together, at the concentration tested, appeared to stimulate penicillin production possibly by inducing expression of *acvA*, but only in one of the minimal media tested. Supplementation of minimal medium with 1 mM methionine or with 1 mM lysine apparently repressed transcription of the IPNS and ACYT genes. 1 % sodium acetate, which apparently stimulates the production of penicillin when used as the sole carbon source in minimal medium, possibly by allowing the expression of the ACVS gene, also appears to stimulate penicillin production when used to supplement the equivalent minimal medium containing 1 % glucose as the carbon source, which unsupplemented is repressed for penicillin biosynthesis. The sodium acetate appears to stimulate penicillin production by causing induced

expression of the ACVS gene and increased expression of the IPNS and ACYT genes.

The expression of the *acvA*, *ipnA* and *acyA* genes could be under the control of general regulatory genes such as those for carbon and nitrogen metabolism or those controlling the pH of the cell, resulting in repression or derepression of penicillin biosynthesis. Additionally, penicillin biosynthesis may be under the control of specific induction genes, such as those for differentiation, which would be an interesting topic for future study. There is also a possibility that there is a transport mechanism for the transportation of penicillin from the cell against a gradient of the antibiotic outside the cell, which might be a rate-limiting step in the production of penicillin. This is another area of study which could be pursued in the future.

We have identified, apart from the IPNS gene (*ipnA*) which had been cloned prior to our studies (Ramon *et al.*, 1987), the *A. nidulans* structural genes for penicillin biosynthesis, *acvA* and *acyA*. These penicillin biosynthetic genes are contiguous and represent the *A. nidulans npeA* locus located on chromosome VI in the genetic order *acvA-ipnA-acyA*. Transcripts have been detected for the three genes, their approximate sizes being *acvA* > 9.5 kb, *ipnA* 1.4 kb and *acyA* 1.6 kb, with the direction of transcription of *acvA* being in the opposite orientation to that of *ipnA* and *acyA*. All three genes are subject to some form of regulation, although

the mechanism is not clear from my studies. The research presented here has produced fundamental scientific information which will provide a better understanding of the ACVS, IPNS and ACYT genes and, consequently, of penicillin biosynthesis itself. Hopefully, this will lead to applications which are of practical use to the industrial scientists working with commercial fungi, such as the recombination of these cloned genes or mutagenised penicillin biosynthetic genes in suitable host organisms, to obtain improved ß-lactam production yields and possibly new products such as hybrid or novel antibiotics.

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APPENDIX

Summary of *A. nidulans* strains.

Strain No.	Genotype
"wild type"	*biA1*
B418	*biA1, puA2, niaD52*
C46	*pabaA1, niiA4, argB2, JA1, creC27*
481	*pabaA1, creA1*
G0049/1	*yA2, npeA0049/1*
G034	*biA1, argB2*
G0156	*biA1, pacC5*
G0215	*biA1, methH2, su-6 methH2*
G0216	*biA1, methH2, su-7 methH2*
G0217	*biA1, methH2, su-8 methH2*
G34	*yA2, methH2, argB2*
G69	*cha, penA1*
G191	*pabaA1, pyrG89, fwA1, uaY9*
G324	*yA2, wA3, methH2, argB2, galA1, ivoA1, sC12*
G415	*biA1, cnxE14, palC4*
GH36	*galA1, pyroA4, facA303, s3, nic8, ribo2, npeA002*
GH44	*yA2, pyroA4, cnxA5, npeA005*
GH79	*yA2, pyroA4, cnxA5, npeA0022*
M1.4	*pabaA1, niaD, pyrG89, npeA0049/1*
MH8	*biA1, niiA4, areA102*
MH205	*biA1, niiA4, areA19*
MH837	*biA1, xprD1*
Birmingham No.	
49	*npeA*$^{-}$ natural isolate
51	"
108	"
112	"
123	"
132	"
136	"
143	"
No strain No. given to us	
"	*yA1, pyroA4, mapA25*
"	*yA1, pyroA4, mapB1*
"	*yA1, pabaA2, mapB2*
"	*yA1, pyroA4, mapC3*
"	*yA1, pyroA4, mapD6*

Summary of plasmids.

Plasmid	Relevant features
pAN923-21B	*trpC* :: *lacZ* fusion plasmid
pAN923-41B	*lacZ* fusion vector
pAN923-42B	*lacZ* fusion vector
pAN923-43B	*lacZ* fusion vector
pDJB2	Contains *N. crassa pyr4* gene
pILJ16	Contains complete *A. nidulans argB* gene
pSTA18	Contains *P. chrysogenum ipnA*
pSTA200	Contains *A. nidulans ipnA* and *acyA* (lacking 3′ terminus)
pSTA201	Contains *A. nidulans ipnA* and *acvA* (lacking 3′ terminus)
pSTA203	Contains *A. nidulans ipnA*
pSTA204	Contains *A. nidulans ipnA*
pSTA207	Contains *A. nidulans acvA* (lacking 3′ terminus)
pSTA230	Contains 3′ end of *A. nidulans acvA* and upstream sequences
pSTA807	Contains *A. nidulans acvA* (lacking 3′ and 5′ termini)
cosmid No. 35	Contains *A. nidulans acvA, ipnA, acyA* and sequences upstream of *acvA*