THE UNIVERSITY OF ALBERTA

TRANSCRIPTIONAL ANALYSIS OF THE ISOPENICILLIN N SYNTHASE GENE OF *STREPTOMYCES CLAVULIGERUS*

BY

ASTRID KARIN PETRICH

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF MICROBIOLOGY

EDMONTON, ALBERTA

Fall, 1993

ISBN 0-315-88198-4

UNIVERSITY OF ALBERTA

RELEASE FORM

NAME OF AUTHOR: ASTRID KARIN PETRICH

TITLE OF THESIS: TRANSCRIPTIONAL ANALYSIS OF THE ISOPENICILLIN N SYNTHASE GENE OF *STREPTOMYCES CLAVULIGERUS*

DEGREE FOR WHICH THIS WAS PRESENTED: DOCTOR OF PHILOSOPHY

YEAR THIS DEGREE GRANTED: 1993

Astrid K. Petrich

(Student's signature)

Student's permanent address:

Box 119

Lister, B.C.

V0B 1Y0

Date: September 7, 1993

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND REARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled TRANSCRIPTIONAL ANALYSIS OF THE ISOPENICILLIN N SYNTHASE GENE OF *STREPTOMYCES CLAVULIGERUS* submitted by Astrid Karin Petrich in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

.....

Dr. S.E. Jensen-Supervisor

.....

Dr. K.L. Roy

.....

Dr. W.R. Addison

.....

Dr. B.K. Leskiw

.....

Dr. J.M. Piret-External Examiner

Date: August 26, 1993

ABSTRACT

Transcriptional regulation of the isopenicillin N synthase (IPNS) gene (*pcbC*) of *Streptomyces clavuligerus* was analyzed using two experimental procedures. Promoter probe vector analyses and mRNA analyses were performed to characterize the DNA region directly upstream of *pcbC* and this led to an examination of the transcriptional regulation of the two genes found immediately upstream of *pcbC*; *lat* and *pcbAB*. These genes encode the cephamycin biosynthetic enzymes lysine ε-amino transferase and δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase respectively, which act prior to IPNS in the biosynthetic pathway.

The promoter probe vector, pIJ4083, which contains the promoter-less catechol-2,3-dioxygenase (C23O) gene (*xylE*) as a reporter gene, was used to analyze the sequence upstream of *pcbC* for promoter activity. Introduction of an *Sph*I endonuclease restriction site at the start codon of *pcbC* by site-directed mutagenesis allowed the cloning of a DNA fragment containing 307 bp of sequence immediately upstream of *xylE* in pIJ4083. C23O activity was detected in both *Streptomyces lividans* and *S. clavuligerus* cultures that contained the upstream fragment, suggesting the presence of a promoter sequence. Deletions of the fragment were generated to further localize sequences important for the initiation of transcription. A series of DNA fragments that varied in the amount of sequence remaining upstream of the *pcbC* start codon were inserted upstream of the *xylE* gene in pIJ4083 and examined for C23O activity. Deletion analysis indicated that DNA sequence important for promoter activity was at least 152 bp upstream of the *pcbC* coding region.

Northern blot transfer and hybridization of total RNA extracted from *S. clavuligerus* with a *pcbC*-specific probe identified a monocistronic 1.2 kb *pcbC* transcript

that was first detected during stationary phase. Analysis of the *pcbC* transcript by primer extension located the transcription start point to a C nucleotide 91 bp upstream of the *pcbC* start codon. S1 nuclease protection assays of the *pcbC* transcript detected not only the transcript initiating 91 bp upstream of *pcbC* but also a second larger transcript suggesting possible cotranscription with the upstream *pcbAB* gene. When the DNA region immediately upstream of *pcbAB* was examined by promoter probe analysis, no promoter activity was detected and S1 nuclease protection experiments failed to identify a *tsp* directly upstream of *pcbAB*. Northern blot analysis showed no distinct *pcbAB* transcript and indicated severely degraded mRNA. Similar results were obtained when Northern blot analysis was used to search for a *lat* transcript. Promoter probe analysis indicated the presence of an active promoter within a DNA fragment that extended 227 bp upstream of *lat* structural gene. S1 nuclease mapping of the 5' end of the *lat* transcript identified the *tsp* to be a T residue 88 bp upstream of the *lat* start codon. Comparisons of the the S1 nuclease protected DNA fragments generated using RNA isolated at various stages of growth indicated a similar transcription pattern for all three genes, suggesting the possibility that *pcbC* is transcribed as part of an operon together with the *pcbAB* and *lat* genes and also as a single monocistronic message.

ACKNOWLEDGEMENTS

I would like to express my sincere thanks and appreciation to my supervisor, Dr. Susan E. Jensen, for her continued patience, guidance and support throughout my years of studentship in her laboratory. Her example of integrity and dedication to research will remain with me as a clear base on which I hope to establish my own career.

I would also like to express my gratitude to my supervisory committee members for their guidance and suggestions towards my research project. Their time and efforts were greatly appreciated. A special thanks to Dr. Brenda Leskiw for her many fruitful suggestions and discussions. I would also like to indicate the special efforts of two individuals, Dr. Jamie Doran and Dr. Xiaoning Wu, who took the time to teach a starting graduate student some of their expertise in molecular genetics. To the other members of my laboratory, Dr. Kwamena Aidoo, Annie Wong, Dr. Ashish Paradakar, Cecilia Anders and Dylan Alexander, I would like to extend my thanks for their help, suggestions and friendship.

Without the continued support and understanding, of my parents and sisters, this degree would not have been possible.

Finally, special thanks to my friends, especially Bob Glaister, for their encouragement, time and empathy, even though they did not always fully understand what I was doing or why I could possibly want to do it.

TABLE OF CONTENTS

Page

Abstract

Acknowledgements

List of Tables

List of Plates

List of Figures

List of Abbreviations

I. INTRODUCTION 1

II. MATERIALS AND METHODS 31

II.1 Materials 31

II.2 Bacterial strains, plasmids, phages and culture conditions 32

II.3 Isolation of Plasmid DNA 34

II.4 Introducing DNA by transformation 35

II.4.1 Transformation of *S. lividans* TK24 35

II.4.2 Protoplast formation and transformation of *S. clavuligerus* 35

II.4.3 Transformation of *E. coli* MV1193 37

II.5 Techniques used for DNA analysis 37

II.5.1 Restriction fragment analysis by agarose gel electrophoresis 37

II.5.2 DNA sequence analysis 38

II.5.3 Hybridization analysis 39

II.5.3.1 *E. coli* colony hybridization 39

II.5.3.2 *Streptomyces* colony hybridization 40

II.5.3.3 Preparation of Southern blots 40

II.5.3.4 Generation of nick-translated ^{32}P labelled probes 41

II.5.3.5 Hybridization of colony and Southern blots 41

II.6 Site-directed mutagenesis 41

II.6.1 Phosphorylation of the mutagenic oligo 41

II.6.2 Annealing the primers and synthesis of the complementary strand 42

II.6.3 Transformation and detection of mutants 42

II.6.4 Insertion of the *Sph*I fragment upstream of *pcbC* from pIPS-1* into pUC119 (pIPS-1s) 43

II.7 Promoter probe constructions 43

II.7.1 Insertion of the *pcbC* gene into pIJ4083 44

II.7.2 Insertion of the DNA region upstream of *pcbC* into pIJ4083 44

II.7.3 Insertion of the *ermE** up promoter into pIJ4083 45

II.7.4 Insertion of the DNA region upstream of *pcbAB* into pIJ4083 45

II.7.4.1 Insertion of the DNA region upstream of *pcbAB* into pUC119 45

II.7.4.2 Transfer of the DNA region upstream of *pcbAB* from pA2-S/119 to pIJ4083 46

II.7.5 Insertion of the DNA sequence upstream of *lat* into pIJ4083 46

II.7.5.1 Construction of a pUC118 recombinant plasmid containing the DNA region upstream of *lat* using the polymerase chain reaction 46

II.7.5.2 Transfer of the DNA region upstream of *lat* from pLat/118 into pIJ4083 47

II.8 Generation of unidirectional deletions in the DNA region upstream of *pcbC* 48

II.8.1 Generating unidirectional deletions of pIPS-1s 48

II.8.2 Transfer of the deleted *pcbC*up fragments into pIJ4083 50

II.9 Catechol dioxygenase assays of *S. lividans* TK24 and *S. clavuligerus* 3585 transformants containing pIJ4083 recombinant plasmids 50

II.10 Total RNA isolation 51

II.11 Northern analysis 52

II.12 Determination of the *tsp* of *pcbC* by primer extension 53

II.13 High resolution S1 nuclease mapping of the 5' end of *pcbC*, *pcbAB*, and *lat* transcripts 54

II.13.1 Generation of DNA probe fragments by PCR 54

II.13.1.1 PCR to generate *pcbC*-specific DNA probe 54

II.13.1.2 PCR to generate *pcbAB*-specific DNA probe 54

II.13.1.3 PCR to generate *lat*-specific DNA probe 54

II.13.2 Kinase labelling and purification of the DNA probes 55

II.13.3 S1 nuclease protection assays 55

II.14 IPNS expression in *S. lividans* TK24 56

II.14.1 Construction of pIJ702-based expression vector 56

II.14.2 Preparation of cell free extracts and partial purification of IPNS 57

II.14.3 Isopenicillin N synthase (IPNS) assays 58

II.14.4 mRNA analysis of *S. lividans* containing the 1.7/4083 plasmid 58

III. RESULTS 59

III.1 Promoter probe plasmid analysis 59

III.1.1 Assessment of the utility of *xylE* as a reporter gene in *S. clavuligerus* 60

III.1.2 Promoter probe analysis of a 1.7 kb fragment of *S. clavuligerus* DNA containing the *pcbC* gene 65

III.1.3 Promoter probe analysis of the region upstream of the *pcbC* gene of *S. clavuligerus* 65

III.1.3.1 Site directed mutagenesis to introduce an *Sph*I site at the *pcbC* start codon 65

III.1.3.2 Promoter probe analysis of *pcbC* 67

III.1.4 Generation of deletions of the sequence upstream of the *pcbC* gene of *S. clavuligerus* (*pcbC*up) and insertion of the deleted DNA fragments into pIJ4083 68

III.1.5 C23O activity of *S. lividans* and *S. clavuligerus* transformed with *pcbC*up deletion recombinant promoter probe plasmids 68

III.2 Northern transfer and hybridization analysis 69

III.2.1 Northern transfer and hybridization to identify the *pcbC* transcript of *S. clavuligerus* 70

III.3 Primer extension to determine the 5' end of the *pcbC* transcript 70

III.4 S1 nuclease protection assays to determine the 5' end of the *pcbC* gene of *S. clavuligerus* 75

III.4.1 Generation of *pcbC*-specific DNA probes for S1 nuclease protection assays 75

III.4.2 Determination of the 5' end of the *pcbC* transcript of *S. clavuligerus* by high resolution S1 nuclease mapping 78

III.5 Promoter probe analysis of the region upstream of the *pcbAB* and *lat* genes of *S. clavuligerus* 81

III.5.1 Insertion of the DNA sequence upstream of the *lat* and

pcbAB genes of *S. clavuligerus* into pIJ4083 81

III.5.2 Promoter probe analysis of the DNA sequence upstream of *pcbAB* 81

III.5.3 Promoter probe analysis of the DNA sequence upstream of the *lat* gene 82

III.6 Northern analysis to identify the *pcbAB* and *lat* transcripts of *S. clavuligerus* 88

III.6.1 Northern transfer and hybridization to detect the *pcbAB* transcript of *S. clavuligerus* 88

III.6.2 Northern transfer and hybridization to detect the *lat* transcript of *S. clavuligerus* 89

III.7 Primer extension to determine the 5' end of the *pcbAB* and *lat* transcripts of *S. clavuligerus* 89

III.8 S1 nuclease protection assays to determine the 5' end of the *lat* and *pcbAB* transcripts of *S. clavuligerus* 94

III.8.1 Generation of DNA probes for S1 nuclease mapping the 5' end of the *lat* and *pcbAB* transcripts 94

III.8.2 S1 nuclease protection assays to determine the 5' end of the *pcbAB* transcript of *S. clavuligerus* 94

III.8.3 S1 nuclease protection assays to determine the 5' end of the *lat* transcript of *S. clavuligerus* 95

III.9 Comparison of the putative promoter regions of *pcbC* and *lat* with consensus promoter sequences and promoters of other *Streptomyces* genes 98

III.10 IPNS expression in *S. lividans* 102

III.10.1 Construction of two vectors containing the *pcbC* gene of

S. clavuligerus for expression of IPNS in *S. lividans* 102

III.10.2 Determination of IPNS activity in partially-purified cell free extracts by HPLC analysis 103

III.10.3 Northern transfer and hybridization to detect the *pcbC* transcript in total RNA isolated from *S. lividans* transformed with 1.7/4083 108

III.10.4 S1 nuclease protection assays to determine the 5' end of the *pcbC* transcript from *S. lividans* transformed with 1.7/4083 108

IV. DISCUSSION 114

V. BIBLIOGRAPHY 133

VI. APPENDIX 144

LIST OF TABLES

Table	Description	Page
I	Catechol dioxygenase (C23O) activity in cell free extracts of *Streptomyces clavuligerus* and *Streptomyces lividans* transformed with pIJ4083 recombinant plasmids for identification of the *pcbC* promoter	66
II	Catechol dioxygenase (C23O) activity in cell free extracts of *Streptomyces clavuligerus* and *Streptomyces lividans* transformed with pIJ4083 recombinant plasmids for identification of the *pcbAB* and *lat* promoters	87
III	Comparison of the putative promoter sequence of *pcbC* and *lat* with the consensus promoter sequences of *Escherichia coli* and *Streptomyces* and with promoters of other *Streptomyces* secondary metabolic genes	101

LIST OF PLATES

Plate	Description	Page
1	Visual assessment of relative C23O activity in *S. lividans* and *S. clavuligerus* transformed with pIJ4083 recombinant promoter plasmids to determine the location and relative activity of the promoter directing transcription of *pcbC*	64
2	Northern analysis of total *S. clavuligerus* RNA using a *pcbC*-specific probe	72
3	Location of the 5' end of the *pcbC* gene of *S. clavuligerus* by 5' primer extension	74
4	Location of the 5' end of the *pcbC* transcript of *S. clavuligerus* by high resolution S1 nuclease mapping	80
5	Visual assessment of relative C23O activity in *S. lividans* and *S. clavuligerus* transformed with pIJ4083 recombinant plasmids for identification of the promoters directing transcription of *pcbAB* and *lat*	86
6	Northern analysis of total RNA preparations from *S. clavuligerus* using a *pcbAB*-specific probe	91
7	Northern analysis of total RNA preparations from *S. clavuligerus* using a *lat*-specific probe	93
8	Location of the 5' end of the *pcbAB* transcript of *S. clavuligerus* by high resolution S1 nuclease mapping	97
9	Location of the 5' end of the *lat* transcript of *S. clavuligerus* by high resolution S1 nuclease mapping	100
10	Northern analysis of a total RNA preparation from *S. lividans* transformed with recombinant plasmid 1.7/4083, using a *pcbC*-specific probe	110
11	Comparisons of the 5' ends of the *pcbC* transcripts from *S. clavuligerus* and from *S. lividans* transformed with the recombinant plasmid 1.7/4083	112

LIST OF FIGURES

Figure	Description	Page
1	Examples of a variety of β-lactam antibiotics	3
2	Pathway of cephamycin biosynthesis in *Streptomyces clavuligerus*	7
3	Pathway of α-aminoadipate biosynthesis in *Streptomyces clavuligerus*	10
4	Cephamycin biosynthetic gene cluster in *Streptomyces clavuligerus*	19
5	Recombinant promoter probe plasmids used to determine the location and relative activity of the promoter directing transcription of *pcbC*	62
6	DNA probe fragments generated by PCR for high resolution S1 nuclease mapping the 5' ends of *pcbC*, *pcbAB* and *lat* transcripts of *Streptomyces clavuligerus*	77
7	Recombinant promoter probe plasmids used to determine the location and relative activity of promoters directing transcription of *pcbAB* and *lat* of *Streptomyces clavuligerus*	84
8	Recombinant plasmids used for IPNS expression in *Streptomyces lividans*	105
9	HPLC analysis of IPNS activity	107

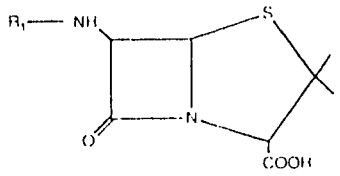
LIST OF ABBREVIATIONS

α-aaa	α-aminoadipic acid
ACV	δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine
ACVS	ACV synthetase
AMV	Avian myeloblastosis virus
C23O	Catechol-2,3-dioxygenase
DTT	Dithiothreitol
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
HPLC	High performance liquid chromatography
IPN	Isopenicillin N
IPNS	IPN synthase
LAT	Lysine ε-amino transferase
MOPS	3-[N-Morpholino]propanesulfonic acid
MYM	Malt extract-yeast extract-maltose
NTG	N'-methyl-N'-nitro-N-nitrosoguanidine
oligo	oligodideoxyribonucleotide
ORF	Open reading frame
PAG	Polyacrylamide gel
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
SDS	Sodium dodecyl sulfate
SSC	Standard Saline-Citrate
TDE	Tris-DTT-EDTA buffer
TE	Tris-EDTA buffer
TEA	Tris-EDTA-acetate buffer
TEB	Tris-EDTA-borate buffer
TSB	Trypticase soy broth
TSBG	Trypticase soy broth-glycerol
TSBS	Trypticase soy broth-starch
tsp	transcription start point
YEME	Yeast extract-malt extract

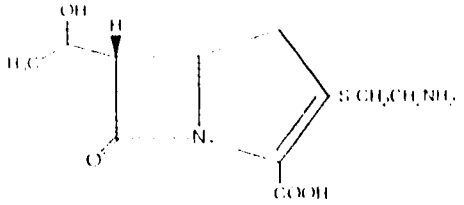
I. INTRODUCTION

Since the discovery of penicillin seventy years ago, β-lactam antibiotics have remained a very active subject of research both at the industrial and academic level. Their role clinically has remained prominent, so that they still account for greater than 50% of the antimicrobial agents prescribed, making them both medically and economically important. Over the past few years the emphasis of research has shifted to genetic characterization of antibiotic biosynthetic pathways in the ongoing effort to improve productivity. The future of research in this field relies on the elucidation of the genetic systems and our ability to exploit these systems to over-produce conventional β-lactams and possibly also produce novel compounds.

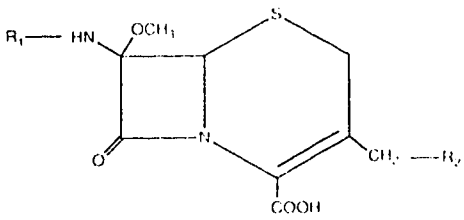
Penicillin was first detected in the fungus *Penicillium notatum* in 1929 (Fleming, 1929) and cephalosporin was first identified from *Cephalosporium acremonium* in 1955 (Newton and Abraham, 1955). Since that time many other fungal producers of penicillins and cephalosporins have been identified (reviewed in Jensen, 1986). The search for other types of β-lactam compounds, together with the screening of novel producing species, has demonstrated that a diverse group of procaryotic Gram-negative and Gram-positive organisms are also able to produce β-lactam antibiotics (Jensen, 1986). Unlike the fungal producers, the Gram-positive mycelial-forming Actinomycetes are able to produce not only those antibiotics with the conventional penam (5- membered sulfur-containing ring) and cephem (6- membered sulfur-containing ring) structures, but also a number of non-classical β-lactam antibiotics including clavams or oxypenams (5-membered oxygen-containing ring) carbapenems (5-membered ring with no heteroatom) and monobactams (monocyclic β-lactam ring only). Examples of naturally occurring antibiotics of the penicillin and cephamycin structure, as well as those containing different nuclei than the classic β-lactam structures, are indicated in Figure 1. These structural variations in the basic β-lactam structure alter the characterisitics of the antibiotic. Clavulanic acid (oxypenam) is a weak antibiotic, but acts as an irreversible β-lactamase



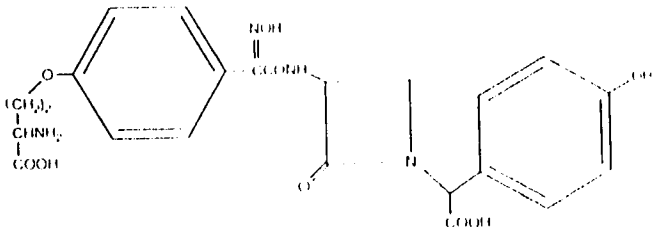
PENICILLIN



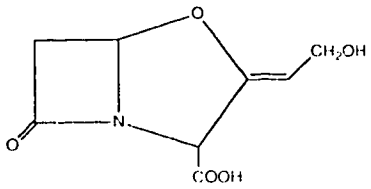
THIENAMYCIN



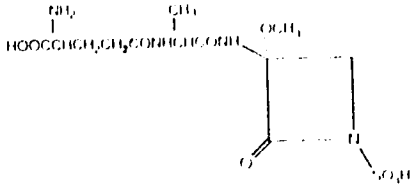
CEPHAMYCIN



NOCARDICIN A



CLAVULANIC ACID



SULFAZECIN

inhibitor. Cephamycins, with the cephalosporin nuclear structure and the substituent methoxy group on the C-7, have a greater intrinsic resistance to β-lactamases than the penicillins and cephalosporins. Thienamycin, a carbapenem, is an exceptionally active antibiotic effective against a broad range of bacteria. It is this ability of the Actinomycetes to produce a diverse group of antibiotics that is intriguing from the aspect of research into novel antibiotic production.

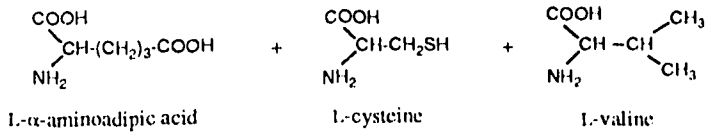
Within the past ten to fifteen years intensive research has been undertaken to elucidate the biosynthetic pathways, characterize the enzymes involved and identify and analyze the genes that determine the production of penicillins and cephamycins. Some excellent reviews have recently been published on the elucidation of the biosynthetic pathway and the regulation of the enzymes and genes involved (Aharonowitz et al., 1992; Queener et al., 1990; Jensen and Demain, 1993). For the sake of brevity, in this study emphasis will be placed on the genetic characterization of the early genes involved in cephamycin production in *Streptomyces*. Although the early steps in penicillin and cephalosporin production are also utilized in cephamycin production, there are significant differences between the fungal and procaryotic systems that have implications for genetic control. Therefore, for the most part, this review will address the genetic systems found in the *Streptomyces* β-lactam producers, with an emphasis on the specific producing organism of interest, *Streptomyces clavuligerus*, and reference to other systems for the sake of clarification.

Initial research into β-lactam production centered around increasing antibiotic production by random mutagenesis, selection of strains with increased production yields and by empirical modification of fermentation medium composition and growth conditions. Much of the information gained at this time had little to do with the genetic characterization of the biosynthetic pathway, but it did lead to increased yields of antibiotic and corresponding increases in the cellular contents of the biosynthetic

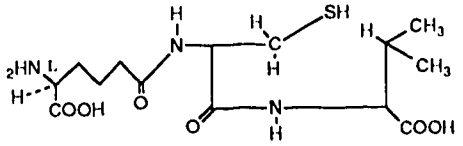
enzymes. The availability of strains producing unnaturally high levels of the antibiotic biosynthetic enzymes made it easier to isolate and purify these enzymes for biochemical studies and to use information obtained from purified proteins to assist in cloning the corresponding genes.

Like the fungal producers, *Streptomyces* produce penicillin and cephalosporins, but unlike the fungi, they have the ability to produce cephamycins. Cephamycins are an unusual form of cephalosporin that contains a methoxy group attatched to the carbon atom at position 7. The universal ability to produce penicillins, seen also in species which produce cephalosporins and cephamycins, including both fungal and procaryotic species, suggested a common biosynthetic pathway. Furthermore, since the converse is not true, ie. penicillin producers do not necessarily produce cephalosporins and cephamycins, this suggested that penicillins might be an early intermediate in the biosynthetic pathway to cephalosporins and cephamycins. It has since been recognized that the pathway up to the level of isopenicillin N is similar in all of the eucaryotic fungi and the procaryotic producers of penicillins, cephalosporins and cephamycins. After this point, the pathways of penicillin producers, and cephalosporin and cephamycin producers diverge (Figure 2). As Figure 2 indicates, the penicillin, cephalosporin and cephamycin pathways begin with the three constituent amino acids, α-aminoadipic acid (α-aaa), cysteine and valine. Cysteine and valine are primary metabolic amino acids, while the origin of α-aaa is different in fungi and procaryotes. In the fungal systems, α-aaa is formed as an obligate intermediate in the synthesis of lysine. However, in procaryotes, lysine is synthesized via the diaminopimelic acid pathway and therefore no α-aminoadipic acid is produced. An early study indicated that in β-lactam producing *Streptomyces* breakdown of lysine is necessary for the production of α-aaa (Whitney et al., 1972). Madduri et al. (1989) indicated that a pathway for the breakdown of lysine via cadaverine and δ-aminovalerate is present in both cephamycin producing and non-

Figure 2: Pathway of cephamycin C biosynthesis in *Streptomyces clavuligerus*. The enzymes and the corresponding genes for each step are indicated. Only the structures of the intermediates of the first two enzymatic steps of the pathway are included.

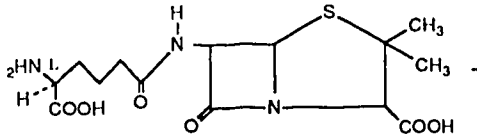


δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine Synthetase (*pcbAB*)



δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine

Isopenicillin N Synthase (*pcbC*)



Isopenicillin N

6-APA

Acyl-CoA: 6-APA Acyl Transferase (*penDE*)

Penicillin G and other hydrophobic penicillins
A. nidulans
P. chrysogenum

Isopenicillin N Epimerase (*cefD*)

Penicillin N

Deacetoxycephalosporin C Synthase (*cefE*)

Deacetoxycephalosporin C

Deacetoxycephalosporin C 3' Hydroxylase (*cefF*)

Deacetylcephalosporin C

Deacetylcephalosporin C acetyltransferase (*cefG*)

Cephalosporin C
C. acremonium
A. niger

O-carbamoyltransferase

O-carbamoyl-deacetylcephalosporin C

7-α-hydroxylase

7-α-O-carbamoyl-deacetylcephaolsporin C

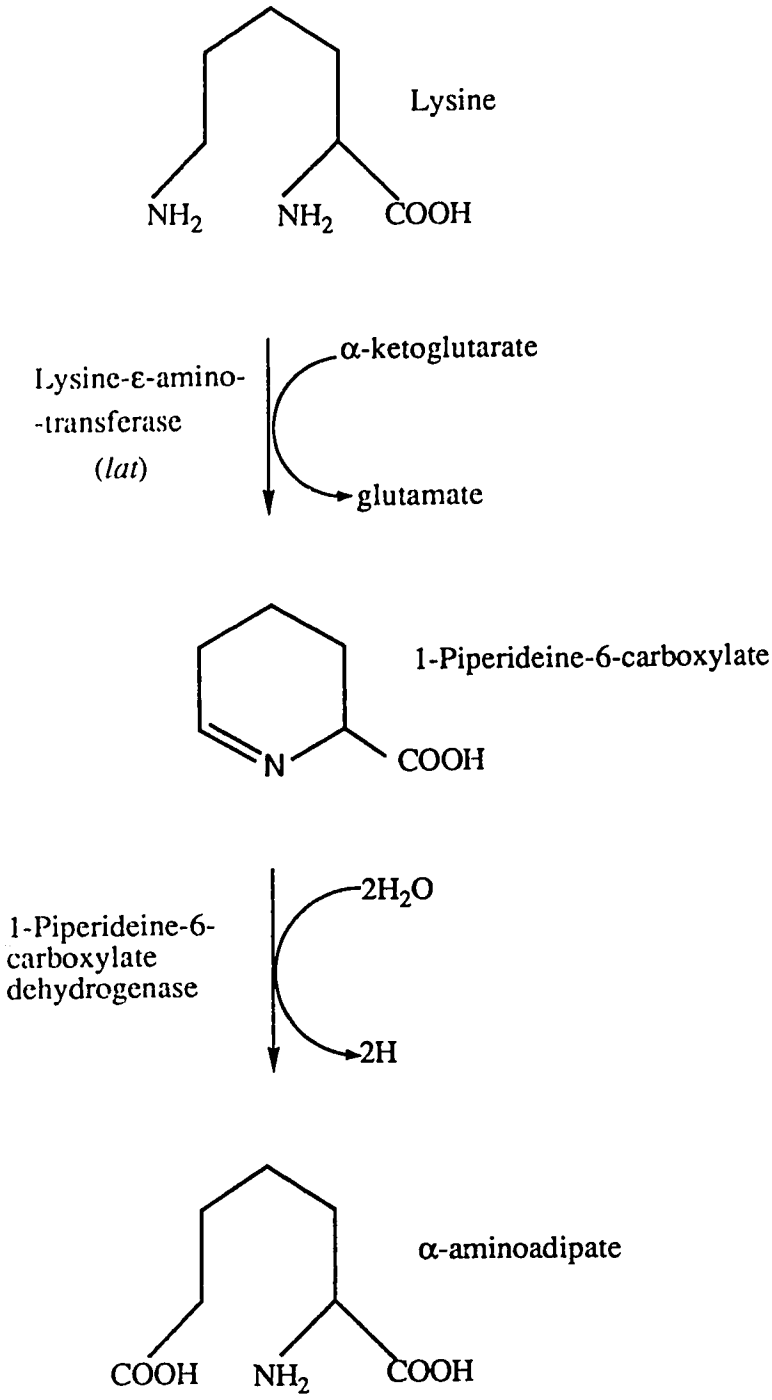
Cephamycin Methyltransferase

Cephamycin C
S. clavuligerus,
N. lactamdurans

producing *Streptomyces*. In cephamycin producing *Streptomyces*, a second pathway of lysine catabolism which converts lysine to α-aaa was found to be necessary for cephamycin production (Madduri et al., 1989). Production of α-aaa is a two step process (Figure 3), with the enzyme L-lysine-ε-aminotransferase (LAT) catalyzing the removal of the ε-amino group of lysine to form the intermediate 1-piperideine-6-carboxylate. The enzyme which catalyzes the dehydrogenation of 1-piperideine-6-carboxylate to α-aaa remains uncharacterized, although it is unlikely to be a spontaneous reaction and could be catalyzed by a primary metabolic enzyme. LAT activity has been shown to be specific to and necessary for cephamycin biosynthesis in *S. clavuligerus* (Madduri et al., 1989) and *Nocardia lactamdurans* (Kern et al., 1980), and is considered to be the first enzyme in the cephamycin biosynthetic pathway.

The second step in the cephamycin biosynthetic pathway (first step for the fungal penicillin and cephalosporin producers) is the joining of the three component amino acids. Condensation of the three amino acids, α-aaa, cysteine, and valine, and isomerization of the valine to form the tripeptide δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV), is catalyzed by the enzyme ACV synthetase (ACVS) (Figure 2). Isolation and characterization of the ACVS enzyme was a difficult process, as the enzyme appeared quite large; estimates range from 220-800 kDa. Stabilizing buffers (Zhang and Demain, 1990) and the development of HPLC assays based on the appearance of ACV using fluorescent derivatives (Jensen et al., 1988; Banko et al., 1987) have permitted biochemical studies on nutritional conditions that affect ACVS enzyme production (reviewed in Demain and Piret, 1990). ACVS is a non-ribosomal peptide synthetase, similar to other peptide synthetases such as tyrocidine synthetase and gramicidin synthetase (Kleinkauf and von Dohren, 1990). The ACVS enzyme requires ATP to activate the substituent amino acids to form adenylates. The activated amino acids are then transferred to the enzyme via thioester linkages between the activated amino acid and

Figure 3: Pathway of α-aminoadipate biosynthesis in *Streptomyces clavuligerus*.


Lysine
NH2
NH2
COOH
Lysine-ε-amino-
-transferase
(lat)
α-ketoglutarate
glutamate
1-Piperideine-6-carboxylate
N
COOH
1-Piperideine-6-
carboxylate
dehydrogenase
2H2O
2H
α-aminoadipate
COOH
NH2
COOH

a sulfhydryl group, either on the enzyme or on a pantetheine cofactor. Studies with cell free extracts from *C. acremonium* indicated that α-aaa is condensed first with cysteine and then valine is added with a change in stereochemistry (from the L-form of valine to the D-form), as indicated by low levels of δ-(L-α-aminoadipyl)-L-cysteine (AC) formation , when the enzyme was provided with α-aaa and cysteine only (Banko et al., 1986). However, AC formation appeared to proceed more efficiently when all three amino acids were present (Banko et al., 1987).

Oxidative cyclization of the linear ACV tripeptide to form the first β-lactam ring-containing intermediate, isopenicillin N (IPN), is catalyzed by IPN synthase (IPNS) (Figure 2). The IPNS enzyme has been purified from both fungal and procaryotic systems and shows considerable similarity in that the co-factors Fe^{2+}, ascorbate, dithiothreitol and molecular oxygen, are all required for optimal activity in cell free extract assays. There are slight differences in the Km and molecular weights, 26.5 kDa (*N. lactamdurans*) to 39 kDa (*P. chrysogenum*), of the IPNS enzymes isolated from different hosts which might be a reflection of changes due to evolution (reviewed in Martín and Liras, 1989). The molecular weight of purified IPNS enzyme isolated from *S. clavuligerus* is in between this range at 33 kDa (Jensen et al., 1986).

The formation of IPN, marks the point at which the pathways diverge for the fungal species which produce penicillin only and the fungal and bacterial species which produce cephalosporins and cephamycins. In the fungal penicillin producers, such as *P. chrysogenum* and *A. nidulans*, the α-aaa side chain of IPN is removed by an acyl transferase complex (AAT) and replaced with non-polar side chains such as phenylacetyl and phenoxyacetyl (to produce penicillin G and penicillin V respectively). In addition to phenylacetic acid and phenoxyacetyl acid, any of a range of substituted acetic acid compounds can take part, yielding a variety of penicillins. The composition of the

growth medium may favour the formation of different penicillins, dependent on the substituted acetyl group that is available. In contrast, cephalosporin and cephamycin biosynthesis have no such counterpart and only a single side chain is possible. This process, called precursoring, has been used industrially to form a range of useful penicillins. It is unclear whether the acyl transferase reaction is occurring as a single step exchange or as a two-step process in which the α-aaa side chain is first cleaved to give the intermediate 6-aminopenicillanic acid (APA), followed by the addition of the hydrophobic side chain from acetyl-CoA activated amino acids (Martín and Liras, 1989). Purification of the enzyme activity indicated that AAT is composed of two subunits and the corresponding AAT genes (*penDE*) from *A. nidulans* (Montenegro et al., 1990) and *P. chrysogenum* (Barredo et al., 1989) have been cloned and sequenced. Expression of the *P. chrysogenum* gene in *E. coli* suggests that AAT is a heterodimer, the two subunits of which are encoded by the *penDE* gene to give a preprotein that is processed following translation (reviewed in Aharonowitz et al., 1992; Queener, 1990).

In contrast to the fungal penicillin producers, the first step that commits the pathway to production of cephalosporin and cephamycin is the conversion of the L-form of the α-aminoadipyl side chain of isopenicillin N to the D-form, to give penicillin N (Figure 2). Isopenicillin N epimerase (IPNE) activity was first detected in *C. acremonium* cell free extracts (Konomi et al., 1979) and appeared to be highly labile, making characterization of the fungal epimerase difficult. The corresponding enzyme from *S. clavuligerus* appears to be less labile, and was partially purified with the addition of pyridoxal phosphate for stability (Jensen et al., 1983). IPNE has since been purified to homogeneity from *N. lactamdurans* (Laiz et al., 1990), and *S. clavuligerus* (Usui and Yu, 1989). IPNE catalyzes a reversible reaction that proceeds at about the same rate in either direction and requires pyridoxal phosphate as a cofactor.

Penicillin N acts as the substrate for an oxidative ring-expansion of the 5-membered thiazolidine ring to a six-membered dihydrothiazine ring to form deacetoxycephalosporin C. This oxidative reaction is catalyzed by deacetoxycephalosporin C synthase (DAOCS), also referred to as expandase, and was first detected in cell free extracts from *C. acremonium* (Kohsaka and Demain, 1976; Yoshida et al., 1978). DAOCS was initially characterized from *C. acremonium* and was shown to require Fe^{2+}, molecular oxygen, DTT, ascorbic acid and α-ketoglutarate as cofactors (Kupka, et al, 1983). The same cofactors were required for DAOCS activity from *S. clavuligerus* (Jensen et al., 1982a), although attempts at purification indicated structural differences between the eucaryotic and procaryotic enzymes. DAOCS purified from *C. acremonium* appeared to be inseparable from the next enzyme in the pathway deacetylcephalosporin C synthase (DACS) and had a molecular weight of 41,000 (Dotzlaf and Yeh, 1987; Scheidegger et al., 1984). Expression of the cloned gene from *C. acremonium* in *E. coli* demonstrated both DAOCS and DACS activities in the host, thereby establishing the bifunctional nature of the enzyme (Samson et al., 1987b). However, the DAOCS and DACS activities were clearly separable upon purification from *S. clavuligerus*, indicating two proteins of molecular weights of 29,500 and 26,200 respectively (Jensen et al., 1985). Two distinct enzymes have also been identified in *N. lactamdurans* (Cortes et al., 1987). The DACS enzyme catalyzes the hydroxylation of the C-3 methyl group on the dihydrothiazine ring of deacetoxycephalosporin C to give deacetylcephalosporin C. Like DAOCS, DACS also acts as an oxygenase and requires the same co-factors; molecular oxygen, DTT, ascorbic acid and Fe^{2+} (Jensen et al., 1985; Dotzlaf and Yeh, 1987).

After the formation of deacetoxycephalosporin C, the biosynthetic pathways between the cephalosporin producers and the cephamycin producers diverge. In *C.*

acremonium, an acetyl group from acetyl-CoA is transferred to the hydroxyl group on the sulfur-containing ring of deacetylcephalosporin C to form cephalosporin C. The enzyme that catalyzes this reaction, cephalosporin C synthetase has not been completely characterized yet and the gene responsible for this activity has not been isolated. In the procaryotic cephamycin C producer *S. clavuligerus*, there appear to be three biochemical steps for the conversion of deacetylcephalosporin C to cephamycin C. The first of these steps is considered to be the carbamoylation of deacetylcephalosporin C to O-carbamoyl-deacetylcephalosporin C (OCDAC). The enzyme O-carbamoyltransferase has been partially purified from *S. clavuligerus* and transfers a carbamoyl group from carbamoyl phosphate to the C-3 hydroxymethyl group of deacetylcephalosporin C in a reaction that is stimulated by Mg^{2+}, Mn^{2+} and ATP (Martín and Liras, 1989). The second reaction is a hydroxylation of OCDAC to form 7-α-hydroxy-OCDAC and is catalyzed by the enzyme 7-α-hydroxylase which has been purified from *S. clavuligerus* to near homogeneity (Xiao et al., 1991). The hydroxylation reaction that converts OCDAC to 7-α-OCDAC (Figure 2) appears very similar in its co-factor requirements to the C-3 methyl hydroxylase, DACS. Both enzymes act as oxygenases and require Fe^{2+}, DTT, molecular oxygen, and α-ketoglutarate (O'Sullivan and Abraham, 1980). The final step leading to formation of cephamycin C is the transfer of a methyl group from S-adenosylmethionine to the C-7 hydroxyl group of 7-α-OCDAC to give cephamycin C.

The enzymes of the cephamycin biosynthetic pathway of *S. clavuligerus* have, for the most part, been well characterized. Purification of the enzymes allowed cloning of the corresponding genes, which has given greater insight into the regulation of antibiotic production. For the early enzymes, many of the corresponding genes were cloned first from the fungal producers, as these organisms had been manipulated to produce larger quantities of enzyme, facilitating their purification. Cloning of the genes specific for the

procaryotic producers has followed at a slower pace, with a number of the later genes remaining uncharacterized.

The gene encoding the first enzyme specific to β-lactam production in procaryotic species, *lat*, has only recently been cloned from both *S. clavuligerus* (Tobin et al., 1991; Madduri et al., 1991) and *N. lactamdurans* (Coque et al., 1991a). In both cases the genes were situated near other genes encoding enzymes of β-lactam biosynthesis. This supports the idea that *lat* be considered part of the β-lactam biosynthetic pathway in procaryotic producers.

The cloning of the gene encoding ACVS (*pcbAB*) from *P. chrysogenum* (Diez et al., 1990; Smith et al., 1990) identified a ORF of 11.5 kb that would correspond to a protein 426 kDa in size. A large mRNA transcript of 9.5-11.5 kb in size was detected (Smith et al., 1990; Diez et al., 1990), implying a single monocistronic message in the fungal system. The gene itself did not contain introns and analysis of the deduced amino acid sequence indicated the presence of three domains that showed significant homology to each other. Presumably these domains are the binding sites where the three amino acids are activated by adenylation and then condense to form ACV. The *pcbAB* gene has also been cloned and sequenced from *N. lactamdurans* (Coque et al., 1991b), *C. acremonium* (Gutierrez et al., 1991), and *A. nidulans* (MacCabe et al., 1991). While the complete sequence of the *pcbAB* gene from *S. clavuligerus* is not yet known, the first 1.8 kb (Tobin et al., 1991) and the last 1 kb (Doran et al., 1990) have been sequenced, and so the location of the gene has been determined. All of the *pcbAB* genes characterized appear to encode proteins with the same three-domain structure as was detected initially in the *P. chysogenum* protein.

The gene encoding IPNS, *pcbC*, was first isolated from *C. acremonium* by Samson et al. (1985) and has now been isolated and sequenced from *P. chrysogenum*

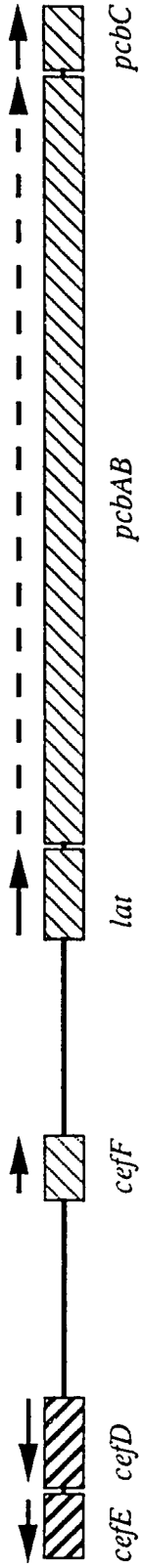
(Carr et al., 1986), *A. nidulans* (Ramon et al., 1987), *Streptomyces jumonjinensis* (Shiffman et al., 1988), *Streptomyces lipmanii* (Weigel et al., 1988), *Streptomyces griseus* (Garcia-Dominguez et al., 1991) as well as from *S. clavuligerus* (Leskiw et al., 1988). There is a high degree of homology between the *pcbC* genes (about 60% at the nucleotide level). No evidence of introns was found in the coding region of the fungal genes and the relatively high G + C content of all *pcbC* genes, regardless of source, suggested that during evolution, the β-lactam coding genes may have originally been transferred from a procaryote producer to the eucaryotes.

Cloning of the genes encoding the isopenicillin N epimerase (IPNE) and the deacetoxycephalosporin C synthetase (DAOCS) genes (*cefD* and *cefE* respectively) of *S. clavuligerus* was achieved using "guessmer" probes generated from information obtained from N-terminal sequence analysis of the purified proteins. "Guessmer" probes are long non-degenerate oligonucleotides, where only the most commonly used of the possible codons at each position is inserted. Codon usage reflects the high GC content of *Streptomyces* DNA, making the use of this type of probe feasible for screening libraries of *Streptomyces* DNA. Cosmids containing *S. clavuligerus* DNA and known to contain the previously identified *pcbC* gene were screened by hybridization using the "guessmer" probes. Two genes were identified, located, and found to be transcriptionally oriented in the same direction, with only 81 bp of intergenic sequence between them (Kovacevic et al., 1989; Kovacevic et al., 1990). RNA studies that included Northern analysis and S1 nuclease mapping indicated that the *cefD* and *cefE* genes constituted an operon and the promoter was located upstream of the *cefD* coding region. A transcript of at least 10 kb was detected and this indicated that the *cefD/cefE* transcript also includes an as yet unidentified gene found downstream of *cefE* (Kovacevic et al., 1990). The *cefD* and *cefE* genes were found in close proximity to the *pcbC* gene and the *pcbAB* genes, however oriented in the opposite direction.

The gene encoding deacetylcepholosporin C synthase (DACS), *cefF*, from *S. clavuligerus* has been cloned and sequenced (Kovacevic and Miller, 1991) and is found upstream of the *cefD/cefE* genes and oriented in the opposite direction. Of the late genes involved in the final steps for cephamycin biosynthesis, to date only the 7-α-hydroxylase, *cmcI*, has been cloned (Xiao et al., 1993). It has been located to a 1.5 kb *SalI* DNA fragment, although the exact location has not yet been identified.

The tendency for antibiotic biosynthetic genes to be clustered in *Streptomyces* has been well documented (Martín, 1992; Seno and Baltz, 1989) and this includes the genes for penicillin and cephamycin biosynthesis. The approximate location and orientation of the known cephamycin biosynthetic genes from *S. clavuligerus* are shown in Figure 4. The *pcbC* genes are located within the biosynthetic gene clusters, adjacent to the *pcbAB* genes in all producing species studied to date (Martín, 1992; Jensen and Demain, 1993). Some degree of clustering is also seen in fungal producers where *pcbAB* is again located adjacent to *pcbC*. Clustering of biosynthetic units is less common in the chromosomes of eucaryotes, further evidence for horizontal gene transfer of the penicillin biosynthetic genes from a procaryote to a eucaryote. However, the orientation of the *pcbAB* gene in regard to the *pcbC* gene is different in *P. chrysogenum* and *A. nidulans* compared to *N. lactamdurans* and *S. clavuligerus*. In the procaryotic producers the orientation of transcription for the two genes is in the same direction, while the fungal genes are adjacent but oriented away from each other. One interesting aspect is that the *lat* gene of *S. clavuligerus* (Figure 3) and *N. lactamdurans* is found immediately upstream of the *pcbAB* gene and is also oriented in the same direction as the two later genes (Tobin et al., 1991; Coque et al., 1991b). Mutants have been isolated from *S. clavuligerus* that appear polar with respect to at least two of the three early genes, *lat*, *pcbAB*, and *pcbC*. Romero et al. (1988) isolated a number of cephamycin deficient mutants that were shown to have decreased levels of both LAT and IPNS enzyme activities. Piret et al. (1990) described a

Figure 4: Linear map of the known cephamycin biosynthetic gene cluster in *S. clavuligerus*. Open boxes represent the the approximate sizes of the coding regions for the genes. Solid arrows indicate the direction of transcription from deduced amino acid sequences (*pcbC* is approximately 1.0 kb). *pcbAB* is indicated as a dashed arrow as DNA sequence has been obtained for the N-terminal (Tobin et al., 1991) and C-terminal ends (Doran et al., 1990) of the gene only.


pcbC
pcbAB
lat
cefF
cefD
cefE

mutant deficient in ACVS and IPNS, and have since found it to be deficient in LAT activity as well (J. Piret, personal communication). Although the intergenic region between *pcbAB* and *pcbC* was scrutinized for possible promoter sequences for *pcbC* and for possible terminator sequences for the upstream *pcbAB* gene, none were readily apparent (Doran et al., 1990). The physical location of the three early genes of the cephamycin biosynthetic pathway, the lack of detectable transcription control signals within the intergenic region of *pcbC* and *pcbAB* and the polarity effect observed between the three enzymes in mutants unable to produce cephamycin, all suggest that the expression of the three genes may be interrelated. It is unclear whether they are regulated in a similar manner due to a regulatory factor that acts on each gene individually or because they are co-transcribed as a single polycistronic message. Positive regulation of gene expression by a small molecular weight intermediate of the biosynthetic pathway, such as ACV, is also a third possibility. Despite these indications that the early genes may be transcriptionally linked, in *S. griseus* a monocistronic message of approximately 1.2 kb was identified for the *pcbC* gene. Transcriptional analysis of the fungal *pcbC* genes has indicated the presence of monocistronic messages of about 1.5 kb (reviewed in Aharonowitz et al., 1992). This finding correlates well with the divergent orientation of the *pcbAB* and *pcbC* genes on the chromosome.

At present most of the cephamycin biosynthetic genes have been cloned and sequenced, and although genetic technical procedures have been developing rapidly, characterization of the transcription and expression of the biosynthetic genes is still in the initial stages. An increased understanding of factors controlling penicillin and cephamycin production and the mechanisms by which this control is exerted promises to have a major impact in several aspects of antibiotic production. Knowledge of the genetic organization of the biosynthetic pathways of antibiotics and the mechanisms by which this control is exerted may allow for increased production yields of antibiotic and may

provide a clearer understanding of a rational approach for generating novel antibiotics. Antibiotic production in *Streptomyces* spp. and other organisms is often associated with the processes of differentiation and sporulation (Chater and Hopwood, 1989). This association is seen at two different levels; organisms which have complex life cycles involving cellular differentiation are frequently also unusual in their ability to produce antibiotics, eg. *Bacillus*, *Myxobacteria*, *Streptomyces* and filamentous fungi. Secondly, in those organisms that produce antibiotics, production is not uniform throughout the life cycle and more usually the appearance of antibiotic coincides with the onset of differentiation. Therefore, an understanding of the mechanisms used to control antibiotic production may be assisted by an understanding of the regulatory processes which control differentiation and sporulation in these complex organisms.

Streptomyces are Gram-positive, filamentous soil bacteria that have been widely studied for their complex morphological differentiation during their life-cycle and for their ability to produce a large variety of secondary metabolites that include exocellular enzymes and antibiotics. The organisms lead a complex life-cycle starting out as actively growing substrate mycelia, and then as nutrients become limiting, their growth slows and differentiation takes place. During the differentiation process they form aerial mycelia and eventually spores which then disperse and begin the process again. Typically the onset of differentiation to aerial mycelia is linked to the initiation of production of secondary metabolites including antibiotics. Both of these processes are presumed to involve the differential and temporally regulated expression of multiple genes and the mechanism involved in controlling the transcription of such genes would be expected to be quite complex. *Streptomyces* genetic analysis has been rapidly progressing with the expanding arsenal of cloning vectors and genetic techniques that have become available (Hopwood et al., 1985).

The development of promoter probe vectors that utilise a variety of useful promoter-less reporter genes for identifying and chararacterizing *Streptomyces* promoter sequences has allowed the complexity of regulation of gene expression in *Streptomyces* to be studied. Promoter-less reporter genes used for analysis of *Streptomyces* promoters have included the aminoglycoside phosphotransferase gene (*aph*) from the transposon Tn*5* that confers resistance to kanamycin and neomycin in *Streptomyces* spp. (Ward et al., 1986), the *galK* gene of *E. coli* that codes for galactokinase (Brawner et al., 1985), the *cat* gene of *E. coli* that confers chloramphenicol resistance (Bibb and Cohen, 1982) and the *luxAB* operon of *Vibrio harveyi* that emits light in the presence of long-chain fatty acids (Schauer et al., 1988). However, not all reporter genes are ideal for characterization of all *Streptomyces* promoters. The *galK* gene requires the use of a special *S. lividans galT*$^-$ host for the detection of promoter activity above background levels. Another serious problem involves the extremely high guanine (G) plus cytosine (C) composition of the DNA of *Streptomyces*, which averages at about 73 mol% G+C (Enquist and Bradley, 1971), thereby biasing the codon usage to those containing C and G residues. Because many of the reporter genes, like *galK* and *lacZ* were isolated from *E. coli*, they will have a higher level of A (adenine) and T (thymine) containing codons, which theoretically may hinder translation of the indicator proteins. However, no clear experimental data has shown that this codon bias has prevented translation of messages in *Streptomyces*. Many *E. coli* reporter genes such as *lacZ* and *ampC*, also contain a number of TTA codons, which have been implicated in translational control in *S. coelicolor*. TTA codons are very rare in *Streptomyces*, are absent from vegetatively expressed genes, and are translated via a developmentally regulated tRNA, the product of the *bldA* gene (Leskiw, et al., 1991). Thus a reporter gene containing a number of these codons may be subject to translational regulation, and therefore the true transcriptional regulation determined by the DNA insert of interest would be masked. The *luxAB*

operon of *Vibrio harveyi* provides a luminescent product in the presence of long chain fatty acids that can be very useful for temporal and spatial monitoring of promoter activity (Schauer et al., 1988). However, special light detecting equipment is necessary for assaying relative activities, and the *luxAB* protein also contains a number of TTA codons that may affect the perceived expression of *luxAB* under the control of the promoter sequence in the DNA insert. Some reporter genes that express antibiotic resistance, such as *cat* and *aph* (conferring resistance to chloramphenicol, and neomycin and kanamycin respectively) have been popular because of their availability and the advantage of being able to select easily those transformants that have an active promoter. However, these types of reporter genes may not be ideal for detecting promoter activity of antibiotic biosynthetic genes which are normally active late in the growth cycle. The promoters may not be active early enough during the life cycle to allow the cells to grow, therefore, giving the incorrect conclusion of no promoter being present. When using these particular reporter genes, false negatives can be minimized by selecting transformants initially for the presence of the vector and then screening for promoter activity on antibiotic gradient plates.

One particularly useful reporter gene has been the *xylE* gene of *Pseudomonas putida*, another organism with DNA of high GC content, which encodes the enzyme catechol-2,3-dioxygenase (C23O) and was first exploited as a reporter gene in *Bacillus subtilis* by Zukowski et al. (1983). C23O rapidly converts colourless catechol to the intensely yellow coloured 2-hydroxymuconic semialdehyde. The presence of C23O activity can be monitored by spraying plate cultures with an aqueous catechol solution and the production of a bright yellow coloured product is indicative of C23O activity. Quantitative measures of specific activity can be carried out by spectrophotometric assays using cell free extracts. This particular reporter gene does not require special hosts, special conditions of growth or expensive equipment to visualize activity, and does not

contain TTA codons to complicate analysis of the transcriptional regulation. The *xylE* gene has been shown to act as a useful reporter gene in *S. lividans* (Ingram et al., 1989) and *S.coelicolor* A3(2) and suitable vectors for promoter probe analysis utilizing the promoter-less *xylE* gene have been developed. The *Streptomyces* high copy promoter probe vector, pIJ4083, contains the promoter-less *xylE* gene downstream of a multiple cloning site (MCS) that facilitates the directional sub-cloning of DNA inserts, to generate transcriptional fusions with the *xylE* gene (Clayton and Bibb, 1990). The vector itself is a broad-host-range, multi-copy vector that contains the thiostrepton resistance gene (*tsr*) of *Streptomyces azureus* for selection of transformants. Upstream of the MCS is a transcriptional terminator from the fd phage of *E. coli*, that has been reported as being efficient at preventing transcriptional read-through from vector promoter sequences (Ward et al., 1986). Translational stop codons are positioned in all three frames upstream of the ribosome binding site of the *xylE* gene, to prevent translational fusions from occurring.

There are a number of problems associated with the use of promoter probe vectors. First of all, the promoter sequence being analyzed is affected by the DNA environment of the insert that has been cloned into the promoter probe vector. This will not be the same environment that the promoter is found in within the chromosome, thereby the regulation of promoter activity may not be completely accurate. Fortuitous promoters may be generated during recombinant vector construction, and depending on the sensitivity of the reporter gene and growth conditions used, weak or highly regulated promoters may go undetected. Comparisons between different promoters can only be made in a general sense, as each promoter is found within its own specific environment. Therefore, the use of promoter probe vectors can give only a relative measure of the strength of a promoter and quantitative measurements of promoter activity are meaningful only when the same promoter construct is being assessed under different conditions.

Promoter probe vectors can be very useful when looking for promoters within a specific region of DNA, however, it is difficult to define the actual transcription start point (*tsp*) or the presence of multiple transcripts using this technique. A number of other procedures have been used successfully in *Streptomyces* that give detailed information about transcription. In vitro run-off transcription experiments have been utilized not only to define promoter regions, but also to indicate the class of sigma factor that may be required for initiation of transcription (Bibb et al., 1985; Buttner et al., 1988; Westpheling and Brawner, 1989). This has been very useful with genes having multiple promoters and has supplied a great deal of information on the heterogeneity of sigma factors in *Streptomyces*. Although this technique is very useful, the isolation of RNA polymerase and reconstitution procedures can be quite technically demanding since standard procedures are still being developed. As an alternative, mRNA analysis can provide a rapid, specific method for determining initial information about the transcriptional regulation of a gene of interest.

Messenger RNA isolated from procaryotes is characteristically metabolically unstable in comparison to many eucaryotic mRNAs. Procaryotic RNA molecules lack the 3' polyA extensions and 5' methylated caps that protect eucaryotic messages from degradation by nucleases. Techniques have been devised to isolate total RNA from *Streptomyces* cells grown from liquid or plate cultures, that involve the disruption of cells in the presence of strong protein denaturants (Hopwood et al., 1985). Northern analysis and hybridization of total RNA gives a direct indication of the number and size of transcripts for a particular gene. This technique can be very useful for a first assessment of the transcription pattern of a gene. One problem that has become evident with *Streptomyces* RNA studies is that RNA transcripts may be polycistronic and therefore very large. Large transcripts have proven to be difficult to isolate in an intact form, which can make Northern transfer and hybridization not particularly useful when

polycistronic messages are being analyzed. Many groups do not routinely use Northern analysis to examine the abundance and transcription pattern of a particular message due to these problems.

A particularly sensitive technique that is routinely used for detecting the presence and determining the *tsp* of RNA molecules is S1 nuclease protection. In this procedure an end-labelled DNA probe fragment that is believed to extend upstream beyond the 5' end of the RNA message of interest is allowed to anneal to total RNA under stringent conditions. S1 nuclease is added and allowed to incubate with the DNA/RNA hybrid to digest away any single-stranded part of the DNA probe fragment that is not protected by annealed RNA molecule. The protected DNA probe fragment is then electrophoresed on a polyacrylamide gel under denaturing conditions and the size of the protected DNA fragment can be directly established if a sequencing ladder is run in adjacent wells. Should the 5' end of the RNA molecule extend beyond the extent of the DNA probe, this can be detected by including non-homologous sequence at the 3' end of the complementary DNA strand (Leskiw et al., 1993). This non-homologous sequence would be removed during the nuclease treatment and would make it possible to differentiate full-length probe reannealing and thus protecting itself, and full-length probe protecting a full-length homologous transcript. S1 nuclease mapping can be a very precise technique and does not necessarily require that the entire RNA transcript be intact, since with S1 nuclease mapping one is assessing a small region of the whole RNA molecule (Hopwood et al., 1985).

A second type of procedure used to map the 5' end of transcripts is primer extension using the enzyme reverse transcriptase (Calzone et al., 1987). An oligodeoxyribonucleotide (oligo) complementary to the mRNA copy of the gene of interest is end-labelled and allowed to anneal to total RNA. The primer is then extended by reverse transcriptase as the annealed mRNA transcript acts as a template for synthesis

of the cDNA. Presumably, the 3' end of the generated DNA fragment will correspond to the 5' end of the mRNA transcript and the exact end-point of the protected DNA fragment can be established by electrophoresis.

The utilization of promoter probe vectors coupled with mRNA analysis has allowed the identification of a number of *Streptomyces* promoter sequences, mainly of secondary metabolic enzymes. A comprehensive review by Strohl (1992) of the 139 *Streptomyces* promoter sequences identified up to the time of the review indicated the striking heterogeneity of the DNA sequences upstream of the *tsp*. Only 29 out of the 139 promoter sequences identified appeared to conform to the *E. coli* consensus sequence (Hawley and McClure, 1983), which would correspond to those promoter sequences that could be recognized by the $E\sigma^{70}$ (vegetatively expressed RNA polymerase holoenzyme). Considering the high G+C content of *Streptomyces* DNA and the knowledge that promoter sequences of *E. coli* and *Bacillus subtilis* are known to be rich in adenine (A) and thymine (T) (Hawley and McClure, 1983), it is understandable that some *Streptomyces* promoters have been identified that are incapable of promoting transcription in *E. coli* (Bibb and Cohen, 1982). Strohl (1992) based his organization of promoter sequences on similar spacing between the -10 and -35 regions for those promoters that appeared to show homology to *E. coli*-like consensus sequences. However, he indicated that many promoters bore little resemblance to consensus sequences or to other promoter sequences in the -10 and -35 regions, suggesting that with at least some *Streptomyces* promoters, regions other than just the -35 and -10 are important for the regulation of transcription. The *Streptomyces* collection of promoters is biased to those genes that have been studied in depth, of which the majority are associated with production of secondary metabolites. This bias may be reflected in the low number of *Streptomyces* promoter sequences that resemble the *E. coli* consensus sequence that is vegetatively expressed in growing cells.

Heterogeneity of *Streptomyces* promoter sequences is consistent with the identification of at least three different σ factors in *S. coelicolor* A3 (2), σ^{35} (appears to recognize the consensus vegetative promoter), σ^{28}, and σ^{49}, which are capable of recognizing different promoter sequences and were identified by holoenzyme reconstitution experiments followed by run-off transcription assays using defined promoter sequences (Westpheling et al., 1985; Buttner et al., 1988). Furthermore, the detection of three genes, *hrdB*, *hrdC*, and *hrdD* in *S. coelicolor* A3(2) each homologous to the *E.coli* vegetative sigma factor gene *rpoD* (Buttner et al., 1990; Tanaka et al., 1988), but showing differential regulation and specificity to promoter sequences, suggests that *Streptomyces* not only produce a number of different classes of σ factors, but also produce multiple forms of each class of σ factor. In addition, genetic studies have indicated the presence of a sigma factor encoded by the *whiG* gene from *S. coelicolor* A3(2), that appears to play a critical role in triggering the onset of sporulation in aerial mycelia (Mendez and Chater, 1987). As stated in the review by Buttner (1989) this indicates the presence of at least 7 different sigma factors in *S. coelicolor* A3(2). Some *Streptomyces* genes contain an extra layer of transcriptional complexity in that they are regulated by multiple promoters (Westpheling and Brawner, 1989; Distler et al., 1987; Buttner et al., 1988). These promoters may be recognized by RNA polymerase possessing different sigma factors, such as the *dagA* gene of *S. coelicolor* which has 4 transcriptional start sites, with at least three of them being transcribed by a different form of RNA polymerase (Buttner et al., 1988). The *dagA* gene codes for an extracellular agarase, the first enzyme in the pathway for agar metabolism. The expression of this enzyme is subject to a number of controls including repression by glucose and other sugars, and induction by agar degradation products. It is postulated that if a series of different sigma factors appears and disappears throughout the growth cycle, this form of complex regulation of *dagA* using multiple promoters recognized by different sigma

factors may be necessary to ensure the expression of the gene throughout the growth cycle. *dagA* is only one example of *Streptomyces* genes with multiple promoter sequences. Strohl (1992) indicated that out of the 87 genes described in the review article, 27 of them (31%) have multiple promoters and that 13 loci were shown to have overlapping, divergent promoters, suggesting complex regulatory patterns. Antibiotic production is generally switched on late in growth, and is often sensitive to particular components of the growth media. As an example of this type of sensitivity to environmental conditions, different enzymes in the cephamycin biosynthetic pathway of *S. clavuligerus* have been shown to be repressed by a readily utilizable carbon source (such as glycerol), excess nitrogen (such as ammonia), and abundant supplies of inorganic phosphate (reviewed in Demain and Piret, 1990). Exhaustion of such repressing constituents of the culture medium may contribute to the switching on of some antibiotic biosynthetic enzymes. This illustrates the fine level of regulation that the genes of these pathways are under, and the possibility of complex regulatory control.

Of all the enzymes of the penicillin, cephalosporin and cephamycin pathways, IPNS has been the most extensively studied to date. Analyses to define its substrate requirements (Baldwin et al., 1983; Jensen et al., 1984; Huffman et al., 1992) and mechanism of action (Samson et al., 1987a; Baldwin and Bradley,1990) have been carried out although the crystal structure of the enzyme is yet to be determined. These types of studies require large quantities of IPNS enzyme, but by giving insights into the regions important for substrate binding and enzyme activity, they may suggest ways to alter the structural gene to encode an enzyme that has an increased specific activity and/or broadened substrate specificity to allow for the synthesis of novel, unnatural antibiotics (Wolfe et al., 1984). Large amounts of purified IPNS are difficult to obtain from natural sources such as *S. clavuligerus*. Typical yields of 0.2 mg of purified IPNS from 2 L of *S. clavuligerus* culture with a specific activity of 1.58 mU/g protein have been reported

(Jensen et al., 1986). Expression of the *pcbC* gene in heterologous hosts, such as *E. coli*, can generate the quantities of enzyme necessary. The *pcbC* genes of *S. jumonjinensis* (Shiffman et al., 1988), *S. lipmanii* (Weigel et al., 1988), *A. nidulans* (Ramon et al., 1987), *C. acremonium* (Samson et al.,1985), *P. chrysogenum* (Carr et al., 1986), and *S. clavuligerus* (Durairaj et al., 1992), have been expressed at high level in *E. coli* and the protein produced is apparently indistinguishable from the native enzyme. For example, IPNS specific activities obtained from high level expression of the *S. clavuligerus pcbC* gene in *E. coli* reached levels of 79.28 x 10^3 mU/g of protein, (Durairaj et al., 1992). However, when the *S. clavuligerus* and *S. griseus pcbC* genes with their native control signals were introduced into the heterologous *Streptomyces* non-producing host, *S. lividans*, no active IPNS enzyme was detected (Jensen et al., 1989; Garcia-Dominguez, 1990). This may indicate some form of species-specific transcriptional regulation.

Because the IPNS enzyme has been so thoroughly studied to date and catalyzes a universal reaction necessary for the biosynthesis of penicillins, cephalosporins and cephamycins, we chose the corresponding gene, *pcbC*, of *S. clavuligerus* to initiate our analysis of the transcriptional controls that regulate antibiotic biosynthesis.

II. MATERIALS AND METHODS

II.1 MATERIALS

Restriction endonucleases were obtained from either Bethesda Research Laboratories, Inc., Burlington, Ont. or Boehringer Mannheim, Laval, Quebec. T4 DNA ligase, polynucleotide kinase from T4 infected *Escherichia coli* B cells, *E. coli* DNA polymerase I, Klenow fragment of *E. coli* DNA polymerase, DNA polymerase from *Thermus aquaticus* (Taq), Exonuclease III, Bal31 nuclease, S1 nuclease and Avian Myeloblastosis Virus (AMV) reverse transcriptase were obtained from Boehringer Mannheim. Sequenase, a chemically modified form of T7 DNA polymerase, was purchased from United States Biochemicals, Cleveland, Ohio. RQ1 RNAase-free DNAaseI was obtained from Pharmacia P-L Biochemicals Inc., Milwaukee, Wisconsin. All enzymes were used according to specifications advised by the manufacturer.

Deoxyribonucleoside triphosphates, dideoxyribonucleoside triphosphates and the modified nucleoside triphosphate 2'-deoxyribo-7-deazaguanosine-5'triphosphate were purchased from Boehringer Mannheim.

Radioactively labelled [α-^{32}P]dATP, [γ-^{32}P]ATP and [α-^{35}S]dATP were obtained from ICN Biochemicals, St.-Laurent, Quebec.

The oligo primer used to generate an *Sph*I site at the *pcbC* start codon by site-directed mutagenesis was obtained from the Regional DNA Synthesis Laboratory, University of Calgary, Calgary, Alberta, Canada. All other oligo primers including specific oligo primers used for polymerase chain reactions (PCR) and universal M13 sequencing primers, were purchased from the Dept. of Microbiology DNA Synthesis Laboratory, University of Alberta, Edmonton, Alberta, Canada.

Thiostrepton was obtained from S. Lucania, Squibb and Sons, Inc.

Bis δ-(L-α-aminoadipyl -L-cysteinyl)-D-valine (bis-ACV) was a generous gift from S. Wolfe, Simon Fraser University, Burnaby, B.C., Canada.

Trypticase soy broth (TSB) was purchased from BBL, Becton Dickinson Microbiology Systems, Cockeysville, Maryland, USA. Yeast extract, Bacto peptone, nutrient broth and malt extract were purchased from Difco Laboratories, Detroit, Michigan, USA.

All other chemicals used in this study were reagent grade.

II.2 BACTERIAL STRAINS, PLASMIDS, PHAGES AND CULTURE CONDITIONS

Streptomyces clavuligerus (NRRL 3585 Northern Regional Research Center, Peoria, Ill.) and *Streptomyces lividans* TK24 (Kieser et al., 1982) were maintained as frozen glycerol stocks of spores, and plasmid-containing strains were maintained as frozen glycerol stocks of mycelia. This difference in procedures for maintenance was a result of observations in our laboratory that indicated rapid plasmid loss when cells were sporulated. *S. clavuligerus* was grown on sporulation media as described by Jensen et al. (1982b) and spores were scraped from plates using a sterile spatula into 20% glycerol (v/v) at 0.5 mL/plate. The spore suspension was filtered through sterile non-absorbant cotton packed into a 10 mL syringe. 0.5 mL aliquots were dispensed into sterile screw-capped tubes and stored at -70°C. *S. clavuligerus* cultures containing plasmids were grown in 25 mL of trypticase soy broth (TSB) + 1% starch (TSBS) in the presence of thiostrepton at a final concentration of 10 µg/mL for 36h at 28°C and 280 rpm and were homogenized using sterile No. 22 or 23 homogenizers (Kimble Kontes Glass Co.). The dispersed mycelia were centrifuged at 12,100 x g for 10 min, resuspended in 5 mL of 20% (v/v) glycerol, and dispensed into 0.5 mL samples to be stored at -70°C. Spore and mycelial stocks of *S. lividans* TK24 were prepared as described previously (Hopwood et al., 1985).

Streptomyces plasmids, pIJ4083 (Clayton and Bibb, 1990) and pIJ702 (Katz et al., 1983) were generous gifts of M.J. Bibb of the John Innes Inst., Norwich, England.

pIJ4070, a pUC18 derivative, that contains the strong constitutively expressed *ermE** promoter mutant form of the erythromycin resistance gene (*ermE*) promoter region of *Saccharopolyspora erythraea* (Bibb et al., 1986; Gramajo et al., 1991) was also received from the John Innes Inst., Norwich, England.

S. clavuligerus and *S. lividans* were grown for the purposes of plasmid isolation according to Hopwood et al. (1985). For small scale preparations, cells were grown in 10 mL of TSB + 1% glycerol (v/v) (TSBG) with a final thiostrepton concentration of 5 μg/mL. For large scale plasmid isolation, seed cultures were started from glycerol stocks and grown at 28°C in 25 mL of TSBG supplemented with 5 μg/mL thiostrepton in 125 mL flasks at 280 rpm. Cells were grown for 36h, homogenized when necessary, and subcultured at 2% (v/v) into 200 mL of TSBG (2 x 100 mL in 500 mL flasks) and further incubated for 36-40h.

For preparation of cell free extracts for enzyme assays, *S. clavuligerus* and *S. lividans* were cultivated on TSBS and TSBG respectively, and supplemented with 5 μg/mL thiostrepton when necessary. Seed cultures were started from glycerol stocks following the same procedure stated for large scale plasmid isolation, and subcultured into 100 mL of liquid media in 500 mL flasks. Samples were further incubated at 28°C and 280 rpm for the stipulated time periods before harvesting.

For purposes of generating protoplasts, *S. clavuligerus* was grown according to Bailey and Winstanley (1986). One 0.5 mL spore stock was used to inoculate a 25 mL seed culture of TSBG in a 125 mL flask. Cells were grown for 36h and then subcultured at 2% (v/v) into 8 x 25 mL amounts of medium composed of: [0.18% (w/v) yeast extract, 0.18% (w/v) malt extract, 0.24% (w/v) Bacto peptone, 0.6% (w/v) glucose, 0.4% (v/v) glycerol, 20.4% (w/v) sucrose, 1.2% (w/v) TSB, 0.5% (w/v) glycine and 0.1% (w/v) $MgCl_2$]. The cultures were grown for an additional 40h at 28°C and 280 rpm. Culture

conditions and media used for preparing *S. lividans* TK24 cells for protoplasting were as described by Hopwood et al. (1985).

Growth conditions for RNA extractions were described previously (Petrich et al., 1992). *S. clavuligerus* and *S. lividans* cultures were started from glycerol spore stocks and grown in TSBS and TSBG respectively for 36h or the specific time indicated. *S. lividans* cultures containing pIJ4083 recombinant plasmids were started from glycerol mycelial stocks and allowed to grow for 36h in the presence of 5μg/mL thiostrepton.

The *E. coli* strain used in this study was MV1193 (Viera and Messing, 1987). This strain, the *E. coli* phagemids pUC118 and pUC119, and the helper phage M13K07 (Viera and Messing, 1987) were all gifts from J. Viera, Department of Biochemistry, University of Minnesota. The *E. coli* cells were grown and competent cells prepared according to Morrison (1979). For all other purposes, *E. coli* strains were grown on 2YT [1.6% (w/v) tryptone, 1.0% (w/v) yeast extract, 0.5% (w/v) NaCl] broth or agar plates (Miller, 1972) supplemented with 50 μg/mL of ampicillin to maintain plasmids. All other *E. coli* maintenance procedures and culture conditions followed standard procedures (Sambrook et al, 1989; Viera and Messing, 1987).

II.3 ISOLATION OF PLASMID DNA

Plasmids replicated in *S. lividans* and *S. clavuligerus* were isolated and purified according to the alkaline lysis method of Kieser et al. (1984) as described by Hopwood et al. (1985) for small and large scale preparations.

E. coli plasmids and phagemids were isolated according to a modification of the method of Birnboim and Doly (1979) recorded in Sambrook et al. (1989). Single-stranded forms of pUC118 and pUC119 based phagemids were purified according to the method reported by Viera and Messing (1987). All plasmid preparations were analyzed by agarose gel electrophoresis. Some recombinant plasmids were subjected to DNA

sequence analysis, described in section II.4.2, to ensure that the correct fragment had been inserted in the desired orientation.

II.4 INTRODUCING DNA BY TRANSFORMATION

II.4.1 Transformation of *S. lividans* TK24

The procedure used to make protoplasts of *S. lividans* TK24 was previously described in Hopwood et al. (1985). There were however, slight modifications to the procedure described for transformation and these are reported below. Instead of a large 50 mL centrifuge tube being used for the transformation procedure, a sterile 1.5 mL centrifuge tube containing a 100 µL aliquot of protoplasts (approx. 1 x 10^9 cells) was centrifuged in an Eppendorf microfuge at 2,940 x g (6000 rpm) for 3 min. The supernatant was discarded and cells were resuspended in the residual liquid by gently tapping the tube with a finger. The volumes used during the transformation procedure were decreased by a factor of 5 for all the procedures, except the DNA which remained at a volume of 10-20 µL. After 24h of regeneration at 30°C, the plates were overlayed with 1 mL of sterile distilled H_2O containing thiostrepton to give a final concentration per plate of 10 µg/mL, taking into account the volume of agar in the plate.

II.4.2 Protoplast formation and transformation of *S. clavuligerus* 3585

The procedure used for making protoplasts from *S. clavuligerus* cells was based on the method outlined by Hopwood et al. (1985) with modifications by Illing et al. (1986). All procedures were performed under aseptic conditions. Cells from 8 x 25 mL cultures were spun down at room temperature in 50 mL centrifuge tubes at 12,100 x g for 15 min. The pellets were washed twice with 10 mL of 10.3% (w/v) sucrose. The mycelium was resuspended in 4 mL of lysozyme buffer which consisted of 1mg/mL of lysozyme dissolved and filter sterilized in P-buffer [0.3 M sucrose, 25 mM MOPS, pH 7.2, 0.057 mM K_2SO_4, 48 mM $CaCl_2$, 0.59 µM $ZnCl_2$, 1.48 µM $FeCl_3$, 0.12 µM $CuCl_2$, 0.10 µM $MnCl_2$, 0.052 µM $Na_2B_4O_7$ and 1% (w/v) bovine serum albumin). Samples

were pooled into 4 tubes and incubated at 28°C with gentle shaking (50 rpm) for 1h. Every 15 min the samples were triturated 3 times with a 10 mL pipette to disperse the mycelia. To determine if the formation of protoplasts was complete, samples were visually scrutinized under the phase-contrast microscope. When filamentous mycelia could no longer be detected and protoplasting was considered to be essentially complete, 10 mL of P-buffer was added to stop the reaction, triturating as above. The protoplast suspension was filtered through non-absorbant cotton into fresh centrifuge tubes. Protoplasts were pelleted at 1,090 x g for 10 min and washed with 10 mL of P-buffer. Protoplasts were resuspended in P-buffer and dispensed in 100 µL aliquots (approx. 1 x 10^9 cells) to be slowly frozen and stored at -70°C.

Because of the highly active restriction system of *S. clavuligerus*, covalently closed circular forms of the desired plasmid constructions were isolated first from *S. lividans*, which is free of DNA modification and restriction systems, and then introduced into *S. clavuligerus*. The procedure used for introducing plasmid DNA into *S. clavuligerus* is based on Bailey and Winstanley (1986) with minor modifications. An aliquot of frozen protoplasts (10^9 cells) was thawed quickly and washed with 1 mL of the *S. clavuligerus* P-buffer described above. Cells were centrifuged in an Eppendorf microfuge at 2,940 x g for 3 min and the pellet was gently resuspended in the drop of P-buffer that remained after decanting. Twenty microlitres of 10.3% (w/v) sucrose was added to help resuspend the pellet and the cells were heat-pulsed at 43°C for 5 min. DNA (up to 20 µL) was added and immediately 100 µL of T-buffer [(P-buffer that contained 25% (w/v) PEG1000 in place of bovine serum albumin)] was added and mixed with the cells and DNA by pipetting. One millilitre of P-buffer was quickly added to stop the reaction. Transformed cells were centrifuged as above and plated onto R2YE regeneration agar composed of: 0.5 M sucrose, 0.58 mM K_2SO_4, 59 mM sodium glutamate, 48 mM $CaCl_2$, 25 mM MOPS, pH 7.2, 0.20 mM $MgSO_4$, 0.29 µM $ZnCl_2$, 0.74 µM $FeCl_3$,

0.059 μM $CuCl_2$, 0.050 μM $MnCl_2$, 0.026 μM $Na_2B_4O_7$, 0.008 μM $(NH_4)_6Mo_7O_{24}$, 1% (v/v) glycerol, 0.1% (w/v) casaminoacids, 0.5% (w/v) yeast extract, and 2% (w/v) agar. Plates were incubated at 21°C for 24h and then at 28°C for 16h before overlaying with 3.0 mL of soft nutrient agar [0.8% (w/v) nutrient broth, 0.7% (w/v) agar] containing thiostrepton to give a final concentration per plate of 5 μg/mL.

II.4.3 Transformation of *E. coli* MV1193

Transformation of competent *E. coli* MV1193 cells followed standard procedures outlined by Sambrook et al. (1989) with minor alterations. One hundred microlitres of 50 mM $CaCl_2$ was placed into a sterile 13 x 100 mm test tube and the desired DNA in a volume up to 20 μL was added. This tube was then placed on ice and the mixture was allowed to cool (about 10 min). A 250 μL aliquot of competent *E. coli* MV1193 cells, prepared as described in section II. 2, was added to the iced mixture and the sample was left to incubate on ice for a further 30 min. The cells were then heat-pulsed at 42°C for 120s, plated out onto 2YT plates supplemented with 50 μg/mL ampicillin and incubated overnight at 37°C. For plasmid constructions that involved inserting a DNA fragment into the polylinker of pUC118 or pUC119, screening for insertion was based on a blue/white color differentiation due to β-galactosidase inactivation. Colonies containing a pUC118 or pUC119 plasmid produce the α-peptide of β-galactosidase, and when grown in the presence of the inducer IPTG (Isopropyl-β-D-thiogalactopyranoside) and the chromogenic substrate X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactoside) would appear blue. Colonies that had lost the ability to produce β-galactosidase due to insertion of a foreign DNA fragment into the polylinker would appear white. Standard levels of IPTG (50 μL of a 100 mM solution per plate) and X-gal (50 μL of a 2% (w/v) solution per plate) were used in 2YT plates for suitable transformations.

II.5 TECHNIQUES USED FOR DNA ANALYSIS

II.5.1 Restriction fragment analysis by agarose gel electrophoresis

DNA fragments in the size range of 75-800 bp were subjected to electrophoresis on 1.25% (w/v) agarose gels using a TEA buffer system (40 mM Tris-Acetate, pH 8.0, 1 mM EDTA). DNA fragments of 0.8-5 kb were analyzed on 1.0% (w/v) agarose gels, and DNA fragments in the size range of 6-14 kb were electrophoresed on 0.6-0.8% (w/v) agarose gels using the TEA buffer conditions described above. Appropriate molecular weight markers were either *Bst*EII or *Pst*I restriction fragments of λ phage DNA.

II.5.2 DNA sequence analysis

DNA sequence analysis was performed using the chain termination method of Sanger et al. (1977) as modified by Tabor and Richardson (1987) for use with Sequenase. Earlier work used [α-^{32}P]dATP as the radioactive marker, but with the purchase of a BioRad ^{35}S sequencing gel apparatus, the labelled deoxynucleotide used was [α-^{35}S]dthioATP. For checking the orientation of small DNA inserts into pUC118 or pUC119 and for determining the extent of deletions to the *pcbC* upstream DNA fragment, the universal M13 sequencing primer was used to generate extension products in sequencing reactions. For the purposes of generating a sequencing ladder in 5' primer extension and S1 nuclease mapping, the same oligo that was used for the actual extension reaction (5' primer extension) or used for PCR to amplify the probe fragment, was used for the sequencing reactions.

Labelled extension products in the sequencing reactions were separated by size on 6% polyacrylamide (38:2, acrylamide: N,N'-methylene bisacrylamide) denaturing gels (8.3 M urea) using a TEB buffer system (100 mM Tris-HCl, pH 8.0, 1 mM EDTA, 60 mM borate). After electrophoresis, gels containing ^{32}P labelled DNA fragments were lifted off the glass plates using old X-ray film and covered with Saran Wrap before placing in the X-ray cassette holder. Sequencing gels containing ^{35}S labelled extension products were placed into a fixing solution (10% methanol, 10% acetic acid) for 10-15 min, the gel was lifted onto Whatman (3MM) filter paper and dried using a Biorad Model

583 Gel Dryer. Radioactive bands were visualized by exposing the frozen ^{32}P gels or the fixed and dried ^{35}S sequencing gels to Kodak X-OMAT AR film at -70°C. Films were developed using a FUJI RGII X-ray film processor.

II.5.3 Hybridization analysis

Depending on the type of samples being analyzed, one of two types of DNA hybridization techniques was used. Colony hybridizations were performed when a large number of transformants were available and when no positive selection system distinguished between the desired plasmid construction and other possible recombinants. When a smaller number of samples was being analyzed and the plasmid DNA was readily available, Southern hybridization was chosen as a rapid detection for the presence of DNA inserts of interest. Stringency of the pre-hybridization and hybridization procedures used was identical in each case (see below).

II.5.3.1 *E. coli* colony hybridization

Colony hybridization using Hybond-N Nylon membranes (Amersham Corp., Oakville, Ont.) was performed according to the Amersham Manual (1985) with minor alterations. Circular nylon membranes were labelled with a graphite pencil and placed on 2YT agar plates containing ampicillin at 50 μg/mL. Colonies were picked onto the filter with sterile toothpicks and were also picked onto another plate. About 90 colonies were picked per plate and the cells were allowed to grow at 37°C for 6-8h until a small distinct patch of growth was visible. The filters were removed from the plate and placed colony side up, onto a pad of Whatman (3MM) filter paper soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 7 min. Filters were then placed, colony side up onto a pad of filter paper soaked in neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.2, 0.001 M Na_2EDTA) for 3 min. This step was repeated with a fresh pad of filter paper. Filters were washed in 2xSSC (0.3 M NaCl, 0.03 M $Na_3Citrate$) and rubbed with a gloved hand

to remove any colony debris. The filters were air dried and baked in an 80°C vacuum oven for 2h.

II.5.3.2 *Streptomyces* colony hybridization

The procedure used for colony hybridization is that described in Hopwood et al. (1985) with minor alterations. Colonies were picked onto nylon filters which had been placed onto MYM agar plates [0.4% (w/v) yeast extract, 1.0% (w/v) malt extract, 0.4% (w/v) maltose, 2.0% (w/v) agar] supplemented with 5 μg/mL of thiostrepton. Plates were incubated at 28°C for about 24h. To weaken the cell wall, nylon filters were placed, colony side up, on filter papers soaked in lysozyme buffer (4 mg/mL lysozyme in TE buffer) for 45 min. Filters were moved to paper soaked with denaturing solution (1 M NaOH, 1% (w/v) SDS) for 20 min. Nylon filters were placed on filter papers soaked in neutralization buffer (7 parts 1 M Tris-HCl, pH 7.5 and 3 parts 5 M NaCl) with two changes (5 min each). Filters were washed and rubbed in 2xSSC + 0.1% sodium dodecyl sulfate (SDS) to remove debris, rinsed in 2xSSC, and baked for 2h in a vacuum oven at 80°C.

II.5.3.3 Preparation of Southern blots

The procedure followed for transferring separated DNA fragments from an agarose gel to a nylon membrane was an adaption of the method of Southern (1975) described by Rigaud et al. (1987). The gel of interest was soaked with gentle shaking in 0.25 N HCl for a total of 30 min (2 x 15 min). DNA fragments were denatured by soaking the gel twice in denaturing solution (described in section II.5.3.1) for a total of 40 min. The gel was allowed to equilibrate in transfer solution (1 M NH_4Acetate, 0.02 N NaOH) for 1h (2 x 30 min). DNA was blotted to a nylon membrane in the above transfer solution with a transfer set-up as described by Sambrook et al. (1989). After overnight transfer, the filter was rinsed in 2xSSC and allowed to air dry before baking in an 80°C vacuum oven for 2h.

II.5.3.4 Generation of nick-translated ^{32}P labelled probes

The method for labelling a DNA fragment by nick-translation is based on the protocol described by Sambrook et al. (1989). Plasmid DNA or DNA restriction fragments (200-500 ng) were labelled with 20 μCi of [α-^{32}P]dATP in a buffer containing; 50 mM Tris-HCl, pH 7.5, 10 mM $MgSO_4 \cdot 7H_2O$, 0.1 mM dithiothreitol (DTT), 50 μg/mL BSA, 0.01 ng DNAaseI, 0.03 mM dTTP, 0.03 mM dCTP, 0.03 mM dGTP, and 1 unit *E. coli* DNA polymerase I at 15°C for 1h. The labelled probe was separated from unincorporated [α-^{32}P]dATP on a 10 mL Sephadex G-50 column.

II.5.3.5 Hybridization of colony and Southern blots

Blots were hybridized using the procedure outlined in the Amersham Manual (1985) with minor alterations. The nylon filters were placed in a plastic Seal-a-Meal bag (Dazey Corp., Industrial Airport, KS.) with prehybridization solution of the following composition: 0.9 M NaCl and 0.09 M sodium citrate (6xSSC), 0.5% SDS, 5x Denhardt's (Denhardt, 1966), and 10 μg/mL denatured salmon sperm DNA. Enough prehybridization solution was added to completely cover the filter when it was laid in a horizontal position and the blot was then incubated at 65°C with shaking for at least 2h.

Hybridization of the desired probe was performed by replacing the prehybridization solution with hybridization solution (prehybridization solution with approx. 20 μCi of denatured ^{32}P-labelled probe). The bag was resealed and allowed to continue to incubate with shaking overnight at 65°C. The filter was removed and washed twice in 2xSSC + 0.1% SDS and twice in 0.2xSSC + 0.1% SDS for 30 min each at 60°C. Filters were air dried, wrapped in plastic wrap, and exposed to Kodak X-OMAT AR film at -70°C.

II.6 SITE-DIRECTED MUTAGENESIS TO INTRODUCE AN *Sph*I SITE AT THE START CODON OF *pcbC*

II.6.1 Phosphorylation of the mutagenic oligo.

To introduce an *Sph*I site by a single base change (T to G) at the start codon of the *pcbC* gene, the two primer procedure of Zoller and Smith (1987) was followed with minor alterations. The 17-mer oligo 5'-CTGGCATGCAACCCTCC-3', homologous to the coding sequence of nt -10 - +7 relative to the *pcbC* start except for the one mismatch located directly in the centre of the oligo, was synthesized for the purpose of generating the 1 bp change. Two hundred pmoles of mutagenic oligo was phosphorylated in a reaction that contained; 100 mM Tris-HCl (pH 8.3), 10 mM $MgCl_2$, 10 mM DTT, 0.5 mM ATP, and 2 units T4 polynucleotide kinase in a final volume of 20 µL and allowed to incubate at 37°C for 1h. The reaction was stopped by heating at 65°C for 10 min.

II.6.2 Annealing the primers and synthesis of the complementary DNA strand.

The single-stranded (ss) form of the pIPS-1 plasmid (Doran et al., 1990), a pUC119-derived plasmid containing a 1.7-kb insert of *S. clavuligerus* DNA which includes the *pcbC* gene together with 335 bp of upstream sequence and 383 bp of downstream sequence, was isolated to use as a template for the mutagenesis experiment. In a small plastic centrifuge tube 10 pmoles each of universal M13 sequencing primer and phosphorylated mutagenic oligo were added to 200 ng of the ss template DNA in a reaction buffer composed of; 20 mM Tris-HCl (pH 7.4), 2 mM $MgCl_2$, and 50 mM NaCl in a final volume of 10 µL. The sample was denatured for 10 min at 55°C, allowed to cool slowly down to 25°C, and then placed on ice. Synthesis of the second strand was initiated with the addition of 2 units T4 DNA polymerase and 5 units T4 DNA ligase in a final reaction buffer composed of; 17.5 mM Tris-HCl (pH 7.4), 0.75 mM ATP, 0.4 mM each dNTP, 3.75 mM $MgCl_2$, and 21.5 mM DTT. The reaction mixture was incubated on ice for 5 min, then at 25°C for 5 min, and finally at 37°C for 90 min.

II.6.3 Transformation and detection of mutants.

Two microlitres of the above DNA extension mixture was transformed into competent *E. coli* MV1193 cells according to procedures described in section II.4.3.

Twenty transformants were picked and grown in 2YT + ampicillin (50 μg/mL) broth for plasmid isolation as described in section II.3. About 1 μg of DNA of each plasmid sample was treated with *Sph*I restriction endonuclease and samples were analyzed on a 1.25 % agarose gel. The non-mutagenized pIPS-1 plasmid contains a single *Sph*I site at one end of the polylinker. Those samples that contained the single bp change would have acquired an additional *Sph*I site 335 bp into the DNA insert. The restriction endonuclease-treated samples with the newly generated *Sph*I site would run as two distinct bands on an agarose gel, as opposed to the wild type sample which would run as a single band. About 20% of the transformants analyzed contained the desired base change and these plasmids were then called pIPS-1*.

II.6.4 Insertion of the *Sph*I fragment upstream of *pcbC* from pIPS-1* into pUC119 (pIPS-1s).

pIPS-1* (1 μg) was digested with *Sph*I and the reaction mixture was analyzed on an agarose gel (1.25%). The 335 bp band was cut out of the gel and extracted from the agarose using the Gene Clean kit (Bio 101, La Jolla, Calif.) according to the manufacturer's specifications. This procedure will hereinafter be referred to as gel purification. The gel purified DNA fragment was then ligated to *Sph*I digested pUC119. The ligated DNA was transformed into *E. coli* MV1193 cells, and the cells were plated onto 2YT plates supplemented with ampicillin (50 μg/mL), IPTG and X-Gal as previously described. White transformants were cultivated for plasmid isolation and production of single-stranded plasmid. The orientation of the *Sph*I restriction fragment was determined by DNA sequencing using the M13 universal sequencing primer. The recombinant plasmid with the desired orientation of the insert (start codon near *Eco*RI site of polylinker) was given the name pIPS-1s, and used for subsequent manipulations.

II.7 PROMOTER PROBE CONSTRUCTIONS

The high copy *Streptomyces* plasmid pIJ4083 contains the promoter-less *xylE* gene which encodes the enzyme catechol-2,3-dioxygenase (C23O). This vector contains a

multiple cloning site (MCS) immediately upstream of the *xylE* gene that readily allows the directional cloning of DNA fragments to ascertain the presence and relative strength of promoters found within these DNA fragments.

II.7.1 Insertion of the *pcbC* gene into pIJ4083 (1.7/4083)

The previously described plasmid, pIPS-1 (2 μg) which is a pUC119 derivative that contains the *pcbC* gene on a 1.7 kb *Sma*I fragment, was digested with *Hin*dIII and *Eco*RI. The reaction mixture was run on an agarose gel (1.0%) and the 1.7 kb insert was gel purified. The purified DNA fragment was ligated to 400 ng of similarly digested pIJ4083 using standard procedures. The ligation mixture was introduced into *S. lividans* TK24 protoplasts and transformants were screened by colony hybridization using nick-translated pIPS-1 as a probe. Plasmid DNA was isolated from those transformants that hybridized strongly to the probe and further screened by restriction endonuclease digestion and gel electrophoresis. pIJ4083 containing the 1.7 kb insert was named 1.7/4083 and approximately 1 μg of plasmid DNA was transformed into *S. clavuligerus* protoplasts. Transformants were analyzed by restriction endonuclease digestion and gel electrophoresis.

II.7.2 Insertion of the DNA region upstream of *pcbC* into pIJ4083 (*pcbC*up/4083)

The plasmid construction pIPS-1s (2 μg), described above in section II.6, was digested with *Hin*dIII and *Eco*RI to remove the DNA fragment which originated upstream of the *pcbC* coding region (*pcbC*up). This 335 bp fragment was gel purified and ligated to *Eco*RI/*Hin*dIII digested pIJ4083 (400 ng). The ligation mixture was transformed into *S. lividans* and transformants that appeared yellow when sprayed with an aqueous 0.5 M catechol solution were further analyzed by restriction endonuclease digestion with *Eco*RI and *Hin*dIII. Plasmid DNA was then isolated from those colonies that contained the correct construction (named *pcbC*up/4083), and introduced into *S. clavuligerus*. Initial screening by the spraying of plates with catechol to detect yellow transformants (ie. those

producing C23O) was confirmed by restriction endonuclease digestion and gel electrophoresis.

II.7.3 Insertion of the *ermE** up-promoter into pIJ4083 (ERME*/4083)

The 380 bp DNA fragment containing the *ermE** up promoter was removed by digesting 2 µg of the plasmid pIJ4070 (M.J, Bibb, personal communication) with *Eco*RI and *Bam*HI. After electrophoresis, on a 1.25 % agarose gel, the fragment of interest was gel purified and ligated to similarly digested pIJ4083 (400 ng) before transformation into *S. lividans*. *S. lividans* transformants were sprayed with 0.5 M catechol. Those colonies that turned yellow were cultivated for plasmid isolation and restriction analysis. pIJ4083 containing the *ermE** promoter region (ERME*/4083) was then introduced into *S. clavuligerus* by transformation. Colonies containing the plasmid construction were detected by their ability to produce C23O.

II.7.4 Insertion of the DNA region upstream of *pcbAB* into pIJ4083

The DNA region upstream of *pcbAB* was first inserted into pUC119 in order to obtain suitable restriction sites to insert the *pcbAB* upstream region directionally into pIJ4083.

II.7.4.1 Insertion of the DNA region upstream of *pcbAB* into pUC119 (pA2-S/119)

The cosmid 8-51, containing a fragment of *S. clavuligerus* DNA inserted into the vector pLAFR3 (Staskawicz et al., 1987), and known to contain the *lat* gene and *pcbAB* gene (K. Aidoo, personal communication) was digested with the restriction enzyme *Kpn*I and the DNA fragments were analyzed on a 1.0% agarose gel. A 687 bp band (nt -670 to +18 relative to the *pcbAB* start) was gel purified and ligated into *Kpn*I digested pUC119. *E. coli* MV1193 competent cells were transformed and white colonies on 2YT plates containing ampicillin, X-gal and IPTG, were selected and cultivated for plasmid isolation. The orientation of the insert was determined by DNA sequence analysis and those plasmids of the desired orientation (with the start codon being nearest the *Eco*RI site of the

polylinker) were named pA2/119. pA2/119 plasmid was digested with *Sal*I, which cleaves on one side of the polylinker and also within the insert (-344 relative to the *pcbAB* start codon). The vector was then re-ligated and transformed into *E. coli* MV1193 cells. Those transformants that had lost the *Sal*I fragment now contained a plasmid (pA2-S/119) with a 358 bp insert (*pcbAB*up; nt -344 to +18 relative to the *pcbAB* start).

II.7.4.2 Transfer of the DNA region upstream of *pcbAB* from pA2-S/119 into pIJ4083 (*pcbAB*up/4083)

Two μg of plasmid pA2-S/119 DNA was digested with *Eco*RI and *Hin*dIII and the fragments were electrophoresed on a 1.25% agarose gel. The 358 bp DNA fragment was gel purified and ligated to 400 ng of similarly digested pIJ4083. *S. lividans* protoplasts were transformed and screened by colony hybridization for those containing recombinant plasmids carrying the *pcbAB* upstream fragment (*pcbAB*up). The probe used for hybridization was the plasmid pA2-S/119 labelled with ^{32}P by nick-translation. Plasmid was isolated from positive colonies, checked by restriction endonuclease treatment followed by gel electrophoresis, and the correct recombinant plasmid (*pcbAB*up/4083) was transformed into *S. clavuligerus* protoplasts. Colonies harbouring *pcbAB*up/4083 were determined by restriction endonuclease digestion and gel electrophoresis of isolated plasmid DNA.

II.7.5 Insertion of the DNA region upstream of *lat* into pIJ4083

To simplify the directional subcloning of the DNA region upstream of *lat* into pIJ4083, the region of interest was first inserted into pUC118.

II.7.5.1 Construction of a pUC118 recombinant plasmid containing the DNA region upstream of *lat* using the polymerase chain reaction (pLat/118)

No useful restriction sites were available for subcloning the DNA region upstream of *lat*. Therefore, the desired DNA fragment was amplified using the polymerase chain reaction (PCR), as outlined by Saiki et al. (1988) with minor modifications described below. Primers used were the M13 reverse sequencing primer (17-mer) and the synthetic

oligo 5'CATGCGGATCCCGTCGACGAGCATATGC-3'. The 3' end of the latter primer, up to the underlined portion (recognition site for *Bam*HI restriction endonuclease), is homologous to the coding strand of *lat* from nt +87 to +103. The template for amplification was a pUC118 subclone containing a 1.08 kb *Eco*RI/*Kpn*I insert of *S. clavuligerus* DNA obtained from the cosmid 8-51 described in section II.7.4.1, that included 230 bp of sequence upstream of the *lat* coding region and 850 bp of the *lat* gene. Reaction conditions used were as follows: 70 mM Tris-HCl, pH 8.8, 2 mM $MgCl_2$, 0.1% (w/v) Triton X-100, 2.5 mM dNTPs, 1% (v/v) DMSO, 1 unit Taq DNA polymerase, 20 pmoles of each oligo, and 10 ng template DNA in a final volume of 100 µL. Fifty microlitres of mineral oil was added and the samples were denatured for 3 min at 92°C. Thirty cycles composed of three components: 92°C for 30s, 55°C for 30s, and 72°C for 60s were performed on a Techne PHC-2 automated thermal cycler (Mandel Scientific). The reaction mixture was extracted with 50 µL of chloroform to remove the mineral oil and the DNA in the aqueous phase was precipitated with 250 µL of ethanol and 10 µL of 3 M Na·Acetate at -20°C for 1h. PCR products were analyzed by gel electrophoresis on a 1.25% agarose gel. The amplified DNA fragment was gel purified, digested with restriction endonucleases *Eco*RI and *Bam*HI, and ligated with similarly digested pUC118. Subsequent *E. coli* MV1193 transformants were analyzed by restriction endonuclease treatment followed by gel electrophoresis and DNA sequence analysis to ensure that the correct fragment was cloned and that no unintended mutations were introduced during amplification by PCR. The pUC118 construction containing the DNA region upstream of *lat* was named pLat/118.

II.7.5.2 Transfer of the DNA region upstream of *lat* from pLat/118 into pIJ4083 (*lat*up/4083)

pLat/118 (2 µg) was treated with the restriction endonucleases *Eco*RI and *Bam*HI, and the 330 bp fragment generated was electrophoretically separated on a 1.25% agarose gel. The band of interest was gel purified and ligated to similarly digested pIJ4083 (400

ng). The ligation mixture was introduced into *S. lividans* protoplasts and transformants were analyzed for the presence of an insert by spraying plate cultures with an aqueous 0.5M catechol solution. Transformants that turned yellow were cultivated for plasmid isolation and restriction analysis. The correct recombinant plasmid (named *lat*up/4083) was introduced into *S. clavuligerus* protoplasts by transformation. Those colonies that turned yellow upon exposure to catechol were analyzed by restriction endonuclease treatment and gel electrophoresis [illegible] t the correct recombinant pIJ4083 plasmid was present.

II.8 GENERATION OF UNIDIRECTIONAL DELETIONS IN THE DNA REGION UPSTREAM OF *pcbC* (*pcb*Cup)

The 335 bp *pcbC*up fragment was used to generate a series of deletions that extended increasingly closer to the coding region of the *pcbC* gene. The extent of each deletion was determined by DNA sequence analysis. The desired DNA fragments were inserted into pIJ4083 and transformed into *S. lividans* and *S. clavuligerus*. Transformants were assayed for promoter activity by C23O activity determination.

II.8.1 Generating unidirectional deletions of pIPS-1s

The protocol followed for generating a series of deletions of the *pcbC*up fragment to define the promoter region was described by Thomas and Surdin-Kerjan (1990). Ten micrograms of CCC pIPS-1s plasmid (described in section II.6.5) was placed in 10 µL of reaction buffer composed of: 20 mM $MgCl_2$, 4 mM Tris-HCl, pH 8.0, 0.06% (w/v) bovine serum albumin, and 0.5% (w/v) ethidium bromide. To introduce a single nick per molecule of DNA randomly throughout the plasmid, 0.1 µg DNAaseI was added [final concentration of 0.001% (w/v)] and the reaction mixture was allowed to incubate at 37°C for 45 min. The volume of the sample was increased by the addition of 90 µL TE and then extracted twice with equal volumes of phenol/chlorofom (1:1), once with a 0.5x volume of chloroform, and then ethanol precipitated (2.5x volume ethanol and 0.1x volume 3 M Na·Acetate). The precipitated DNA was recovered by centrifugation and the

pellet was dissolved in 50 µL of TE. This procedure was repeated throughout the deletion experiment and will be referred to as DNA purification and precipitation. To enlarge the nicks into small gaps, Exonuclease III (20 units) was added with 5 µL of 10x Exonuclease III buffer to give a final reaction mixture composed of: 60 mM Tris-HCl, pH 8.0, 77 mM NaCl, 5 mM $MgCl_2$, and 10 mM DTT. The reaction mixture was incubated at 21°C for 7 min and then inactivated by heating at 70°C for 15 min. Protein was removed by DNA purification and precipitation. The precipitated DNA was dissolved in TE and the single strand bridging the gap was removed by the addition of 2.5 units of Bal 31 nuclease in a final volume of 50 µL. Digestion conditions were as follows: 20 mM Tris-HCl, pH 8.0, 12 mM $MgCl_2$, 12 mM $CaCl_2$, 1 mM EDTA, 600 mM NaCl for 5 min at 21°C. The digestion was terminated with the addition of 25 µL 0.2 M EGTA [Ethylene glycol-bis(β-aminoethyl ether) N, N, N', N'-tetraacetic acid]. Enzyme was physically removed from the sample by DNA purification and precipitation. Treated plasmid was then digested with *Pst*I which cleaves at one end of the polylinker adjacent to the -335 end of the *pcbC*up insert and thereby removes the piece of DNA from the *Pst*I restriction site to the gap introduced by the nuclease treatments. The ends obtained from restriction digestion and nuclease treatment were made blunt by S1 nuclease treatment followed by incubation with the Klenow fragment of *E. coli* DNA polymerase I. These procedures followed the standard protocol found in Sambrook et al. (1989). Blunt-ended plasmid was ligated overnight. A second digestion with the restriction enzyme *Bam*HI which cleaves within the polylinker between the *Pst*I restriction site and the -335 end of the *pcbC*up insert, linearized those molecules where the deletion was not in the desired direction. The ligation mixture was transformed into competent *E. coli* MV1193 cells with the expectation that those plasmid molecules that had been linearized would be at a disavantage for transformation. Plasmid DNA was isolated from transformants and the size of the inserts was analyzed on a 1.0% agarose gel. Plasmids that had deletions of up

to 300 bp of DNA were digested with *Sph*I and the reaction mixtures were analyzed on 1.25% agarose gels. DNA fragments ranging in size from 80 bp to 200 bp were gel purified and ligated with *Sph*I digested pUC119. The extent of deletion and the orientation of insertion was determined by DNA sequence analysis using the M13 universal sequencing primer. Plasmids with the desired orientation (start codon near the *Eco*RI of the polylinker) and with sequence upstream of the start codon of *pcbC* ranging from 85 bp to 168 bp were used for subsequent subcloning into pIJ4083.

II.8.2 Transfer of the deleted *pcbC*up fragments into pIJ4083

Plasmids containing deleted *pcbC*up DNA fragments (2 μg) were digested with the restriction endonucleases *Eco*RI and *Hin*dIII. The reaction mixtures were analyzed on a 1.25% agarose gel and the DNA bands of interest were cut out and gel purified. Each purified DNA fragment was ligated to *Eco*RI/*Hin*dIII digested pIJ4083 (400 ng). Ligation mixtures were introduced into *S. lividans* protoplasts by transformation. Transformants were screened by colony blot hybridization and those that appeared positive were confirmed by restriction endonuclease treatment and gel electrophoresis. Desired recombinant plasmids were then transformed into *S. clavuligerus* protoplasts and plasmid isolated from transformants was analyzed by restriction endonuclease digestion followed by gel electrophoresis.

II.9 CATECHOL DIOXYGENASE ASSAYS OF *S. LIVIDANS* TK24 AND *S. CLAVULIGERUS* 3585 TRANSFORMANTS CONTAINING pIJ4083 RECOMBINANT PLASMIDS

Cell free extracts of *S. clavuligerus* and *S. lividans* containing the desired recombinant plasmids were prepared according to Ingram et al. (1989). Mycelia grown for 36h were harvested by filtration through Whatman No.1 filter paper and washed with 50 mL of wash buffer (20 mM KPO_4 buffer, pH7.2). Cells were scraped into a small beaker and resuspended in 10 mL of sample buffer (100 mM KPO_4, pH 7.5, 20 mM EDTA, 10% (v/v) acetone). Cell free extracts were prepared by sonication for 3x 10s at

setting 7 of a Sonifier Cell Disruptor 350 with a 0.75 inch diameter probe (Branson Sonic Power Co.). Broken cell suspensions were centrifuged at 27,000 x g for 15 min and supernatants were put on ice to be assayed immediately.

C23O activity was determined as described by Zukowski et al. (1983). Up to 100 µL of cell free extract was added to pre-warmed reaction mixture composed of: 2.9 mL 100 mM KPO_4, pH 6.8 and 10 µL 20 mM catechol (in ethanol), in a final volume of 3.0 mL. The reaction mixture was mixed and the change in absorbance at 375 nm was measured over time at 30°C. Under these conditions the molar absorption coefficient of 2-hydroxymuconic semialdehyde is reported as 3.3×10^4 (Sala-Trepat and Evans, 1971). One milliunit (mU) is defined as the formation at 30°C of 1 nmol of 2-hydroxymuconic semialdehyde per min. Total protein was measured by the method described by Bradford (1976) using the microassay procedure described by the supplier, Bio-Rad Laboratories, Inc. Bovine gamma globulin was used as a standard.

II.10 TOTAL RNA ISOLATION

Total RNA was isolated from *S. clavuligerus* and *S. lividans* according to Hopwood et al. (1985) as modified by Wu and Roy (1992). All solutions and glassware coming in contact with RNA samples were treated with diethyl pyrocarbonate to inhibit ribonucleases according to Hopwood et al. (1985). Cultures were cooled rapidly by the addition of ice and then filtered through Whatman No.1 filter paper. Mycelia were washed with cold water and ground in a mortar and pestle in the presence of liquid nitrogen, 5 mL Kirby's mix [50 mM Tris-HCl, pH 8.3, 1% (w/v) Tri-isopropylnapthalene sulphonate, 6% (w/v) sodium-4-amino-salicylate, 6% (v/v) phenol mixture] and 0.5 g alumina (Fisher Scientific Co.). The mixture was scraped into a 50 mL centrifuge tube. An equal volume of phenol mixture (phenol containing 0.1% (w/v) 8-hydroxyquinoline and equilibrated with 50 mM Tris-HCl, pH 8.3) was added and the broken cell suspension was vortexed vigorously. The broken cell suspension was forced

through a 18 1/2 gauge needle to shear the chromosomal DNA, and then centrifuged at 17,400 x g for 20 min. The aqueous phase was removed to a fresh centrifuge tube and extracted with an equal volume of phenol/chloroform mixture (50: 50: 1; phenol mixture: chloroform: isoamyl alcohol). This procedure was repeated until the interface between the two phases was clear. Nucleic acid was precipitated with an equal volume of isopropyl alcohol and 0.10 volume of 3 M Na·Acetate. Chromosomal DNA was removed by spooling onto a glass rod and the remaining precipitate was collected by centrifugation. The pellet was washed in 70% ethanol and dissolved in TE buffer. Remaining DNA was removed by incubation with RNAase-free DNAaseI, followed by phenol/chloroform extraction and chloroform extraction to remove the enzyme. Quantity of RNA was determined by spectrophotometric analysis at 260 and 280 nm (Sambrook et al., 1989). A small quantity of RNA was checked for quality (ie. extent of degradation) by electrophoretic separation on a 1.0% agarose gel. Final purified RNA samples were stored in ethanol at -70°C at a final concentration of 1 µg/µL.

II.11 NORTHERN ANALYSIS

Northern transfers and hybridizations were performed according to Williams and Mason (1985) with minor modifications described in Petrich et al. (1992). RNA (40 µg) was denatured at 50°C in the presence of deionized glyoxal and DMSO. Denatured samples were separated by electrophoresis on a 1.25% agarose gel using a 10 mM $Na{\cdot}PO_4$, pH 6.5, recirculating buffer system. Phage λ DNA (500 ng) digested with *ClaI* was treated in a similar manner and run in adjacent wells as size markers. After electrophoresis at 4 v/cm, nucleic acid was transferred from the gel to nylon membrane in 20xSSC overnight. The filter was removed, allowed to air dry, and baked in an 80°C vacuum oven for 2h. The pieces of filter containing the λ markers were cut off and hybridized separately to nick-translated λ DNA using conditions described in section II.5.3.5 for DNA hybridizations. The RNA blot was hybridized using similar conditions,

except 50% deionized formamide was added to the pre-hybridization and hybridization solutions, and the temperature used for incubation and washing was 50°C. Probes used for hybridization were ^{32}P labelled nick-translated DNA fragments that were specific for the gene of interest.

II.12 DETERMINATION OF THE *tsp* OF *pcbC* BY PRIMER EXTENSION

The method used for primer extension was based on the procedure reported by Calzone et al. (1987) with minor modifications. 10 pmol of the oligo primer 5'-GAACAGCGGCGACATGT-3' (complementary to nt +47to +57 relative to the *pcbC* start codon) was end-labelled by T4 polynucleotide kinase treatment at 37°C for 1h in a reaction buffer containing: 100 mM Tris-HCl, pH 8.3, 10 mM $MgCl_2$, 10 mM DTT, 50 µCi [$\gamma^{32}P$]ATP, and 1unit T4 polynucleotide kinase in a final volume of 10 µL. The end-labelled oligo was stored on ice until its use.

Eighty micrograms of RNA isolated from *S. clavuligerus* 3585 and *S. lividans* TK24 grown for 36h was sedimented and the pellet was dissolved in 2 µL H_2O. The RNA was combined with 5 µL of the labelled oligo primer (5 pmol) in a reaction buffer composed of: 50 mM Tris-HCl, pH 8.3, 6 mM $MgCl_2$, 40 mM KCl, 0.5 mM each dNTP, 0.02% (w/v) actinomycin D in a final volume of 10 µL. The reaction mixture was incubated at 70°C for 5 min, then at 50°C for 30 min. AMV reverse transcriptase (8 units) was added and the sample was incubated for a further 60 min at 50°C. RNA was removed by RNAse treatment following heat denaturation at 95°C for 3 min. Protein was removed with a phenol/chloroform extraction followed by ethanol precipitation of the remaining DNA probe fragment. The DNA probe pellets were dissolved in 10 µL of sequencing loading dye and 2 µL samples of each reaction mixture were analyzed by electrophoresis on 6% polyacrylamide sequencing gels. Sequencing reactions generated using the same oligo and single-stranded pIPS-1 as template were used to determine the

exact site of transcription initiation. Sequencing gels were exposed to X-OMAT AR film at -70°C and films were developed using a FUJI RGII X-ray film processor.

II.13 HIGH RESOLUTION S1 NUCLEASE MAPPING OF THE 5' END OF *pcbC*, *pcbAB*, AND *lat* transcripts

II.13.1 Generation of DNA probe fragments by PCR

II.13.1.1 PCR to generate the *pcbC*-specific DNA probe

Primers used for amplification of the *pcbC*-specific DNA probe fragment were the M13 reverse sequencing primer and the same oligo primer used for 5' primer extension (section II.12). The template used for amplification was the pIPS-1 plasmid described in section II.6.2. PCR reaction conditions used were as stated in section II.7.5.1 for amplification of the *lat*up DNA fragment, with one modification, that DMSO was omitted from the reaction buffer. The resulting *pcbC*-specific probe was a 419 bp DNA fragment that included 307 bp of sequence upstream of the *pcbC* start codon, 57 bp of the *pcbC* gene, and 59 bp of *E. coli* sequence at the end opposite to the *pcbC* coding sequence.

II.13.1.2 PCR to generate the *pcbAB*-specific DNA probe

Primers used for amplification of the *pcbAB*-specific DNA probe fragment consisted of the M13 reverse sequencing primer and 5'-TCCACTCCGCTGCGGTC-3' (homologous to nt +24 to +40 relative to the *pcbAB* start codon). The template used for the amplification was a pUC119 recombinant plasmid which contained a 919 bp DNA fragment that included 344 bp of sequence from upstream of the *pcbAB* coding region. PCR reaction conditions used were as stated in section II.7.5.1. for amplification of the *lat*up sequence, except that the final DMSO concentration was changed from 1% (v/v) to 10% (v/v). The resulting *pcbAB*-specific probe consisted of a 425 bp fragment that contained 344 bp of sequence upstream of the *pcbAB* start codon, 40 bp of *pcbAB* coding sequence, and 41 bp of non-homologous *E. coli* sequence at the end opposite to the *pcbAB* coding region.

II.13.1.3 PCR to generate the *lat*-specific DNA probe

Primers used for amplification of the *lat*-specific DNA probe fragment were the M13 reverse sequencing primer and 5'-CGTCGACGAGCATATGC-3' (homologous to nt +87 to +103 relative to the *lat* start codon). The template used to generate the specific DNA fragment was a pUC118 recombinant plasmid which contained a 1080 bp *Eco*RI/*Kpn*I DNA fragment that included 227 bp of sequence from upstream of the *lat* coding region. PCR reaction conditions used were as stated in section II.7.5.1. The resulting *lat*-specific DNA probe consisted of a 351 bp fragment that contained 227 bp of sequence upstream of the *lat* start codon, 103 bp of the *lat* gene, and 21 bp of non-homologous *E. coli* sequence at the end opposite to the *lat* coding sequence.

II.13.2 Kinase labelling and purification of the DNA probes

Five pmol of each PCR generated DNA probe fragment was labelled with [γ^{32}P]ATP by kinase treatment as described for primer extension in section II.7.2. To stop the reaction, 90 µL of TE was added and the reaction mixture was extracted with 100 µL phenol/chloroform. The aqueous phase was precipitated in ethanol at -20°C. The precipitate was collected by centrifugation and the pellet was washed with 70% ethanol, and dried under vacuum. Cerenkov counting in a scintillation counter was used to determine the efficiency of labelling. The pellet was dissolved in an appropriate volume of TE buffer and precipitated in ethanol at a final concentration of 25,000 cpm/µL.

II.13.3 S1 nuclease protection assays

Reaction mixtures were set up in triplicate, one for each gene-specific probe fragment. Fifty micrograms of RNA isolated from cells grown for 24h, 30h, 36h, 48h and 60h was combined with 50,000 cpm of desired probe fragment and the mixture was sedimented by centrifugation. Pellets were washed with 70% ethanol, dried and dissolved in 20 µL of formamide buffer (40 mM PIPES buffer, pH 6.4, 400 mM NaCl, 1mM EDTA, 80% (v/v) deionized formamide). Samples were denatured at 85°C for 30 min and

then slowly cooled down to the annealing temperature of 50°C. After overnight annealing, samples were put on ice and 300 µL of S1 digestion mixture [200 units S1 nuclease, 280 mM NaCl, 30 mM Na·Acetate, pH 4.4, 4.5 mM Zn·Acetate, 2% (w/v) calf thymus DNA] was added to each sample. Samples containing the *pcbC*- and *pcbAB*-specific DNA probes were incubated at 37°C for 45 min. Samples containing *lat*-specific cDNA probe were incubated at 21°C to compensate for the high level of AT residues in the region of the *tsp*. S1 nuclease digestion was stopped with the addition of 75 µL S1 termination mixture (2.5 M NH_4·Acetate, 50 mM EDTA). Samples were precipitated at –20 with 400 µL isopropyl alcohol, 40 µL 3 M Na·Acetate, and 10 µg yeast tRNA. S1 nuclease protected DNA fragments were collected by centrifugation. Pellets were washed with 70% ethanol and dissolved in 3 µL of sequencing loading dye. Samples were analyzed on 6% polyacrylamide sequencing gels. Adjacent sample wells contained 1000 cpm of labelled DNA probe fragment and ^{35}S sequencing reactions generated using the same oligo primer and the same template in single-stranded form that was used for PCR.

II.14 IPNS EXPRESSION IN *S. LIVIDANS* TK24

IPNS expression in *S. lividans* TK24 was attempted using two different plasmids containing the *pcbC* gene. Construction of the 1.7/4083 plasmid was described in section II.7.1 and the insert includes the *pcbC* gene together with the transcriptional and translational signals in the 335 bp of upstream sequence of the *pcbC* gene. The second plasmid 1.35/702, has the *pcbC* structural gene replacing the *S. antibioticus* structural gene of ORF438 (*melC1*), a trans-acting copper transfer protein of pIJ702 (Lee et al., 1988). Therefore, the expression of *pcbC* in plasmid 1.35/702 is under the control of the transcriptional and translational signals of the *melC1* gene.

II.14.1 Construction of the pIJ702-based expression vector (1.35/702)

The pIPS-1* plasmid contains the *pcbC* gene altered by site-directed mutagenesis to have an *Sph*I site at the translation initiation site. pIPS-1* (2 µg) was digested with the

restriction endonucleases *Sst*I and *Sph*I. The reaction mixture was analyzed on a 1.0% agarose gel and the approximately 1.35 kb band, that contained the entire *pcbC* structural gene starting at the start codon and 350 bp of downstream sequence, was gel purified and ligated to pIJ702 (400 ng) that had been similarly digested. pIJ702 treated in this manner will have the *melC1* gene removed precisely at the start codon, as an *Sph*I site is located at the start of *melC1* (Leu, et al., 1989). *S. lividans* protoplasts were transformed and colonies were screened for their inability to produce melanin (loss of brown colour). This distinguished colonies containing the plasmid with the *pcbC* construct from those that had the *melC1* gene reinserted into pIJ702. Melanin minus transformants were analyzed by restriction endonuclease treatment and gel electrophoresis.

II.14.2 Preparation of cell free extracts and partial purification of IPNS

S. lividans TK24 cultures containing the 1.7/4083 and 1.35/702 plasmids, and *S. clavuligerus* cultures were grown for 36h in 100 mL TSBG with 5 µg/mL thiostrepton (*S. lividans*) and TSBS (*S. clavuligerus*). Mycelia were harvested by filtration through Whatman No.1 filter paper and washed with TDE buffer (50 mM Tris-HCl, pH 7.2, 0.1 mM DTT and 0.01 mM EDTA). The washed mycelia were resuspended in 10 mL TDE buffer and disrupted by sonication at 3x 10s at a setting of 7 (Sonifier Cell Disruptor 350, 0.75 inch diameter probe, Branson Sonic Power Co.). To remove cellular debris, broken cell suspensions were centrifuged at 17,400 x g for 20 min.

Cell free extracts were stirred at 4°C as streptomycin sulfate was gradually added to a final concentration of 1% (w/v). Stirring was continued at 4°C for an additional 15 min. Suspensions were centrifuged at 17,400 x g for 15 min and the pellet was discarded. Ammonium sulphate was added in the manner described above and the material precipitating between 50% and 70% saturated ammonium sulphate was collected by centrifugation at 17,400 x g for 15 min and resuspended in 3 mL TDE buffer.

Samples were desalted on a Econo-Pak 10 DG desalting column (BioRad) using the manufacturer's recommended procedures.

II.14.3 Isopenicillin N synthase (IPNS) assays

To determine the level of IPNS activity in partially purified IPNS preparations, the conversion of ACV to isopenicillin N was monitored by HPLC analysis according to the procedure outlined in Jensen et al. (1982c). The reaction mixture was composed of: 0.287 mM bis-ACV, 4 mM DTT (these two were incubated first at 21°C for 15 min to reduce the ACV), 2.8 mM Na·ascorbate, 45 μM $FeSO_4$ and partially purified IPNS preparation to a final volume of 40 μL. Reaction mixtures were incubated for 60 min at 21°C and the reactions were terminated with the addition of 40 μL of methanol. Negative controls used were zero time samples (ie. the methanol was added to the samples before the cell free extract). The chromatographic equipment used for analysis of reaction mixtures included Waters Model 6000A and Model 45 HPLC pumps, a Waters 710 Wisp™ Sample Processor, a Waters 490 Programmable Multi-wavelength Detector, a Waters 840 Data and Chromatography Control Station, and a Waters μBondapak™ C_{18}Radial-Pak™ Cartridge in a Waters Z-Module™ Radial Compression Separation System. Twenty microlitre samples were analyzed with isocratic elution in a mobile phase consisting of 0.05 M KPO_4 (adjusted to pH 4.0 with H_3PO_4): methanol, 95:5 at a flow rate of 2 mL/min.

II.14.4 mRNA analysis of *S. lividans* containing the 1.7/4083 plasmid

Total RNA was isolated from *S. lividans* cultures containing the plasmid 1.7/4083 as described in section II.10. Northern analysis and S1 nuclease protection experiments specific for *pcbC* were conducted using procedures similar to those described previously for *S. clavuligerus* in sections II.11 and II.13.

III. RESULTS

Antibiotic production in *Streptomyces* is not uniform throughout the growth cycle, beginning after active growth has stopped, presumably in response to changing environmental conditions. Transcriptional regulation of the genes encoding the biosynthetic enzymes is at least partly responsible for the delayed appearance of antibiotic. We decided to study the transcriptional regulation of the *pcbC* gene of *S. clavuligerus*, which encodes the enzyme that catalyzes the formation of the β-lactam ring of penicillins, cephamycins and cephalosporins, as a first step to understanding the regulation of antibiotic production in *S. clavuligerus*.

Characterization of the transcriptional regulation of the *pcbC* gene of *S. clavuligerus* involved two approaches; promoter probe plasmid studies and mRNA analysis. The above approaches were also used to examine the transcription of the *pcbC* gene from *S. clavuligerus* in the heterologous species *S. lividans* .

III.1 PROMOTER PROBE PLASMID ANALYSIS

The *Streptomyces* promoter probe vector, pIJ4083 (Clayton and Bibb, 1990), contains the promoter-less catechol-2,3-dioxygenase (C23O) gene (*xylE*) downstream of the fd transcriptional terminator and a polylinker cloning site. Insertion of DNA fragments of interest into pIJ4083 allows identification of active promoter sequences by comparing the relative levels of C23O activity present in cultures transformed with pIJ4083 recombinant plasmids. Spraying plates containing transformants with an aqueous catechol solution, provided a rapid means for detecting the presence of an active promoter. This procedure could indicate differences between high promoter activity and relatively low activity, but appeared to be very dependent on the age and state of the colony. Therefore this rapid test was most useful as an indicator for the presence of an active promoter and is reproducible when cells are able to grow well. Spectrophotometric assays of C23O activity of cell free extracts were generally more useful for comparing relative promoter

activites. Cells from different samples could be maintained in a more homogeneous manner during growth and would thereby ensure a greater reproducibility in C23O specific activity.

Ligation reaction mixtures (pIJ4083 and the insert of interest) were introduced first into *S. lividans* protoplasts and then the correct recombinant plasmids were transformed into *S. clavuligerus* protoplasts. The recombinant plasmids of interest were confirmed to be present in both *S. lividans* and *S. clavuligerus* mycelia by plasmid isolation, restriction endonuclease treatment, and agarose gel electrophoresis.

III.1.1 Assessment of the utility of *xylE* as a reporter gene in *S. clavuligerus*

The pUC18 derivative, pIJ4070 was digested with the restriction enzymes *Eco*RI and *Bam*HI to remove a 380 bp DNA fragment (ERME*) that contained a mutated form of the erythromycin resistance gene (*ermE*) promoter region of *S. erythraea*. The ERME* promoter region was inserted into similarly digested pIJ4083. The recombinant promoter probe plasmid (illustrated in Figure 5) was transformed into *S. lividans* and *S. clavuligerus* and the resulting transformants were analyzed for C23O activity. C23O in the presence of the substrate catechol, catalyzes the production of 2-hydroxymuconic semialdehyde, a bright yellow coloured product. The relative C23O activity of the *S. lividans* and *S. clavuligerus* cultures was demonstrated by spraying the plate cultures with catechol solution. The mutated form of the *ermE* promoter region, ERME*, is generally considered to be a strong promoter in *Streptomyces* (Bibb, M.J., personal communication). *S. lividans* and *S. clavuligerus* transformants that contained the recombinant plasmid ERME*/4083 turned yellow when sprayed with a catechol solution, illustrating that the *xylE* gene would serve as an efficient reporter gene in *S. clavuligerus* (Plate 1).

Figure 5: Recombinant promoter probe plasmids used to determine the location and relative activity of the promoter directing transcription of *pcbC*. *S. clavuligerus* DNA fragments (except ERME*, from *S. erythraea*) were introduced into the MCS of pIJ4083 by digestion with the indicated restriction endonucleases and ligation to similarly digested pIJ4083 to form a transcriptional fusion with the downstream *xylE* gene. DNA insert fragments were obtained from the following pUC119 recombinant plasmids (in brackets): 1.7 (pIPS-1), *pcbC*up (pIPs-1s), DEL168 (DEL168/119), DEL158 (DEL158/119), DEL152 (DEL152/119), and DEL85 (DEL85/119). The ERME* DNA fragment was obtained from a pUC18 derivative named pIJ4070. Coding regions of genes are indicated by closed boxes and single lines represent the intergenic region between *pcbAB* and *pcbC*.

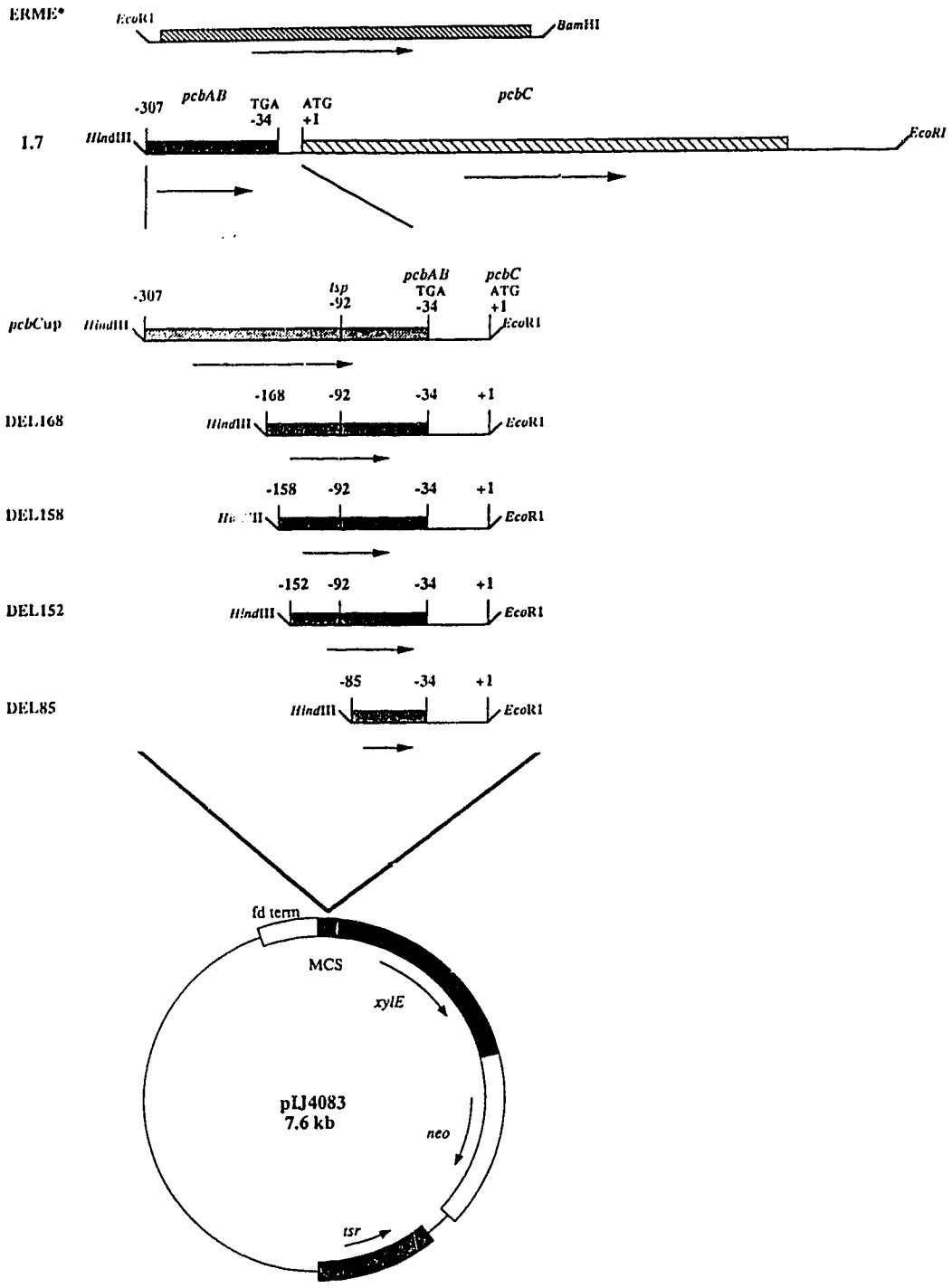
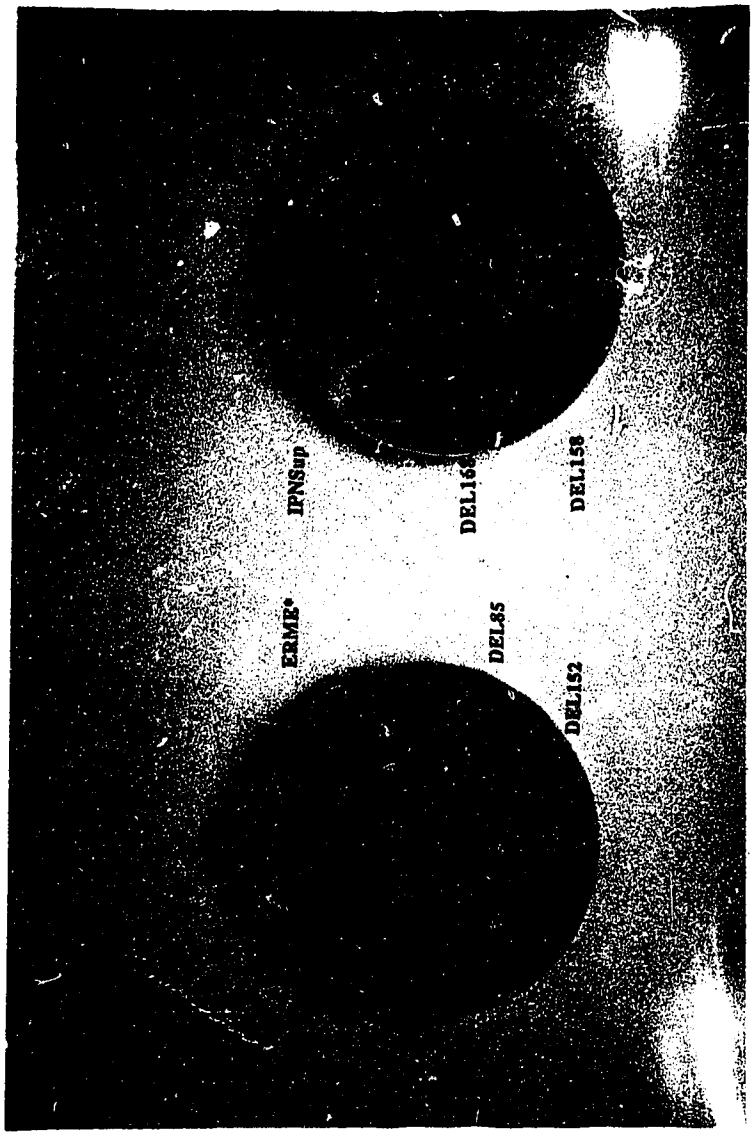

ERME*
EcoR1
BamHI
1.7
-307
pcbAB
TGA
-34
ATG
+1
pcbC
HindIII
EcoRI
pcbCup
tsp
-92
-168
-158
-152
-85
DEL168
DEL158
DEL152
DEL85
fd term
MCS
xylE
pLJ4083
7.6 kb
neo
tsr

Plate 1: Visual assessment of relative C23O activity in patches of *S. lividans* and *S. clavuligerus* cells transformed with pIJ4083 recombinant promoter probe plasmids containing deletion products (DEL168, DEL158, DEL152, and DEL85) of the DNA fragment upstream of the *pcbC* gene (IPNSup) of *S. clavuligerus*. Plasmid constructions are illustrated in Figure 5. Patched cells were grown for 48h on MYM agar plates supplemented with 5 μg/mL thiostrepton. Plates were sprayed with a 0.5 M catechol solution and colour was allowed to develop for at least 10 min. Transformants containing pIJ4083, the promoter-less vector, and the ERME*/4083 construction were included as negative and positive controls respectively.


ERME*
IPNSup
DEL85
DEL165
DEL152
DEL158

III.1.2 Promoter probe analysis of a 1.7 kb fragment of *S. clavuligerus* DNA containing the *pcbC* gene (1.7/4083)

A 1.7 kb *S. clavuligerus* DNA fragment that included the intact *pcbC* gene, 307 bp of upstream DNA sequence, and 383 bp of downstream DNA sequence was previously cloned into pUC119 (pIPS-1; Doran et al., 1990). Digestion of pIPS-1 with the restriction endonucleases, *Eco*RI and *Hin*dIII, removed the 1.7 kb DNA insert for ligation to similarly digested pIJ4083. The recombinant plasmid (1.7/4083) is illustrated in Figure 5. A spectrophotometric assay at 375 nm allows quantification of C23O levels present in cell free extracts of *S. lividans* and *S. clavuligerus* cultures transformed with pIJ4083 promoter probe recombinant plasmids. Cell free extracts from *S. lividans* and *S. clavuligerus* cells transformed with 1.7/4083 were analyzed for C23O activity (Table I). Cell extracts from both *S. lividans* and *S. clavuligerus* cultures carrying the 1.7/4083 recombinant plasmid showed low promoter activity. C23O activities of 8 and 10 mU/mg respectively were only slightly higher than the background levels seen in control extracts from cells containing the pIJ4083 plasmid with no insert. In contrast the positive control plasmid ERME*/4083 gave cell extracts with C23O activities 150-380 times higher than background levels. These results suggested that the 1.7 kb DNA fragment found in 1.7/4083 does not contain an active promoter. However, the 1.7 kb fragment included downstream sequence which contained an area of inverted repeats that have the potential to form a hairpin loop structure resembling a transcriptional terminator (Jensen et al., 1989). Detection of an active promoter upstream of the inverted repeat sequences may be prevented if transcription is terminated before it enters the *xylE* gene. A promoter probe construction of the *pcbC* upstream sequence that lacks any downstream sequence would remedy this situation.

III.1.3 Promoter probe analysis of the region upstream of the *pcbC* gene of *S. clavuligerus*

III.1.3.1 Site directed mutagenesis to introduce an *Sph*I site at the *pcbC* start codon (pIPS-1*)

TABLE I

Catechol dioxygenase (C23O) activity in cell free extracts of *Streptomyces clavuligerus* and *Streptomyces lividans* transformed with pIJ4083 recombinant plasmids for identification of the *pcbC* promoter

Plasmid[a]	Host	
	S. clavuligerus	*S. lividans*
	Catechol dioxygenase specific activity (mU/mg protein)[b]	
pIJ4083	2	2
ERME*/4083	764	295
1.7/4083	10	8
*pcb*Cup/4083	77	29
DEL168/4083	68	18
DEL158/4083	33	15
DEL152/4083	6	1
DEL85/4083	1	1

[a]Recombinant plasmids indicated are illustrated in Figure 5.

[b]C23O activity was determined as described by Zukowski et al. (1983) using a molar absorption coefficient of 3.3×10^4 (Sala-Trepat and Evans, 1971). One milliunit (mU) corresponds to the formation at 30°C of 1 nmol of 2-hydroxymuconic semialdehyde per min.

No useful restriction sites were available to remove the DNA sequence upstream of the *pcbC* coding region for cloning into pIJ4083. Analysis of the DNA sequence near the start codon indicated that a single base change from a T nt to a G nt would introduce an *Sph*I restriction site at the start of the *pcbC*gene. The two primer method of site directed mutagenesis (Zoller and Smith 1987) using the mutagenic oligo described in Section II.6 and the universal sequencing primer, was performed with the pIPS-1 plasmid. Transformants were analyzed by plasmid isolation, *Sph*I endonuclease digestion, and gel electrophoresis, and this analysis indicated that approximately 20% of the transformants obtained contained the new restriction recognition sequence. Those plasmids containing the single base change were named pIPS-1*.

III.1.3.2 Promoter probe analysis of *pcbC* (*pcbC*up/4083)

*Sph*I endonuclease treatment of the pIPS-1* plasmid released a 335 bp DNA fragment that included 307 bp of sequence found immediately upstream of the *pcbC* start codon (*pcbC*up). This fragment was inserted into *Sph*I digested pUC119 and transformed into *E. coli*. Single-stranded plasmid isolated from transformants containing pUC119 with inserts was subjected to DNA sequence analysis to determine the orientation of the insert relative to the polylinker. Plasmids containing the *pcbC*up fragment in the desired orientation (start codon nearest *Eco*RI site of polylinker) were named pIPS-1s. pIPS-1s was digested with *Eco*RI and *Hin*dIII and the *pcbC*up DNA fragment was ligated into similarly digested pIJ4083. The *pcbC*up/4083 plasmid construction is illustrated in Figure 5. Cell extracts from both *S. lividans* and *S. clavuligerus* cultures containing the *pcbC*up/4083 recombinant plasmid indicated promoter activity (Table I). C23O activities of 29 and 77 mU/mg protein respectively were 15-38 times higher than background levels seen in cells containing the pIJ4083 vector with no insert, indicating the presence of a promoter sequence in the 307 bp fragment found immediately upstream from *pcbC*. A comparison between C23O specific activities of cell extracts from cultures containing the

*pcbC*up/4083 and ERME*/4083 recombinant plasmids, indicates that specific activity of the *pcbC*up-derived extract is about ten-fold lower than that of the mutant *ermE**. To localize the region promoting transcription in the *pcbC*up/4083 recombinant plasmid, deletions of the *pcbC*up fragment were generated and tested for promoter activity.

III.1.4 Generation of deletions of the sequence upstream of the *pcbC* gene of *S. clavuligerus* (*pcbC*up) and insertion of the deleted DNA fragments into pIJ4083

To further characterize the promoter region directly upstream of *pcbC*, unidirectional deletions were generated using the method described in Materials and Methods II.8. Using this procedure, deletions of the *pcbC*up insert could be selected which had material removed from the furthest upstream end of the insert moving progressively closer to the *pcbC* start codon. Deleted *pcbC*up fragments were removed by *Sph*I restriction endonuclease treatment and ligated to similarly digested pUC119. The exact size and orientation of insertion of the deleted upstream DNA fragments was determined by DNA sequence analysis and deletion constructions were named according to the amount of DNA sequence remaining upstream of the *pcbC* gene (ie. DEL168/119, DEL158/119, DEL152/119 and DEL85/119 had 168 bp, 158 bp, 152 bp and 85 bp of sequence upstream of the *pcbC* coding region respectively). For promoter probe analysis, the deleted *pcbC*up fragments were removed from their respective plasmids by digestion with the restriction endonucleases *Eco*RI and *Hin*dIII, and ligated into similarly digested pIJ4083. The deletion pIJ4083 recombinant plasmids (DEL168/4083, DEL158/4083, DEL152/4083 and DEL85/4083) are illustrated in Figure 5.

III.1.5 C23O activity of *S. lividans* and *S. clavuligerus* transformed with *pcbC*up deletion recombinant promoter probe plasmids

Relative C23O activities of plate cultures of *S. lividans* and *S. clavuligerus* patches transformed with pIJ4083 recombinant plasmids that contain the *pcbC*up DNA fragment (labelled as IPNSup) and *pcbC*up deletion products (illustrated in Figure 5) are

demonstrated in Plate 1. A comparison of the yellow colour produced by the patches of cells containing *pcbC*up/4083 and *pcbC*up deletion recombinant plasmids does not demonstrate a quantitative measure of promoter activity, rather a general indication of the presence and strength of a sequence promoting transcription can be determined. Many factors may affect the reproducibility of yellow colour produced by plate cultures, such as amount and age of mycelia used to streak patches and the condition of the agar plates. An example of this inconsistency in the amount of yellow colour produced by cell patches is seen in Plate 1. The culture containing DEL152/4083 appears to have greater promoter activity than that seen for the cultures containing DEL168/4083 or the culture containing *pcbC*up/4083, but in several previous trials DEL152/4083 gave a less intense yellow colour. Plates tests for C23O activity are most beneficial when used as a screening method for the presence of an active promoter. Spectrophotometric assays that determine C23O specific activity of cell free extracts from plasmid containing cultures grown under similar conditions (Table I), give a more consistent indication of the relative promoter strength and results are reproducible when cells are grown in a similar manner. The recombinant plasmids carrying deletions of *pcbC*up that show promoter activity have at least 152 bp of sequence upstream of the *pcbC* start codon. The recombinant plasmid that contains the deletion product with only 85 bp of upstream sequence does not show significant promoter activity (no C23O activity) above background (pIJ4083 itself). This implies that sequence important for transcription initiation is located between the -168 and -152 region relative to the *pcbC* coding region.

III.2 NORTHERN TRANSFER AND HYBRIDIZATION ANALYSIS

Northern transfer and hybridization to a gene-specific probe is a direct method for determining the size of specific transcripts. Total RNA preparations from *S. clavuligerus* grown in liquid media to various stages during growth were denatured and separated by electrophoresis on a 1.25% agarose gel under conditions that would maintain the

denatured state of the RNA molecules. RNA was transferred to nylon membranes and hybridized to ^{32}P-labelled gene-specific probes under conditions favourable for formation of a stable DNA/RNA hybrid.

III.2.1 Northern transfer and hybrization to identify the *pcbC* transcript of *S. clavuligerus*

An autoradiograph of the Northern hybridization analysis of *pcbC*, Plate 2, shows a distinct radioactive band indicating the presence of a transcript approximately 1.2 kb in size. This band was specific to *S. clavuligerus* RNA samples prepared from cells grown to stationary phase of the growth cycle. Total RNA prepared from *S. lividans* cultures grown for 36h, included as a negative control, showed no specific hybridization to the *pcbC* probe. The presence of a monocistronic *pcbC* transcript supports the results of the promoter probe experiments and indicates that a promoter must be present just upstream of *pcbC*.

III.3 PRIMER EXTENSION TO DETERMINE THE 5' END OF THE *pcbC* TRANSCRIPT

S. clavuligerus and *S. lividans* RNA preparations from 36h cultures were annealed to an end-labelled 17-nt oligo designed to be complementary to the RNA copy of the *pcbC* gene at nt positions +41 to +57. The primer was extended with AMV reverse transcriptase to generate a cDNA fragment ending at the 5' end of the *pcbC* transcript. Reaction mixtures were analyzed on a 6% polyacrylamide denaturing gel to determine the *tsp* of the *pcbC* gene (Plate 3). Extension products from *S. lividans* (lane 1) and *S. clavuligerus* (lane 2) RNA samples were run adjacent to ^{32}P sequencing reactions generated using the same oligo used for primer extension, with the single-stranded form of the pIPS-1 plasmid as template. The arrow in Plate 3 indicates the *tsp* which corresponds to a C nt in the coding strand 91 bases upstream of the *pcbC* start codon. No extension products were seen in the *S. lividans* RNA sample indicating that no non-specific annealing of primer occurred.

Plate 2: Northern analysis of total *S. clavuligerus* RNA using a *pcbC*-specific probe. Total RNA isolated from *S. clavuligerus* grown for 24h, 30h, 36h, 48h, 60h and 72h (lanes 2 through 7, respectively) and total RNA isolated from *S. lividans* grown for 36h (lane 1) was denatured in the presence of glyoxal and DMSO before separation by electrophoresis on a 1.25% agarose gel. Lambda DNA digested with *Cla*I was treated in a similar manner and run in adjacent lanes as mwt. size markers. Hybridization was carried out using a ^{32}P-labelled pIPS-1 probe.

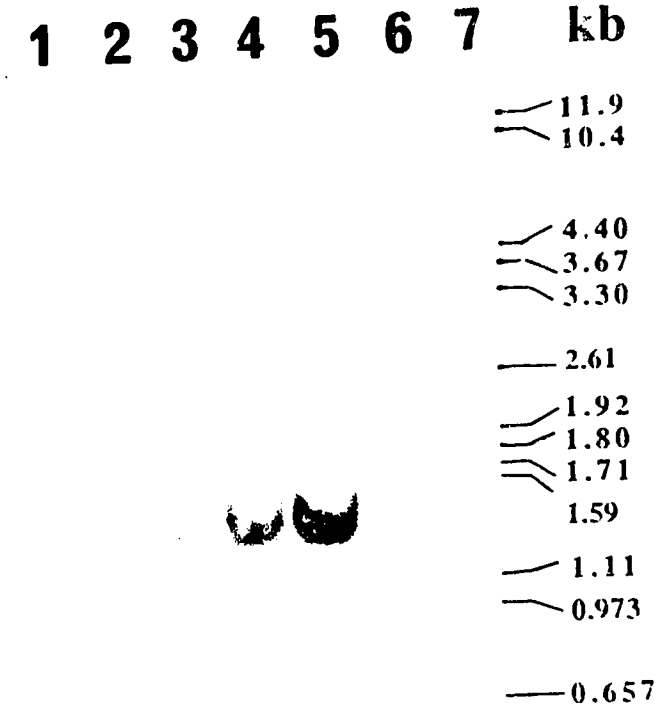
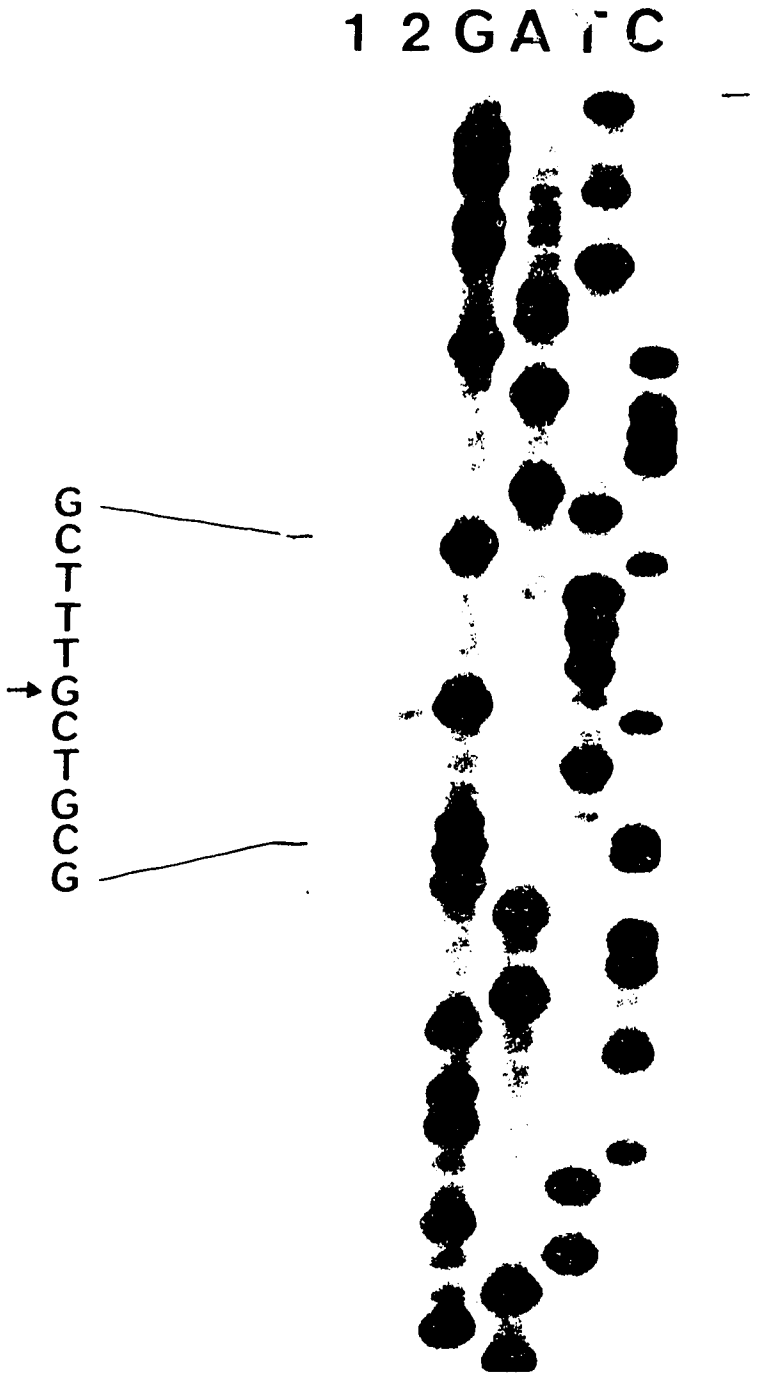

1 2 3 4 5 6 7
kb
11.9
10.4
4.40
3.67
3.30
2.61
1.92
1.80
1.71
1.59
1.11
0.973
0.657

Plate 3: Location of the 5' end of the *pcbC* gene of *S. clavuligerus* by 5' primer extension. Primer extension reactions were carried out using a 17-mer oligo homologous to nt +41 to +57 of the coding strand of *pcbC* as a primer. Labelled oligo was annealed to total RNA preparations from *S. lividans* (lane 1) and *S. clavuligerus* (lane 2) grown for 36h in liquid media. cDNA extension products were separated by PAG electrophoresis in wells adjacent to a ^{32}P DNA sequencing ladder generated using the same oligo used for primer extension (lanes G, A, T and C). An arrow indicates the *tsp* that corresponds to a C residue 91 bp upstream of the *pcbC* start codon.


1 2 G A T C
G
C
T
T
T
G
C
T
G
C
G

III.4 S1 NUCLEASE PROTECTION ASSAYS TO DETERMINE THE 5' END OF THE *pcbC* TRANSCRIPT OF *S. CLAVULIGERUS*

High resolution S1 nuclease protection assays would confirm the *tsp* of the *pcbC* gene of *S. clavuligerus* as identified by 5' primer extension, and detect the presence of larger transcripts if they existed. Total RNA preparations of *S. clavuligerus* grown in liquid medium to various stages during the growth cycle were analyzed by S1 nuclease protection assays to determine when *pcbC* transcripts first appear in the growth cycle and when they appear to be most abundant.

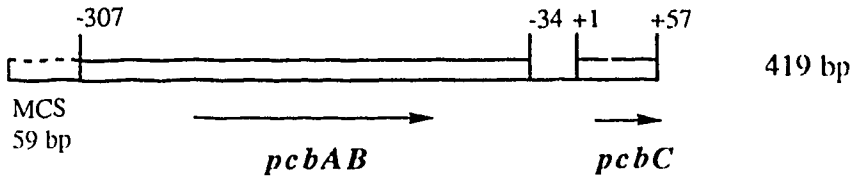
III.4.1 Generation of *pcbC*-specific DNA probes for S1 nuclease protection assays

PCR was used to generate the specific DNA probe fragment used for high resolution S1 nuclease mapping of the 5' end of the *pcbC* transcript of *S. clavuligerus*. One of the primers in the PCR reaction was specific for the *pcbC* gene and the primer is described in detail in section II.13.1 of Materials and Methods. The second primer used was the reverse sequencing primer. The reverse sequencing primer anneals to a part of the template which contains non-*Streptomyces* DNA, and as a result the DNA probe fragment will contain a short stretch of non-homologous sequence. The benefits of using the reverse sequencing primer were two-fold. Should there be a transcript from the other strand of DNA being transcribed in the opposite direction, this could potentially confuse the interpretation of results. However, the use of the reverse sequencing primer to generate the probe ensures that protected DNA fragments resulting from the opposite strand would not be detected, as the label would be removed with the non-homologous DNA sequence. A second benefit to using the reverse sequencing primer is that the non-homologous sequence allows differentiation between probe/probe reannealing and full-length homologous probe protection. Figure 6A illustrates the gene-specific DNA probe fragment generated by PCR for mapping of the 5' end of the *pcbC* transcript. The DNA probe fragment was end-labelled with [γ-^{32}P]ATP by polynucleotide kinase treatment.

Figure 6: DNA probe fragments generated by PCR for high resolution S1 nuclease mapping the 5' ends of *pcbC*, *pcbAB* and *lat* transcripts of *S. clavuligerus*. The DNA probe fragments generated by PCR were specific for *pcbC* (A), *pcbAB* (B), and *lat* (C). Closed boxes represent the coding regions of the indicated genes and solid lines indicate intergenic regions (between *lat* and *pcbAB*, and between *pcbAB* and *pcbC*) or uncharacterized DNA sequence (upstream of *lat*). Boxes enclosed with a dashed line represent non-homologous DNA sequence, the size of which is indicated in bp. Total size of each DNA probe fragment is indicated in bp at the far right.

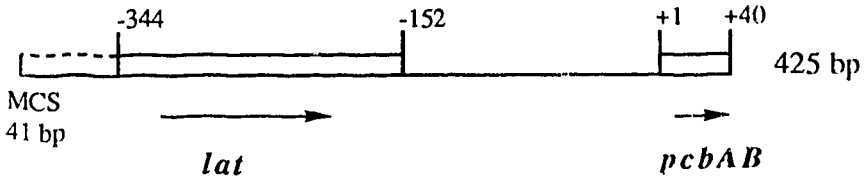
A

***pcbC* probe**



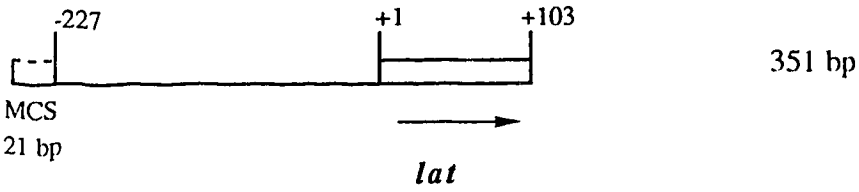
B

***pcbAB* probe**



C

***lat* probe**

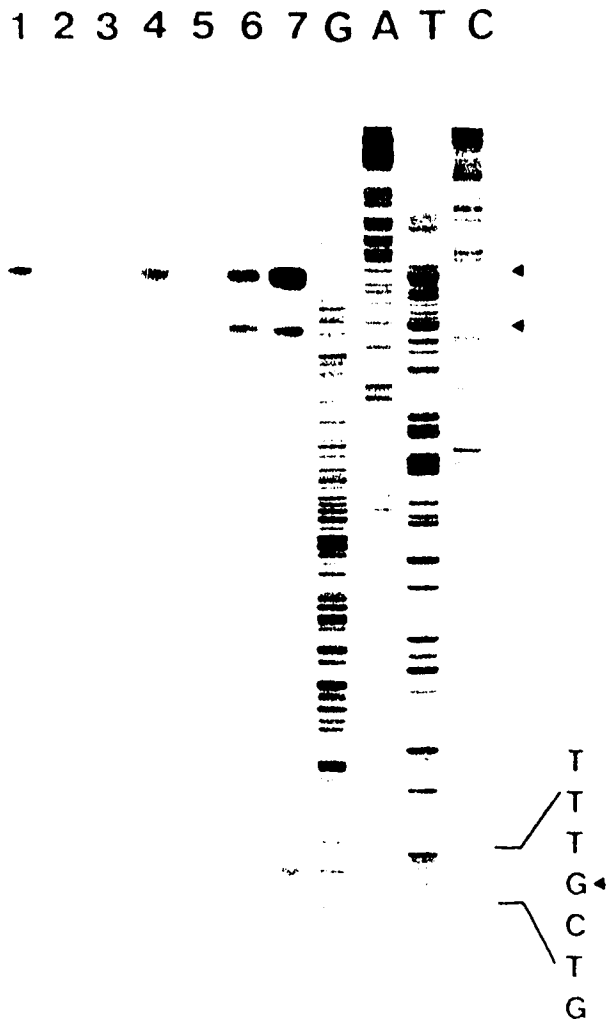


III.4.2 Determination of the 5' end of the *pcbC* transcript of *S. clavuligerus* by high resolution S1 nuclease mapping

Total RNA preparations from *S. clavuligerus* cultures harvested at various times during the growth cycle (24h, 30h, 36h, 48h and 60h) and total RNA isolated from *S. lividans* grown for 36h were annealed to the end-labelled *pcbC*-specific DNA probe fragment. S1 nuclease treatment of samples and electrophoresis were performed as described in section II.13.3 of Materials and Methods (Plate 4). Adjacent wells on the polyacrylamide gel contained full-length *pcbC* probe fragment to indicate the size of the expected band which would result from probe/probe reannealing, and ^{35}S sequencing reactions generated using the same *pcbC*-specific oligo and template in single-stranded form (pIPS-1) used for PCR amplification of the *pcbC* DNA probe fragment. There were three major bands of radioactivity apparent in the *S. clavuligerus* RNA samples (Plate 4). Protected fragments first appear faintly in the 36h RNA samples and appear strongest in the 48h and 60h samples. The faster running protected fragment (lowest arrow) corresponds to the *tsp* identified by 5' primer extension, a C nt 91 bases upstream of the *pcbC* start codon. The second fragment (middle arrow) corresponds to the full length DNA probe fragment that has had the non-homologous *E. coli* sequence at the 3' end removed (59 bases) by S1 nuclease treatment. This indicates the presence of a larger transcript that originates upstream of the DNA probe fragment (at least 307 bases upstream of the *pcbC* start codon). The full length DNA probe fragment (top arrow) can also be detected in samples as the slowest moving radioactive band that runs adjacent to the probe control included in lane 1.

RNA samples used in this S1 mapping experiment were from a different set of samples than those samples used for Northern transfer and hybridization to detect the *pcbC* transcript. Slight differences in the spore inoculum for each time course could result in a shift in appearance of *pcbC* transcript by as much as 24h when comparing one set of RNA samples to another. Repeat experiments with a single set of RNA samples indicated

Plate 4: Location of the 5' end of the *pcbC* transcript of *S. clavuligerus* by high resolution S1 nuclease mapping. S1 nuclease protection assays were performed using the ^{32}P end-labelled *pcbC*-specific DNA probe indicated in Figure 6 (A). The probe was annealed to total RNA isolated from *S. clavuligerus* grown for 24h, 30h, 36h, 48h, and 60h (lanes 3, 4, 5, 6, and 7 respectively) and total RNA isolated from *S. lividans* grown for 36h (lane 2) and then subjected to S1 nuclease digestion. S1 nuclease protected DNA probe fragments were separated by electrophoresis on a 6% polyacrylamide denaturing gel. Adjacent wells contained full-length DNA probe fragment (lane 1) to indicate the size of DNA fragment expected from probe/probe reannealing, and ^{35}S DNA sequencing reactions generated using the same gene specific oligo as was used for PCR to obtain the *pcbC*-specific DNA probe (lanes G, A, T and C). Arrows from top to bottom indicate: full-length protected DNA probe fragment, full-length DNA probe fragment minus the non-homologous DNA sequence (59 bases), and a DNA probe fragment corresponding to a G nt (a C nt in the coding strand) 91 bases upstream of the *pcbC* start codon.


1 2 3 4 5 6 7 G A T C
T
T
T
G
C
T
G

that appearance of *pcbC*-specific signals was reproducible for both S1 mapping experiments and Northern transfers and hybridizations.

It is unclear from this experiment where the second, larger *pcbC* transcript originates. From the perspective of efficiency in antibiotic production, the most likely origin of the larger transcript would be upstream of *pcbAB* (perhaps even upstream of *lat*), however, the *pcbAB* gene is approximately 12 kb and it would be a difficult task to perform S1 nuclease protection experiments that would rule out the presence of a second *tsp* for *pcbC* initiating within the *pcbAB* gene. An indirect approach would be to determine the *tsp* of *pcbAB* itself and determine if the expression profile of S1 protected DNA fragments resembles that of the *pcbC* transcripts.

III.5 PROMOTER PROBE ANALYSIS OF THE REGION UPSTREAM OF THE *pcbAB* AND *lat* GENES OF *S. CLAVULIGERUS*

III.5.1 Insertion of the DNA sequence upstream of the *lat* and *pcbAB* genes of *S. clavuligerus* into pIJ4083

The possibility that *pcbC* was being transcribed as a polycistronic message with *pcbAB* or with both *pcbAB* and *lat* was explored indirectly by analyzing the transcriptional regulation of *pcbAB* and *lat*. DNA sequences upstream of each of these genes were introduced into pUC119 or pUC118 to gain useful restriction sites that would permit directional subcloning of the upstream DNA fragments into pIJ4083.

III.5.2 Promoter probe analysis of the DNA sequence upstream of *pcbAB* (*pcbAB*up/4083)

A *Kpn*I restriction fragment (688 bp) that included 673 bp of sequence upstream of the *pcbAB* start codon and 15 bp of the *pcbAB* coding region, was obtained from a cosmid clone known to include the *lat* and *pcbAB* genes of *S. clavuligerus*. The DNA fragment was ligated to *Kpn*I digested pUC119. The resulting recombinant plasmid (pA2/119) was digested with *Sal*I to shorten the length of the DNA sequence upstream of *pcbAB* by removal of a 330 bp DNA fragment and the plasmid was then re-ligated. A smaller fragment was desirable, as we were most interested in looking for promoter

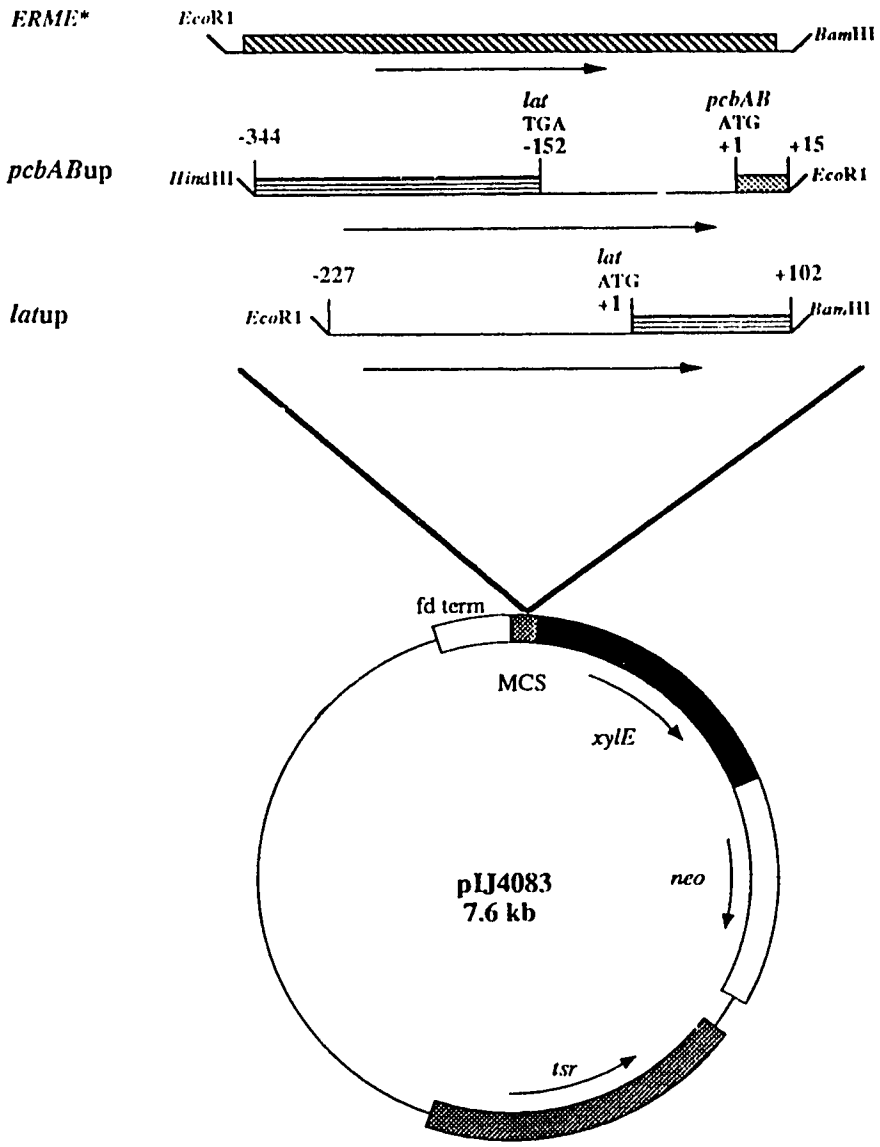
activity downstream of the possible transcriptional terminaator. The final recombinant plasmid (pA2-S/119) contained a 342 bp insert that included 327 bp of sequence upstream of the *pcbAB* start codon and 15 bp of the *pcbAB* coding region. The recombinant plasmid pA2-S/119 was treated with the restriction endonucleases *Eco*RI and *Hin*dIII to remove the insert, *pcbAB*up. *pcbAB*up was ligated to similarly digested pIJ4083, and transformed into *S. lividans* and *S. clavuligerus*. Figure 7 illustrates the *pcbAB*up/4083 recombinant plasmid. Patches of *S. lividans* and *S. clavuligerus* cells transformed with the *pcbAB*up/4083 plasmid (labelled as ACVSup) and grown on MYM agar plates, did not show yellow colour production when sprayed with catechol solution (Plate 5) and patches appeared very similar to those of cells containing the negative control vector pIJ4083. Cell extracts from both *S. lividans* and *S. clavuligerus* cultures carrying the *pcbAB*up/4083 recombinant plasmid showed no evidence of promoter activity. C23O specific activities of 5 and 3 mU/mg protein respectively were not significantly higher than the background levels seen in control extracts from cells containing the pIJ4083 plasmid with no insert (Table II). In comparison cell free extracts from cultures containing the *pcbC*up/4083 plasmid, known to contain an active promoter found immediately upstream of *pcbC*, show levels C23O activity 15-38 times higher than background levels. These results implied that the *pcbAB*up DNA fragment found in *pcbAB*up/4083 does not contain an active promoter. This suggested the possibility that *pcbAB* may be co-transcribed with the *lat* gene or that the promoter of *pcbAB* may be located further within the *lat* gene.

III.5.3 Promoter probe analysis of the DNA sequence upstream of the *lat* gene (*lat*up/4083)

No useful restriction sites were available near the start codon of the *lat* gene to allow the DNA sequence upstream of *lat* to be subcloned into pIJ4083. Therefore, the upstream region of *lat* was amplified by PCR using the reverse sequencing primer and a *lat*-specific primer (described in section II.7.5.1) that included a *Bam*HI restriction site at the 5' end. The template for amplification was a pUC118 subclone containing a 1080 bp

Figure 7: Recombinant promoter probe plasmids used to determine the location and relative activity of promoters directing transcription of *pcbAB* and *lat*. *S. clavuligerus* DNA fragments (except ERME*, from *S. erythraea*) were introduced into the MCS of pIJ4083 by digestion with the indicated restriction endonucleases and ligation to similarly digested pIJ4083 to form a transcriptional fusion with the downstream *xylE* gene. DNA insert fragments were obtained from the following pUC119 or pUC118 recombinant plasmids (in brackets): *pcbAB*up (pA2-S/119) and *lat*up (pLAT/118). The ERME* DNA fragment was obtained from a pUC18 derivative named pIJ4070.

Coding regions of genes are indicated by closed boxes and single lines represent intergenic regions between genes (*lat* and *pcbAB*) or uncharacterized DNA sequence (upstream of *lat*).


ERME*
EcoR1
BamHI
lat
TGA
-152
pcbAB
ATG
+1
+15
-344
pcbABup
HindIII
EcoR1
lat
ATG
+1
-227
+102
latup
EcoR1
BamHI
fd term
MCS
xylE
neo
pLJ4083
7.6 kb
tsr

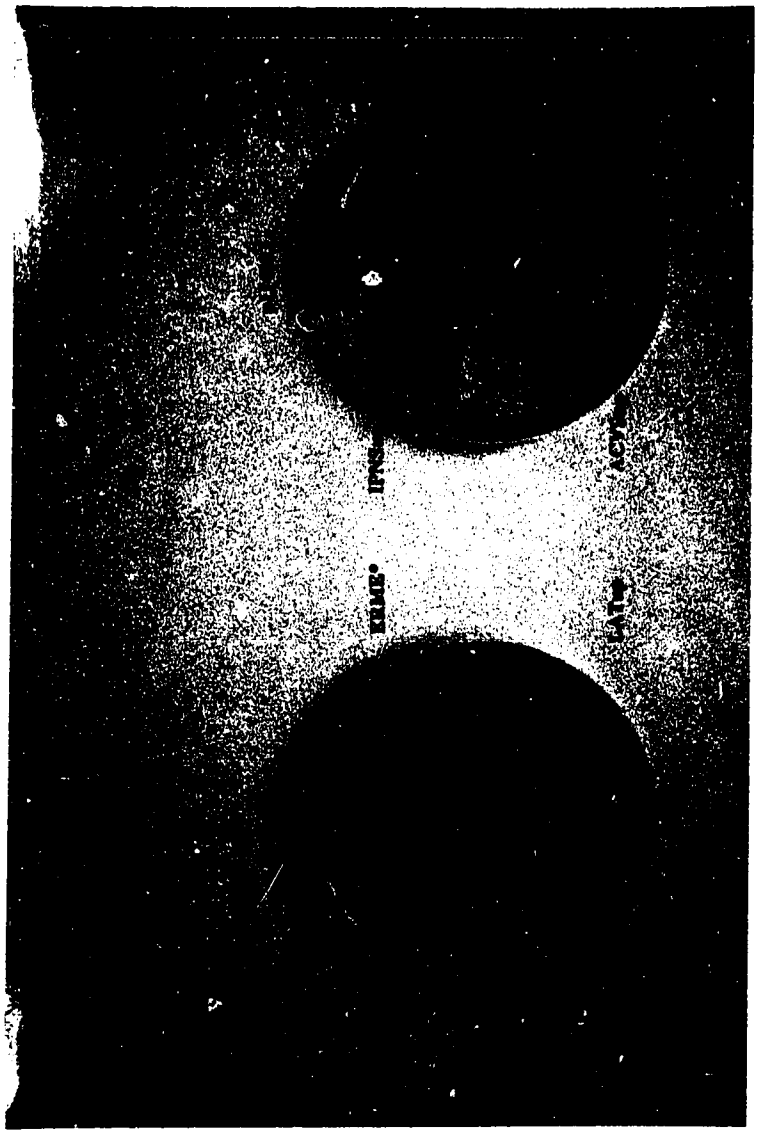

IPNS
KEK

TABLE II

Catechol dioxygenase (C23O) activity in cell free extracts of *Streptomyces clavuligerus* and *Streptomyces lividans* transformed with pIJ4083 recombinant plasmids for identification of the *pcbAB* and *lat* promters

Plasmid[a]	Host	
	S. clavuligerus	*S. lividans*
	Catechol dioxygenase specific activity (mU/mg protein)[b]	
pIJ4083	2	2
ERME*/4083	764	295
*pcbAB*up/4083	3	5
*lat*up/4083	5260	34

[a]Recombinant plasmids indicated are illustrated in Figure 7.

[b]C23O activity was determined as described by Zukowski et al. (1983) using a molar absorption coefficient of 3.3×10^4 (Sala-Trepat and Evans, 1971), where one milliunit (mU) corresponds to the formation at 30°C of 1 nmol of 2-hydroxymuconic semialdehyde per min.

insert of *S. clavuligerus* DNA that included 230 bp of sequence upstream of the *lat* start codon and 850 bp of the *lat* gene. PCR reactions were performed as described in section II.7.5.1 and the amplified DNA fragment, *lat*up (354 bp), was digested with the restriction endonucleases *Pst*I and *Bam*HI. The *lat*up fragment was ligated to similarly digested pUC118 and the resulting recombinant plasmid was named pLat/118. For directional cloning of the *lat*up DNA fragment into pIJ4083, pLat/118 was digested with the restriction endonucleases *Eco*RI and *Bam*HI, and the *lat*up DNA fragment was ligated to similarly digested pIJ4083. The *lat*up/4083 recombinant plasmid illustrated in Figure 7 was transformed into *S. lividans* and *S. clavuligerus*, and cell extracts were analyzed for C23O activity. *S. lividans* and *S. clavuligerus* cells transformed with *lat*up/4083 cultured on MYM agar plates clearly indicated intense yellow colour (especially *S. clavuligerus*) when sprayed with catechol solution (Plate 5). Similarly, cell extracts from both *S. lividans* and *S. clavuligerus* cultures carrying the *lat*up/4083 recombinant plasmid showed evidence of promoter activity (Table II). C23O specific activities of 34 and 5260 respectively were 16 and 2500 times higher than background C23O levels of control extracts from cells transformed with pIJ4083 with no insert. The C23O activity level of *S. lividans* containing the *lat*up/4083 plasmid is very similar to the C23O activity level seen from cell free extracts of cells carrying the plasmid *pcbC*up/4083. However, C23O activities of *S. clavuligerus* cells transformed with *lat*up/4083 is approximately 70 times greater than C23O levels seen for *S. clavuligerus* cells containing *pcbC*up/4083. This indicates a dramatic difference between the expression of the two promoters in the two hosts.

III.6 NORTHERN ANALYSIS TO IDENTIFY THE *pcbAB* AND *lat* TRANSCRIPTS OF *S. CLAVULIGERUS*

III.6.1 Northern transfer and hybridization to detect the *pcbAB* transcript of *S.clavuligerus*

Total RNA isolated from *S. clavuligerus* cells grown in liquid medium for 12h, 24h, 36h, 48h, 60h and 72h was analyzed by Northern transfer and hybridization (Plate 6). Hybridization was performed using a ^{32}P-labelled 3 kb *Kpn*I/*Bam*HI DNA fragment internal to the *pcbAB* gene as a probe. No discrete *pcbAB* hybridzation signals were observed. Rather a smear of degraded RNA was seen which included quite large fragments in early time samples, but which became more degraded as the cultures aged. Although total RNA isolated from *S. lividans* was not included on this particular blot, previous Northern hybridization analyses with *pcbAB*-specific probes that included *S. lividans* RNA as a negative control did not show any hybridization signals under the stringent conditions used.

III.6.2 Northern transfer and hybridization to detect the *lat* transcript of *S. clavuligerus*

Total RNA preparations from *S. clavuligerus* cells cultured in liquid media for 24h, 30h, 36h, 48h and 60h and total RNA isolated from *S. lividans* grown for 36h in liquid media were subjected to Northern analysis using a *lat*-specific probe (Plate 7). Hybridization was performed with a ^{32}P-labelled *Nde*I/*Kpn*I 768 bp DNA fragment found internal to the *lat* gene as a probe. The specific smears of radioactive signal detected for *lat* appear very similar to those detected for *pcbAB*. The size of the degraded RNA transcript indicated by the radioactive signal appears to become progressively smaller as the age of the cultures increases. The *S. lividans* total RNA preparation (lane 1) does not show any non-specific hybridization to the *lat* probe. As seen for *pcbAB*, Northern analysis of *lat* in *S. clavuligerus* total RNA samples did not detect a discrete hybridization signal to indicate an intact mRNA transcript. RNA samples used for the *lat* and *pcbAB* Northern transfer and hybridization experiments were isolated from two different growth curves, and this may account for the slight difference in the time of appearance of hybridization signal.

III.7 PRIMER EXTENSION TO DETERMINE THE 5' END OF THE *pcbAB* AND *lat* TRANSCRIPTS OF *S. clavuligerus*

Plate 6: Northern analysis of total RNA preparations from *S. clavuligerus* using a *pcbAB* specific probe. Total RNA isolated from *S. clavuligerus* grown for 12h, 24h, 36h, 48h, 60h and 72h (lanes 1 through 6 respectively) was separated by electrophoresis and transferred to a nylon membrane as described in Plate 2. Hybridization was carried out using a ^{32}P-labelled 3 kb *Kpn*I/*Bam*HI DNA internal fragment obtained from restriction digestion of the *pcbAB* gene of *S. clavuligerus*.

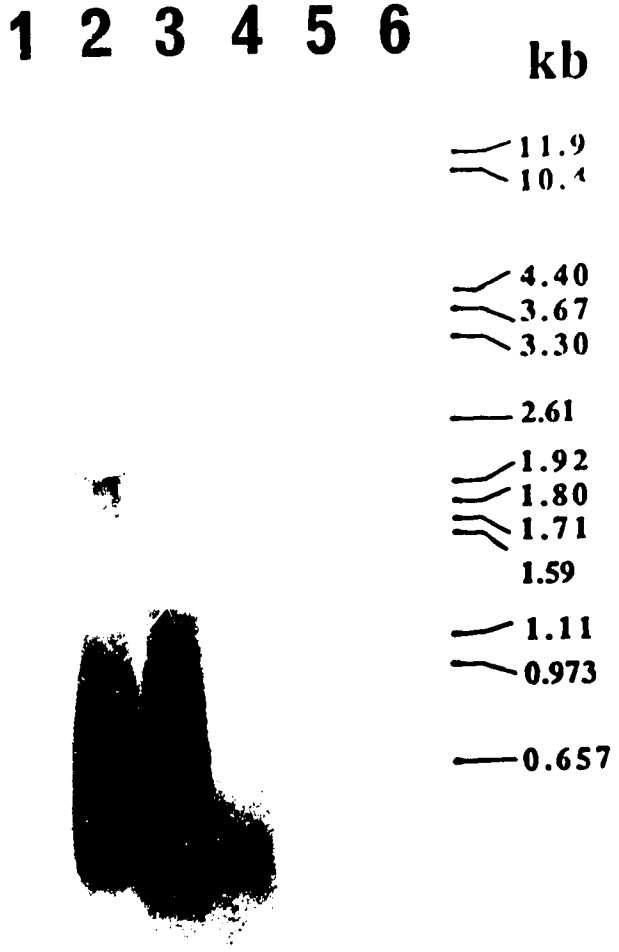
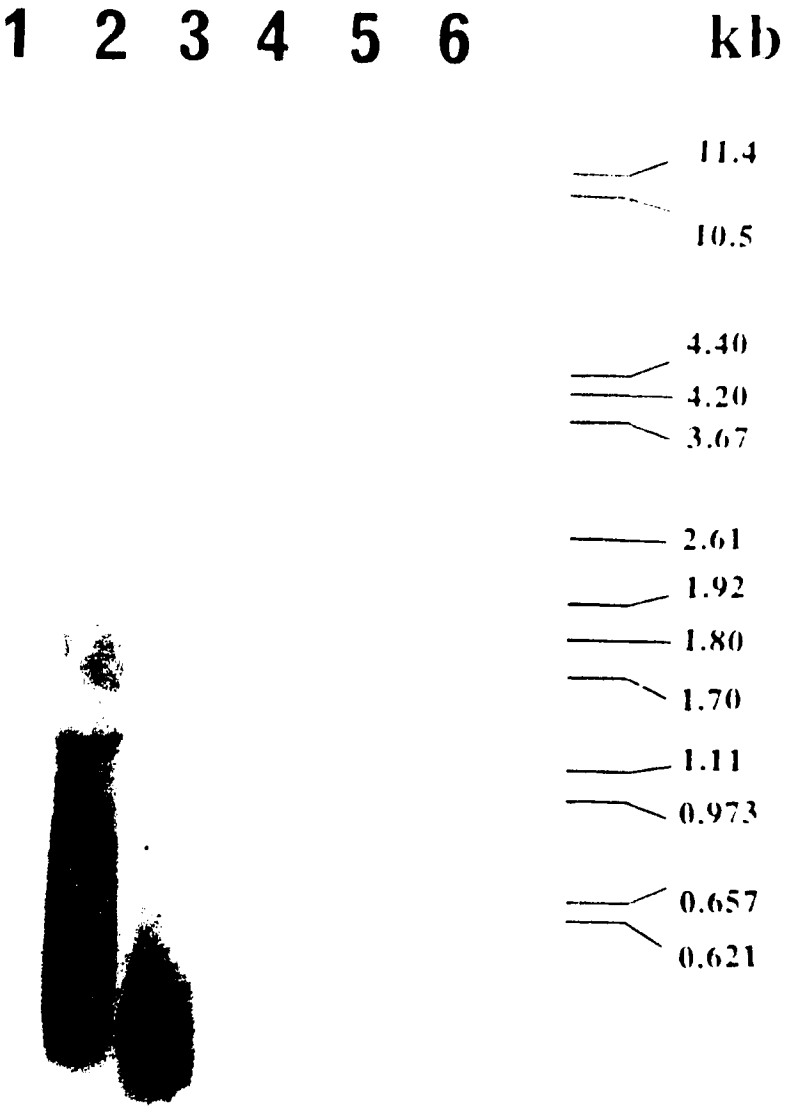

1 2 3 4 5 6
kb
11.9
10.4
4.40
3.67
3.30
2.61
1.92
1.80
1.71
1.59
1.11
0.973
0.657

Plate 7: Northern analysis of total RNA preparations of *S. clavuligerus* using a *lat* specific probe. Total RNA isolated from *S. clavuligerus* grown for 24h, 30h, 36h, 48h, and 60h (lanes 2 through 6 respectively) and total RNA isolated from *S. lividans* grown for 36h (lane 1) was separated by electrophoresis and transferred to a nylon membrane as described in Plate 2. Hybridization was carried out using a ^{32}P-labelled 768 bp *NdeI/KpnI* DNA fragment internal to the *lat* gene of *S. clavuligerus*.


1 2 3 4 5 6
kb
11.4
10.5
4.40
4.20
3.67
2.61
1.92
1.80
1.70
1.11
0.973
0.657
0.621

Primer extension reactions were attempted to determine the 5' end of the *lat* and *pcbAB* transcripts of *S. clavuligerus* using a procedure similar to that described for determination of the *pcbC tsp* (section II.12). Oligos used for annealing and extension were 17-mers (described in section II.13.1), homologous to the RNA copy of the specific gene of interest. Experimental results of primer extension of the *pcbAB* and *lat* transcripts are not included as the experimental conditions could not be found which were stringent enough to prevent non-specific annealing. For this reason high resolution S1 nuclease protection mapping assays were performed to determine the *tsp* of the *lat* and *pcbAB* transcripts of *S. clavuligerus*.

III.8 S1 NUCLEASE PROTECTION ASSAYS TO DETERMINE THE 5' END OF THE *lat* AND *pcbAB* TRANSCRIPTS OF *S. CLAVULIGERUS*

III.8.1 Generation of DNA probes for S1 nuclease mapping the 5' end of the *lat* and *pcbAB* transcripts

PCR was used to generate the specific DNA probe fragments used for high resolution S1 nuclease mapping of the 5' ends of the *pcbAB* and *lat* transcripts of *S. clavuligerus*. The reverse sequencing primer was used as a primer to generate one end of the DNA fragment. The second primer in each reaction was specific for the gene of interest and these primers are described in detail in section II.13.1 of Materials and Methods. The gene-specific DNA probe fragments generated by PCR for mapping of the 5' end of *pcbAB* (B) and *lat* (C) transcripts are illustrated in Figure 6 together with the DNA probe fragment for determining the *tsp* of *pcbC*. The DNA probe fragments were end-labelled with [γ-^{32}P]ATP by polynucleotide kinase treatment.

III.8.2 S1 nuclease protection assays to determine the 5' end of the *pcbAB* transcript of *S. clavuligerus*

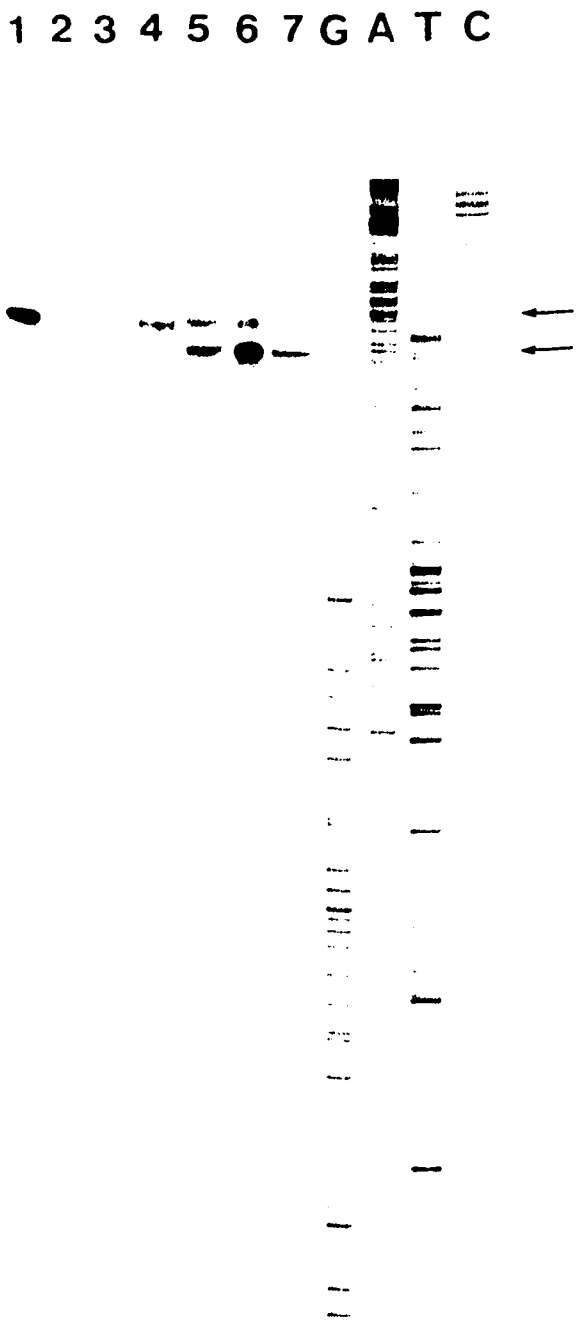
Total RNA isolated from *S. clavuligerus* cultures grown in liquid media for 24h, 30h, 36h, 48h and 60h and total RNA isolated from *S. lividans* 36h cultures were annealed to end-labelled *pcbAB*-specific DNA probe fragment illustrated in Figure 6. S1 nuclease treatment of samples and electrophoresis was performed as described in section

II.13.3 of Materials and Methods. ^{35}S sequencing reactions were generated using the same oligo and template used for PCR of the *pcbAB* DNA probe fragment (section II.13.1.2). Two sizes of radioactive bands were detected on the autoradiograph used to determine the 5' end of the *pcbAB* transcript (Plate 8). The slower running radioactive band (top arrow) detected is full length DNA probe fragment that annealed to itself and therefore was protected from nuclease digestion. The faster running radioactive band (lower arrow) corresponds to full length homologous probe fragment minus the *E. coli* non-homologous tail of 41 bases that was removed during the nuclease digestion. The lower band appears initially in the 36h *S. clavuligerus* RNA sample and peaks in intensity at 48h before dropping slightly in the 60h RNA sample. No other faster moving bands were detected to indicate a *tsp* for the *pcbAB* gene within the DNA probe fragment. The *pcbAB* probe fragment extended 344 bp upstream of the *pcbAB* start codon and 192 bp into the upsteam *lat* gene. Therefore, there does not appear to be a *tsp* or promoter immediately upstream of the *pcbAB* gene, which is consistent with the promoter probe studies. The expression pattern indicated by protected fragments was similar to the expression pattern seen for both of the *pcbC* transcripts detected by S1 nuclease mapping (Plate 7). To determine, indirectly, if the *pcbAB* gene may be being transcribed as part of an operon with the upstream *lat* gene, the *tsp* and expression pattern obtained for *lat* protected DNA probe fragments can be compared to those obtained for the *pcbC* and *pcbAB* genes, keeping in mind that slight differences may be the result of different RNA preparations. In the case of the S1 nuclease mapping the same RNA samples were used for both the *lat*- and the *pcbAB*- specific experiment, however a different set of RNA samples was used for the *pcbC* experiments.

III.8.3 S1 nuclease protection assays to determine the 5' end of the *lat* transcript of *S. clavuligerus*

Total RNA preparations of *S. clavuligerus* isolated from liquid cultures grown for 24h, 30h, 36h, 48h, and 60h and total RNA isolated from *S. lividans* grown for 36h were

Plate 8: Localization of the 5' end of the *pcbAB* transcript of *S. clavuligerus* by high resolution S1 nuclease mapping. S1 nuclease protection assays were performed using the end-labelled *pcbAB*-specific DNA probe illustrated in Figure 6 (B). The probe was annealed to total RNA isolated from *S. clavuligerus* cultures grown for 24h, 30h, 36h, 48h, and 60h (lanes 3, 4, 5, 6, and 7 repectively) and total RNA isolated from an *S. lividans* culture grown for 36h (lane 2) and then subjected to S1 nuclease digestion. S1 nuclease protected DNA probe fragments were separated by electrophoresis on a 6% polyacrylamide denaturing gel. Adjacent wells contained full length DNA probe fragment (lane 1) and ^{35}S DNA sequencing reactions generated using the same *pcbAB*-specific oligo as was used for PCR to obtain the *pcbAB* DNA probe fragment (lanes G, A, T and C). Arrows from top to bottom indicate: full length DNA *pcbAB* probe fragment and the full length DNA probe fragment minus the non-homologous DNA sequence (41 bases).


1 2 3 4 5 6 7 G A T C

annealed to end-labelled *lat*-specific DNA probe illustrated in Figure 6. S1 nuclease treatment of samples and electrophoresis was performed as described in section II.13.3 of Materials and Methods (Plate 9). Adjacent wells on the polyacrylamide gel contained full length DNA probe fragment to distinguish probe/probe reannealing and ^{35}S sequencing reactions generated using the same gene-specific oligo and template in single-stranded form used for PCR to generate the *lat*-specific DNA probe fragment (section II.13.1.3). Determination of the 5' end of the *lat* transcript and its transcription profile by S1 nuclease protection assays indicated the presence of a protected DNA fragment (Plate 9). The one main *tsp* of the *lat* gene is indicated by the arrow and corresponds to a T nt in the coding strand 88 bases upstream of the *lat* start codon. The radioactive band for the protected probe fragment first appears faintly in the 36h RNA sample, and has its greatest intensity in the 48h and 60h RNA samples. This pattern of expression is very similar to those observed for both of the *pcbC* transcripts and for the *pcbAB* transcript. A faint band is also detected in the RNA sample showing the strongest intensity band (48h) that corresponds to a full length homologous probe fragment. This observation was reproducible, although it is unclear what significance this holds. It is possible that transcription of *lat* is initiating at a site upstream of the DNA probe fragment used for S1 nuclease mapping under certain conditions and this observation requires further investigation.

III.9 COMPARISON OF THE PUTATIVE PROMOTER REGIONS OF *pcbC* AND *lat* WITH CONSENSUS PROMOTER SEQUENCES AND PROMOTERS OF OTHER *STREPTOMYCES* GENES

Determination of the *tsp* of the *pcbC* and *lat* genes of *S. clavuligerus* has allowed comparison of the sequence directly upstream of the *tsp* to known consensus sequences and promoters of other *Streptomyces* secondary metabolic genes. Table III shows an alignment of the -35 and -10 regions of the *pcbC* and *lat* promoters to the *E. coli* and *Streptomyces* consensus sequences. Also included in this table is the promoter region of

Plate 9: Localization of the 5' end of the *lat* transcript of *S. clavuligerus* by high resolution S1 nuclease mapping. S1 nuclease mapping experiments were performed using the end-labelled *lat*-specific DNA probe fragment illustrated in Figure 6 (C). The probe was annealed to total RNA isolated from *S. clavuligerus* grown for 24h, 30h, 36h, 48h, and 60h (lanes 3, 4, 5, 6, and 7) and total RNA isolated from *S. lividans* grown for 36h (lane 2) and then subjected to S1 nuclease digestion. S1 nuclease protected DNA fragments were electrophoresed on a 6% polyacrylamide denaturing gel in adjacent wells to DNA *lat* probe fragment (lane 1) and to ^{35}S DNA sequencing reactions generated using the same *lat*-specific oligo as was used for PCR to obtain the *lat*-specific DNA probe fragment (lanes G, A, T and C). Arrows from top to bottom indicate: full length *lat* DNA probe fragment, and a protected DNA probe fragment that corresponds to an A nt (T nt in the coding strand) 88 bases upstream of the *lat* start codon.

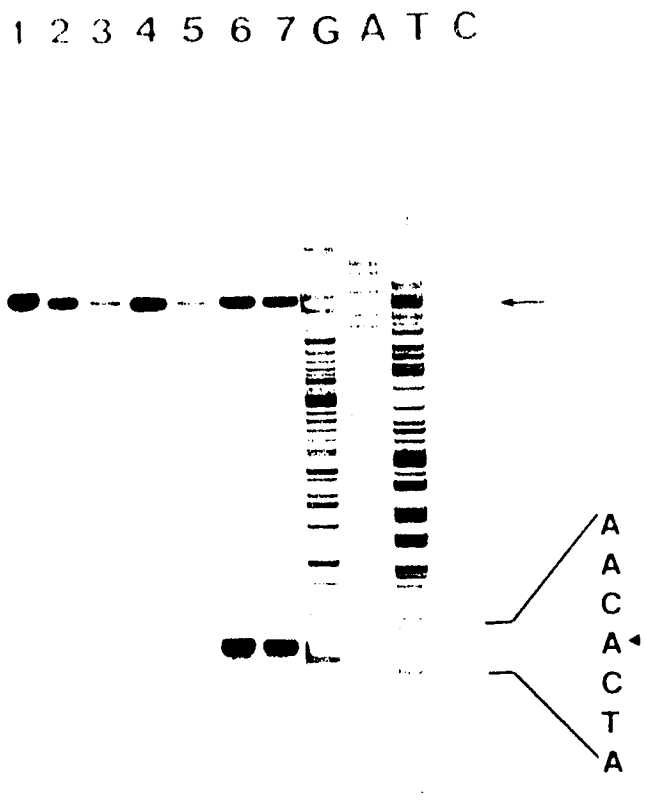

1 2 3 4 5 6 7 G A T C
A
A
C
A
C
T
A

TABLE III

Comparison of the putative promoter sequence of *pcbC* and *lat* with the consensus promoter sequences of *Escherichia coli* and *Streptomyces* and with the promoters of other *Streptomyces* secondary metabolic genes

Promoter[a]	Sequence[b]	Reference
E. coli	TTGACa-17bp-TAtAaT	Hawley and McClure (1983)
Streptomyces	TTGaca-18bp-tAGgaT	Hopwood et al. (1986)
pcbC	TGCACG-18bp-GTGGGT	This study
lat	TGGAAG-17bp-GTGGGT	This study
cefD/cefE	TTGAAG-18bp-CAGAAT	Kovacevic et al. (1990)
tsr P1	GGCAGC-17bp-TAGGGT	Janssen et al. (1985)
ermE P2	TTGACG-18bp-GAGGAT	Bibb et al. (1985)

[a]The *cefD/cefE* promoter was identified from the cephamycin biosynthetic genes of *S. clavuligerus*. The *tsr P1* promoter was identified from the thiostrepton resistance-encoding gene of *S. azureus* and the *ermE P2* promoter was characterized from the *S. erythraea* erythromycin resistance-encoding gene.

[b]For consensus sequences (first two lines), the upper-case letters indicate nt that are highly conserved and lower-case letters show nt that are significantly conserved.

the *cefD/cefE* genes of the cephamycin biosynthetic pathway of *S. clavuligerus* and the promoter regions of two *Streptomyces* antibiotic resistance genes that showed some sequence similarity (*tsr P1* from *S. azureus* and *ermE P2* from *S. erythraea*). Greatest similarity in sequence was seen between the promoter sequences of the two early cephamycin biosynthetic genes, *lat* and *pcbC*. There was also significant homology between the -35 regions of the *lat* and *pcbC* genes and the later cephamycin biosynthetic genes *cefD/cefE*.

III.10 IPNS EXPRESSION IN *S. LIVIDANS*

An indication of the role that the 1.2 kb *pcbC* transcript plays in antibiotic production in *S. clavuligerus* may be obtained from examing its transcription and IPNS activity in a heterologous host. Promoter probe plasmid studies of the *pcbC* upstream promoter in pIJ4083 (*pcbC*up/4083) indicated that the promoter located immediately upstream of the gene was active in *S. lividans* cells. However, previous reports of IPNS expression in *S. lividans* indicated that IPNS activity had never been detected in significant levels when the native transcriptional and translational signals were controlling expression (Jensen et al., 1989). Two *pcbC*-containing recombinant plasmids were constructed to determine if expression of IPNS in *S. lividans* is restricted at the transcriptional level.

III.10.1 Construction of two vectors containing the *pcbC* gene of *S. clavuligerus* for expression of IPNS in *S. lividans* (1.7/4083 and 1.35/702)

The first recombinant plasmid contained the *pcbC* gene on a 1.7 kb fragment inserted into the polylinker of pIJ4083 (1.7/4083), the same recombinant plasmid as was used to assess promoter activity of *pcbC* (Table I, Figure 5). This *S. clavuligerus* DNA fragment contained the *pcbC* structural gene, 307 bp of sequence upstream of the *pcbC* coding region and 383 bp of downstream sequence. The 307 bp of upstream sequence included the promoter region identified in this study and a previously identified DNA sequence that resembled a ribosome binding site (Doran et al., 1990). Specific details for

construction of 1.7/4083 are given in section II.7.1 of Materials and Methods. The second recombinant plasmid contained a 1.35 kb *S. clavuligerus* DNA fragment that included the *pcbC* structural gene and 383 bp of downstream sequence, but lacked any *pcbC* upstream sequence. The 1.35 kb DNA fragment was inserted into the *Streptomyces* vector pIJ702 so that the *pcbC* structural gene precisely replaced the *melC1* structural gene (1.35/702). Thus, in this construction *pcbC* expression was under the transcriptional and translational control signals of the *melC1* gene of pIJ702. The specific procedure for the construction of the recombinant plasmid 1.35/702 is indicated in section II.14.1. Figure 8 illustrates the 1.7 kb and 1.35 kb inserts and their sites of insertion into pIJ4083 and pIJ702 respectively. The recombinant plasmids were introduced into *S. lividans* for the purposes of examining IPNS expression.

III.10.2 Determination of IPNS activity in partially purified cell free extracts by HPLC analysis

Cell free extracts were prepared from *S. lividans* cultures containing the recombinant plasmids 1.7/4083 and 1.35/702, and from an *S. clavuligerus* culture grown for 36h in liquid media. Partial purification of IPNS with streptomycin sulfate and ammonium sulfate precipitation is described in section II.14.2 of Materials and Methods. IPNS activity was detected by HPLC analysis as the conversion of ACV to isopenicillin N. Partially purified IPNS preparations were added to reduced ACV in the presence of cofactors and allowed to incubate for 60 min. IPNS preparations from *S. lividans* transformed with 1.7/4083 showed no detectable IPNS activity (Figure 9B) when compared to corresponding preparations from *S. clavuligerus* (Figure 9A). Since the 1.7 kb insert of *S. clavuligerus* DNA in 1.7/4083 is known to contain the promoter and ribosome binding site for *pcbC*, this suggested two possibilities. Either the promoter sequence is not recognized, resulting in a failure to transcribe the gene, or the transcript cannot be translated. When the *pcbC* gene is downstream of the *melC1* regulatory region,

Figure 8: Recombinant plasmids used for IPNS expression in *S. lividans*. (A) 1.7/4083. A 1.7 kb *S. clavuligerus* DNA fragment that contained the intact *pcbC* gene, 307 bp of upstream sequence, and 365 bp of downstream sequence was removed from the plasmid pIPS-1 by restriction endonuclease treatment and ligated into the multiple cloning site of pIJ4083. (B) 1.35/4083. A 1.35 kb *S. clavuligerus* DNA fragment that contained the intact *pcbC* gene and 365 bp of downstream sequence was removed from the pIPS-1* plasmid by restriction endonuclease treatment and ligated into *SphI/SstI* digested pIJ702 (replacing the *melC1* structural gene of pIJ702). Closed boxes indicate coding regions of the indicated genes of interest.

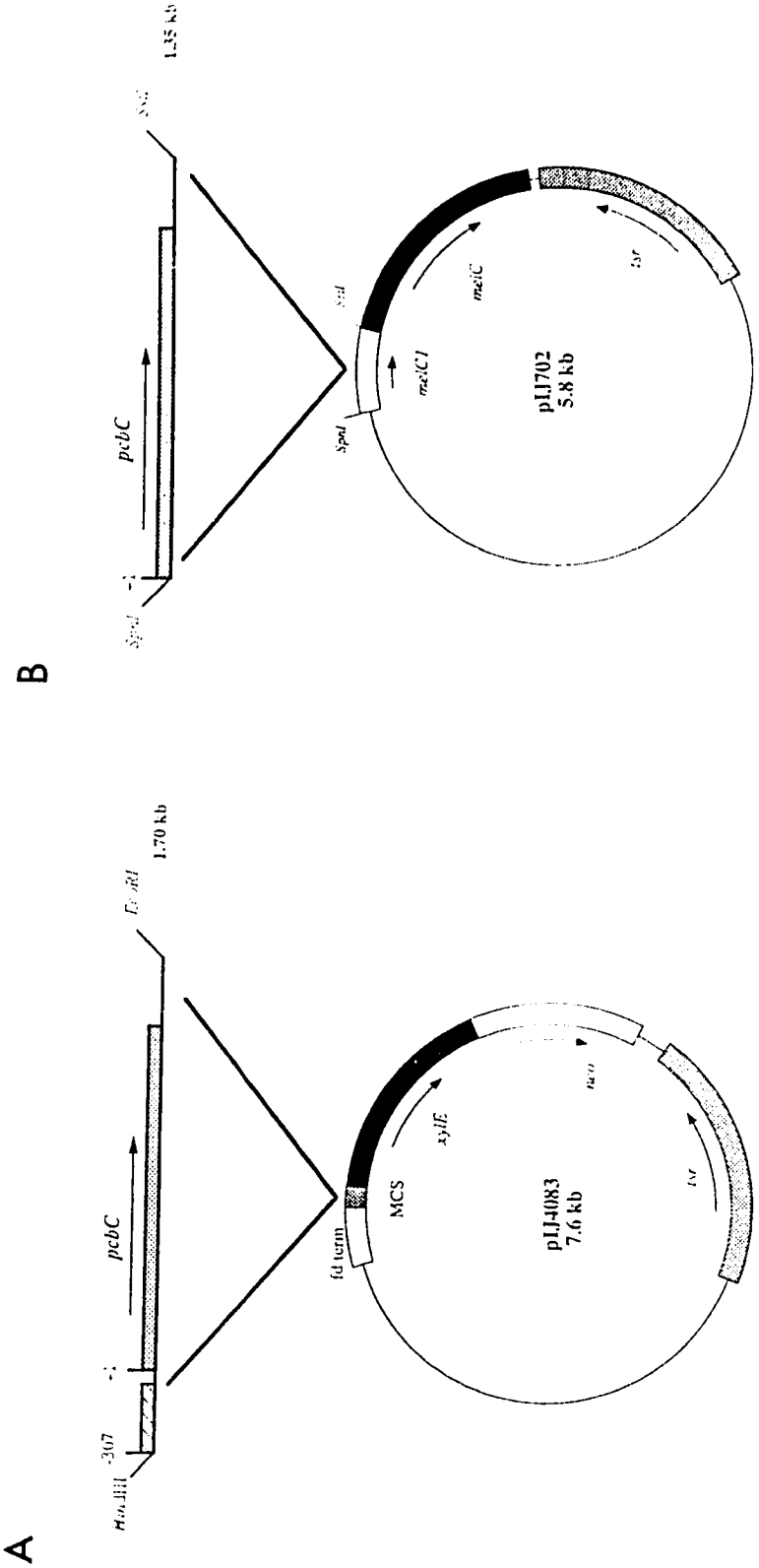
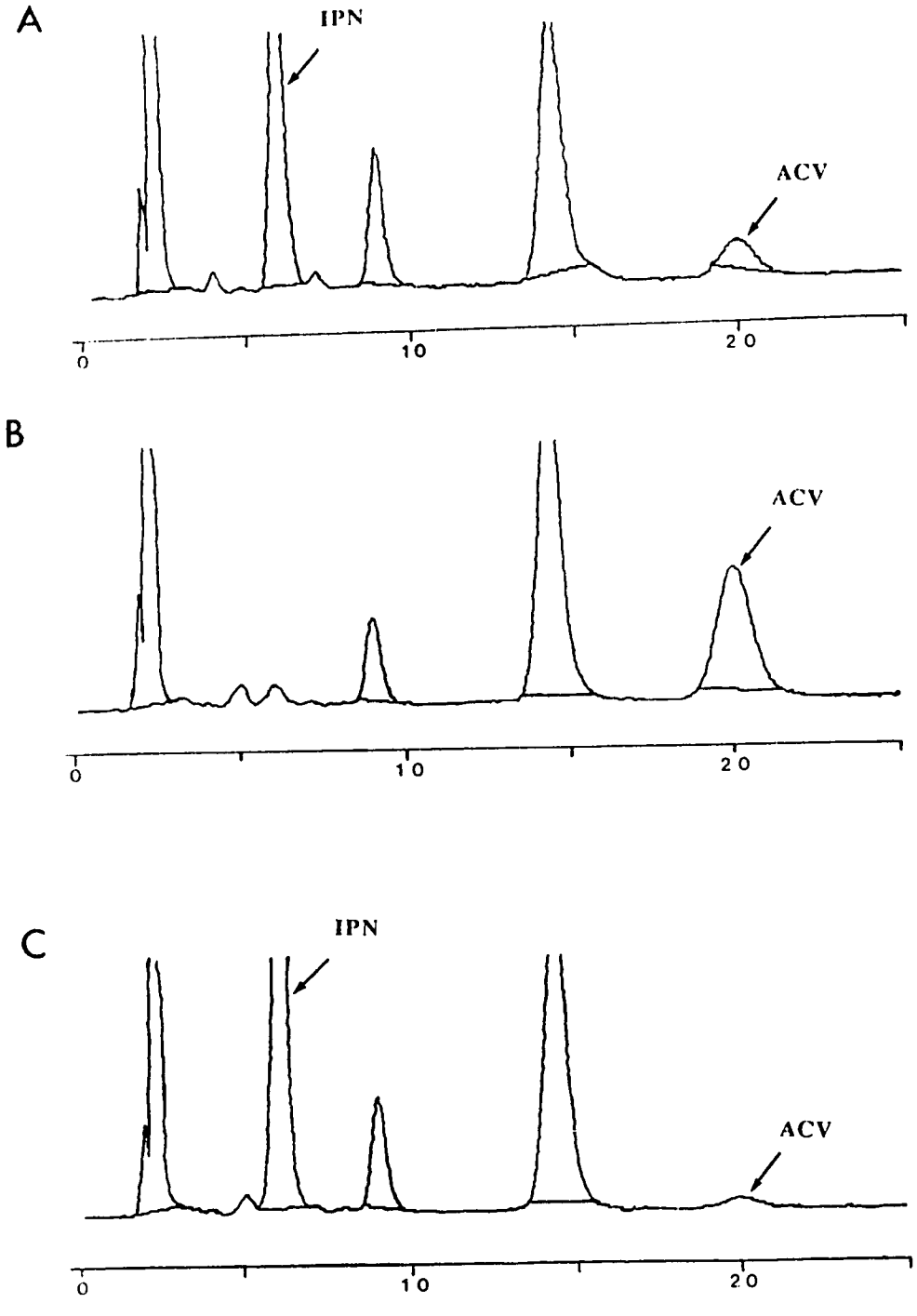

A
1.70 kb
pcbC
MCS
xylE
pLJ40B3
7.6 kb
fd term
B
1.35 kb
pcbC
melC1
melC
pLI702
5.8 kb

Figure 9: HPLC analysis of IPNS activity. Partially-purified cell free extracts of *S. clavuligerus* (A), *S. lividans* transformed with the recombinant plasmid 1.7/4083 (B), and *S. lividans* transformed with the recombinant plasmid 1.35/702 (C) all grown for 36h in liquid media, were assayed for IPNS activity levels by observing the disappearance of ACV (arrows labelled ACV) and the appearance of isopenicillin N (arrows labelled IPN). The retention time is marked in minutes.


A
IPN
ACV
0
10
20
B
ACV
0
10
20
C
IPN
ACV
0
10
20
RETENTION TIME, MINUTES

as in 1.35/702, IPNS activity is detected and the level of activity is comparable to that seen in IPNS preparations from *S. clavuligerus* (Figure 9C). These results suggest that the translation of the structural gene itself is not a problem in *S. lividans*. HPLC traces of zero time samples (reactions stopped before the addition of cell free extract) resembled the test trace for *S. lividans* transformed with 1.7/4083 (B). Peaks corresponding to ACV (ACV) and isopenicillin N (IPN) are identified by arrows. IPNS activity is indicated by a marked decline in the peak corresponding to the substrate ACV and appearance of an isopenicillin N peak in reaction mixtures.

III.10.3 Northern transfer and hybridization to detect the *pcbC* transcript in total RNA isolated from *S. lividans* transformed with 1.7/4083

S. lividans transformed with the 1.7/4083 plasmid showed no IPNS activity despite the presence of the complete *pcbC* gene and transcriptional and translational control regions. Northern transfer and hybridization with a *pcbC*-specific probe was performed to determine if the lack of IPNS activity results from a lack of transcription of the *pcbC* gene in *S. lividans*. Northern transfer and hybridization of total RNA isolated from *S. lividans* transformed with the 1.7/4083 plasmid (lane 2), *S. clavuligerus* (lane 3) and *S. lividans* (lane 1) grown for 36h in liquid medium clearly demonstrates the presence of a 1.2 kb *pcbC* transcript in *S. lividans* transformed with 1.7/4083 that is similar in size to the *pcbC* transcript from the natural host, *S. clavuligerus* (Plate 10).

III.10.4 S1 nuclease protection assays to determine the 5' end of the *pcbC* transcript from *S. lividans* transformed with 1.7/4083

Although no active IPNS was detected in *S. lividans* cultures transformed with the *pcbC*-containing vector 1.7/4083, a transcript specific to *pcbC* was detected in total RNA samples. One possibility is that in the *S. lividans* host, transcription is not initiating at a site that allows translation to follow. To check this possibility, the 5' end of the *pcbC* transcript from *S. lividans* transformed with 1.7/4083 was determined by S1 nuclease protection mapping (Plate 11). Total RNA isolated from *S. lividans* (lane 1), *S. lividans*

Plate 10: Northern analysis of a total RNA preparation from *S. lividans* transformed with recombinant plasmid 1.7/4083, using a *pcbC*-specific probe. Total RNA isolated from *S. lividans* (lane 1), *S. lividans* transformed with the recombinant plasmid 1.7/4083 (lane 2) and *S. clavuligerus* (lane 3) after 36h of growth was separated by electrophoresis and transferred to nylon membrane as described in Plate 3. Hybridization was carried out using ^{32}P-labelled pIPS-1 plasmid as a probe.

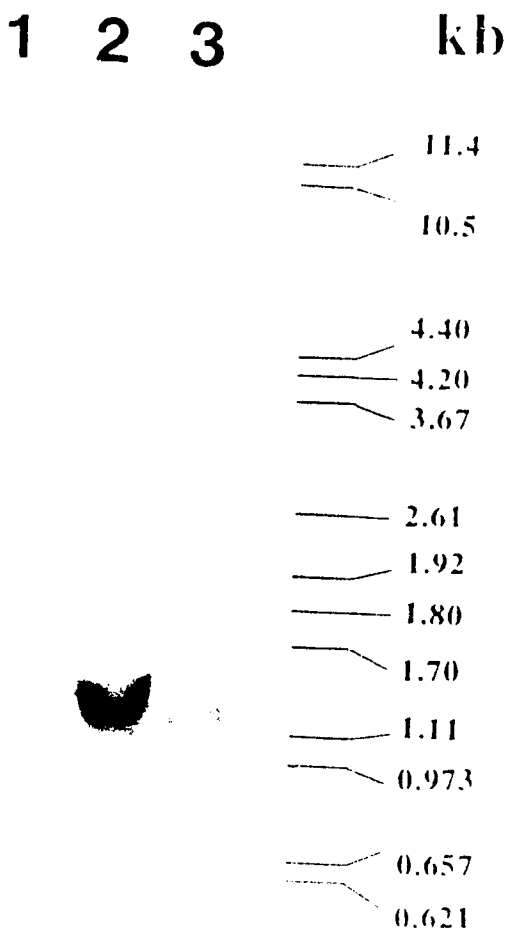
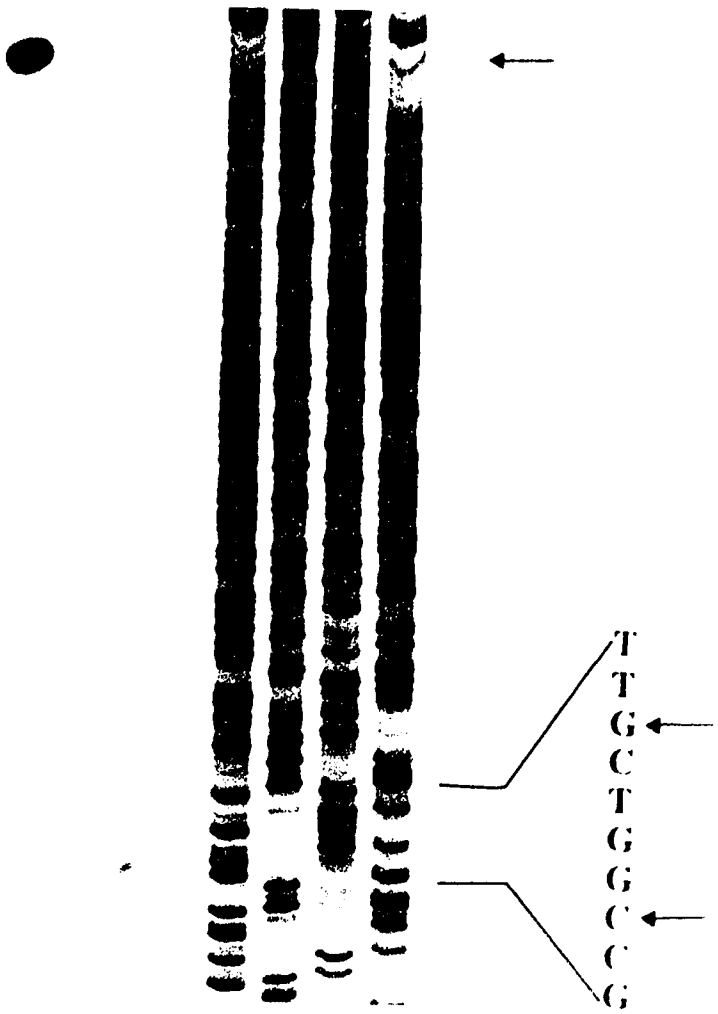

1 2 3
kb
11.4
10.5
4.40
4.20
3.67
2.61
1.92
1.80
1.70
1.11
0.973
0.657
0.621

Plate 11: Comparison of the 5' ends of the *pcbC* transcripts from *S. clavuligerus* and *S. lividans* transformed with the recombinant plasmid 1.7/4083. High resolution S1 nuclease mapping experiments were performed using the *pcbC* DNA probe fragment described in Figure 6 (A). The probe was annealed to total RNA prepared from *S. lividans* (lane 2), *S. lividans* transformed with the recombinant plasmid 1.7/4083 (lane 3), and *S. clavuligerus* (lane 4) all grown for 36h in liquid media and then subjected to S1 nuclease digestion. S1 nuclease protected DNA fragments were analyzed on a 6% polyacrylamide denaturing gel in adjacent wells to *pcbC* DNA probe fragments (lane 1) and ^{35}S sequencing reactions generated using the same *pcbC*-specific oligo as was used to generate the *pcbC*-specific DNA probe by PCR (lanes G, A, T and C). Arrows from top to bottom indicate; full-length DNA probe fragment, a DNA probe fragment that corresponds to the previously identified *tsp* of *pcbC* in *S. clavuligerus* (C nt in coding strand 91 bases upstream of the *pcbC* start codon), and a DNA probe fragment that corresponds to a C nt (G nt in the coding strand) 86 bases upstream of the *pcbC* start codon.


1 2 3 4 G A T C
T
T
G
C
T
G
G
C
C
G

transformed with 1.7/4083 and *S. clavuligerus* (lane 3) cultures grown for 36h in liquid media were annealed to end-labelled *pcbC*-specific DNA probe fragment illustrated in Figure 6. S1 nuclease treatment of samples and electrophoresis was performed as described in section II.13.3 of Materials and Methods. Wells adjacent to the S1 nuclease mapping reactions contained the DNA probe fragment and ^{35}S sequencing reactions generated using the same *pcbC*-specific oligo and template in single-stranded form (pIPS-1) used for PCR of the *pcbC* DNA probe fragment. Three radioactive bands were detected by the S1 protection reactions. The top arrow points to fully protected DNA probe fragment from probe/probe reannealing. The single detected *tsp* of the *pcbC* transcript in *S. lividans* transformed with 1.7/4083, indicated by the bottom arrow, corresponds to a C nt (G nt in the coding strand) 86 bases upstream of the translational start codon. There is a difference of 5 nt from the *tsp* of the *pcbC* transcript in *S. clavuligerus*, indicated by the middle arrow, which corresponds to a G nt (C nt in the coding strand) 91 bases upsteam of the translational start codon, to the *tsp* of *pcbC* in *S. lividans*. The location of the *tsp* for *pcbC* in *S. lividans* transformed with 1.7/4083 was reproducible when the experiment was repeated. Whether this slight change in the location of transcription initiation is reponsible for the lack of IPNS expression in *S. lividans* transformed with 1.7/4083, requires further investigation, such as Western blot analysis for the detection of IPNS. The faint unmarked band found in lane 4 which contains the *S. clavuligerus* RNA sample represents the *pcbC* transcript that originates further upstream, as indicated by the full length homologous probe. A second faint band can be seen in lane 3, just below where the full length probe fragment would be found, in the lane containing the RNA sample from *S. lividans* transformed with 1.7/4083. However, this radioactive signal was not reproducible and was not detected when other S1 nuclease protection experiments of the *pcbC* transcript were performed using RNA isolated from *S. lividans* containing the 1.7/4083 plasmid.

IV. DISCUSSION

Characterization of the transcriptional regulation of the *pcbC* gene of *S. clavuligerus* involved two main approaches; promoter probe recombinant plasmids and mRNA analysis. Initial experimentation centred around the DNA sequence upstream of the *pcbC* gene itself, however, this was later extended to the DNA sequence upstream of the other early genes in the cephamycin biosynthetic pathway, *lat* and *pcbAB*.

The *Streptomyces* promoter probe vector pIJ4083 contains the *xylE* gene coding for the enzyme C23O. This enzyme has previously been shown to be highly effective as a reporter gene in both *S. coelicolor* and *S. lividans* (Ingram et al, 1989; Guthrie and Chater, 1990). The ERME*/4083 plasmid, which contained a mutated form of the promoter region of the gene encoding erythromycin resistance from *S. erythraea*, was constructed as a positive control to ensure that the *xylE* gene would be expressed and that the enzyme would be active in *S. clavuligerus*. This construction indicated that promoter activity could be detected in *S. clavuligerus* when a suitable DNA insert was transcriptionally fused to the *xylE* gene.

The DNA sequence immediately upstream of *pcbC* includes a 31 bp intergenic region and then the *pcbAB* gene transcriptionally oriented in the same direction as *pcbC*. Regions important for initiating transcription typically extend at least 35 bp upstream of the *tsp*. With such a short intergenic region between *pcbC* and *pcbAB*, if a promoter was to be present upstream of *pcbC* it would necessesarily be located at least partially within the *pcbAB* coding gene which might indicate complex regulation. Inspection of the DNA sequence upstream of *pcbC* failed to detect a sequence resembling a possible promoter (Doran et al., 1990). However, *Streptomyces* promoter sequences identified to date are very heterogeneous (Strohl et al., 1992), therefore the inability to detect a promoter by sequence alone does not rule out its presence. Similarly, no sequence resembling a transcription terminator for the upstream *pcbAB* gene was detected in the intergenic

region (Doran et al., 1990). Piret et al. (1990) showed that a mutant of *S. clavuligerus* produced lower than normal levels (about 35% of normal) of both ACVS and IPNS. This suggested an interrelationship between the expression of the two genes. The genetic arrangement of the genes, the lack of easily identifiable promoter or terminator sequences in the short intergenic region, and the existence of a mutant showing possible polarity all made us initially presume that *pcbC* was part of an operon and that its transcription was regulated together with the upstream *pcbAB* gene. Nonetheless, an initial promoter probe construction was prepared to determine whether a *pcbC* promoter might be present within a 335 bp insert of *S. clavuligerus* DNA that included 307 bp of sequence immediately upstream of *pcbC* (*pcbC*up). Surprisingly this construction, *pcbC*up/4083, demonstrated significant promoter activity in both *S. lividans* and *S. clavuligerus* compared to the cultures containing the promoter-less vector itself (pIJ4083), suggesting that *pcbC* has an active promoter directly upstream.

Deletions of the *pcbC*up DNA fragment were generated to further localize the sequences necessary for transcription of *pcbC* from the upstream promoter. The deletion process generated a collection of fragments ranging in amount of DNA sequence found directly upstream of the *pcbC* coding region. These DNA fragments, when ligated to pIJ4083, showed a marked decrease in promoter activity with a decreasing fragment length in both *S. lividans* and *S. clavuligerus* when compared to the complete *pcbC*up fragment. Between the pIJ4083 recombinant plasmids, *pcbC*up/4083 (307 bp of upstream sequence) and DEL168/4083 (168 bp) little promoter activity is lost (85% remains in *S. clavuligerus* and about 66% remains in *S. lividans* cell extracts). Cell extracts from cultures transformed with recombinant plasmids that contained 158 bp of upstream sequence had only 50% of specific activity remaining. All promoter activity was lost when only 152 bp of upstream sequence remained. This indicates that the promoter region or some DNA sequence that is absolutely necessary for transcription

initiation of *pcbC* is located within the *pcbAB* gene, at least 152 bp upstream of the coding region of *pcbC*.

The specific activity of cell free extracts from *S. lividans* transformed with *pcbC*up/4083 and ERME*/4083 is lower than that seen from *S. clavuligerus* transformants. Because this is observed with both promoters, it is most likely the result of differences in growth characteristics rather than differences in promoter strength from one species to the other. The pattern of transcription of the *pcbC* promoter constructions, as indicated by the relative activities in *S. clavuligerus* are very simlar to those seen in *S. lividans*. This raised an interesting point, as previous reports indicated that expression of the *pcbC* gene of *S. clavuligerus* and *S. griseus* had not been detected in *S. lividans* when the gene was under the control of its native control signals (Garcia-Dominguez et al., 1991; Jensen et al.,1989). The original explanation proposed to explain this observation was that *S. lividans* was unable to recognize the transcription control signals upstream of *pcbC*. However, the promoter probe recombinant plasmids studied here indicated that the *pcbC* promoter is capable of initiating transcription in *S. lividans* and this *tsp* was mapped. Therefore, the failure to observe *pcbC* expression in *S. lividans* from the native control signals apparently involves something other than a block at the transcriptional level.

Northern analysis and hybridization of RNA preparations from *S. clavuligerus* using a *pcbC*-specific probe identified a 1.2 kb transcript. A 1.2 kb transcript for *pcbC* has also been identified in *S. griseus* (Garcia-Dominguez et al., 1991). The 1.2 kb size of transcript, together with the identification of a possible transcriptional terminator positioned 7 bp downstream of the translational stop codon of *pcbC* (Jensen et al., 1989) suggested that *pcbC* is transcribed singly rather than as part of an operon and that the *pcbC* promoter sequence lies within the upstream ORF of *pcbAB*. The appearance of the 1.2 kb transcript is first evident after 30h of growth and appears to reach its peak at about

48h. This delay in appearance of transcript during the growth cycle is consistent with the time course of appearance of IPNS activity in cell free extracts of *S. clavuligerus* grown in a similar manner (Jensen et al., 1982b). No evidence of a larger transcript, as might be expected if *pcbC* were co-transcribed together with the upstream ORF, was seen. However, since a *pcbC/pcbAB* transcript would necessarily be very large, it is unclear whether the transcript does not exist or whether the transcript is just technically difficult to detect.

Primer extension to determine the 5' end of the 1.2 kb *pcbC* transcript identified the *tsp* to be a C residue 91 bp upstream of the *pcbC* translational start codon. Only one major band was detected for the *tsp* of the *pcbC* gene of *S. clavuligerus*. The *tsp* is in the same region but not in the same position as the *tsp* of the *pcbC* gene of *S. griseus*, which was determined by S1 nuclease mapping to be 44 bases upstream of the translational start codon (Garcia-Dominguez et al., 1991). The upstream regions of these two *pcbC* genes show high homology and stretches of sequence corresponding to both putative *tsp* were present in each species. Garcia-Dominguez et al. (1991) used an unrelated DNA sequence ladder (ie. M13 sequencing ladder), while this study used a DNA sequence ladder generated from the same oligo primer used for the extension reaction. This may result in slight discrepancies in the localization of the *tsp*, however, a 47 bp difference in the *tsp* most likely indicates actual differences between the two species. The *tsp* of *pcbC* of *S. clavuligerus* correlates reasonably well with the recombinant promoter probe studies to localize the promoter region upstream of the *pcbC* coding region. Deletion analysis implied that the DNA sequence between 168 bp and 85 bp was necessary for initiation of transcription. The greatest loss of activity appeared to come between 158 bp and 152 bp upstream of the start codon. This implies that sequence 67 bp upstream of the tsp is important for full promoter activity. It also emphasizes that many *Streptomyces*

promoters require sequence which extends further upstream than the -35 region for full promoter activity and regulation (Strohl et al., 1992).

In *S. clavuligerus* the *tsp* is located 60 bases within the upstream *pcbAB* gene, which means that the *pcbAB* and *pcbC* transcripts must overlap. One would expect that the transcription of the upstream *pcbAB* gene could influence the initiation of transcription of the 1.2 kb transcript of *pcbC*. Studies in *E. coli* have demonstrated that the passage of RNA polymerase I can prevent the binding of transcription initiation factors, thereby interfering with initiation of transcription of a downstream gene (Adhya and Gottesman, 1982). This could conceivably mean that when *pcbAB* was being actively transcribed, *pcbC* transcription would be depressed, which would seem counter productive for antibiotic production. However, the S1 nuclease mapping studies do not indicate that this type of interference is occurring. ACVS acts immediately prior to IPNS during biosynthesis and since no other function is known for either enzyme, synchronous expression of the enzymes would seem to be most efficient for antibiotic production. Whether these overlapping transcripts play a role in regulation *in vivo* remains to be determined.

Although primer extension can be a rapid method for mapping the 5' ends of transcripts, there are a number of factors which may limit its use. Because primer extension relies on an extension reaction using RNA as a template, the RNA molecule itself must be reasonably intact. The specificity of the reaction is also limited by the need to provide a buffer composition and maximum temperature which will support AMV reverse transcriptase activity. Because of the high GC ratio of *Streptomyces* DNA, it may be difficult to obtain primers that will be specific enough during extension. The enzyme AMV reverse transcriptase is known to have significant RNAase H activity, which is known to suppress the yield of cDNA and restrict its length (Sambrook et al., 1989). It is for this reason that it would be difficult to detect a transcript originating far

upstream of the primer. As an alternative, S1 nuclease mapping can be used to locate *tsp* and this technique may be less sensitive to some of the factors which limit the utility of the 5' primer extension procedure. For these reasons S1 nuclease protection was attempted with the remaining mapping experiments.

S1 nuclease mapping of the 5' end of the *pcbC* gene was performed to confirm the location of the previously detected *tsp* immediately upstream of the gene as determined by primer extension. In addition, S1 nuclease mapping would allow detection of a larger transcript, initiating too far upstream to be detected by primer extension, if one should exist. Excess end-labelled probe fragment was used in these studies to allow some probe/probe reannealing. The size of probe was then easily distinguishable from full length homologous probe annealing to a transcript that continues further upstream than the probe. Addition of excess probe also gave an indication of the relative levels of transcript present. S1 nuclease protection assays performed using a *pcbC*-specific probe indicated the presence of a second larger transcript in addition to the previously identified transcript initiating 91 bases upstream of the *pcbC* coding region. This second transcript originates at least 273 bp within the *pcbAB* gene and extends through the intergenic region and into *pcbC*. This was the first clear evidence of the presence of a larger transcript, since neither primer extension nor Northern analysis had shown any indication of a larger species. From the growth curve it appeared that both transcripts are similarly regulated, first appearing faintly at 36h and increasing in the 48h and 60h samples, and that both transcripts are present in about equal amounts. Multiple *tsp* may allow differential expression of a gene under variable nutritional conditions, but in the case of *pcbC*, the relative amounts of the two transcripts remained constant throughout the growth curve under the conditions used. It is unlikely that the small transcript of *pcbC* is a degradative product of the large transcript, as promoter activity immediately upstream of the *pcbC* gene has been detected in both *S. lividans* and *S.*

clavuligerus. The presence of a 1.2 kb transcript in *S. lividans* initiating within this upstream region also indicates a true *tsp* immediately upstream. It will take further investigation to determine how the cell is able to generate two overlapping transcripts at the same time and whether transcription from the promoter immediately upstream of *pcbC* is only initiating during a small break between passing RNA polymerases.

Because *pcbAB* is such a large gene (about 11 kb), it is difficult to rule out the possibility that the larger transcript originates somewhere within the *pcbAB* coding region. However, it seems more likely that the entire *pcbAB* gene would be transcribed together with the *pcbC* gene as a single message, allowing for more efficient control of antibiotic production. An indirect approach was taken to examine the possiblity of linked transcription of *pcbC* and *pcbAB*. Characterization of the transcriptional regulation of *pcbAB*, may indicate a relationship between the two genes.

The coding regions of the *lat* and *pcbAB* genes are separated by an intergenic region of 152 bp that contains an inverted repeat sequence proposed to be acting as a transcriptional terminator (Tobin et al., 1991). The *pcbAB*up DNA fragment tested for promoter activity included the last 192 bp of the *lat* gene, the 152 bp intergenic region and 19 bp of the *pcbAB* gene. The potential terminator sequence is located immediately downstream of the *lat* stop codon and there were approximately 120 bp of sequence between the inverted repeats and the start codon of *pcbAB*. If transcription initiates immediately upstream of *pcbAB*, one would expect the promoter to be located within the 120 bp region downstream of the putative transcriptional terminator. Somewhat surprisingly, *S. lividans* and *S. clavuligerus* cells transformed with the *pcbAB*up/4083 plasmid did not show significant levels of C23O activity. This indicates that under the growth conditions used, conditions favourable for antibiotic production (Jensen et al., 1982b), no active promoter is present in the *pcbAB*up region. The *pcbAB* transcript must be initiating upstream of the *pcbAB*up DNA fragment, either within the *lat* gene

itself or upstream of the *lat* gene and therefore be co-transcribed with *lat*. It is unlikely that the inverted repeats found in the intergenic region are acting as a terminator (at least not under these conditions), as any transcript initiating upstream of the *pcbAB gene* would terminate before it reached the structural gene. Inverted repeats that have the potential to form hairpin loop structures have also been implicated in mRNA stability and translational regulation of some bacterial genes (reviewed in Platt, 1986). The failure to observe any evidence of termination occurring at this inverted repeat suggests that its *in vivo* function may be in one of these other possible capacities. Site directed mutagenesis of the inverted repeat sequence may give some indication of its role, if any, in regulation of antibiotic biosynthesis.

Since no active promoter was detected immediately upstream of *pcbAB* in the *pcbAB*up fragment, this suggested the possibility that a polycistronic message may be originating upstream of *lat* and extending through all three of the early genes. The region immediately upstream of the *lat* coding region (*lat*up) was introduced into pIJ4083 to determine if a promoter was present. Remarkably strong C23O activity was seen in *S. clavuligerus* transformed with the *lat*up/4083 plasmid. A comparison between C23O activity from *S. clavuligerus* cell extracts containing the positive control vector, ERME*/4083, and cell extracts containing containing *lat*up/4083, showed that the *lat*up/4083 containing cell extracts had approximately 7 times greater activity than cell extracts containing ERME*/4083. The high C23O activity of the *lat*up/4083 construction is specific for *S. clavuligerus* and since only moderate promoter activity is seen in *S. lividans*, this could indicate the requirement for a positive regulatory protein, transcription factor, or specific sigma factor found only in the natural host. Examples of these types of regulation have been observed in *Streptomyces*. Sigma factor heterogeneity has been well documented with as many as seven different sigma factors already identified (Buttner et al., 1989). It is possible that a particular sigma factor might be required to

transcribe antibiotic biosynthetic genes and that this particular sigma factor may be absent or in low abundance in cephamycin non-producers. Other antibiotic biosynthetic pathways in *Streptomyces* like the actinorhodin (Hopwood et al., 1986) and the streptomycin (Pissowotzki et al., 1991) biosynthetic pathways require positive acting transcription activator proteins encoded by the *actII* ORFIV and *strR* genes respectively, to express the antibiotic genes. Such regulatory genes would not be expected to exist in non-producing organisms. In addition to *S. lividans*, *Streptomyces venezuelae* 13s, a cephamycin non-producer, has also been transformed with the *lat*up/4083 construction. Like *S. lividans*, *S. venezuelae* did not show the remarkably high C23O levels seen in *S. clavuligerus*. Studies are currently in progress to introduce the *lat*up/4083 construction into other *Streptomyces sp.* to see if the high promoter activity evident in *S. clavuligerus* is characteristic of cephamycin producing species only.

It is difficult to interpret these C23O specific activities as absolute measures of promoter strength and regulation as they might occur *in vivo* because the promoter sequences are present on a high copy plasmid and the local conformation of the DNA is limited to the size of DNA fragment being inserted. A particular promoter region present in high copy number may not give a fair assessment of promoter regulation and activity, because certain regulatory factors, such as repressors or specific sigma factors may be titrated out. To understand fully the regulation of a particular promoter, a low copy plasmid may give a clearer indication of the level of promoter activity. Studies are in progress to move the various promoter containing fragments, together with the *xylE* reporter gene, into the *Streptomyces* low copy number plasmid pIJ941 (Lydiate et al., 1985). The first of these plasmid constructions, *pcbC*up/*xylE* in pIJ941, displays weak C23O activity in *S. lividans* but has yet to be transformed into *S. clavuligerus*. In the case of the *pcbC*up fragment, use of a low copy number promoter probe system may give a clearer understanding of the possible role and significance of the upstream promoter

sequence. The most accurate assessment of transcriptional regulation could be determined using the ϕC31 phage-based promoter probe *att*⁻ vectors that contain the *xylE* gene and are capable of inserting site-specifically into the chromosome by recombination (Bruton, 1991). This would allow an assessment of the promoter strength when coupled to a reporter gene at single copy in the same context as the promoter exists *in vivo*. Although attempts were made to use one of these phage vectors, KC858, carrying a 1.05 kb *S. clavuligerus* DNA fragment (307 bp upstream sequence and 750 bp of the 5' end of the *pcbC* gene) for site-specific integration, a stable *S. clavuligerus* lysogen could not be obtained.

Northern analysis had indicated the presence of a 1.2 kb *pcbC*-specific transcript, but had failed to detect the presence of the larger transcript seen subsequently in S1 nuclease protection studies. To determine if the failure was due to the instability of the larger transcript, Northern analysis with the *pcbAB*-specific probe was carried out. Since ACVS, the product of the *pcbAB* gene, is essential for antibiotic production, the gene must be being transcribed in antibiotic producing cells. The large size of the *pcbAB* transcript, even if it were being transcribed singly, provides a means of assessing the stability of very large transcripts and their ability to be detected by Northern anlysis.

Northern transfer and hybridization of *S. clavuligerus* RNA indicated only a diffuse smear of radioactive signal specific to the *pcbAB* internal probe. As the cultures grew older the smear became increasingly more degraded. Hybridizing material first appeared at 24h and the location of the top of the smear corresponded to material of quite large size (at least 11 kb) but it was not possible to estimate the actual size of the original transcript before degradation, nor was it possible to determine whether a transcript large enough to include *pcbC* was being produced. It is unclear whether the size of the transcript makes it difficult to detect or whether this specific mRNA molecule is particularly unstable. Intact transcripts up to 11 kb in size from other regions of the *S.*

clavuligerus chromosome that remain stable throughout the growth curve have been detected in RNA preparations prepared in this manner (Aidoo et al., 1991), suggesting that it is this particular mRNA molecule that is being degraded more rapidly than most. An interesting aspect is that the hybridizing material detected on the autoradiograph of a Northern blot hybridized with a probe specific for the *lat* transcript is very similar to that detected for the *pcbAB* hybridization. In each case the hybridizing smears indicated a rapidly degraded transcript that was initially quite large (at least 11 kb). Even with shorter exposure times, no distinct bands were detected. In the data presented here *lat*-specific hybridizing material first appeared at a slightly later sample time (30h) compared to the *pcbAB*-specific hybridizing smear which appeared at 24h. However, the *pcbAB* and *lat* Northern analyses were conducted using two separate sets of RNA preparations. From one growth curve to another there may have been minor variations in the spore inoculum which may account for variations in growth and regulation of antibiotic production. Therefore, blots made using different RNA preparations may show slightly different transcription patterns. The same RNA samples were used for Northern analysis and S1 nuclease mapping experiments of the *lat* gene. The differences between the first appearance of hybridization signal in the Northern blot and in the S1 nuclease mapping experiments was as much as 24h. There are a number of possible reasons for these differences. The samples in both procedures were treated differently, ie. samples prepared for electrophoresis are manipulated to a greater extent and may be exposed to RNAses or be in a situation where they are more susceptible to degradation. Perhaps later samples are more susceptible to degradation during the preparation procedure for Northern analysis. The X-ray film for the *lat* Northern transfer and hybridization was exposed for longer periods of time than was the X-ray film for the *pcbC* Northern analysis, while times of exposure for S1 nuclease mapping experiments specific for the two genes was similar, suggesting much lower levels of *lat* transcript in the Northern

blots. In S1 nuclease protection experiments, mRNA transcripts are allowed to anneal and be protected from nuclease digestion. Different regions of the RNA molecule are also being examined with the two techniques, which may indicate slight differences in stability. If *lat* and *pcbAB* were transcribed as a monocistronic message, the *pcbAB* message would be over 11 kb in length but the *lat* message would be only about 1.4 kb. Failure to detect a specific *lat* transcript suggests that it may be co-transcribed with *pcbAB* or that it is remarkably unstable.

S1 nuclease protection experiments were conducted to examine the time course of the appearance of the *pcbAB* transcript during growth and to determine the location of the *pcbAB tsp*. The lower radioactive band indicates that the *pcbAB* transcript extends at least 192 bases into the upstream *lat* gene and through the intergenic region. An inverted repeat in the intergenic region between *pcbAB* and *lat* was proposed to be acting as a transcriptional terminator (Tobin et al., 1991). However, the results of the S1 nuclease protection experiment suggest that the transcript hybridizing to the *pcbAB*- specific probe extends through the putative transcriptional terminator and into *pcbAB*. A preliminary experiment was performed to determine if transcription of the *lat* gene is terminated in the *lat/pcbAB* intergenic region after the inverted repeats. A 3' end-labelled restriction DNA fragment that spanned the intergenic region of the *lat* and *pcbAB* genes and included coding sequence of the two genes was used for S1 nuclease protection experiments. By introducing the label on the 3' end of the probe fragment, the S1 nuclease protection experiments described previously can also enable transcription stop points to be mapped. Only full length protection of the probe was observed and no smaller protected fragments were detected, using RNA samples isolated from cultures grown in media optimized for antibiotic production. These data were not incorporated into the main body of the Results section because the DNA probe fragment used did not contain any non-homologous DNA. Therefore, it was not possible to rule out unequivocally the possibility that the

observed protected fragment was solely due to probe/probe reannealing and that a genuine shorter transcript was present but not detected for technical reasons. However, in the absence of those unlikely explanations the results suggest that, under the conditions used in this experiment, termination was not occurring at the putative hairpin loop structure.

The lack of a detectable *tsp* for *pcbAB* within the DNA sequence encompassed by the *pcbAB* probe fragment further corroborates the lack of observed promoter activity from *pcbAB*up/4083 in *S. clavuligerus*, and suggests that *pcbAB* is co-transcribed with *lat*. However, the possibility that there may be a promoter sequence within the *lat* gene, but upstream of the *pcbAB* probe used, that directs the transcription of *pcbAB* cannot be ruled out by the S1 nuclease protection experiments performed to date. There is a preliminary report, based on promoter probe studies using the pIJ486 promoter probe vector, that a DNA fragment located further within the *lat* gene than the *pcbAB* probe used, showed significant promoter activity (J. Piret, personal communication). Additional S1 nuclease protection experiments using DNA probe fragments that extend further into the *lat* gene, would clarify the role of this promoter for *pcbAB* transcription. However, for coordinated control of the onset of antibiotic production, it seems more logical that the entire *lat* gene would be co-transcribed with the *pcbAB* and *pcbC* genes. On the other hand, the presence of a second *tsp* for *pcbC* immediately upstream indicates that a second transcript for *pcbAB* could also be possible.

S1 nuclease protection experiments were conducted to determine the *lat tsp* and to compare the time course of expression on a growth curve of the *lat* transcript to those obtained for *pcbAB* and *pcbC*. A single major *tsp* was detected that corresponded to a T residue 88 bases upstream of the *lat* start codon. In the 48h sample, when the main transcript band is strongest, there is also a faint band that corresponds to full length protection of the homologous probe sequence. Whether this indicates the possibility of

an additional *tsp* still further upstream would require more S1 nuclease protection studies of this region. Approximately 4 kb upstream of *lat* is the *cefF* gene, which encodes deacetylcephalosporin C synthase, a late enzyme in the production of cephamycin. The region between the two genes remains uncharacterized, though one would propose that regulatory or resistance genes specific to the pathway may be located within this uncharacterized region of the gene cluster. Alternatively, several of the late genes of the cephamycin pathway have yet to be located.

A comparison of the intensity of the S1 nuclease protected bands generated for *lat*, *pcbAB* and *pcbC* indicates a very similar transcription profile for all three genes. Faint signals appear in the 36h samples and in all cases the strongest signals appear in the 48h and 60h samples. Similar patterns of DNA protected fragments for all three genes support the premise that one long transcript initiates upstream of *lat* and continues through the *pcbAB* and *pcbC* genes. Furthermore, the transcription profile for the two *pcbC* transcripts also follows the same pattern. A number of differential growth characteristics, known to affect the production of enzymes of the cephamycin biosynthetic pathway, were tested in an attempt to dissociate this seemingly linked transcription and identify conditions under which the individual *pcbC* promoter would predominate. The conditions tested were ammonia excess, phosphate excess and carbon source catabolite repression. Transcription profiles for all three genes as determined by S1 nuclease protection assays remained very similar regardless of the conditions used. Similarly, the time course of appearance and relative intensities of the large and small *pcbC* transcripts were the same under all growth conditions tested. In separate studies underway in the laboratory, cephamycin and penicillin non-producing mutants of *S. clavuligerus*, generated by treatment with N'-methyl-N'-nitro-N-nitrosoguanidine, were subjected to transcriptional analysis of the three early genes. A pattern that indicated a polar effect of the mutations became apparent. Mutants which lacked a *lat* transcript also

lacked a *pcbAB* transcript and both the small and large transcripts of *pcbC*. Also, those mutants that had a *lat* transcript but did not show evidence of a *pcbAB* transcript, did not contain the large or small *pcbC* transcripts. No mutants were detected that had the small but not the large *pcbC* transcript. Romero et al. (1988) isolated a number *S. clavuligerus* mutants that were deficient in cephamycin production and were shown to have decreased levels of both LAT and IPNS. Unfortunately these mutants were not tested for ACVS activity, although from the results of the present study, one would suspect that these mutants would have lost ACVS activity as well. Using mutants generated by random mutagenesis experiments is not an ideal situation, in that the nature and extent of the mutations are unknown. Some ongoing experiments in our laboratory include attempts to block transcription within the operon by inserting transcription terminators into the coding region of the individual genes. These defined mutants can then be assessed as to the effect of the mutation on transcription of the remaining genes. These experiments will also indicate if a small molecule such as ACV or cephamycin is necessary for wild type levels of transcription and translation to occur. Blocking specific genes in the pathway allows one to assess the role that the corresponding gene product may play in the regulation of enzyme production.

Results obtained from the mutant studies suggest that the transcription of the large *pcbC* transcript is somehow linked to the transcription of the small *pcbC* transcript. This in turn suggests that the promoter region upstream of *lat* which directs formation of a large polycistronic transcript and the individual promoter upstream of *pcbC* may be similar. The DNA sequences upstream of the *tsp* of the *pcbC* and *lat* genes were analyzed to determine if there were any sequence similarities between the two. Also the -10 and -35 regions of the *pcbC* and *lat tsp* were compared to the promoter region of the *cefD/cefE* genes of *S. clavuligerus*, to promoters of other *Streptomyces* secondary metabolic genes and to the consensus promoters of *E. coli* and *Streptomyces*. The two

early cephamycin biosynthetic genes appear to have very similar promoter sequences based only on the -35 and -10 region. The -10 region of the two genes is exactly alike and the -35 region shows some similarity (4 out of 6 nt). Although the -10 region of both the *pcbC* and *lat* genes showed little similarity to the -10 region of the other cephamycin biosynthetic genes, *cefD/cefE*, the *lat* -35 region matched the *cefD/cefE* -35 region almost exactly (5 out of 6 nt match) and the *pcbC* -35 region matched the *cefD/cefE* -35 region less significantly (3 out of 6 nt). The -35 and -10 regions of the *pcbC* and *lat* genes did not show significant homology to either of the consensus sequences. There is some homology to two *Streptomyces* antibiotic-resistance gene promoter sequences where 4 out of 6 nt match in the -10 region but the similarity in the -35 region varies from a match in 2 out of 6 nt to a match in 4 out of 6 nt. The *pcbC* and *lat* -35/-10 regions show greater similarity to each other and to the *cefD/cefE* gene of the same biosynthetic pathway than to the promoter sequences of other *Streptomyces* genes identified to date (Strohl et al., 1992). Homology in promoter sequences may reflect similar patterns of expression. However, it is still unclear from the putative promoter sequences why *S. clavuligerus* cultures containing the *lat*up/4083 construction have such high levels of C23O activity in comparison to *pcbC*up/4083-containing cultures. Some additional factor acting specifically on the region upstream of *lat* must be present to explain such high transcriptional activity.

An indirect method for determining the role of the small 1.2 kb transcript for antibiotic production is the introduction of the *pcbC* gene together with its upstream promoter sequence in *trans* to a non-producing host to determine the relative amount of IPNS produced. However when such experiments were carried out, no expression of IPNS in *S. lividans* was observed. Lack of expression of IPNS from the *S. clavuligerus* *pcbC* gene in *S. lividans* was initially thought to be due to a failure to recognize the control signals and therefore a lack of transcription. The two *pcbC*-containing plasmids,

1.7/4083 and 1.35/702 were constructed to determine if transcription of *pcbC* was the point at which expression of IPNS was blocked. The control signals of the *melC1/melC* gene of *S. antibioticus* were known to be functional in *S. lividans* (Leu et al., 1989). When the *pcbC* structural gene was placed behind the promoter and ribosome binding site of the *melC1/melC* gene (1.35/702), IPNS activity was detected in levels comparable to the wild type levels seen in *S. clavuligerus*. This indicated that there was no fundamental problem with the *pcbC* structural gene which would prevent its expression in *S. lividans*, given compatible control signals. However, partially purified cell free extracts of *S. lividans* transformed with 1.7/4083 failed to demonstrate detectable levels of IPNS activity. The only significant difference between the two recombinant plasmids is the transcriptional/translational control regions found within the upstream DNA sequences. When *S. venezuelae* 13s, a cephamycin non-producer, was transformed with the 1.7/4083 plasmid, it also failed to show IPNS enzyme activity. Therefore, the lack of IPNS expression may be something specific to non-producing organisms that prevents the production of a functional enzyme when expression is controlled by the native *pcbC* upstream sequence.

Northern transfer of RNA isolated from *S. lividans* containing 1.7/4083 and hybridized to a *pcbC*-specific probe identified a 1.2 kb transcript, similar to that detected in *S. clavuligerus*. This indicated that the inability to observe IPNS enzyme activity in cell free extracts from *S. lividans* transformed with 1.7/4083 does not appear to be due to a failure to produce the 1.2 kb transcript. One possible explanation would be that transcription was not initiating at the correct site, and that this may be interfering with translation. S1 nuclease mapping to determine the 5' end of the *pcbC* transcript from *S. lividans* containing 1.7/4083 indicated that the *tsp* was 5 nt further downstream of the *tsp* of the *pcbC* message from *S. clavuligerus*, but it seems unlikely that this would interfere with the initiation of translation. In related studies ongoing in our laboratory, Western

blot analysis has been performed on cell free extracts to determine if a protein is formed. It appears that the IPNS protein is present in cell free extracts of *S. lividans* transformed with 1.7/4083, albeit not in very large amounts. One possible explanation that remains is that translation is initiating at another site, thereby giving a non-functional IPNS protein in *S. lividans* (and presumably also in *S. venezuelae*). Within the *pcbC* upstream sequence is another possible start codon (-39 relative to the putative *pcbC* start), that would be in frame with the coding region of the *pcbC* gene. Translation initiation at this start codon would add on 13 amino acids that could possibly result in incorrect folding or some other effect that would render the protein non-functional. However, a sequence resembling a ribosome binding site is not present upstream of this alternative start codon making this explanation unlikely. Because the 1.35/702 construction gave functional IPNS protein in *S. lividans*, it implies that some feature in the upstream DNA sequence of the *pcbC* gene is preventing the formation of an active IPNS enzyme in *S. lividans*. Further work must be done to provide an explanation for a lack of activity in the wake of what appears to be fully active transcription and translation.

Our current understanding of the transcriptional regulation of the *pcbC* gene of *S. clavuligerus* is that there is evidence for a large polycistronic transcript that appears to be responsible for the majority of the *in vivo* production of LAT, ACVS and IPNS. A second transcript initiating immediately upstream from *pcbC* is also evident, and transcription from this promoter seems to be tightly linked to the production of the larger transcript. The promoter directing transcription of the large polycistronic message from upstream of *lat* appears to be unusually strong in its natural host, suggesting the presence of a positive species-specific regulatory protein or factor that is increasing the level of *lat* transcription. *S. lividans* containing the *pcbC* gene of *S. clavuligerus* with its native upstream sequence failed to produce functional IPNS protein, although transcription was definitely occurring. It remains unclear as to what is preventing the formation of active

IPNS in this system. There are numerous avenues for further studies into this interesting system before regulation of β-lactam production is fully understood.

V. BIBLIOGRAPHY

Adhya, S. and Gottesman, M.: Promoter occlusion: transcription through a promoter may inhibit its activity. Cell 29 (1982) 939-944.

Aharonowitz, A., Cohen, G. and Martín, J.F.: Penicillin and cephalosporin biosynthetic genes: structure, organization, regulation, and evolution. Annu. Rev. Microbiol. 46 (1992) 461-495.

Aidoo, K. and Jensen, S.E.: Molecular analysis of an ACVS-related protein of *Streptomyces clavuligerus*. International Symposium on the Biology of Actinomycetes, '91. Madison, Wisconsin, August 18-23, 1991.

Amersham Manual. Membrane transfer and detection methods. Amersham International plc., Oakville, Ont. January 1985.

Bailey, C.R. and Winstanley, D.J.: Inhibition of restriction in *Streptomyces clavuligerus* by heat treatment. J. Gen. Microbiol. 132 (1986) 2945-2947.

Baldwin, J.E., Abraham, E.P., Adlington, R.M., Chakravarti, B., Green, N.B., Ting, H.H., and Usher, J.J.: Penicillin biosynthesis. On the stereochemistry of carbon-sulfur bond formation with modified substrates. J. Chem. Soc. Chem. Commun. (1983) 1319-1320.

Baldwin, J.E. and Bradley, M.: Isopenicillin N synthase: mechanistic studies. Chem. Rev. 90 (1990) 1079-1088.

Banko, G., Demain, A.L. and Wolfe, S.: δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (ACV synthetase): a multifunctional enzyme with broad substrate specificity for the synthesis of penicillin and cephalosporin precursors. J. Am. Chem. Soc. 109 (1987) 2858-2860.

Banko, G., Wolfe, S. and Demain, A.L.: Cell-free synthesis of δ-(L-α-aminoadipyl)-L-cysteine, the first intermediate of penicillin and cephalosporin biosynthesis. Biochem. Biophys. Res. Commun. 137 (1986) 528-535.

Barredo, J.L. Cantoral, J.M., Alvarez, E., Diez, B. and Martín, J.F.: Cloning, sequence analysis and transcriptional study of the isopenicillin N synthase of *Penicillium chrysogenum* AS-P-78. Mol. Gen. Genet. 216 (1989) 91-98.

Bibb, M.J., Bibb, M.J., Ward, J.M. and Cohen, S.N.: Nucleotide sequences encoding and promoting expression of three antibiotic resistance genes indigenous to *Streptomyces*. Mol. Gen. Genet. 199 (1985) 26-36.

Bibb, M.J. and Cohen, S.N.: Gene expression in *Streptomyces* : construction and application of promoter-probe plasmid vectors in *Streptomyces lividans*. Mol. Gen. Genet. 187 (1982) 265-277.

Bibb, M.J., Janssen, G.R. and Ward, J.M.: Cloning and analysis of the promoter region of the erythromycin resistance gene (*ermE*) of *Streptomyces erythraeus*. Gene 38 (1986) E357-E368.

Birnboim, H.C. and Doly, J.: A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7 (1979) 1513-1523.

Bradford, M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72 (1976) 248-254.

Brawner, M.E., Auerbach, J.I., Fornwald, J.A., Rosenberg, M. and Taylor, D.P.: Characterization of *Streptomyces* promoter sequences using the *Escherichia coli* galactokinase gene. Gene 40 (1985) 191-201.

Bruton, C.J., Guthrie, E.P. and Chater, K.F.: Phage vectors that allow monitoring of transcription of secondary metabolism genes in *Streptomyces*. Biotechnology 9 (1991) 652-656.

Buttner, M.J.: RNA polymerase heterogeneity in *Streptomyces coelicolor* A3(2). Mol. Microbiol. 3 (1989) 1653-1659.

Buttner, M.J., Chater, K.F. and Bibb, M.J.: Cloning, disruption, and transcriptional analysis of three RNA polymerase sigma factor genes of *Streptomyces coelicolor* A3(2). J. Bacteriol. 172 (1990) 3367-3378.

Buttner, M.J., Smith, A.M. and Bibb, M.J.: At least three different RNA polymerase holoenzymes direct transcription of the agarase gene (*dagA*) of *Streptomyces coelicolor* A3(2). Cell 52 (1988) 599-607.

Calzone, F.J., Britten, R.J. and Davidson, E.H.: Mapping of gene transcripts by nuclease protection assays and DNA primer extension. Methods Enzymol. 152 (1987) 611-632.

Carr, L.G., Skatrud, P.L., Scheetz II, M.E., Queener, S.W. and Ingolia, T.D.: Cloning and expression of the isopenicillin N synthetase gene from *Penicillium chrysogenum*. Gene 48 (1986) 257-266.

Chater, K.F. and Hopwood, D.A.: Antibiotic biosynthesis in *Streptomyces*. *In*: Hopwood, D. A., and Chater, K. F. (Eds.), Genetics of Bacterial Diversity. Academic Press, London, 1989, pp. 129-150.

Clayton, T.M. and Bibb, M.J.: *Streptomyces* promoter-probe plasmids that utilise the *xylE* gene of *Pseudomonas putida*. Nucleic Acids Res. 18 (1990) 1077.

Coque, J.J.R., Liras, P., Laiz, L. and Martín, J.F.: A gene encoding lysine 6-aminotransferase which forms α-aminoadipic acid, a precursor of beta-lactam antibiotics, is located in the cluster of cephamycin biosynthetic genes in *Nocardia lactamdurans*. J. Bacteriol. 173 (1991a) 6258-6264.

Coque, J.J.R., Martín, J.F., Calzada, J.G. and Liras, P.: The cephamycin biosynthetic genes *pcbAB*, encoding a large multidomain peptide synthetase, and *pcbC* of *Nocardia lactamdurans* are clustered together in an organization different from the same genes in *Acremonium chrysogenum* and *Penicillium chrysogenum*. Mol. Microbiol. 5 (1991b) 1125-1133.

Cortes, J., Martín, J.F., Castro, J.M., Laiz, L. and Liras, P.: Purification and characterization of a 2-oxoglutarate-linked ATP-dependent deacetoxycephalosporin C synthase of *Streptomyces lactamdurans*. J. Gen. Microbiol. 133 (1987) 3165-3174.

Demain, A.L.and Piret, J.M.: Cephamycin production by *Streptomyces clavuligerus*. *In* Baumberg, S., Krugel, H. and Noack, D. (Eds.), Genetics and Product Formation of *Streptomyces*. Plenum, New York, 1990, pp. 87-103.

Denhardt, D.T.: A membrane-filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Comm. 23 (1966) 641-646.

Diez, B., Gutierrez, S., Barredo, J.L., van Solingen, P., Van der Voort, L.H. M., and Martin, J.F.: The cluster of penicillin biosynthetic genes. Identification and characterization of the *pcbAB* gene encoding the α-aminoadipyl-cysteinyl-valine synthetase and linkage to the *pcbC* and *penDE* genes. J. Biol. Chem. 265 (1990) 16358-16365.

Distler, J., Ebert, A., Mansouri, K., Pissowotzki, Stockman, M. and Piepersberg, W.: Gene cluster for streptomycin biosynthesis in *Streptomyces griseus*: nucleotide sequence of three genes and analysis of transcriptional activity. Nucleic Acids Res. 15 (1987) 8041-8056.

Doran, J.L., Leskiw, B.K., Petrich, A.K., Westlake, D.W.S. and Jensen, S.E.: Production of *Streptomyces clavuligerus* isopenicillin N synthase in *Escherichia coli* using two-cistron expression systems. J. Ind. Microbiol. 5 (1990) 197-206.

Dotzlaf, J.E. and Yeh, W.-K.: Copurification and characterization of deacetoxycephalosporin C synthetase/hydroxylase from *Cephalosporium acremonium*. J. Bacteriol. 169 (1987) 1611-1618.

Durairaj, M., Doran, J.L. and Jensen, S.E.: High-level expression of the *Streptomyces clavuligerus* isopenicillin N synthase gene in *Escherichia coli*. Appl. Environ. Microbiol. 58 (1992) 4038-4041.

Enquist, L.W. and Bradley, S.G.: Characterization of deoxyribonucleic acid from *Streptomyces venezuelae* spores. Dev. Ind. Microbiol. 12 (1971) 225-236.

Fleming, A.: On the antibacterial action of cultures of a penicillin, with special reference to their use in the isolation of *B. influenzae*. Br. J. Exp. Pathol. 10 (1929) 226.

Garcia-Dominguez, M., Liras, P. and Martín, J.F.: Cloning and characterization of the isopenicillin N synthase gene of *Streptomyces griseus* NRRL 3851 and studies of expression and complementation of the cephamycin pathway in *Streptomyces clavuligerus*. Antimicrob. Agents Chemother. 35 (1991) 44-52.

Gramajo, H.C., White, J., Hutchinson, C.R. and Bibb, M.J.: Overproduction and localization of the components of the polyketide synthase of *Streptomyces glaucescens* involved in the production of tetraenomycin C. J. Bacteriol. 173 (1991) 6475-6483.

Guthrie, E.P. and Chater, K.F.: The level of a transcript required for production of a *Streptomyces coelicolor* antibiotic is conditionally dependent on a tRNA gene.

J. Bacteriol. 172 (1990) 6189-6193.

Gutierrez, S., Diez, B., Montenegro, E. and Martín, J.F.: Characterization of the *Cephalosporium acremonium pcbAB* gene encoding α-aminoadipyl-cysteinyl-valine synthetase, a large multidomain peptide synthetase: linkage to the *pcbC* gene as a cluster of early cephalosporin biosynthetic genes and evidence of multiple functional domains. J. Bacteriol. 173 (1991) 2354-2365.

Hawley, D.K. and McClure, W.R.: Compilation and analysis of *Escherichia coli* promoter DNA sequences. Nucleic Acids Res. 11 (1983) 2237-2255.

Hopwood, D.A., Bibb, M.J., Chater, K.F., Janssen, G.R., Malpartida, F. and Smith, C.P.: Regulation of gene expression in antibiotic-producing *Streptomyces*. *In*: Booth, I. R. and Higgins, C. F. (Eds.), Regulation of Gene Expression 25 Years On. Cambridge University Press, London, 1986, pp. 251-276.

Hopwood, D.A., Bibb, M.J., Chater, K.F., Kieser, T., Bruton, C.J., Kieser, H.M., Lydiate, D.J., Smith, C.P., Ward, J.M. and Schrempf, H.: Genetic Manipulation of *Streptomyces*: a Laboratory Manual. The John Innes Foundation, Norwich, 1985.

Huffman, G.W., Gesellchen, P.D., Turner, J.R., Rothenberger, R.B., Osborne, H.E., Miller, F.D., Chapman, J.L.and Queener, S.W.: Substrate specificity of isopenicillin N synthase. J. Med. Chem. 35 (1992) 1897-1914.

Illing, G.T., Normansell, I.D. and Peberdy, J.F.: Towards an efficient recombination system for *S. clavuligerus*. Fifth International Symposium on the Genetics of Industrial Organisms, abstract #W1-P28. Sept. 14-20 Split, Yugoslavia.

Ingram, C., Brawner, M., Youngman, P. and Westpheling, J.: *xylE* functions as an efficient reporter gene in *Streptomyces spp.*: use for the study of *galP1* , a catabolite-controlled promoter. J. Bacteriol. 171 (1989) 6617-6624.

Janssen, G.R., Bibb, M.J., Smith, C.P., Ward, J.M., Kieser, T. and Bibb, M.J.: Isolation and analysis of *Streptomyces* promoters. *In* Leive, L. (Ed.) Microbiology-1985. American Society for Microbiology, Washington, DC, 1985, pp. 392-396.

Jensen, S.E.: Biosynthesis of cephalosporins. CRC Crit. Rev. Biotechnol. 3 (1986) 277-301.

Jensen, S.E. and Demain, A.L.: β-lactams. *In*: Vining, L.C. (Ed.), Biochemistry and Genetics of Antibiotic Biosynthesis. Stoneham, MA., Butterworth Heinemann, in press, (1993).

Jensen, S.E., Leskiw, B.K., Doran, J.L., Petrich, A.K. and Westlake, D.W.S.: Expression of the *Streptomyces clavuligerus* isopenicillin N synthase gene in *Escherichia coli* and *Streptomyces lividans*. In Hershberger, C.L., Queener, S.W. and Hegeman, G (ed.), Genetics and Molecular Microbiology of Industrial Microorganisms. American Society for Microbiology, Washington, DC, 1989, pp. 239-245.

Jensen, S.E., Leskiw, B.K., Vining, L.C., Aharonowitz, Y., Westlake, D.W.S. and Wolfe, S.: Purification of isopenicillin N synthetase from *Streptomyces clavuligerus*. Can. J. Microbiol. 32 (1986) 953-958.

Jensen , S.E., Westlake, D.W.S, Bowers, R.J. and Wolfe, S.: Cephalosporin formation by cell-free extracts from *Streptomyces clavuligerus*. J. Antibiotics 35 (1982a) 1351-1360.

Jensen, S.E., Westlake, D.W.S., Bowers, R.J., Ingold, C.F., Jouany, M., Lyubechansky, L. and Wolfe, S.: Penicillin formation by cell-free extracts of *Streptomyces clavuligerus*. Behavior of aminoadipyl-modified analogs of the r atural peptide precursor δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV). Can. J. Chem. 62 (1984) 2712-2720.

Jensen, S.E., Westlake, D.W.S., and Wolfe, S.: Cyclization of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine to penicillins by cell-free extracts of *Streptomyces clavuligerus*. J. Antibiotics 35 (1982b) 483-490.

Jensen, S.E., Westlake, D.W.S. and Wolfe, S.: High performance liquid chromatographic assay of cyclization activity in cell-free systems from *Streptomyces clavuligerus*. J. Antibiotics 35 (1982c) 1026-1032.

Jensen, S.E., Westlake, D.W.S. and Wolfe, S.: Partial purification and characterization of isopenicillin N epimerase activity from *Streptomyces clavuligerus*. Can J. Microbiol. 29 (1983) 1526-1531.

Jensen, S.E., Westlake, D.W.S. and Wolfe, S.: Deacetoxycephalosporin C synthetase and deacetoxycephalosporin C hydroxylase are two separate enzymes in *Streptomyces clavuligerus*. J. Antibiot. 38 (1985) 263-265.

Jensen, S.E., Westlake, D.W.S. and Wolfe, S.: Production of the penicillin precursor δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) by cell free extracts from *Streptomyces clavuligerus*. FEMS Microbiol. Lett. 49 (1988) 213-218.

Katz, E., Thompson, C.J. and Hopwood, D.A.: Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. J. Gen. Microbiol. 129 (1983) 2703-2714.

Kern, B.A., Hendlin, D. and Inamine, E.: L-lysine ε-aminotransferase involved in cephamycin C synthesis in *Streptomyces lactamdurans*. Antimicrob. Agents Chemother. 17 (1980) 676-685.

Kieser, T.: Factors affecting the isolation of cccDNA from *Streptomyces lividans* and *Escherichia coli*. Plasmid 12 (1984) 19-36.

Kieser, T., Hopwood, D.A., Wright, H.M. and Thompson, C.J.: pIJ101, a multi-copy broad host-range *Streptomyces* plasmid: functional analysis and development of DNA cloning vectors. Mol. Gen. Genet. 185 (1982) 223-238.

Kleinkauf, H. and von Dohren, H.: Nonribosomal biosynthesis of peptide antibiotics. Eur. J. Biochem. 192 (1990) 1-15.

Kohsaka, M. and Demain, A.L.: Conversion of penicillin N to cephalosporin(s) by

cell-free extracts of *Cephalosporium acremonium*. Biochem. Biophys. Res. Commun. 70 (1976) 465-473.

Konomi, T., Herchen, S., Baldwin, J.E., Yoshida, M., Hunt, N.A. and Demain, A.L.: Cell-free conversion of δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine into an antibiotic with the properties of isopenicillin N in *Cephalosporium acremonium*. Biochem. J. 184 (1979) 427-430.

Kovacevic, S. and Miller, J.R.: Cloning and sequencing of the β-lactam hydroxylase gene (*cefF*) from *Streptomyces clavuligerus*: gene duplication may have led to separate hydroxylase and expandase activities in the actinomycetes. J. Bacteriol. 173 (1991) 398-400.

Kovacevic, S., Tobin, M.B. and Miller, J.R.: The β-lactam biosynthesis genes for isopenicillin N epimerase and deacetoxycephalosporin C synthetase are expressed from a single transcript in *Streptomyces clavuligerus*. J. Bacteriol. 172 (1990) 3952-3958.

Kovacevic, S., Weigel, B.J., Tobin, M.B., Ingolia, T.D. and Miller, J.R.: Cloning, characterization, and expression in *Escherichia coli* of the *Streptomyces clavuligerus* gene encoding deacetoxycephalosporin C synthetase. J. Bacteriol. 171 (1989) 754-760.

Kupka, J., Shen, Y.Q., Wolfe, S. and Demain, A.L.: Partial purification and properties of the α-ketoglutarate-linked ring-expansion enzyme of β-lactam biosynthesis of *Cephalosporium acremonium*. FEMS Microbiol. Lett. 16 (1983) 1-6.

Laiz, L., Liras, P., Castro, J.M. and Martín, J.F.: Purification and characterization of the isopenicillin N epimerase from *Nocardia lactamdurans*. J. Gen. Microbiol. 136 (1990) 663-671.

Lee, Y.-H.W., Chen, B.-F., Wu, S.-Y., Leu, W.-M., Lin, J.-J., Chen, C.W. and Lo, S.J.: A trans acting gene is required for the phenotypic expression of a tyrosinase gene in *Streptomyces*. Gene 65 (1988) 71-81.

Leskiw, B.K., Aharonowitz, Y., Mevarech, M., Wolfe, S., Vining, L.C., Westlake, D.W.S. and Jensen, S.E.: Cloning and nucleotide sequence determination of the isopenicillin N synthetase gene from *Streptomyces clavuligerus*. Gene 62 (1988) 187-196.

Leskiw, B.K., Bibb, M.J. and Chater, K.F.: The use of a rare codon specifically during development? Mol. Microbiol. 8 (1991) 243-252.

Leskiw, B.K., Mah, R., Lawlor, E.J. and Chater, K.F.: Accumulation of *bldA*-specified tRNA is temporally regulated in *Streptomyces coelicolor* A3(2). J. Bacteriol. 175 (1993) 1995-2005.

Leu, W.-M., Wu, S.-Y., Lin, J.-J., Lo, S.J. and Lee, Y.-H. W.: Analysis of the promoter region of the melanin locus from *Streptomyces antibioticus*. Gene 84 (1989) 267-277.

Lydiate, D.J., Malpartida, F. and Hopwood, D.A.: The *Streptomyces* plasmid SCP2*: its functional analysis and development into useful cloning

vectors. Gene 35 (1985) 223-235.

MacCabe, A.P., van Liempt, H., Palissa, H., Unkles, S.E., Riach, M.B.R., Pfiefer, E., van Dohren, H. and Kinghorn, J.R.: δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase from *Aspergillus nidulans*: molecular characterization of the *acvA* gene encoding the first enzyme of the penicillin biosynthetic pathway. J. Biol. Chem. 266 (1991) 12646-12654.

Madduri, K., Stuttard, C. and Vining, L.C.: Lysine catabolism in *Streptomyces* spp. is primarily through cadaverine: β-lactam producers also make α-aminoadipate. J. Bacteriol. 171 (1989) 299-302.

Madduri, K. Stuttard, C. and Vining, L.C.: Cloning and location of a gene governing lysine ε-aminotransferase, an enzyme initiating β-lactam biosynthesis in *Streptomyces* spp. J. Bacteriol. 173 (1991) 985-988.

Martin, J.F.: Clusters of genes for the biosynthesis of antibiotics: regulatory genes and overproduction of pharmaceuticals. J. Ind. Microbiol. 9 (1992) 73-90.

Martin, J.F. and Liras, P.: Enzymes involved in penicillin, cephalosporin and cephamycin biosynthesis. Adv. Biochem. Eng. 39 (1989) 153-187.

Mendez, C. and Chater, K.F.: Cloning of *whiG*, a gene critical for sporulation of *Streptomyces coelicolor* A3(2). J. Bacteriol. 169 (1987) 5715-5720.

Miller, J.: Experiments in Molecular Genetics. Cold Spring Harbor Laboratory. Cold Spring Harbor, New York. 1972.

Montenegro, E., Barredo, J.L., Gutierrez, S., Diez, B., Alvarez, E. and Martin, J.F.: Cloning, characterization of the acylCoA:6-amino penicillanic acid acyltransferase gene of *Aspergillus nidulans* and linkage to the isopenicillin N synthase gene. Mol. Gen. Genet. 221 (1990) 322-330.

Morrison, D.A.: Transformation and preservation of competent bacterial cells by freezing. Methods in Enzymology 68 (1979) 326-331.

Newton, G.G.F. and Abraham, E.P.: Cephalosporin C, a new antibiotic containing sulphur and D-α-aminoadipic acid. Nature 175 (1955) 548.

O'Sullivan, J. and Abraham, E.P.: The conversion of cephalosporins to 7-α-methoxycephalosporins by cell-free extracts of *Streptomyces clavuligerus*. Biochem. J. 186 (1980) 613-616.

Petrich, A.K., Wu, X., Roy, K.L. and Jensen, S.E.: Transcriptional analysis of the isopenicillin N synthase-encoding gene of *Streptomyces clavuligerus*. Gene 111 (1992) 77-84.

Piret, J., Resendiz, B., Mahro, B., Zhang, J.-Y., Serpe, E., Romero, J., Connors, N. and Demain, A.L.: Characterization and complementation of a cephalosporin-deficient mutant of *Streptomyces clavuligerus* NRRL 3585. Appl. Microbiol. Biotechnol. 32 (1990) 560-567.

Pissowotzki, K., Mansouri, K. and Piepersberg, W.: Genetics of streptomycin production in *Streptomyces griseus*: molecular structure and putative function of genes *strELMB2N*. Mol. Gen. Genet. 231 (1991) 113-123.

Platt, T.: Transcription termination. Ann. Rev. Biochem. 55 (1986) 339-372.

Queener, S.W.: Molecular biology of penicillin and cephalosporin biosynthesis. Antimicrob. Ag. Chemother. 34 (1990) 943-948.

Ramon, D., Carramolino, L., Patino, C., Sanchez, F. and Penalva, M.A.: Cloning and characterization of the isopenicillin N synthetase gene mediating the formation of the β-lactam ring in *Aspergillus nidulans*. Gene 57 (1987) 171-181.

Rigaud, G., Grange, T and Pictet, R.: The use of NaOH as transfer solution onto nylon membrane decreases the hybridization efficiency. Nucleic Acids Res. 15 (1987) 857.

Romero, J., Liras, P. and Martin, J.: Isolation and biochemical characterization of *Streptomyces clavuligerus* mutants in the biosynthesis of clavulanic acid and cephamycin C. Appl. Microbiol. Biotechnol. 27 (1988) 510-516.

Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B. and Erlich, H.A.: Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. Science 239 (1988) 487-491.

Sala-Trepat, J.M. and Evans, W.C.: The meta cleavage of catechol by *Azotobacter* species: 4-oxalocrotonate pathway. Eur. J. Biochem. 20 (1971) 400-413.

Sambrook, J., Fritsch, E.F. and Maniatis, T.: Molecular Cloning - A Laboratory Manual. Second Edition. Cold Spring Harbor Laboratory, Cold Spring Harbor Press, NY, 1989.

Samson, S.M., Belagaje, R., Blakenship, D.T., Chapman, J.L., Perry, D., Skatrud, P.L., VanFrank, R.M., Abraham, E.P., Baldwin, J.E., Queener, S.W. and Ingolia, T.D.: Isolation, sequence determination, and expression in *E. coli* of the isopenicillin N synthetase gene from *Cephalosporium acremonium*. Nature 318 (1985) 191-194.

Samson, S.M., Chapman, J.L., Belagaje, R., Queener, S.W. and Ingolia, T.D.: Analysis of the role of cysteine residues in isopenicillin N synthetase activity by site directed mutagenesis. Proc. Natl. Acad. Sci. USA 84 (1987a) 5705-5709.

Samson, S.M., Dotzlaf, J.E., Slisz, M.L., Becker, G.W., VanFrank, R.M., Veal, L.E., Yeh, W.-K., Miller, J.R., Queener, S.W. and Ingolia, T.D.: Cloning and expression of the fungal expandase/hydroxylase gene involved in cephalosporin biosynthesis. Bio/Technol. 5 (1987b) 1207-1214.

Sanger, F., Nicklen, S. and Coulson, A.R.: DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA. 74 (1977) 5463-5467.

Schauer, A., Ranes, M., Santamaria, R., Guijarro, J., Lawlor, E., Mendez, C., Chater, K.and Losick, R.: Visualizing gene expression in time and space in the filamentous bacterium *Streptomyces coelicolor*. Science 240 (1988) 768-772.

Scheidegger, A., Kuenzi, M.T. and Nuesch, J.: Partial purification and catalytic properties of a bifunctional enzyme in the biosynthetic pathway of beta-lactams in *Cephalosporium acremonium*. J. Antibiot. 37 (1984) 522-531.

Seno, E.T. and Baltz, R.H.: Structural organization and regulation of antibiotic biosynthesis and resistance genes in Actinomycetes. *In*: Shapiro, S. (Ed.), Regulation of Secondary Metabolism in Actinomycetes. CRC Press. Boca Raton, Fla., 1989 pp. 1-48.

Shiffman, D., Mevarech, M., Jensen, S.E., Cohen, G. and Aharonowitz, Y.: Cloning and comparative sequence analysis of the gene coding for isopenicillin N synthase in *Streptomyces*. Mol. Gen. Genet. 214 (1988) 562-569.

Smith, D.J., J., Earl, A.J. and Turner, G.: The multifunctional peptide synthetase performing the first step in penicillin biosynthesis in *Penicillium chrysogenum* is a 421,073 dalton protein similar to *Bacillus brevis* peptide antibiotic synthetases. EMBO J. 9 (1990) 2743-2750.

Southern, E.: Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98 (1975) 503-517.

Staskawicz, B., Dahlbeck, D., Keen, N. and Napoli, C.: Molecular characterization of cloned avirulence genes from Race 0 and Race 1 of *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. 169 (1987) 5789-5794.

Strohl, W.R.: Compilation and analysis of DNA sequences associated with apparent streptomycete promoters. Nucleic Acids Res. 20 (1992) 961-974.

Tabor, S. and Richardson, C.C.: DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. Proc. Natl. Acad. Sci. USA. 84 (1987) 4767-7771.

Tanaka, K., Shiina, T. and Takahashi, H.: Multiple principal sigma factor homologs in eubacteria: identification of the 'rpoD' box. Science 242 (1988) 1040-1042.

Thomas, D. and Surdin-Kerjan, Y.: An improved strategy for generating a family of unidirectional deletions on large DNA fragments. GATA 7 (1990) 87-90.

Tobin, M.B., Kovacevic, S., Madduri, K., Hoskins, J., Skatrud, P.L., Vining, L.C. and Miller, J.R.: Localization of the lysine ε-aminotransferase (*lat*) and δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine synthetase (*pcbAB*) genes from *Streptomyces clavuligerus* and production of lysine ε-aminotransferase activity in *Escherichia coli*. J. Bacteriol. 173 (1991) 6223-6229.

Usui, S., Yu, C.-A.: Purification and properties of isopenicillin N epimerase from *Streptomyces clavuligerus*. Biochim. Biophys. Acta 999 (1989) 78-85.

Viera, J. and Messing, J.: Production of single-stranded plasmid DNA. Methods Enzymol. 153 (1987) 3-11.

Ward, J.M., Janssen, G.R., Kieser, T., Bibb, M.J., Buttner, M.J. and Bibb, M.J.: Construction and characterization of a series of multi-copy promoter-probe

plasmid vectors for *Streptomyces* using the aminoglycoside phosphotransferase gene from Tn5 as indicator. Mol. Gen. Genet. 203 (1986) 468-478.

Weigel, B.J., Burgett, S.G., Chen, V.J., Skatrud, P.L., Frolik, C.A., Queener, S.W. and Ingolia, T.D.: Cloning and expression in *Escherichia coli* of isopenicillin N synthetase genes from *Streptomyces lipmanii* and *Aspergillus nidulans*. J. Bacteriol. 170 (1988) 3817-3826.

Westpheling, J. and Brawner, M.: Two transcribing activities are involved in the expression of the *Streptomyces* galactose operon. J. Bacteriol. 171 (1989) 1355-1361.

Westpheling, J., Ranes, M. and Losick, R.: RNA polymerase heterogeneity in *Streptomyces coelicolor*. Nature 313 (1985) 22-27.

Whitney, J.D., Brannon, D.R., Mabe, J.A. and Wicker, K.J.: Incorporation of labelled precursors into A16886B, a novel β-lactam antibiotic produced by *Streptomyces clavuligerus*. Antimicrob. Agents Chemother. 1 (1972) 247-251.

Williams, J.G., and Mason, P.J.: Hybridisation in the analysis of RNA. *In* Hames, B. D. and Higgins, S. J. (Eds.), Nucleic Acid Hybridisation - A Practical Approach. IRL Press, Oxford, 1985, pp. 139-178.

Wolfe, S., Demain, A.L., Jensen, S.E. and Westlake, D.W.S.: Enzymatic approach to syntheses of unnatural beta-lactams. Science 226 (1984) 1386-1392.

Wu, X. and Roy, K.L.: Complete nucleotide sequence of a linear plasmid from *Streptomyces clavuligerus* and characterization of its RNA transcripts. J. Bacteriol. 175 (1993) 37-52.

Xiao, X., Wolfe, S. and Demain, A.L.: Purification and characterization of cephalosporin 7-α-hydroxylase from *Streptomyces clavuligerus*. Biochem. J. 280 (1991) 471-474.

Xiao, X., Hinterman, G., Hausler, A., Barker, P.J., Foor., F., Demain, A.L. and Piret, J.: Cloning of a *Streptomyces clavuligerus* DNA fragment encoding the cephalosporin 7-α-hydroxylase and its expression in *Streptomyces lividans*. Antimicrob. Agents Chemother. 37 (1993) 84-88.

Yoshida, M., Konomi, T., Kohsaka, M., Baldwin, J.E., Herchen, S., Singh, P., Hunt, N.A. and Demain, A.L.: Cell-free ring expansion of penicillin N to deacetoxycephalosporin C by *Cephalosporium acremonium* CW-19 and its mutants. Proc. Natl. Acad. Sci. USA 75 (1978) 6253-6257.

Zhang, J.Y. and Demain, A.L.: Purification of ACV synthetase from *Streptomyces clavuligerus*. Biotechnol. Lett. 12 (1990) 649-654.

Zoller, M.J. and Smith, M.: Oligonucleotide - directed mutagenesis: a simple method using two oligonucleotide primers and a single-stranded DNA template. Methods Enzymol. 154 (1987) 329-349.

Zukowski, M.M., Gaffney, D.F., Speck, D., Kauffmann, M., Findeli, A., Wisecup, A. and Lecocq, J.-P.: Chromogenic identification of genetic regulatory signals in *Bacillus subtilis* based on expression of a cloned *Pseudomonas* gene. Proc. Natl. Acad. Sci. USA. 80 (1983) 1101-1105.

VI. APPENDIX

Partial DNA sequence of the *lat*, *pcbAB* and *pcbC* genes from *Streptomyces clavuligerus* that includes upstream sequence used for promoter probe analysis and S1 nuclease mapping experiments.

```
                                          -227
                                          GAATTCCCCTGAACACG
                                          EcoRI
-210
AAGCTGAGCAACAGCTCGTCACGCGCTCCCGAGCTGGCCATTCAGGGCAG

-160
TTCACAAAGAGCCATCGAGAGGCGTCCGAGAGAGCTGGAAGAGGGGTCCA

-110                              tsp
AGAGCATGGTGGGTCATTATTGTGATCCTAAAATGTCCAGTTCACCGCCA

-60
TGACAGCAGAGGCTGGAAAGTCCCCCATAATTCAGCCTGATCCCCCAGGA

-10          >lat
GTTCTCACCCATGGGCGAAGCAGCACGCCACCCCGACGGCGATTTCTCGG

+40
ACGTGGGAAACCTCCACGCTCAGGACGTGCACCAGGCACTTGAGCAGCAT

+90
ATGCTCGTCGACGGGTACGACCTCGTTCTCGACCTCGACGCCAGCTCCGG
                    -344
.................GTCGACCTGCCGGACACCCGGACCCGCAATGAGG
                   SalI
-310
TGCTGCGGCTCATGTACACGGAGCACCAGGTCATCGCCCTGCCCTGCGGC

-260
GGGCGCAGCCTCCGGTTCCGCCCCGCGCTGACGATCGCGGAGCACGAGAT

-210
CGACCAGGCCCTTCAGGCGCTGGCGAGCAGTGTCACGCCGGTCGCCGAGA

-160
GCGTCTGACGCCCGGCCGGCCGGGTGCCCCAGCGGGGCCCGGCCGGCCGC

-110
ACCGCGGAACGGAACACCCCTGGCGCGTCGCCCGGTGACACGCTCTCCGG

-60
GCGGCTCAACCGCGCCACCCCCACGGCACGTTCGCCTTCACCCGCACGGA

-10          >pcbAB
GAGCCCACGAATGATGTCAGCACGGTACCCGAGGACCGCAGCGGAGTGGA
                                  KpnI
+40
CCACTCGCATTCAAGGAGTGTCGAGCGAGCGTTGCGATCTTGAAATCTGC

+90
TGAAGGACGAGTGGCGCAACAGGATCGCGGTACGGGACGACGACCCCGGT
      -307
....CCCGGGAGGATCTGGAGCGGCTGCGCGCGAACACCGGCGATGTGGT
    SmaI
-260
GCTGTTCCGGGCGGACGAGCCGAACGAGATCGTCCGGGACGAGGAGCAGC

-210
GCCGGCTGTTCGACTTCTACCAGCGGTCCTCCTTCAACGGGCTGGACGCG

-160
CTGCTGCCCGCGGAGTCCATCGAGGTCCACCGGCTGCACGGTGAGACCCA

-110                              tsp
CCATTCGTGGGTACGAAACGACCGGCTGGTCGCCGACATCTGTGCGCGTG

-60
TCTCGGCGTCGTCGCCGGATGCCCGGTGAACGGCCGGGCGCTGAGTCCAA

-10       * >pcbC
GGAGGGTTTCATGCCAGTTCTGATGCCGTCGGCCCACGTTCCGACCATCG

+40
ACATCTCGCCGCTGTTCGGAACCGACGCCGCCGCGAAGAAGCGCGTCGCC

+90
GAGGAGATACACGGGGCCTGCCGCGGCTCGGGCTTCTTCTACGCCACGAA
```

* Base changed from T to G by site-directed mutagenesis to introduce an *SphI* site.